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From Neurons to Neuroblastoma:

Dietary Flavonoids as Regulators of Life, Death & Differentiation

Thesis presented by

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for the degree of

Doctor of Philosophy

University College Cork

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under the supervision of

Dr. Gerard O'Keeffe, Prof. Aideen Sullivan, Dr. Shane Hegarty

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

_____

Amnah Alshangiti
February 2020

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Publications arising from this work

Abstracts

Examination of the Neurotrophic Effects of Small Molecule Activators of BMP-Smad Signaling in SH-SY5Y cells.

Amnah M. Alshangiti, Shane V. Hegarty, Aideen M. Sullivan, Gerard W. O'Keeffe.

Neuroscience Ireland Conference August 2017

Development of small molecule therapy for paediatric neuroblastoma.

Amnah M. Alshangiti, Katie L. Togher, Shane V. Hegarty, Aideen M. Sullivan, Gerard W. O'Keeffe.

INFANT Research day October 2017.

Papers

Published

PAPER 1

The dietary flavonoid isoliquiritigenin is a potent cytotoxin for human neuroblastoma cells.

Amnah M. Alshangiti, Katie L. Togher, Shane V. Hegarty, Aideen M. Sullivan, Gerard W. O'Keeffe.

Neuronal Signaling (2019), 3 (1): NS20180201.

PAPER 2

4-Hydroxychalcone Induces Cell Death via Oxidative Stress in MYCN-Amplified Human Neuroblastoma Cells.

Amnah M. Alshangiti , Eszter Tuboly, Shane V. Hegarty, Cathal M. McCarthy, Aideen M. Sullivan, and Gerard W. O'Keeffe.

Oxidative Medicine and Cellular Longevity (2019), https://doi.org/10.1155/2019/1670759

PAPER 3

Association of distinct type 1 bone morphogenetic protein receptors with different molecular pathways and survival outcomes in neuroblastoma.

Amnah M. Alshangiti, Sean L. Wyatt, Shane V. Hegarty, Aideen M. Sullivan, Gerard W. O'Keeffe.

Neuronal Signaling (2020) 4 (1): NS20200006.

Abbreviations

4HC	4-hydroxychalcone
6-OHDA	6-hydroxydopamine
ALK	Activin Receptor-like Kinases
BDNF	Brain-Derived Neurotrophic Factor
BMP	Bone Morphogenetic Protein
BMPR	Bone Morphogenetic Protein Receptor
Boc-D-FMK	Caspase inhibitor III
BSA	Bovine Serum Albumin
CNS	Central Nervous System
CSCs	Cancer Stem Cells
DAPI	4'-6-Diamidino-2-phenylindole
DIV	Days In Vitro
DMSO	Dimethyl sulfoxide
DTNB	5,5'-dithio-bis(2-nitrobenzoic acid)
E	Embryonic Day
EFS	Event-Free Survival
EMT	Epithelial to Mesenchymal Transition
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
GCL	Glutamate Cysteine Ligase
GD2	Disialoganglioside
GDF5	Growth Differentiation Factor 5
GFP	Green Fluorescent Protein
GO	Gene Ontology

GRN	Gene Regulatory Network
GSH	Glutathione
GSSG	Glutathione Disulfide
H_2O_2	Hydrogen Peroxide
HBSS	Hank's Balanced Salt Solution
IM-54	Necrosis Inhibitor
INPC	International Neuroblastoma Pathology Classification
INSS	International Neuroblastoma Staging System
INRG	International Neuroblastoma Risk Group
ISLQ	Isoliquiritigenin
LDH	Lactate dehydrogenase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
MEM	Minimum Essential Medium
MET	Mesenchymal-to-Epithelial Transition
MLKL	Mixed Lineage Kinase Domain-Like Protein
MRI	Magnetic resonance imaging
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetyl-L-cysteine
NB	Neuroblastoma
NC	Neural Crest
NEAA	Non-Essential Amino Acid Solution
NEC-1	Necrostatin-1
NGF	Nerve Growth Factor
PC-12	Pheochromocytoma cell line
PCP	Planar Cell Polarity
P	Post-Natal Day

RA	Retinoic Acid
RIPK	Receptor-Interacting Protein Kinase
R-Smads	Receptor-regulated Smads
ROS	Reactive Oxygen Species
SCG	Superior Cervical Ganglion
SNS	Sympathetic Nervous System
TGF-ß	Transforming Growth Factor-Beta
TICs	Tumor-Initiating Cells
TVD	Topotecan-Vincristine-Doxorubicin

Abstract

Neuroblastoma (NB) is an embryonal malignancy that arises from cells of sympathoadrenal lineage during the development of the nervous system. Patients classified as stage 4 with aggressive tumors are considered to belong to a high-risk group. *MYCN*-amplification is detected as a hallmark of NB in 20% of high-risk cases. Based on the International Neuroblastoma Risk Group (INRG), the relapse survival rate of high-risk patients with *MYCN*-amplification is only 4%. Despite extensive studies, metastasis and tumor relapse lead to debilitating morbidity and frequently death, in high-risk NB patients. Moreover, NB chemotherapy can result in very debilitating long-term sequelae.

Errors in the molecular mechanisms that control the development of cell of the sympathetic lineage have been linked to NB development. One group of signals, known as the bone morphogenetic proteins (BMPs), plays an important role in sympathetic neuritogenesis, by signaling through bone morphogenetic protein receptor (BMPR)2 and either BMPR1A or BMPR1B. Alterations in BMPR2 expression have been reported in NB, but it is unknown if the expression of BMPR1A or BMPR1B is altered. The use of neurotrophic factors such as nerve growth factor (NGF) and growth differentiation factor (GDF)5 to promote cell differentiation has been reported in postnatal day (P)1 mouse NC cultures and *MYCN*-amplified NB SK-N-BE(2), respectively. GDF5 induces cell differentiation in NB cells through activation of BMP-Smad signaling. However, recombinant BMPs are expensive to produce and their clinical use is limited, due to several factors.

Therefore, there is an urgent need to identify novel therapeutic agents that could induce cell differentiation in post-chemotherapy tissue with the minimum side effects. Recently, several screening studies have identified small molecules that modulate the BMP pathway. These include natural dietary compounds, including flavonoids and in particular the chalcone subfamily. Interestingly, flavonoids have potential anti-tumor properties with selective cytotoxicity to cancer cells. Additionally, chalcones, which are the most potent flavonoid subclass, have also demonstrated anti-tumor activity in some contexts.

This thesis first investigated, in chapter 2, the association of distinct type1 BMPRs with different molecular pathways and survival outcomes in NB. Lower expression of *BMPR2* and *BMPR1B*, and higher *BMPR1A* expression, were observed in stage 4 and in *MYCN*-amplified NB, and they were associated with poor survival. In contrast, higher expression of *BMPR2* and *BMPR1B* were associated with better survival. Regarding *MYCN* status, the correlation

between *BMPR2* and *BMPR1A* was strengthened, while the correlation between *BMPR2* and *BMPR1B* was lost, in *MYCN*-amplified NB. This study therefore showed that expression of distinct BMPRs is associated with different survival outcomes in NB.

In chapter 3, the effects of low doses of the chalcones isoliquiritigenin (ISLQ) and 4-hydroxychalcone (4HC), small molecule activator of BMP-Smad signaling, on the morphological differentiation of NB cells, as well as the molecular mechanism of their action, was investigated. ISLQ and 4HC promoted neurite growth in NB cells by activation of BMP-Smad signaling. Moreover, treatment with these chalcones lead to a significant reduction of cell aggregates (tumor-like aggregations) in SK-N-BE(2) cells.

After these findings, in chapter 4, the cytotoxic effect of ISLQ in *MYCN*-amplified NB cells was investigated in detail. ISLQ induced cell death in *MYCN*-amplified NB cells SK-N-BE(2) and IMR-32 through an elevation of oxidative stress and necroptosis. Combined treatment of ISLQ with the chemotherapy agent cisplatin led to a greater cell toxicity in *MYCN*-amplified NB cells.

In chapter 5, the cytotoxic effect of 4HC on NB cells, and its mode of action, was examined. 4HC demonstrated selective cytotoxicity on *MYCN*-amplified NB cells, which were more sensitive to 4HC than non-amplified SH-SY5Y cells. Cell death induced by 4HC was shown to result from glutathione (GSH) depletion, elevation of oxidative stress and mitochondrial impairment.

This thesis, therefore, demonstrates the role of various types of BMPRs in different molecular pathways and survival outcomes in NB, It also highlights the potential of dietary chalcones for either the induction of NB cell differentiation at lower concentrations, through the activation of BMP-Smad signaling, or induction of selective NB cell death at higher concentrations, by elevation of oxidative stress.

Chapter 1 : Introduction

1.0 General Introduction

1.1 Neuroblastoma pediatric cancer

Neuroblastoma (NB) is a developmental tumor arising from sympatho-adrenal lineage of the neural crest (NC), in the sympathetic nervous system (SNS) (Louis and Shohet 2015). Historically, NB tumors are described as "enigmatic" due to their unpredictable clinical behavior, which includes spontaneous regression, that is now considered to be strongly associated with tumor genetic profile (Brossard, Bernstein et al. 1996, Pugh, Morozova et al. 2013). James Wright first described NB and named it due to the presence of fibrils in arrangements similar to those of neuroblast (Wright 1910). NB is the third most common childhood cancer, after leukemia and brain tumors, and the most common extracranial solid tumor in childhood (Kaatsch 2010), and accounts for 15% of cancer-related deaths in childhood (Thorner 2014).

The median age at diagnosis is around 22 months, although this tumor can regress in some infants who may never present to medical attention (Colon and Chung 2011). Magnetic resonance imaging (MRI) scans of pregnant women can reveal NB diagnosis in uterus (Figure 1.1). One rare case of NB presenting in a 19-year-old child was reported (London, Castleberry et al. 2005). The vast majority of children diagnosed with NB are younger than 5 years old and almost all of these were diagnosed by the age of ten (Shohet and Foster 2017).

The factors lead to the initiation of NB tumor growth or trigger its regression are not known, nor any factors that may contribute to or lessen disease progression (Shimada and Ikegaki 2019). NB offers one of the best models to investigate the relationship between tumor genes or molecular properties, and morphologic manifestations (Shimada, Ambros et al. 1999), and also to investigate potential therapies (Shastry, Basu et al. 2001).

1.1.1 NB staging system

NB is remarkable for its broad spectrum of clinical symptoms that closely correlate with a number of biological features. These include disease stage, age of patient, grade of tumor differentiation, state of *MYCN* oncogene, chromosome 11q status, and DNA ploidy (Louis and Shohet 2015). According to the International Neuroblastoma Staging System (INSS), Stage 1 tumor is localized, and can be completely removed along with the lymph node.

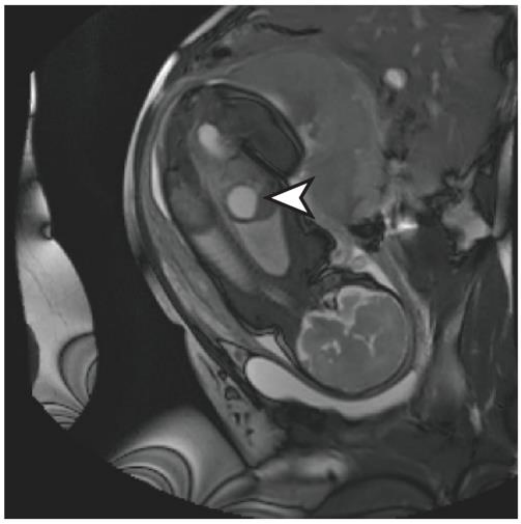


Figure 1.1: Cystic adrenal mass identified on a non-contrast fetal MRI obtained at 36 weeks and 2 days of gestation. The white arrows indicate a 3.1×3×2.8 cm lesion in the left adrenal gland (Louis and Shohet 2015).

In stage 2A NB, the tumor is also localized, but gross excision is not possible with negative ipsilateral nonadherent lymph nodes. Stage 2B could involve either complete or incomplete excision with positive ipsilateral nonadherent lymph nodes, yet without enlarged contralateral lymph nodes. High-risk stage 4 NB tumor is considered to start from stage 3, which is unresectable tumor that infiltrates unilaterally across the midline, with or without regional lymph node attachment. Unless it classified as stage 4S, any tumor that spreads to a distant organ such as skin, liver, bone marrow or lymph node is considered to be stage 4. Stage 4S occurs when there are both localized tumors at stages 1, 2A or 2B, as well as tumors that have spread to other organs such as skin, liver, and/or bone marrow, in infants less than 12 months old (Brodeur, Seeger et al. 1988). Another staging system was developed to taking into account image-defined risk factors in place of degree of surgical resection, named International Neuroblastoma Risk Group (INRG) (Cohn, Pearson et al. 2009). The use of image-defined risk factors system included perinatally diagnosed infants that could not be classified by INSS. L1 refers to a localized tumor with no image-defined risk factors, L2 to a localized tumor with one or more image-defined risk factors, M distant metastatic disease, and finally MS is metastatic disease in children under 18 months with metastases limited to skin, liver, and/or bone marrow (Cohn, Pearson et al. 2009, Sokol and Desai 2019). This classification that is based on histological and biologic characteristics is used, NB patients are also divided into low, intermediate and high-risk categories (Louis and Shohet 2015). The use of this staging system and risk classification have profoundly facilitated the comparison of risk-based clinical trials from regions around the world. (Monclair, Brodeur et al. 2009).

It is highly recommended to obtain biopsied samples before starting chemotherapy or irradiation, and the international neuroblastoma pathology classification (INPC) system is then applied to these samples (Shimada, Umehara et al. 2001). This is mainly for diagnostic purposes but it also can help in comparisons between pre and post-chemotherapy gene mutations in cases of tumor regression. However, diagnosis primarily depends on clinical examination, as there are no risk factors.

Thirty-five percent of NB cases arise in the abdominal region, particularly from adrenergic cells in the adrenal medulla. An equivalent percentage arises from paraspinal ganglia, 20% in the posterior mediastinum, 5% in the pelvis and 5% in the neck (Hallett and Traunecker 2012). Occasionally, tumors can be detected prenatally. Younger children can develop bluish skin metastases, and newborns may present with abdominal enlargement and

respiratory complications due to a mass presence in the chest region, or liver metastases causing massive hepatomegaly.

NB is distinctive in its biological heterogeneity that range from spontaneous regression to highly aggressive metastatic tumors that do not respond to the standard or investigational chemotherapy (Louis and Shohet 2015). Fifty percent of NB patients have haematogenous metastatic disease and these patients are often have unfavorable tumors. The most common symptoms include: fever, anemia, weight loss and sever pain which make the child withdrawn (Hallett and Traunecker 2012). Symptoms of paraneoplastic phenomena (tumor secretions that can damage tissues far from the mass location (Alavi 2013)) include sporadic hypertension due to catecholamine secretion, or diarrhea (Hallett and Traunecker 2012).

1.1.2 Neural Crest

The NC is an embryonic cell population forming in a transient stage of nervous system development, which migrates extensively and differentiates into a wide variety of derivatives. This diversity allows the study of embryonic induction, specification, migratory potential and cell-fate determination using NC culture. The NC cells of the fourth germ layer are multipotent and give rise to many types of cells, due to their stem-cell-like properties (Hall 2000). NC cells can give rise to many cell types such as glia of sensory, autonomic and enteric ganglia, medullary secretory cells, smooth muscle cells, melanocytes, and bone and cartilage cells. Moreover, it has been reported that NC cells can regenerate after ablation, residual neural tube cells can compensate for the cells that are lost (Scherson, Serbedzija et al. 1993, Sechrist, Nieto et al. 1995, Sauka-Spengler and Bronner-Fraser 2008). NC cells were first discovered by Wilhelm His (1868) and ever since they have occupied a prominent place in developmental biology due to their extensive migratory and remarkable developmental properties (Simões-Costa and Bronner 2013). This interest in NC cells has been increased because of their medical importance in underlying disorders and evolutionary importance in giving rise to a wide variety of different cell types. For instance, it has been reported that many birth defects conditions and neoplastic diseases can be linked to errors that occur during development of the NC and its derivatives (Simões-Costa and Bronner 2013) (Iwashita, Kruger et al. 2003). NB is a NC-derived malignancy of the peripheral nervous system (Johnsen, Dyberg et al. 2019).

During vertebrate development, the most extensive morphogenetic movements are observed in the initial phases of NC formation. NC formation spans from gastrulation to late

organogenesis in response to intrinsic and extrinsic factors. This formation takes place at the border of a territory known as the neural plate, the embryonic region directed to form the central nervous system (CNS), in a process known as neurulation. Then, an elevation of the neural plate edges (neural folds) starts to convert the flat neural plate into a cylindrical structure called the neural tube, which will ultimately form the brain and the spinal cord. During the neural tube closure and when its folds converge toward the midline, pre-migratory NC cells resides in the dorsal aspect of the neural tube. Interestingly, shortly after this formation, NC cells start to lose their intercellular connections to undergo a special process called epithelial to mesenchymal transition (EMT), which allows them to earn mesenchymal, migratory characteristics that allow them to leave the neural tube (Gammill and Bronner-Fraser 2003, Sauka-Spengler and Bronner-Fraser 2008, Simões-Costa and Bronner 2013). NC cells fall into four categories that give rise to different other types of tissue around the body depending on their site formation of the body axis. Carinal NC cells give rise to most of the facial skeleton, cranial ganglia, smooth muscle, and pigment cells. Vagal NC cells contribute to the heart valves and septa, and almost all of the gut enteric nervous system, as well as NC cells that form in the sacral regions. Trunk NC cells are the origin of dorsal root and sympathetic ganglia of the peripheral nervous system, some secretory cells, and melanocytes (Figure 1.2). Finally, sacral NC cells which cooperates with the vagal crest to form a small portion of the enteric nervous system (Simões-Costa and Bronner 2013). Many tissues that developed from NC cells have been reported to retain NC stem cells that are multipotent (Crane and Trainor 2006).

1.1.3 NC development signaling and NB development

The precise mechanism by which NC formation occurs is based on a series of regulatory steps arranged hierarchically. Theses regulatory steps define each cell's identity and behaviour at specific time points, and also control smooth transitions to the next regulatory phase (Sauka-Spengler and Bronner-Fraser 2008, Simões-Costa and Bronner 2013). This processes is called Cranial NC gene regulatory network (GRN). The induction events lead to the formation of the neural plate border, which is dependent on an interaction between various signaling molecules, such as Wingless (Wnt), bone morphogenetic protein (BMP), fibroblast growth factor (FGF) and Notch/Delta. These first three are required for NC migration, as they regulate the EMT that facilitate cell mobility. The non-canonical Wnt-planar cell polarity (PCP) signaling cascade is necessary for NC cell mobilization and the

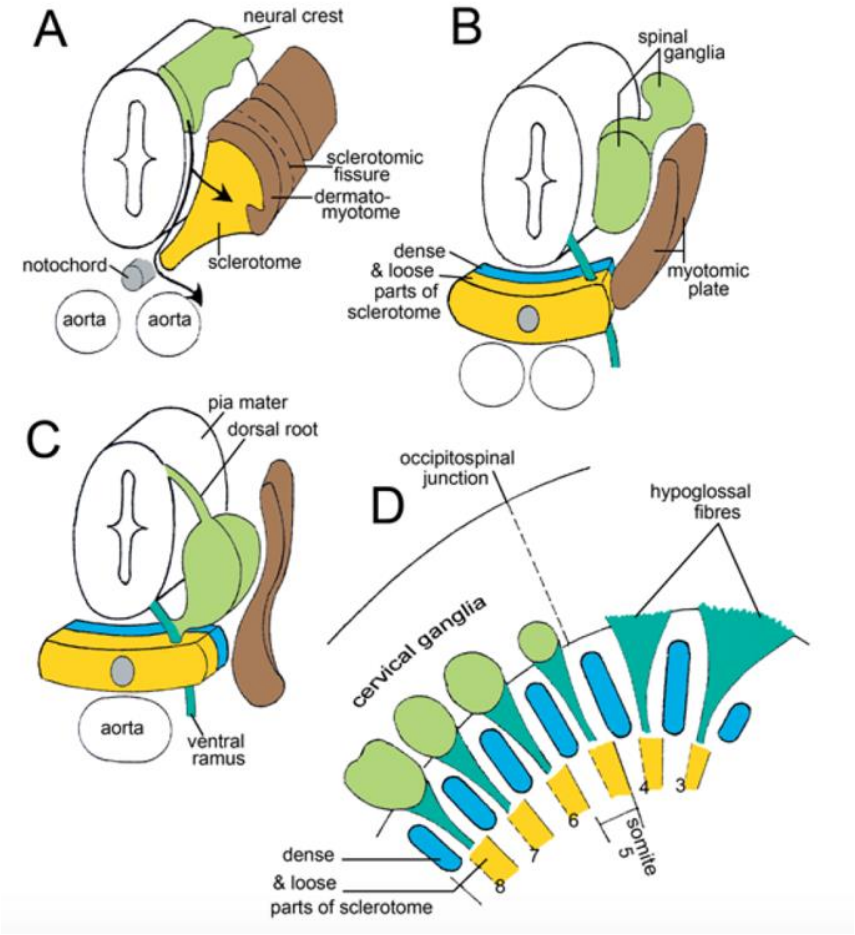


Figure 1.2: Trunk NC cells give rise to all of dorsal root, spinal ganglia (sympathetic ganglia) and cervical ganglia (green part) (taken from (O'Rahilly and Müller 2007).

initiation of their migration through the inhibition of their locomotion (Johnsen, Dyberg et al. 2019). PCP proteins have been found to control Rho GTPases activity locally by activation or inhibition of RhoA and Rac, which facilitate NC cell migration (Sebbagh and Borg 2014). The activation of Rho/Rac signaling leads to the activation of downstream serine/threonine kinases called Rho-Associated Coiled-Coil Containing Protein Kinases, ROCK1 and ROCK2 (Anastas and Moon 2013). Then, ROCK1 and ROCK2 phosphorylate downstream substrates which control several cellular functions predominately through rearrangement of the actin cytoskeleton (Hahmann and Schroeter 2010). Studies of NB genetic profile have shown that genes associated with Rho/Rac signaling are frequently mutated in NB and also that high expression of ROCK2 is strongly linked to poor prognosis (Molenaar, Koster et al. 2012) (Dyberg, Fransson et al. 2017). Deregulation or any disturbance of the highly active Rho signaling during NC initiation and migration stages could severely be an oncogenic hit that cause sever turning toward NB development (Johnsen, Dyberg et al. 2019).

Cell division control protein 42 homolog (Cdc42) is a small GTPase of the Rho family, it regulates cell morphology, cell migration, endocytosis and cell cycle progression (Qadir, Parveen et al. 2015). RhoA, Rac1 and Cdc42 involvement in Eph/ephrin internalization and signaling has been reported (Baker and Antin 2003). Eph/ephrin signaling has a substantial roles in NC cell migration, as they are involved in cytoskeleton re-modelling and axon guidance, but they also stimulate angiogenesis and tissue separation (Xu and Wilkinson 2013) (Wislet, Vandervelden et al. 2018).

Basic-loop-helix transcription factor (NeuroD1) is a neuronal differentiation transcriptional factor that is implicated in the neurogenesis and early differentiation of pancreatic endocrine cells (Huang, Kishida et al. 2011). However, high expression of NeuroD1 was found to promote cell motility in NB by suppressing the expression of Slit2 (Lu, Kishida et al. 2015), and subsequently associated with poor prognosis in both NB patients and also in 2 weeks-old TH-MYCN mice which developed NB (Huang, Kishida et al. 2011). Slit proteins (Slit1-Slit3) are extracellular matrix glycoproteins that function in axon guidance. They provide repulsive guidance that regulates migration of NC cells by binding to the repulsive guidance receptor Roundabout (Robo1-Robo4) family (Wong, Ren et al. 2001).

During embryogenesis, dorsal aorta smooth muscles express members of the family of BMPs such as BMP2, BMP4 and BMP7, that promote the differentiation of sympathetic neurons in the NC (Varley and Maxwell 1996). This happens through recruitment of a group

of transcriptional factors such as Phox2b, Phox2a, Ascl1, Insm1, Hand2, Gata2/3, Sox4 and Sox11(Howard, Stanke et al. 2000) (Tsarovina, Pattyn et al. 2004). The knockout of Phox2b in mice results in complete absence of the entire peripheral autonomic nervous system (Pattyn, Morin et al. 1999), hence it has been identified as a master gene of autonomic neuron development. The block of NC cells differentiation caused by Phox2b mutations has been found to allow continuous proliferation of neural precursor cells that could finally lead to NB development (Reiff, Tsarovina et al. 2010). Somatic and heterozygous germline mutations of Phox2b are found in some cases of NB, as well as in NC disorders such as Hirschsprung disease and congenital hypoventilation syndrome, suggesting a link between NB and NC development defects (Johnsen, Dyberg et al. 2019). Collectively, all of these data strongly highlight the strong relationship between NC development errors and NB development.

1.1.4 EMT in NC and Cancer

Epithelial cells are adherent cells that form intercellular adhesion complexes (tight junctions, adherent junctions, desmosomes, and gap junctions) in their lateral membranes. They also show apico-basal polarity morphology, with distinctive basally-located basement membrane that separates the epithelium from other tissues. In contrast, mesenchymal are mobile cells, they are nonpolarized and they lack intercellular junctions, so they can move individually through an extracellular matrix (Thiery and Sleeman 2006). The transformation from the first type to the second is known as EMT, which is in fact reversible, since mesenchymal cells can revert to epithelial cells via mesenchymal-to-epithelial transition (MET). EMT has three types: the one mediating normal development, wound healing, and the one that mediate carcinogenesis, those types and their biomarkers are reviewed by (Scanlon, Van Tubergen et al. 2013). It is important to mention that EMT is a critical process for normal germ layer formation and cell migration in the early development such as NC cell migration (Acloque, Adams et al. 2009).

Although EMT is a technique that normal cells use to promote different morphology, it is also involved in tumor progression with metastatic expansion, and the initiation of cancer cells that have pluripotent properties, which in turn has an essential impact in chemotherapy resistance (Roche 2018). NC cells' EMT process is morphologically quite similar to that which occurs in cancer progression. Interestingly, for cancer cells the EMT is an incomplete process, since the cells are present in different transitional stages that makes them move in

a cluster-like group, which expresses a mixture of both epithelial and mesenchymal genes. These characteristics result in hybrid cells that are more aggressive than those with complete EMT (Jolly, Boareto et al. 2015). Cancer cells also take advantage of the reverse process, so they can revert to become epithelial cells when they have reached a desirable metastatic location to develop secondary tumors (Roche 2018).

In cancer, metastasis is a complicated and controlled process that consists of several and overlapped stages, starting from exit from the primary tumor, then the access to the circulation, then being transported to a distant location, exiting the circulation, to ultimately regrow in a different microenvironment. This journey starts with the EMT process when cells lose connection to the primary location. Next, those invasive cells penetrate the endothelium of blood or lymphatic vessels and enter the corresponding circulations. Circulating cancer cells need to overcome the circulating first line of defense to reach their remote distention in a live state. In case any cells have been arrested by the circulating leukocytes and platelets, cancer cells could extravasate blood and lymphatic vessels to form colonies and subsequently a metastatic site. Moreover, when appropriate environmental circumstances are present in that secondary metastatic site, cancer cells will undergo more genetic and epigenetic alterations that allow them to undergo MET, proliferation, and the angiogenic generation necessary for the formation of even larger metastatic tumors (Ara and DeClerck 2006).

Among the genes that trigger this shift of EMT in tumor microenvironment are the transcriptional controllers SNAI1 and SNAI2, ZEB1 and ZEB2, Twist and E12/E47, non-coding RNAs (miRNAs and long non-coding RNAs) (De Craene and Berx 2013). In *in vitro* studies, *Snail, Twist, and Sip1/Zeb2/ZFHX1B,* have been identified as essential mediators of EMT in metastasis (Kerosuo and Bronner-Fraser 2012) (Rogers, Saxena et al. 2013). Furthermore, the expression of pro-survival and pluripotent factors in NC migrating cells render them highly proliferative and as contributors to apoptosis resistance (Rogers, Saxena et al. 2013).

1.1.5 EMT and NB metastasis

Metastasis is one of the major causes of death in NB patients, and it has a wide variety of presentations and results in hugely variable prognosis (Hallett and Traunecker 2012). Transforming growth factor (TGF)-ß was found to be implicated in the induction of EMT reviewed here (Wendt, Allington et al. 2009). NB cells can colonize and invade remote

organs such as bone morrow using a mechanism of interaction between tumor cells and the microenvironment, this invasion mechanism is reviewed by (Ara and DeClerck 2006). Another study that investigated the invasion mechanism in NB have found that TGF-ß levels are elevated and this is involved in NB migration (Zhen, Guo et al. 2016) (Lynch, Fay et al. 2012). TGF- ß was found to induce EMT in NB cells by a reduction of the cytoplasmic epithelial marker E-Cadherin in an manner independent from the Smad signaling pathway (Shao, Gao et al. 2017).

NC development is a carefully orchestrated progress that consists of a wide variety of stages, signaling pathways and cell fates. These massive variations can affect NB tumors' pathological heterogeneity. That is, during the initiation stages, NB tumor cells may gain a recognized tumor phenotype, which matches the formation level of their NC precursors (Howk, Voller et al. 2013) (Louis and Shohet 2015). For example, a population of highly tumorigenic stem cells that are found in NB expresses a high level of pre-migratory early NC markers, such as SOX10 (Hsu, Agarwal et al. 2013). Metastases generally arise from tumors that contain more mesenchymal progenitors and lower levels of growth factors. The programmed inhibition but not the silencing of P53 was reported to coordinate cell growth and EMT of normal NC cells (Rinon, Molchadsky et al. 2011). P53 is wild type in NB at the diagnosis stages (Chen, Malcolm et al. 2007). MDM2, a primary inhibitor of the P53 tumor suppressor, is positively regulated by *MYCN* (Chen, Lin et al. 2009). Aberrant regulation of MDM2 by the *MYCN* transgene is an important component of overall suppression of P53 in NB (Chen, Lin et al. 2009). Thus, *MYCN* and MDM2 work together to inhibit apoptosis. This was found to be consistent with the observation that NB express wild-type P53 in almost all cases at the first diagnosis, despite the fact that NB with P53 wild-type are still resistant to apoptosis (Kim and Shohet 2009). On the other hand, NB tumors that arise from further differentiated NC cells in later stages show less aggressive behaviour and more favourable prognosis (Figure 1.3) (Louis and Shohet 2015).

1.1.6 NB Current treatment

Mainly, high-risk NB therapies fall into three phases- induction, consolidation, and post-consolidation therapy (maintenance phase) (Armideo, Callahan et al. 2017). After disease diagnosis, based on surgical tissue sample biopsy for biomarker and cytogenetic analysis, high-risk patients are typically treated with multimodal therapy (Mazur 2010). This includes intensive induction chemotherapy, followed by harvesting of patient's own stem cells for

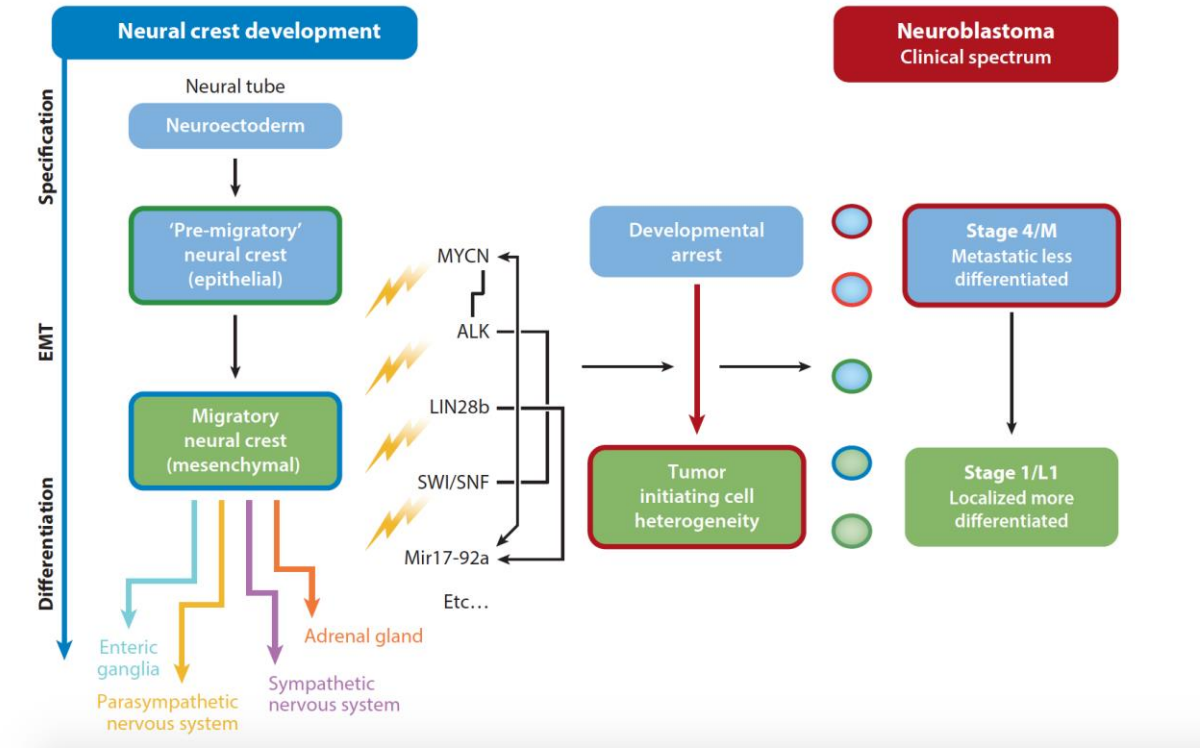


Figure 1.3 : Disruption of normal developmental process in NC cells with distinct oncogenic drivers (e.g., *MYCN* or ALK oncogenes), at different times, can lead to multiple subtypes of NB tumors (Louis and Shohet 2015).

(autologous) transplantation to bone marrow, since the chemotherapy can decrease bone marrow activity, i.e. fewer red blood cells, white blood cells, and platelets (myeloablation) (Kushner, O'Reilly et al. 1991). Then patients are treated with myeloablative consolidation chemotherapy and subsequent surgical resection and radiation at the primary site to optimize local control (Yalcin, Kremer et al. 2010). Myeloablative chemotherapy with autologous stem-cell transplantation has been reported to enhance the outcome for high-risk NB patients despite the raised risk of treatment-associated death (Berthold, Boos et al. 2005). Sometimes, chemotherapy and/or irradiation are used on the primary site and metastases sites before the tumors are ultimately removed by surgery (neoadjuvant chemotherapy). This is done to help decrease the local tumor sizes and as a precaution to prevent further chemo-resistance (Bowen and Chung 2009). NB chemotherapy regimens significantly vary, depending on the risk group (Johnsen, Dyberg et al. 2018). Doxorubicin and cisplatin are two of the most commonly-used drugs in chemotherapeutic regimens in high-risk NB treatment (Ganeshan and Schor 2011, Hara 2012, Smith and Foster 2018). High-risk NB neoadjuvant chemotherapy (treatment given as a first step to shrink a tumor before the main treatment, which is usually surgery) regimens include anthracycline antibiotics such as doxorubicin, epipodophyllotoxins (etoposide), camptothecins (topotecan/irinotecan), alkylating agents (cyclophosphamide / ifosfamide), vinca alkyloids (vincristine), and heavy metals (cisplatin /carboplatin) (Kurt, Armstrong et al. 2008, Mazur 2010, Ganeshan and Schor 2011). In the maintenance phase or post-consolidation, therapy includes immunotherapy and a differentiating agent. Differentiation agent inducers, such as retinoic acid (RA) (Kohler, Imeson et al. 2000), are used to clear any minimal residual tumor that may survive the induction therapy, which could cause relapses, the main challenges following chemotherapy and surgery application. Unfortunately, the side effects of this treatment are quite challenging (for review see, Armideo, Callahan et al. 2017), and these will be discussed in the following section (1.1.8).

1.1.7 NB Immunotherapy

In 2015, the US Food and Drug Administration (FDA) approved dinutuximab (Unituxin; formerly known as ch14.18), aldesleukin (Proleukin; also known as IL-2), granulocyte macrophage colony-stimulating factor (GM-CSF), and isotretinoin, as a post-consolidation immunotherapy for high-risk NB (Armideo, Callahan et al. 2017). The aim of giving those drugs along with dinutuximab is to stimulate the immune system response.

Disialoganglioside (GD2) is an antigen that is ubiquitously expressed on the surface of NB cells, making it an ideal target for immunotherapy. Dinutuximab is a monoclonal antibody that targets GD2, and allows the immune system cells to lyse NB targeted cells; this drug effect is enhanced by the agents GM-CSF and IL-2. IL-2 works to encourage natural killer cells and GM-CSF triggers granulocyte and macrophage cytotoxicity (Schulz, Cheresh et al. 1984, Yu, Gilman et al. 2010, Armideo, Callahan et al. 2017). Isotretinoin is a differentiation agent, that induces cell maturation in NB cells (Armideo, Callahan et al. 2017).

1.1.8 NB Chemotherapy side effects

The high level of oxidative stress in cancer cells has been recognized as a tumor-specific target for the design of chemotherapeutic agents. Therefore, both the effectiveness and limitation of many chemotherapy agents or ionizing radiation therapy, are based on the generation of reactive oxygen species (ROS) or changes in their quality, and consequent increases in oxidative stress, which exceed the reduction capacity of cancerous tissue, leading ultimately to apoptosis and necrosis (Castaldo, Freitas et al. 2016). Unfortunately, this mechanism can lead to the damage of healthy tissues also, resulting in numerous deleterious effects. For example, cardiotoxicity mediated by anthracycline and nephrotoxicity triggered by platinum compounds are long-term sequelae of these commonly-used chemotherapy agents (Angsutararux, Luanpitpong et al. 2015, Karasawa and Steyger 2015).

A study conducted on records of 61 high-risk NB patients reported modestly enhanced survival after three consecutive cycles of high-dose therapy and autologous stem cell transplants, between 1991 and 2008 (Armstrong, Danner-Koptik et al. 2018). The study results indicated that the vast majority of late side-effects included endocrine-related issues such as growth failure, hypothyroidism (low thyroid activity), and hypogonadism (reduced functional activity of the gonads). Secondary malignant neoplasms (secondary malignant tumor results specifically from chemo- or radiation therapy) and skeletal deformities were also reported in those patients (Armstrong, Danner-Koptik et al. 2018). High-risk NB survivors treated with multimodal chemotherapy are 2.2-fold more likely to develop chronic health problems, in comparison to those who are treated with surgery alone (Friedman and Henderson 2018).

The main dose-limiting side-effect of treatment with anthracyclines such as doxorubicin is cardiotoxicity, leading to heart failure in the most severe cases (Cavaletti 2007). One in

every ten children treated with a cumulative anthracycline dose of 300 mg/m^2 body surface area or more eventually develops anthracycline-induced clinical heart failure (van Dalen, van der Pal et al. 2006). Doxorubicin has also been reported to lead to cardiomyopathy, resulting in a form of congestive heart failure that is usually refractory to common treatment (Takemura and Fujiwara 2007). Moreover, cisplatin has dose-dependent side-effects including nephrotoxicity, ototoxicity (hear loss) and neurotoxicity (Oh, Kim et al. 2014). Hearing loss is a common problem due to the frequent use of platinum-based chemotherapy for high-risk NB children (Simon, Hero et al. 2002, Kushner, Budnick et al. 2006). Defects in pulmonary function has also been reported among NB survivals (Huang, Hudson et al. 2011).

Dosing regimen and method of administration of the retinoid drug, 13-cis-retinoic acid (13-cisRA; isotretinoin), the maintenance treatment that combined with antibody therapy, have a marked influence on its plasma concentrations (Veal, Errington et al. 2013). 13-cisRA standard doses have been found to not reflect its plasma concentrations in some patients (Veal, Cole et al. 2007). That is, the major metabolite 4-oxo-13-cisRA has been shown to accumulate above the original concentrations used of treatment, in approximately 70% of patients studied (Veal, Errington et al. 2013). Furthermore, it has been shown to be less active than the parent RA in various tumor cell lines which could lead to a diminished efficacy of 13-cisRA (van der Leede, van den Brink et al. 1997) (Kim, Yoo et al. 2002). Unfortunately, even after decades of studies, the efficacy of All-*trans*-retinoic acid (ATRA) to treat other acute myeloid leukaemia (AML) subtypes and solid tumors remains poor (Schenk, Stengel et al. 2014).

Immunotherapy with dinutuximab has severe side effects as well, including pain, fever, infection, hypokalemia, hypersensitivity reaction, capillary leak syndrome, hyponatremia, transaminitis (elevation in liver enzymes, alanine transaminase (ALT) and aspartate transaminase (AST)), gastrointestinal side-effects (nausea, vomiting, diarrhoea), hypotension, hypoxia, and urticaria (skin problem). (Yu, Gilman et al. 2010, Armideo, Callahan et al. 2017).

Understanding NB molecular heterogeneity has hugely contributed to the development of therapeutic agents and this has subsequently contributed to increasing 5-year survival rates for children with NB (Friedman and Henderson 2018). However, the severe side-effects of some current NB therapies demand that there are further investigations into novel therapies

with potential selective abilities, and less toxicity, to target malignant cells only and to enhance survival outcomes.

1.1.9 NB Drug resistance

Generally, cancer therapy regimes often result in decrease of tumor mass size but rarely succeeds in eliminating all cancer cells. The main known strategies cancer cells use to overcome treatments include; persistence of cancer stem cells (CSCs) , EMT, and down-regulation of apoptosis (Aleksakhina, Kashyap et al. 2019). However, tumor resistance could also involve pathway-specific mechanisms, mutations, changes in gene expression, or post-translational protein modifications (Aleksakhina, Kashyap et al. 2019). Therefore, drug resistance may not be a result of the metastasis, as many studies reported that primary tumor may contain a small cell population of CSCs that have the ability to survive therapeutic toxicity and facilitating the disease progression (Aleksakhina, Kashyap et al. 2019).

In the case of NB, emerging evidence suggesting that drug resistance might be due to this subpopulation of CSCs or tumor-initiating cells (TICs) found inside the bulk of NB tumor (Bahmad, Chamaa et al. 2019). The molecular profile of CSCs of NB tumors has been reported in details (Bahmad, Chamaa et al. 2019). Wnt/Beta-catenin pathway increased expression was found to be involved in promoting chemo-resistance, while decreasing those pathways activity decreased the viability of NB cells, indicating the protective effect of Wnt signaling in the CSC population of NB (Vangipuram, Buck et al. 2012). Apoptotic resistance mechanism has been reported to occur in *MYCN*-amplified tumors, and it confers cell resistance to apoptosis induced by the tumor necrosis factor-related apoptosis-inducing ligand system (Adhikary and Eilers 2005).

1.1.10 NB Relapses

Despite the massive efforts of researchers into NB diagnosis, and the advanced therapy methods that are used, the vast majority of children die from recurrent and/or metastatic disease, reviewed here (Ramaswamy and Taylor 2015). Less than half of patients with high-risk disease remain relapse-free at 3 years after diagnosis (Kreissman, Seeger et al. 2013). The main obstacles to prevent NB recurrent and metastatic treatment are the heterogenous genetic mutations, which are unidentified at the stage of diagnosis and/or which can appear later as a result of chemotherapy. At the diagnosis stage of NB the primary tumor may possess a large population of malignant cells that is composed of multiple clones, while

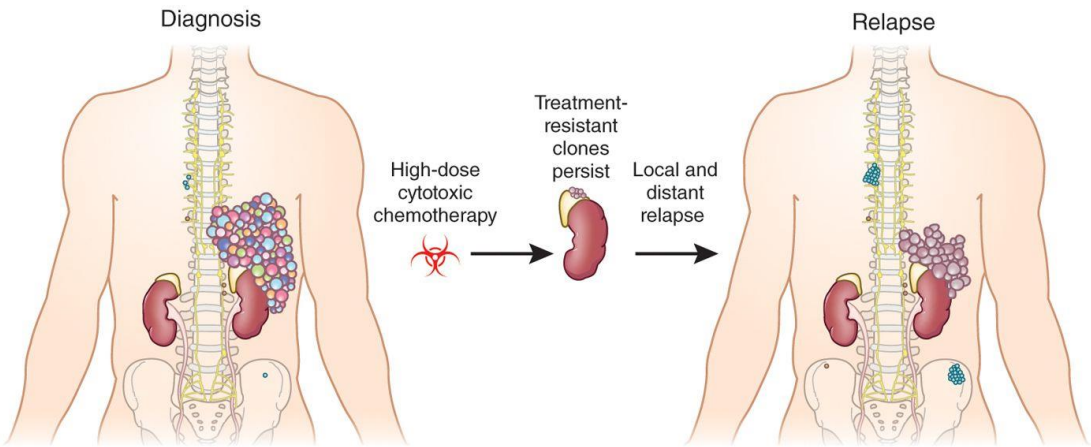


Figure 1.4 : Treatment-resistant populations of NB cell in the primary tumor, that give rise to NB recurrent (Ramaswamy and Taylor 2015).

microscopic metastatic post-chemotherapy consist of small groups of treatment-resistant clones that has expanded at the time of recurrence (Figure 1.4) (Ramaswamy and Taylor 2015). It was thought that analysis of the NB genome at the diagnosis stage adequately predicts the genome at recurrence, but this is not the case (Ramaswamy and Taylor 2015). However, two independent, non-overlapping sequencing studies of 23 and 16 recurrent NB cases with primary-relapse germline have clearly demonstrated the huge difference between NB genome at diagnosis and after relapse (Eleveld, Oldridge et al. 2015) (Schramm, Koster et al. 2015). Moreover, it is not clear yet whether those mutations found in relapsed cancers were absent from the diagnostic tissue or not (Gerlinger, Horswell et al. 2014). This could be due to the limitation of the surgical biopsy and heterogeneity between the primary tumor and other sites (relapsed or metastatic). In addition, it is not known whether those mutations are a result of the chemotherapy or arise *de novo* during tumor progression, reviewed by (Gerlinger, Horswell et al. 2014, Bosse and Maris 2016). For example, P53 has an antiproliferative effects including cell cycle arrest and apoptosis. When P53 loses its oncosuppressor function during chemotherapy in NB cell lines, this loss of function was found to be strongly linked to chemotherapy resistance (Keshelava, Zuo et al. 2001). Post-chemotherapy mutant P53 has an additional oncogenic function which have been reviewed here (Muller and Vousden 2013). This gain of function includes promoting invasion, migration, angiogenesis and proliferation in vitro (Lang, Iwakuma et al. 2004, Olive, Tuveson et al. 2004). Mutant P53 is also involved in increasing expression of genes involved in chemoresistance in cancer in general (Muller and Vousden 2013) and specifically NB (Petroni, Veschi et al. 2012). However, samples from recurrent NB that had been treated with surgery alone were similar to those taken at the stage of diagnosis than post chemotherapy samples, which support the idea that chemotherapy can results in gene mutations (Eleveld, Oldridge et al. 2015, Schramm, Koster et al. 2015).

1.1.11 Treatment of relapsed NB

Understanding relapsed tumor molecular profile is a substantial aid in discovering new tumor targets that help developing novel drugs to prevent genetic changes that lead to cancer recurrence. The chemotherapy of relapsed NB patients include agents distinctive from those previously used such as topotecan and irinotecan which demonstrate successful activities in both preclinical and clinical NB studies (Blaney, Needle et al. 1998, Carol, Houghton et al. 2010). In clinical trial studies of 13 relapsed NB children, a combination of topotecan and

cyclophosphamide demonstrated objective responses in ~ 50% of patients (Saylors, Stine et al. 2001) . Another study demonstrated 64% overall response rate in 25 patients treated with topotecan combined with doxorubicin and vincristine (TVD), 4 of them having a complete response (Garaventa, Luksch et al. 2003). Subsequently, TVD regimen was combined to high-risk NB chemotherapy protocol as salvage therapy for relapsed cases that have ineffective induction therapy (Zage 2018). However, long-term benefits of TVD regiment need to be determined in further clinical trials (Amoroso, Erminio et al. 2018). Another studies suggested that relapsed NB children may benefit from more aggressive chemotherapy that consists of myeloablative (High-dose) chemotherapy followed by autologous stem cell rescue, similar to induction therapy yet with distinctive agents (Zage 2018).

1.1.12 NB Genetic profile

NB is characterized by a remarkable heterogeneity of histology and molecular biology that is reflected in its clinical outcome ranging from spontaneous regression to lethal metastatic disease. Genetic changes in NB are used mainly for prognosis rather than diagnosis (Thorner 2014). DNA content or ploidy is one of the oldest genetic prognostic markers for NB (Look, Hayes et al. 1984). The prognostic value of DNA content is more significant in patients <12 months of age, and after 2 years becomes not significant. Ploidy is one of the genetic variables currently used in risk identifications (Thorner 2014).

NB pathogenesis has always linked to identification of a few genes defects which are limited to *MYCN* amplification (20%) and ALK activations (7%)(Mossé, Laudenslager et al. 2008). However, Molenaar, Jan J et. al. have conducted a whole genome sequencing analysis of 87 NB cases of all stages and they found local shredding of chromosomes, known as chromothripsis, in 18% of high-stage NB (Molenaar, Koster et al. 2012). In addition to that they have found neuritogenesis gene alterations, which frequently occur in high-risk tumors.

MYCN

MYCN is normally expressed in developing neural tissues, and acts as a transcription factor to regulate neural progenitor cell proliferation and differentiation. It is located on chromosome 2p24, and increased copies of this gene (amplification) are seen in around 25% of NB tumors (Huang and Weiss 2013). Patients with amplification are considered to be high-risk in the most recent system regardless of other prognostic factors (Cohn, Pearson et

al. 2009). Amplification is defined as >10 gene copies per diploid cell. Values of 3–10 are referred to as *MYCN*-gained, and often reflect increased copies of chromosome 2. Level of *MYCN* amplification does not change post treatment or when the tumor spreads to other sites (Thorner 2014). Previously, it has been reported that overexpression of MYCN alone is sufficient to cause NB in mice (Weiss, Aldape et al. 1997). This model was specifically developed by targeting expression of MYCN to the NC lineage using the rat tyrosine hydroxylase (TH) promoter. This promoter was reported to be active in the migrating cells of NC in early development (Banerjee, Hoppe et al. 1992). Later on, this model was profoundly helpful in understanding the cellular and molecular changes that may occur in the genesis of *MYCN*-amplified NB (Hansford, Thomas et al. 2004) (Alam, Cui et al. 2009). It also found that those tumors show typical histopathological features similar to those from human NB (Weiss, Godfrey et al. 2000, Moore, Wood et al. 2008).

The multiple roles *MYCN* plays in malignant tumors and maintaining stem like state include; the activation of metastatic genes transcription (Harenza, Sussman et al. 2016), survival, proliferation, pluripotency (Solari, Borriello et al. 2014, Kramer, Ribeiro et al. 2016), self-renewal (Kaneko, Suenaga et al. 2015), and angiogenesis (Ribatti, Raffaghello et al. 2002).

As mentioned before P53 wild-type, but not mutant, suppression is important in tumor progression. Interestingly, *MYCN* was found to interact directly with tetrameric form of P53 and transcriptionally co-regulate it in high-risk *MYCN* amplified NB. This interaction was found to led to regulation of alternative P53 targets were not regulated in low *MYCN* levels (Agarwal, Milazzo et al. 2018). Bleeker, et. al. have identified two patterns of metastasis in 249 newly diagnosed stage 4 NB patients that associated with *MYCN* copy number. In details, patients with *MYCN* amplification tumors show limited-focal lesions, which means here that the imaged lesions appear as hot-spots with clear margins, distinguishable from the background. In contrast, tumors with single copy *MYCN* show extensive and diffuse lesions without clear margins and uptake spread throughout the body segments, which indicating *MYCN* role in NB metastasis (Bleeker, van Eck-Smit et al. 2015). Another study conducted to investigate *MYCN* copy number in the primary tumor, pre- and post-chemotherapy, and in metastatic specimens in 102 samples taken from 30 patients with *MYCN*-amplified NB, showed a biologic significance of *MYCN* heterogeneity (Marrano, Irwin et al. 2017).

Down-regulation of *MYCN* was reported in NB cells differentiated via retinoic acid, even before the observation of morphological changes (Thiele, Reynolds et al. 1985). *MYCN* amplification was also reported to repressed cellular immunity in NB patients (Zhang, Wu

et al. 2017). *MYCN* maintains an aggressive and undifferentiated NB phenotype by deregulation of estrogen and NGF signaling (Dzieran, Rodriguez Garcia et al. 2018). The knockdown of *MYCN* mRNA by *MYCN* siRNA led to apoptosis and differentiation in *MYCN*-amplified NB cells (Maeshima, Tagalakis et al. 2016).

MYCN amplification and Glutathione

Aerobic glycolysis " the Warburg effect" is well-known as a hull mark of cancer (Vander Heiden, Cantley et al. 2009). *MYCN* amplification has been shown to be associated with enriched glutaminolysis (Wang, Liu et al. 2018). Aerobic glycolysis and glutaminolysis are tightly controlled in cancer cells to meet the demand of the highly proliferative function in cancer cells. Glutamine is a critical amino acid for many fundamental functions of cancer cells, including synthesis of metabolites that maintain mitochondrial metabolism and generation of antioxidants, such as glutathione (GSH), to control ROS levels (Wang, Liu et al. 2018). Reduced GSH is ubiquitous low-molecular thiol antioxidant synthesized inside cells and has substantial role in cellular detoxification from ROS (Forman, Zhang et al. 2009). In cancer cells, the constant proliferation is a highly metabolic process that leads to an enormous accumulation of ROS. Glutaminolysis itself has been reported to result in excessive amounts of ROS in *MYCN*-amplified NB cells, (Wang, Liu et al. 2018). Therefore, as an adaptive response, cancer cells harbour elevated levels of ROS-scavenging molecules such as GSH (Bansal and Simon 2018). MYC-driven tumors support the citric acid cycle, which provides the carbon backbone required for macromolecule biosynthesis, through enhancing glutamine transport and glutamate metabolism (Le, Lane et al. 2012, Carter, Sutton et al. 2016).

MYC contributes to production of both reduced forms of GSH and nicotinamide adenine dinucleotide phosphate (NADPH), which is a mechanism to overcome potentially damaging metabolic byproducts, such as ROS in cancer cells (Gao, Tchernyshyov et al. 2009, Cairns, Harris et al. 2011). Moreover, *MYCN*-amplified NB have been shown to have a two-fold enrichment of GSH levels in comparison to NB cells without *MYCN* amplification (SH-SY5Y) (De Tudela, Delgado-Esteban et al. 2010).

Targeting *MYCN* inhibition is one of the therapeutic approach in *MYCN* amplified NB reviewed here (Greengard 2018). *MYC*, *MYCN*, and *MYCL* are the *MYC* gene family, and their proteins serves as a transcriptional factor that form a heterodimeric complex with MAX, their functional partner. This complex in tern recruits other transcriptional co-factors

and activates or suppress gene expression (Eilers and Eisenman 2008). Inhibiting this link between *MYCN* and MAX has been investigated for suppressing *MYCN* amplification tumor progression. Two unrelated compounds has been identified to block *MYCN*-MAX interaction, 10074-G5 and 10058-F4 (Müller, Larsson et al. 2014).

Inhibition of glutamate cysteine ligase (GCL), the rate-limiting step in GSH biosynthesis was reported to contribute to make *MYCN*-amplified NB cells more vulnerable to apoptosis (Marengo, De Ciucis et al. 2008). In cancer cells GSH has been reported to regulate drug resistance mechanism to cisplatin chemotherapy (Chen and Kuo 2010) and doxorubicin (Gottesman, Fojo et al. 2002). GSH depletion has been reported to increase glioma cancer cell sensitivity to both cisplatin and temozolomide (Rocha, Garcia et al. 2014), and leukaemia cell sensitivity to doxorubicin and etoposide (Mahbub, Le Maitre et al. 2015). Collectively, this highlights the role of *MYCN* amplification and GSH production in cancer cell survival and their suppression or depletion respectively in cancer cell death.

1.2 BMP signaling

1.2.1 BMP signaling in NC development

One key group of signals for sympathetic development is the BMPs, which is the largest subgroup of the TGF-β superfamily of ligands (Hegarty, O'Keeffe et al. 2013). BMPs are produced by cells in the wall of the dorsal aorta and they act by inducing expression of transcription factors in NC cells, which promote the differentiation of sympathetic progenitors, towards a catecholaminergic neuronal fate (Kléber, Lee et al. 2005). These transcription factors include paired homeodomain factors Phox2b and Phox2a, the basic helix-loop-helix factors (bHLH) Mash1 and Hand2, and the zinc-finger factors Gata 2/3 and Insm1, which have been shown to form a regulatory network rather than a linear cascade (Kameda 2014). Other important nuclear factors include HuD, *MYCN*, Id2, and p73, which may also be involved in cell fate determination, directing progenitor cell differentiation to sympathetic neurons (Nakagawara 2005). Among BMPs ligands that induce NC cells differentiation are BMP2, BMP4 and BMP7 (Reissmann, Ernsberger et al. 1996).

Wnts are also expressed in the dorsal neural tube at the time of NC cell emigration (Lee, Kléber et al. 2004), while BMPs are secreted by the ectoderm and the dorsal neural tube (Liem, Tremml et al. 1995).

NC induction includes two phases: the first one when FGF and Wnt signaling induce NC progenitors at the border of the neural plate, and the second phase is when BMP, Wnt, and Notch signaling maintain these progenitors and induce the expression of definitive NC markers including Snail2, FoxD3, and Sox9/10 reviewed here (Stuhlmiller and Garcia-Castro 2012) and here (Rogers, Jayasena et al. 2012). There are a progressive pattern of BMP expression from anterior to posterior ectoderm during gastrulation (Tucker, Mintzer et al. 2008). At early stages of development, BMP and Wnt signaling act synergistically to suppress NC cell differentiation and maintain marker expression and multipotency (Kléber, Lee et al. 2005). Gradients of these signaling molecules explain why some NC cells adopt a sensory neuronal fate as they migrate, and the rest of them keep their multipotent phenotype and can still differentiate into other NC derivatives (Kléber, Lee et al. 2005). Another study suggested that neural and epidermal cell fates require continued inhibition or activation of BMP signaling, respectively, while the NC is generated by an early phase of inhibition and a late phase of activation in chick embryos (Patthey, Edlund et al. 2009). Moreover, BMP signaling has been shown to accelerate NC cell migration in the dorsal neural tube that is induced by BMP4 treatment and regulated by its inhibitor noggin in vivo (Sela-Donenfeld and Kalcheim 1999). BMP4 has been shown to interact with noggin along the rostral-to-caudal axis of the neural tube, with BMP4 mRNA remaining constant, while noggin transcription gradually decreases, correlating with somite development. The gradient in noggin expression progressively relieves the inhibition of BMP4, which triggers EMT in NC cells (Burstyn-Cohen, Stanleigh et al. 2004, Kalcheim 2015). BMP signaling has also been shown to promote cell differentiation in adrenergic trunk NC cells, that is controlled by BMP2 and BMP4, but not by BMP6 ligands (Varley and Maxwell 1996). Moreover, other studies have shown that BMP signaling initiates NC cell differentiation in rats, that is controlled by BMP6, BMP7, growth differentiation factor (GDF)5, and GDF6 (Gajavelli, Wood et al. 2004). Collectively these studies show that BMPs play important roles in developing sympathetic neurons and for this reason there has been an intensive research interest in characterising the receptors and downstream mediators of their effects.

BMP ligands signal by binding a heteromeric complex bone morphogenetic protein receptor (BMPR2) complexed with one of two type 1 BMPRs, called BMPR1A (Activin Receptor-like Kinases (ALK)3) and BMPR1B (ALK 6), which are transmembrane serine/threonine kinase receptors (Weiss and Attisano 2013). In the canonical BMP pathway BMP receptors activate transcription factors known as the R-Smads (Smad1/5/8) which complex with

Smad4 to translocate to the nucleus and regulate gene expression (Hegarty, O'Keeffe et al. 2013). Receptor-regulated Smads (R-Smads) act as substrates for BMPs, while Smad4 serves as a common partner for all R-Smads (Schmierer and Hill 2007). Type 2 BMP receptors are the primary ligand-binding component, while type 1 are responsible of transducing the signal into the cell (Kawabata, Imamura et al. 1998). Following a ligand binding, the BMPR2 transphosphorylates the intracellular domain of the BMPR1 , which in turn recruits and phosphorylates Smad proteins (Miyazono, Kamiya et al. 2009). BMPR1s interact with co-Smad4 and phosphorylate R-Smads, resulting in the formation of a heterotrimeric complex, which can then regulate gene expression in the nucleus. (Zhang, Musci et al. 1997). BMP signaling was found to regulate the development of sympathetic neurons through both Smad4-dependent and -independent pathways (Morikawa, Zehir et al. 2009). BMP signaling regulates the survival of sympathetic progenitors by Phox2b activation, which is a Smad4-independent pathway. However, they also control noradrenergic differentiation and regulate NC cells proliferation by maintaining Mash1 expression, which is a Smad4-dependent pathway (Morikawa, Zehir et al. 2009). Knockout of BMPR1A in mice did not affect NC cell migration but these cells died once they reached the dorsal aorta. However, deletion of Smad4 does not affect NC cell neurogenesis or ganglia formation, but it leads to a reduction in proliferation of immature sympathetic neurons (Morikawa, Zehir et al. 2009). In addition, deletion of Smad4 caused a reduction in noradrenergic cells differentiation and downregulation of Mash1 expression (Morikawa, Zehir et al. 2009). Moreover, deletion of both of BMPR1A and Smad4 in adult mice immature noradrenergic sympathetic neurons led to a reduction in dendrite length, complexity and neuron cell body size (Majdazari, Stubbusch et al. 2013). No further reduction in dendritic features was shown after combined deletion of BMPR1A and BMPR1B, and Smad1/5/8 phosphorylation was normal in these animals. Smad4 knockout in the same study resulted in no effect on cell dendrite or cell body size, while dendrite complexity and number were increased, suggesting a main role of BMPR1 in dendrite complexity in sympathetic neurons (Majdazari, Stubbusch et al. 2013).

BMPR1A has been found to be always expressed in neuronal precursors, while BMPR1B expression is found in the dorsal neuronal tube, the source of BMP ligands, starting from embryonic day 9 (Panchision, Pickel et al. 2001). BMPR1A activation induces BMPR1B expression and promotes precursor cells proliferation, while hedgehog signaling prevents the latter expression (Panchision, Pickel et al. 2001). BMPR1B activation leads to mitotic

arrest and therefore limits precursor cell numbers. BMPR1B was also found to induce apoptosis in early gestation (E11.5) while it promotes differentiation in later gestation (E13.5) in mice (Panchision, Pickel et al. 2001).

When both BMPR1s and BMPR2 are preassembled in the cell membrane, BMP ligands can bind with high affinity to both of them. However, ligands can also act by binding to one subunit and then recruiting the other subunit to the complex (Bond, Bhalala et al. 2012). Different BMP ligands have different affinities for BMPR complexes. For example; BMP2, BMP7 and GDF5 (BMP14) can all bind BMPR2 (Weber, Kotzsch et al. 2007, Yin, Yeh et al. 2008). However while BMP2 and BMP7 can bind to BMPR2 with BMPR1A or BMPR1B receptors (Koenig, Cook et al. 1994), GDF5 binds BMPR2 with BMPR1B, but not BMPR1A with high affinity (Nishitoh, Ichijo et al. 1996, Nickel, Kotzsch et al. 2005).

The vast majority of sensory neurons in the peripheral nervous system, including sympathetic neurons, depend for their survival on expression of NGF in their targets (Ye, Kuruvilla et al. 2003), but not for their axon growth or branching. However, GDF5, a widely-expressed member of TGF-β superfamily, is a novel factor that newly discovered to promotes axon growth and branching in sympathetic nerves through Smad 1/5/8 phosphorylation (O'Keeffe, Gutierrez et al. 2016).

BMPRs can also activate a number of non-canonical pathways that include GTPases, MAPK and PI3K pathways, depending on the cellular context (Lee-Hoeflich, Causing et al. 2004, Guan, Li et al. 2014, Zhang 2016). BMP2 and BMP4 play important roles in CNS development in vertebrates, and BMP6, BMP7 and BMP9 are also critical during certain developmental events (Bond, Bhalala et al. 2012).

1.2.2 BMP signaling in NB development

Given that BMP signaling controls neuritogenesis in the developing sympathetic nervous system, alterations in expression of the BMPRs may contribute to the defects in neuritogenesis in NB (Molenaar, Koster et al. 2012). In support of this, a previous study has shown that there is a significant down-regulation of BMPR2 expression in NB, that BMPR2 knockdown increased cell growth and tumorigenicity in mice, and that patients with high BMPR2 expression have better overall survival (Cui, Yang et al. 2017). In addition, increased expression of BMPR2 led to a reduction in NB cultured cells proliferation and colony formation, while knockdown of BMPR2 caused an opposite effect (Cui, Yang et al. 2017). It has also been shown that Smad4 is required for the inhibition of growth, invasion

and metastasis of NB and that patients with high Smad4 expression have greater survival probability (Qu, Zheng et al. 2016). Moreover, a number of BMPs have been shown to promote the neurite growth and differentiation of human NB cells, including BMP4 (Ferlemann, Menon et al. 2017), BMP9 (Lauzon, Drevelle et al. 2017), BMP2 (Du and Yip 2010), BMP6 (Sumantran, Brederlau et al. 2003) and GDF5 (Hegarty, Sullivan et al. 2013). Despite this, although BMP-BMPR2 binding requires a type 1 BMPR for signal transduction, the alterations that occur in BMPR1A and BMPR1B expression in NB, and whether the expression of these receptors is associated with patient outcome and or neuritogenesis, is unknown.

1.2.3 *MYCN* and NB differentiation

As mentioned in section 1.1.12, *MYC*, *MYCN*, and *MYCL* make up the *MYC* gene family, and their proteins serve as transcriptional factors that form a heterodimeric complex with MAX, their functional partner. This complex recruits other transcriptional co-factors and activates or suppress gene expression (Eilers and Eisenman 2008). N-MYC protein is encoded by *MYCN* and it has an essential role in NB differentiation. N-*MYCN* overexpression has been found in around 25% of primary NB tumors (Muñoz, Vendrell et al. 2006). *MYCN* expression is first detected at the seventh day of pregnancy, it increases in the ninth and eleventh day, then starts to decrease after the twelfth day (Hui, Lo et al. 2001). This is important as alteration of *MYCN* expression was found to be associated with birth defects, reviewed here (Ruiz-Pérez, Henley et al. 2017). Reduction in *MYCN* expression is necessary for differentiation process, otherwise, increased level would induce neoplastic phenotype (Guglielmi, Cinnella et al. 2014). In agreement, the elevation of *MYCN* expression after the initiation of differentiation (by retinoic acid) in LAN-5 NB cells leads to a positive modulation of various differentiation markers. However, when *MYCN* was silenced the differentiation was inhibited and the markers were negatively modulated (Guglielmi, Cinnella et al. 2014). This indicates the importance of *MYCN* in NB differentiation.

MYCN amplification, on the other hand, is the hallmark of high-risk NB and it is well recognized to be related to poor prognosis (Huang and Weiss 2013). High-risk NB without *MYCN* amplification frequently expresses elevated levels of c-*MYC* (Westermann, Muth et al. 2008). This is strongly correlated with undifferentiated phenotype. For example, microarray analysis of NB, showed that poor survival, independent of *MYCN* amplification,

was significantly correlated with genes in the *MYC* pathway as well as low expression of differentiation markers of the sympathoadrenal neural lineage (Fredlund, Ringnér et al. 2008). It was also observed that more aggressive type of NB tumor were found in younger infants, with early stages of NC undifferentiated precursors (Louis and Shohet 2015). It has been reported that a high number of low-risk NB show a significant ability of spontaneous differentiation of regression (Ruiz-Perez, Henley et al. 2017) (Brodeur and Bagatell 2014). This was the reason why differentiation therapy was targeted for NB, for example, retinoic acid is used as a maintenance therapy reagent to clear high-risk NB minimal residual cancer cells, which results in an improvement in event-free survival (EFS) (Matthay, Villablanca et al. 1999, Matthay, Reynolds et al. 2009).

MYCN expression was found predominantly in the developing peripheral NC (Westermark, Wilhelm et al. 2011). TrkB neurotrophin receptor, as well as its ligand brain-derived neurotrophic factor (BDNF), was found to be co-expressed in the unfavorable *MYCN*-amplified NB (Nakagawara, Azar et al. 1994). However, expression of NGF's TrkA receptor is associated with differentiated tumors with good prognosis, and negatively correlates with *MYCN* amplification (Nakagawara, Arima-Nakagawara et al. 1993). Amplified *MYCN* was also found to inhibit cell differentiation in high-risk NB by deregulation of estrogen receptor alpha (ERα) and thereby NGF signaling, maintaining an aggressive phenotype (Dzieran, Rodriguez Garcia et al. 2018). The importance of *MYC* signaling in controlling an undifferentiated phenotype and the inhibition of this signaling to promote a less aggressive phenotype has been considered as a target for therapy of high-risk NB (Westermark, Wilhelm et al. 2011).

1.3 Oxidative stress

Cells are normally supplied with antioxidants to work as the first defense line against the ROS either neutralizing them by transforming them into stable molecules or blocking their formation. GSH is a substantial antioxidant that maintains intracellular redox in both hypoxia and oxidative stress (Jones, Carlson et al. 2000). In physiological conditions it is found in two balanced forms; 90% reduced GSH that sequestrates ROS, and 10% oxidized, glutathione disulfide (GSSG), the imbalanced ration between those two forms result in oxidative stress (Griffith 1999, Kumari, Badana et al. 2018).

ROS are a group of highly reactive molecules including singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) (Teppo, Soini et al. 2017).

They are a battery of radical and non-radical molecules which can be produced by both cellular metabolism or exogenous source (Gào and Schöttker 2017). In physiological conditions, the cellular ROS level convenient to the activity of mitochondrial electron transport and tightly controlled by endogenous antioxidant system. However, energy metabolism and ROS homeostasis is totally different in cancer cells (Kang, Lee et al. 2015). ROS have crucial roles in both physiological functions and tumor development (Weinberg and Chandel 2009). Oxidative stress is a term used to describe the imbalance between pro-oxidant and anti-oxidant defeat system (Gào and Schöttker 2017). In malignant tumors oxidative stress could be a results of increasing metabolic rate, oncogene activation and defective vasculature which leads to a hypoxic area. It has been reported that cancer tissues upregulate levels of antioxidant enzymes to counteract this increased oxidative stress (Castaldo, Freitas et al. 2016). In addition, this could induce interruption in cell signaling pathways that might promote tumor cell survival, invasion, proliferation, the formation of new blood vessels (angiogenesis), inhibition of apoptosis and make those cells resistant to radio and chemotherapy (Reuter, Gupta et al. 2010, Zhou, Shao et al. 2014).

1.3.1 Cancer cells survival in oxidative stress:

Cancer cells have a high level of metabolism which is both require and produce high level of ROS compared to normal cells. ROS are essential to both cancer initiation and progression and they are elevated mainly during cancer transformation due to the high metabolic rate in the mitochondria, endoplasmic reticulum and cell membranes. The initiation phase of cancer development includes respiratory disfunction, raising electron leakage and then increased ROS generation (Rohrmann, Overvad et al. 2013). To maintain survival in this high energy environment, support their rapid proliferation and expand around the body (metastases), cancer cells alter their metabolism mechanism (Fadaka, Ajiboye et al. 2017). Several decades ago Nobel laureate Otto Warburg proved that cancer cells use a primitive ineffective reaction , aerobic glycolysis (Warburg effect) to produce most of their energy, reviewed here (Fadaka, Ajiboye et al. 2017). The Warburg effect has been detected with and without adequate oxygenation, however, some researchers tend to consider cancer cells aerobic glycolysis as a type of adaptation to hypoxic conditions, mitochondrial defect and elevated ROS generation (Kumari, Badana et al. 2018).

1.3.2 Necroptosis and oxidative stress:

ROS is usually associated with the principle of oxidative stress. However, it is well established now that they serve as signaling molecules that regulate biological and physiological processes (Finkel 2011). Oxidative stress has long been considered as a driving force that triggers apoptosis and necroptosis cell death depending on the cell type (Schulze-Osthoff, Bakker et al. 1992, Vanlangenakker, Vanden Berghe et al. 2011). Necroptosis is regulated caspase-independent necrotic cell death. At the core of the necroptosis pathway are receptor-interacting protein kinase 1 (RIPK1), RIPK3, and mixed lineage kinase domain-like protein (MLKL). Necroptosis pathways precise mechanism includes; TNF, FASL, and TRAIL ligands that bind to their specific receptors that cause receptors trimerization and subsequently those receptors interact with RIPK1. The activation of RIPK1 cause its dissociation from the plasma membrane (Vanlangenakker, Vanden Berghe et al. 2012). RIPK1 is specifically responsible for the phosphorylation of RIPK3 and subsequently the latter phosphorylation further causes MLKL phosphorylation and trimerization. MLKL homotrimer then to the plasma membrane and causes necrotic plasma membrane permeabilization through nonspecific pore formation or through interactions with calcium channels to promote calcium and sodium influx, and induce cell death (Sun, Wang et al. 2012, Cai, Jitkaew et al. 2014, Su, Yang et al. 2016).

The complex formed of activation of these three proteins called “necrosome” that upstream the cell death receptors and downstream executive molecules such as ROS, and also cause plasma membrane permeabilization, and cytosolic ATP reduction (Jouan-Lanhouet, Riquet et al. 2014, Su, Yang et al. 2016).The elevation of oxidative stress as a potential cell death mechanisms induced by necroptosis trigger, might be the reason why several studies have indicated necroptosis accompanies increased ROS production in human (Ma, Peng et al. 2016) and murine (Wang, Wu et al. 2015) cancer cell types. Some necrosis inducers skip RIPK1 activation to RIPK3 or MLKL directly (Dillon, Weinlich et al. 2014). Necroptosis can be inhibited by blocking any of its signaling molecules using small molecule inhibitors such as necrostatin-1 (Nec-1) (RIPK1 inhibitor), GSK'872 (RIPK3 inhibitor), and necrosulfonamide (MLKL inhibitor)(Su, Yang et al. 2016). Necrostatin-7 has also been reported as necroptosis inhibitor but it does not inhibit RIPK1, suggesting the possibility that it may target an additional and yet unknown regulatory molecule in the pathway (Zheng, Degterev et al. 2008).

1.4 Flavonoids

Flavonoids are a group of natural low-molecular-weight compounds with varying phenolic structures, that have been isolated from fruits, vegetables, grains, bark, roots, stems, flowers, tea and wine, hence they are termed dietary flavonoids (Panche, Diwan et al. 2016). Many of them are flower pigments, most belonging to the angiosperm families (Panche, Diwan et al. 2016). There are a number of known subgroups in the flavonoid family, including chalcones, flavones, flavonols and isoflavones. Flavonoids are abundant in human and animal diets, and while they also possess therapeutic potential, they can only be biosynthesized by plants; therefore, they have to be obtained by humans from the diet (Kozlowska and Szostak-Wegierek 2014). In animals, bacteria and plants, flavonoids have many biological activities. They are responsible for colour and aroma of flowers and fruits, and in this way they contribute to attracting pollinators and consequently help seed and spore germination (Griesbach 2005). They are also found to function as a unique ultraviolet (UV) filter in plants (Takahashi and Ohnishi 2004), and to function as signaling molecules in plants (Woo, Kuleck et al. 2002), detoxifying agents and antimicrobial defensive compounds (Mathesius 2018). Interestingly, plants were found to synthesize flavonoids as a reaction to microbial attack, and flavonoids were found to possess antimicrobial properties against a wide range of pathogenic microorganisms in vitro (Górniak, Bartoszewski et al. 2019).

1.4.1 Flavonoids and oxidative stress

In humans, flavonoids have been reported to possess antioxidant activity meaning that they may be important for human health by working as protective antioxidants. Specifically, this antioxidant ability was found to be due to the ability of flavonoids to transfer free radical electrons, to activate antioxidant enzymes (Ferrali, Signorini et al. 1997), and inhibit oxidases (Hirano, Sasamoto et al. 2001). Flavonoids have also been reported to directly scavenge free radicals, to scavenge transition metal ions, to inhibit enzymes responsible for antioxidant production, and to possess other antioxidant mechanisms (Alzand 2012).

Despite these reported antioxidant abilities of flavonoids, under certain conditions, flavonoids can act as pro-oxidants that can induce oxidative damage by reacting with various biomolecules, such as lipids, proteins and DNA (Procházková, Boušová et al. 2011). Interestingly, this pro-oxidant activity was found to be linked to the number of hydroxyl groups in the particular flavonoid molecule (Cao, Sofic et al. 1997). Specifically, molecules

with multiple hydroxyl groups, especially in the B ring, were found to significantly increase the production of hydroxyl radicals (Hanasaki, Ogawa et al. 1994). In addition, some flavonoid compounds containing pyrogallol structure (organic compound with the formula $C_6H_3(OH)_3$) in the A-ring, were found to promote hydrogen peroxide production (Procházková, Boušová et al. 2011). The pro-oxidant activity of flavonoids acts in a concentration-dependent manner, with highest concentrations inducing the highest H_2O_2 production in human lymphocytes (Yen, Duh et al. 2003). Moreover, all of the tested flavonoids (quercetin, naringenin, hesperetin and morin) in the study by Yen et al. were found to induce DNA strand breakage in a concentration-dependent manner and those with a higher -OH group number were most effective. That is to say, uercetin, which has five OH groups, was found to have the most pronounced pro-oxidative activity, while hesperetin and naringenin which each have three -OH groups, exerted lower effects (Yen, Duh et al. 2003). Interestingly, these effects of flavonoids can be cell type-specific. For example, flavonoids induced ROS that activated caspases and subsequently induced apoptotic cell death in human glioblastoma T98G and U87MG cells, but not in human normal astrocytes (Das, Banik et al. 2010). Chrysin is a flavonoid extracted from Propolis (bee glue), which possesses multiple anticancer activities and was found to enhance sensitization of A549 and HeLa human cancer cell lines to tumor necrosis factor-related apoptosis-inducing ligand (Lirdprapamongkol, Sakurai et al. 2013). This suggests that flavonoids may be beneficial in combating chemotherapy resistance by enhancing the sensitivity of cancer cells to cytotoxic agents.

1.4.2 Flavonoids as agents of cell death or cell differentiation

Flavonoids have also been reported to have anticancer activity, whether they are used as a mixed plant extract or in their purified form. An example of a purified plant extract is genus Linaria, which belongs to the family of Scrophulariaceae and comprises 200 species that are mainly distributed in Europe (Cheriet, Mancini et al. 2015). The anticancer activity of flavonoids has also been reported in studies on some cancer cell lines, including large cell lung carcinoma COR-L23, colorectal adenocarcinoma Caco-2, and amelanotic melanoma C32 cell lines (Tundis, Loizzo et al. 2011). Interestingly, flavonoid compounds did not inhibit the growth of the normal human foetal lung MRC-5 cell, suggesting they are potentially selective against cancer cells (Raffa, Maggio et al. 2017). The neurotrophic potential of flavonoids has also been investigated through testing their ability to induce cell

differentiation. Sagara et. al. have demonstrated that a small subset of flavonoids that were neuroprotective could induce neurite outgrowth in PC12 cells (a pheochromocytoma of the rat adrenal medulla), by an extracellular signal-regulated kinase-dependent process (Sagara, Vanhnasy et al. 2004). Another ten citrus flavonoids were found to induce the differentiation of human promyelocytic leukaemia cells (HL-60) into mature monocytes/macrophages, and to exert their activity in a dose-dependent manner. This differentiation mechanism was found to be linked to the structure of the individual flavonoids, as the presence of an ortho-catechol moiety in ring B and C2-C3 double bond both had important impacts on the ability of the flavonoid to induce differentiation of HL-60 cells (Kawaii, Tomono et al. 1999).

1.4.3 Chalcones

One group of flavonoids are the chalcones, molecules which are the precursors of flavonoid biosynthesis that are a major class of natural compounds that is abundant in edible plants. Tomatoes, pears, strawberries, bearberries and certain wheat products are rich in chalcones. While not all plants accumulate chalcones, those that have some accumulation of chalcones have been used in traditional medicine and this has shed light on the biological activities of chalcones (Rozmer and Perjési 2016). Recently, chalcones have been reported to possess a wide range of useful properties, including anti-tumor, anti-oxidant, anti-microbial, anti-inflammatory properties, and to be cytotoxic (Nowakowska 2007, Božić, Milenković et al. 2014, Lahsasni, Al Korbi et al. 2014, Gupta and Jain 2015). The basic chemical structure of a chalcone is 1,3-diphenyl-2-propene-1-one, that is, chalcones consist of open-chain flavonoids in which the two aromatic rings, known as the A and B rings, are joined by a three-carbon α,β-unsaturated carbonyl system (Nowakowska 2007, Rozmer and Perjési 2016, Zhuang, Zhang et al. 2017) (Figure 1.5). Chalcones are unique in the flavonoid family as they do not possess the heterocyclic C ring (Zhuang, Zhang et al. 2017). They exist as cis and trans isomers; cis indicates that the functional groups are on the same side of the carbon chain, while trans conveys that functional groups are on opposing sides of the carbon chain, with the trans isomer being thermodynamically more stable (Zhuang, Zhang et al. 2017). Several extracted and purified chalcones from plants have been approved for clinical trials for treatment of cancer, viral and cardiovascular disorders, or have been included as ingredients in cosmetic preparations (Ni, Meng et al. 2004). The simple chemical structure of chalcones, the ease of hydrogen atom manipulation, the fact that they are straightforward to synthesize, and the variety of promising biological activities, has meant that chalcones

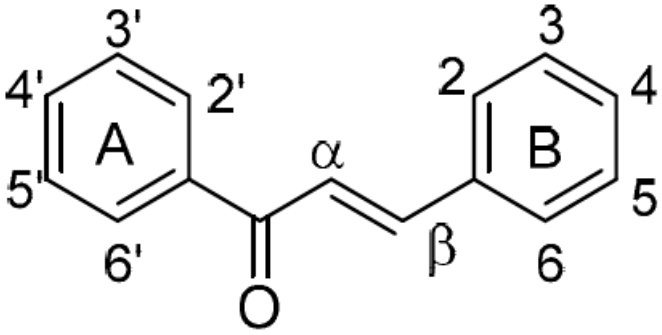


Figure 1.5 : Diagram showing the chemical structure of a chalcone molecule (Rosa et al. 2019).

and their derivatives have been the subject of intensive research (Gomes, Muratov et al. 2017).

1.4.4 The anticancer activity of chalcones

As the structure of chalcones can be modified with relative ease (Liu, Ge et al. 2013, Jandial, Blair et al. 2014), and since chalcones have been reported to be the most potent flavonoid subclass in terms of anti-tumor activity (Cabrera, Simoens et al. 2007, Loa, Chow et al. 2009, Liu, Ge et al. 2013), there has been significant interest in chalcone-based anti-cancer drug development (Mahapatra, Bharti et al. 2015, Sharma, Kumar et al. 2015).Previous reports have suggested that the anti-cancer effects of chalcones are associated with the presence of hydroxyl groups, and consequently, hydroxylated chalcone derivatives have displayed the greatest anti-cancer activity (De Vincenzo, Scambia et al. 1995, Cabrera, Simoens et al. 2007, Loa, Chow et al. 2009). Additionally, the position of the hydroxyl group has been shown to be important in regulating the biological activity of chalcones (Calliste, Le Bail et al. 2001, Nerya, Musa et al. 2004).

Chalcones have also been reported as anti-oxidants, oxygen scavengers, and anti-inflammatory agents in a variety of experimental systems (Nakamura, Watanabe et al. 2003) (Alcaraz, Vicente et al. 2004). The anti-cancer effects of chalcones have been reviewed by (Go, Wu et al. 2005). This strategy of using diet-derived pharmacological agents to prevent, inhibit and delay carcinogenesis has been derived from epidemiological studies which suggest that diets rich in fruit and vegetables can reduce the risk of cancer (Key 2011, Bradbury, Appleby et al. 2014). The multiple mechanisms that have been investigated regarding the mechanism by which chalcones exert anti-cancer effects include, but are not limited to, cell cycle disruption, inhibition of angiogenesis, tubulin polymerization inhibition, apoptosis induction and many others (Karthikeyan, Moorthy et al. 2015).

Of all the different types of chalcones, hydroxychalcones have been found to modulate mitochondrial function as they were confirmed to inhibit phosphorylation, increase ROS, and cause possible covalent interaction with cellular thiols, resulting in GSH depletion, which ultimately leads to cell death (Guzy, Vašková-Kubálková et al. 2010). There is some evidence for cytotoxic effects of other hydroxylated chalcones in melanoma (Navarini, Chiaradia et al. 2009), colon (Pande, Biswas et al. 2017), glioma (Ku, Ryu et al. 2010), breast (Padhye, Ahmad et al. 2010) and leukaemia (Orlikova, Tasdemir et al. 2011) cancer cells. Additionally, chalcones have also been reported to have an indirect anti-cancer effect

through the inhibition of angiogenesis, which is a key process for tumor progression. Specifically, chalcones have been reported to have the ability to inhibit angiogenesis in tumors by suppression of "angiogenic switching" which in turn leads to the prevention of pro-angiogenic factor activation (Bergers and Benjamin 2003, Mahapatra, Bharti et al. 2015).

1.4.5 Flavonoids and chalcones as regulators of BMP-Smad signaling

In sections 1.2.1 and 1.2.2, we discussed the importance of the BMP-Smad pathway in the development of the sympathetic nervous system and how this pathway is altered in NB. This raises the possibility that small molecules that modulate the activity of the BMP-Smad pathway may be able to act as modulators of cell differentiation and or cell death in NB cells. Interestingly in recent years a number of screens has been carried out to identify small molecules that modulate the BMP pathway, leading to the identification of a select number of flavonoids and chalcones, as discussed below.

Flavonoids from Herba Epimedii herbs have been studied in in human mesenchymal stem cells and have been shown to enhance the mRNA expression of BMP-2, BMP-4, Runx2, β-catenin and cyclinD1, all of which are BMP or Wnt-signaling pathway-related regulators (Zhang, Li et al. 2010). The flavonoids of Rhizoma drynariae have been reported to improve the growth performance of chickens with tibial dyschondroplasia, an abnormality of the growth cartilage, via the regulation of BMP-2 and Runx2, a dominant gene for osteoblast differentiation (Yao, Zhang et al. 2018). Chalcone derivatives were reported to promote osteogenic differentiation of C2C12 cells. Those derivatives were demonstrated to activate BMP-2 and also inhibit the activity of noggin, a BMP antagonist (Choi, Park et al. 2018). In parallel to these studies, work has also focused on identifying small molecule activators of BMP signaling. A study by Vrijens, et al. aimed to identify small molecule activators of BMP signaling (Vrijens, Lin et al. 2013). This was because recombinant BMPs are expensive to produce, and their clinical use is limited because the supra-physiological doses required for therapeutic efficacy can cause severe side effects, and due to their molecular size, they are unlikely to cross the blood-brain barrier without a delivery system. This study screened a drug library of 5,600 FDA-approved drugs and chemicals for their ability to activate BMP-Smad signaling in a human cervical carcinoma reporter cell line (Vrijens, Lin et al. 2013). Four out of the 5,600 tested compounds identified were flavonoids, of which two were chalcones, that is, isoliquiritigenin (ISLQ), which had a potency of 10 μM with

80% efficacy, and 4′-hydroxychalcone, which had a potency of 10 µM with 90% efficacy. The other two were flavones, that is apigenin, which had a potency of 3 µM with 75% efficacy, and diosmetin, which had a potency of 1.5 µM with 80% efficacy (Vrijens, Lin et al. 2013). Only the two chalcones were found to activate Smad1/ 5 phosphorylation, which was measured after 24 hours. This study also found that ISLQ was the only flavonoid that induced the partial differentiation of C2C12 mouse myoblast cells into osteoblasts, which is an assay used to measure BMP-induced differentiation (Vrijens, Lin et al. 2013). The two chalcones were also found to induce ventralisation of zebrafish embryos, which is a hallmark of BMP activation during development (Vrijens, Lin et al. 2013).

1.4.6 Isoliquiritigenin

ISLQ (4,2′,4′-trihydroxychalcone), with the formula C15H12O4 (Figure 1.6), is a chalcone that is one of the bioactive components in extracts from plants belonging to the liquorice family, including Glycyrrhiza uralensis, Mongolian glycyrrhiza and Glycyrrhiza glabra (Peng, Du et al. 2015). ISLQ and the flavanone liquiritigenin are the main bioactive constituents of Radix Glycyrrhizae, which are composed of the dried roots and rhizomes of three Glycyrrhiza species (Ramalingam, Kim et al. 2018). Liquorice has been used for a long time in China as a traditional medicine reviewed by (Wang, Zhang et al. 2013), is used for the treatment of gastric and duodenal ulcers (Rahnama, Mehrabani et al. 2013), bronchial asthma (Sadek, Tawfik et al. 2019), and many other conditions reviewed by (Fiore, Eisenhut et al. 2005). Extensive research has now confirmed that liquorice root possesses anti-oxidant, anti-inflammatory, anti-viral, anti-tumor and other protective bioactivities (Bode and Dong 2015). One key bioactive component of liquorice extract appears to be ISLQ, which has demonstrated biological activity in studies on several different tissues and cell types, but for the purposes of this review we will focus on its anti-cancer effects (Ramalingam, Kim et al. 2018).

The anti-cancer properties of ISLQ have been attributed to its ability to inhibit cell cycle progression, induce oxidative stress, apoptosis, autophagy and/or necrosis. However, the precise mechanism of cytotoxicity is dependent on cell type and usually involves a combination of cell death pathways (Jung, Chung et al. 2006, Wu, Chen et al. 2016, Si, Yang et al. 2017, Yang, Zhang et al. 2017). For example, ISLQ was reported to have an antineoplastic effect on human prostate cancer cells, which was linked to G2/M cell cycle arrest and apoptosis (Zhang, Lai et al. 2018).

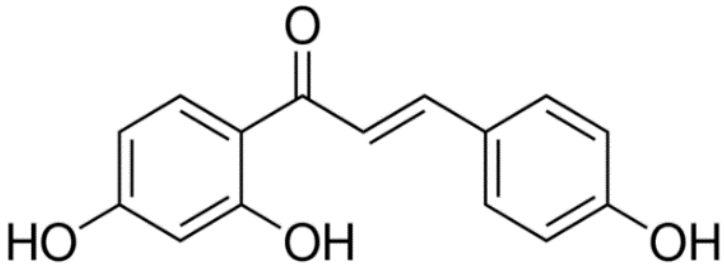


Figure 1.6: Diagram showing the chemical structure of ISLQ molecule (Peng, Du et al. 2015).

It has also been reported to inhibit ovarian cancer cell growth by autophagy (Chen, Huang et al. 2017). ISLQ was also demonstrated to inhibit tumorigenesis and metastasis in breast cancer (Peng, Tang et al. 2017). ISLQ inhibits tumor growth and protects the kidney and liver against chemotherapy-induced toxicity in a mouse xenograft model of colon carcinoma (Lee, Son et al. 2008). Moreover, ISLQ has been reported to have selectively induce cytotoxicity in cancer U87 glioma cells, while displaying minimal toxicity against normal brain cells (Zhao, Chang et al. 2015). A recent study showed that ISLQ had cytotoxic activity on SH-SY5Y NB cells by inducing cell cycle arrest and necroptosis-induced cell death (Escobar, Munoz et al. 2016). A follow-on study by the same group showed that ISLQ has anti-proliferative and cytotoxic activity on SH-SY5Y cells via the loss of ATP, induction of cell cycle arrest, and that it induced cell death through a necroptotic mechanism in the absence of apoptosis (Escobar, Fong et al. 2019). In terms of chemotherapy resistance, it has been previously reported that treatment of human bladder cancer T24 cells with ISLQ increases the expression of bax and caspase 3 apoptotic transcripts (Si, Yang et al. 2017). The apoptosis is triggered by activation of the tumor necrosis factor (TNF) receptor. Then the intrinsic apoptosis start by the release of cytochrome c and caspases/direct IAP-binding protein with low pI (Smac/DIABLO) from the mitochondria into the cytoplasm. This happen as a results of mitochondrial outer membrane permeabilization (MOMP). MOMP is controlled through modifying the interactions of proteins of the Bcl-2 family. Once members of Bcl-2 activated, they promote oligomerization of Bax and Bak in the mitochondrial outer membrane. In the cytoplasm, cytochrome c induces the formation of the apoptosome complex containing the adaptor protein Apaf-1 and caspase-9. This cause the activation of caspase-9, which in turn lead to activation of effector caspases then cell death eventually (Sayers 2011). ISLQ treatment has been found to enhance the chemosensitivity of multidrug-resistant human uterine sarcoma cancer cell lines (MES-SA/Dx5, MES-SA//Dx5-R) to doxorubicin (Lin, Wu et al. 2017). In addition to its cytotoxic effects, ISLQ has reported to promote differentiation in mouse melanoma B16F0 cells in a dose-dependent manner (Chen, Zhang et al. 2012). Collectively, these data suggest ISLQ may be a potent cytotoxin for NB cells, however it is currently unknown whether ISLQ induces cell death and / or differentiation in *MYCN*-amplified NB cells.

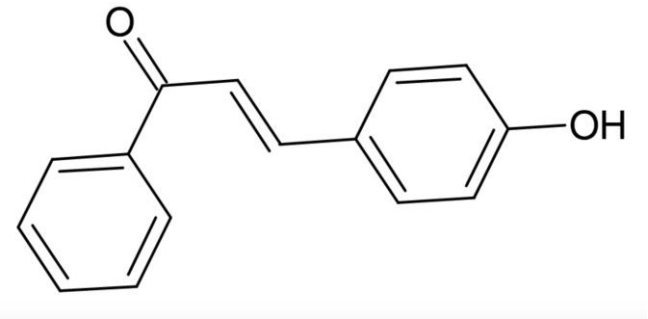


Figure 1.7: Diagram showing the chemical structure of 4-HC (SHUBHALAXMI 2013).

1.4.7 4-Hydroxychalcone

4-Hydroxychalcone (4HC) with the formula C15H12O2, (Figure 1.7), is a trans-chalcone with a hydroxyl group at position 4, that can be extracted from Glycyrrhiza glabra (licorice) roots. Structurally, 4HC is the most simple hydroxylated chalcone, and has been known for its role as a plant metabolite and an antihypertensive agent (Qu, Dai et al. 2014). It is a member of the chalcone family and classified as a phenol by the PubChem database. Anti-angiogenic effects of 4HC have been reported in endothelial cells (Varinska, van Wijhe et al. 2012). Another study found that 4HC treatment of human SH-SY5Y NB cells resulted in mitochondrial dysfunction Additionally, an isomer of 4HC, 4′-hydroxychalcone, was found to inhibit the proliferation and migration of HeLa, MCF-7 and A549 cells (de Moura Escobar, Simone et al. 2019). In the same study, 4HC inhibited angiogenesis in vitro by suppression of growth factor pathways (Varinska, van Wijhe et al. 2012). In addition, 4′-hydroxychalcone has been reported to induce the conversion of cytotoxic GSH reductase to GSH disulphide (Zhang, Yang et al. 1997). Targeting GSH is a well-established approach in developing novel chemotherapeutic agents that also can overcome drug resistance (Zhu, Du et al. 2018, Desideri, Ciccarone et al. 2019). To date, however, it is unknown whether 4HC can induce cell death and /or differentiation in *MYCN*-amplified NB cells.

1.5 Aims of the thesis

Given the link between NC development errors and the development of NB, and the alterations in BMP-Smad signaling components and NB patients outcomes, this thesis aimed to examine the association of distinct type 1 BMP with different molecular pathways and survival outcomes in NB, and testing small molecule BMP-Smad activator chalcones on NB cells differentiation. Moreover, the thesis also examined those chalcones cytotoxicity on NB cells and the mode of cell death.

Chapter 2

Chapter 2 Association of distinct type 1 bone morphogenetic protein receptors with different molecular pathways and survival outcomes in neuroblastoma.

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2.1 Abstract

NB is a paediatric cancer that arises in the sympathetic nervous system. Patients with stage 4 tumours have poor outcomes and 20% of high-risk cases have *MYCN* amplification. The bone morphogenetic proteins (BMPs) play roles in sympathetic neuritogenesis, by signalling through bone morphogenetic protein receptor (BMPR)2 and either BMPR1A or BMPR1B. Alterations in BMPR2 expression have been reported in NB; it is unknown if the expression of BMPR1A or BMPR1B is altered. We report lower *BMPR2* and *BMPR1B*, and higher *BMPR1A*, expression in stage 4 and in *MYCN*-amplified NB. Kaplan-Meier plots showed that high *BMPR2* or *BMPR1B* expression was linked to better survival, while high *BMPR1A* was linked to worse survival. Gene ontology enrichment and pathway analyses revealed that *BMPR2* and *BMPR1B* co-expressed genes were enriched in those associated with NB differentiation. *BMPR1A* co-expressed genes were enriched in those associated with cell proliferation. Moreover, the correlation between *BMPR2* and *BMPR1A* was strengthened, while the correlation between *BMPR2* and *BMPR1B* was lost, in *MYCN*-amplified NB. This suggested that differentiation should decrease *BMPR1A* and increase *BMPR1B* expression. In agreement, nerve growth factor treatment of cultured sympathetic neurons decreased *Bmpr1a* expression and increased *Bmpr1b* expression. Overexpression of dominant negative BMPR1B, treatment with a BMPR1B inhibitor, and treatment with GDF5 which signals via BMPR1B, showed that BMPR1B signalling is required to promote neuritogenesis in *MYCN*-amplified SK-N-BE(2) NB cells, suggesting that loss of *BMPR1B* may alter neuritogenesis. This study shows that expression of distinct BMPRs is associated with different survival outcomes in NB.

2.2 Introduction

NB is a paediatric cancer that arises in the sympathetic nervous system and is responsible for 15% of all pediatric cancer mortality (Colon and Chung 2011). The INSS classifies NB into stages 1-4 and stage 4s (Monclair, Brodeur et al. 2009). Patients with stage 1 or 2 tumours have an excellent prognosis, while those with stage 3 or 4 tumours have a particularly poor outcome. Around 20% of the high-risk NB tumours present with amplification of *MYCN* (Molenaar, Koster et al. 2012). While the molecular drivers of NB progression are multi-factorial, a whole-genome analysis of 87 NB samples identified frequent alterations in the expression of genes associated with neuritogenesis, in high-risk tumours (Molenaar, Koster et al. 2012). This suggests that alterations in the molecular mechanisms that control normal neuritogenesis may occur in cells of the sympathetic lineage in NB.

One key group of signals that control sympathetic development is the bone morphogenetic proteins (BMPs), the largest subgroup of the transforming growth factor (TGF)-β superfamily of ligands (Hegarty, O'Keeffe et al. 2013). BMP ligands signal by binding a heteromeric complex of bone morphogenetic protein receptor (BMPR)2 and one of two type 1 BMPRs, BMPR1A and BMPR1B, which are transmembrane serine/threonine kinase receptors (Weiss and Attisano 2013). Individual BMP ligands have different affinities for BMPR complexes. For example, BMP2, BMP7 and GDF5 (BMP14) all bind to BMPR2 (Weber, Kotzsch et al. 2007, Yin, Yeh et al. 2008). However, BMP2 and BMP7 bind to BMPR1A or BMPR1B as the second receptor in the complex (Koenig, Cook et al. 1994), while GDF5 binds with high affinity to BMPR1B, and but not to BMPR1A (Nishitoh, Ichijo et al. 1996, Nickel, Kotzsch et al. 2005). In the canonical BMP pathway BMP receptors activate transcription factors known as the R-Smads (Smad1/5/8), which form a complex with Smad4, then translocate to the nucleus and regulate gene expression (Hegarty, O'Keeffe et al. 2013). BMPRs can also activate a number of non-canonical pathways, including MAPK and PI3K pathways, depending on the cellular context (Lee-Hoeflich, Causing et al. 2004, Guan, Li et al. 2014, Zhang 2016).

It is well established that BMPs and BMPRs play key roles in the development of sympathetic neurons (Reissmann, Ernsberger et al. 1996, Rohrer 2003). For example, BMPs acting through BMPRs, have been shown to promote the specification and dendritic growth of sympathetic neurons (Beck, Drahushuk et al. 2001, Guo, Lin et al. 2001, Saito, Takase et

al. 2012, Majdazari, Stubbusch et al. 2013), while GDF5 controls sympathetic axon growth and target innervation (O'Keeffe, Gutierrez et al. 2016). Given that BMP signalling controls neuritogenesis in the developing sympathetic nervous system, alterations in expression of the BMPRs may contribute to the defects in neuritogenesis see in NB (Molenaar, Koster et al. 2012). In support of this, a previous study has shown that there is a significant down-regulation of BMPR2 expression in NB, that BMPR2 knockdown increased cell growth and tumourigenicity in mice, and that patients with high BMPR2 expression have better overall survival (Cui, Yang et al. 2017). It has also been shown that Smad4 is required for growth inhibition, invasion and metastasis of NB cells, and that patients with high Smad4 expression have greater survival probability (Qu, Zheng et al. 2016). Moreover, several BMPs have been shown to promote the neurite growth and differentiation of human NB cells; these include BMP4 (Ferlemann, Menon et al. 2017), BMP9 (Lauzon, Drevelle et al. 2017), BMP2 ((Du and Yip 2010), BMP6 (Sumantran, Brederlau et al. 2003) and GDF5 (Hegarty, Sullivan et al. 2013). There is little knowledge on any alterations of BMPR1A and BMPR1B expression that may occur in NB, nor is it known whether the expression of these receptors is associated with NB patient outcomes and/or neuritogenesis.

2.3 Methods

2.3.1 Gene expression analysis of human NB samples.

Gene expression data of human NB samples from GSE: 62564 (Munro, Lund et al. 2014, Su, Fang et al. 2014, Wang, Gong et al. 2014), GSE: 45547 (Kocak et al., 2013) and GSE: 120572 (Ackermann, Cartolano et al. 2018) data sets were analysed. Expression data and Kaplan-Meier plots were generated using the R2: Genomics Analysis and Visualisation Platform (http://r2.amc.nl). For gene co-expression analysis, all genes that displayed a significant correlation with *BMPR2*, *BMPR1A* or *BMPR2* expression after a Bonferroni multiple testing correction were analysed. Gene ontology (GO) and Panther pathway enrichment analysis was carried out using tools available at www.geneontology.org. All gene expression data are presented as mean rank expression values. Differences in expression were analysed using a Kruskal Wallis test with a Dunn's *post-hoc* test, or Mann-Whitney test, as appropriate.

2.3.2 Quantitative Real-Time PCR (RT-qPCR)

The levels of *Bmpr1a* and *Bmpr1b* mRNAs in P0 mouse sympathetic neurons were quantified by RT-qPCR relative to a geometric mean of reference mRNAs for the three housekeeping enzymes, glyceraldehyde phosphate dehydrogenase (*Gapdh*), succinate dehydrogenase (*Sdha*) and hypoxanthine phosphoribosyltransferase-1 (*Hprt1*). Briefly, 5 µl of total RNA from cultured sympathetic neurons was reverse transcribed for 1 h at 45°C using the AffinityScript kit (Agilent Technologies, Berkshire, United Kingdom) in a 25 µl reaction volume, according to the manufacturer's instructions. 2 µl of cDNA was amplified in a 20 µl reaction volume using Brilliant III ultrafast qPCR master mix reagents (Agilent Technologies) with 150 nM of primers and 300 nM of dual-labeled (FAM/BHQ1) hybridization probes specific to each of the cDNAs (MWG/Eurofins, Ebersberg, Germany) using the Mx3000P platform (Agilent Technologies). The PCR primers were: *Bmpr1b* forward: 5′-AGT GTA ATA AAG ACC TCC A-3′ and reverse: 5′-AAC TAC AGA CAG TCA CAG-3′; *Bmpr1a* forward: 5′-TAC GCA GGA CAA TAG AAT-3′ and reverse: 5′-AAC TAT ACA GAC AGC CAT-3′; *Gapdh* forward: 5′-GAG AAA CCT GCC AAG TAT G-3′ and reverse: 5′-GGA GTT GCT GTT GAA GTC-3′; *Sdha* forward: 5′-GGA ACA CTC CAA AAA CAG-3′ and reverse: 5′-CCA CAG CAT CAA ATT CAT-3′; *Hprt1* forward: 5′-TTA AGC AGT ACA GCC CCA AAA TG-3′ and reverse: 5′-AAG TCT GGC CTG TAT CCA ACA C-3′. Dual labelled probes were: *Bmpr1b* 5′-FAM-ACC TAC ACC CTA CAC TGC CTC-BHQ1-3′; *Bmpr1a*: 5'-FAM-TGA GCA CAA CCA GCC ATC G-BHQ1-3'; *Gapdh*: 5′-FAM-AGA CAA CCT GGT CCT CAG TGT-BHQ1-3; *Sdha*: 5′-FAM-CCT GCG GCT TTC ACT TCT CT-BHQ1–3, *Hrpt1*: 5′-FAM-TCG AGA GGT CCT TTT CAC CAG CAA G-BHQ1–3′. Levels of *Bmpr1a*, *Bmpr1b* and reference mRNAs in experimental samples were quantified by reference to a standard curve generated for each of the mRNAs that was constructed from a serial dilution of reverse transcribed adult mouse brain RNA.

2.3.3 Cell culture and analysis.

Sympathetic neurons were dissected from the superior cervical ganglion (SCG) P0 CD1 mice, then trypsinized and plated onto poly-ornithine- and laminin-coated 35-mm tissue culture dishes (Greiner, Gloucestershire, UK) in serum free Ham's F14 medium supplemented with 0.25% Albumax I (Invitrogen, Paisley, UK), as described previously (Davies, Lee et al. 1993). Neuronal cultures were grown with 0 to 10 ng/ml NGF and 25 µM

of pan-caspase inhibitor Boc-D-FMK (Calbiochem) for 24h before RNA extraction. SK-N-BE(2) cells (ATTC) were cultured in Minimum Essential Medium supplemented with 100 nM L-glutamine, 1% penicillin-streptomycin, 1% (1:1) non-essential amino acid solution: Ham's F-12, and 15% foetal bovine serum (FBS). All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. For both primary cultures and cell lines, 2 x 10^5 cells were plated per well of a 24-well plate and treated for 72 h with 0.5 µM K02288, a highly selective 2-aminopyridine-based BMP signalling inhibitor, or with GDF5 (0 to 50 ng/ml) where indicated. For analysis of neurite length, cultures were labelled with the vital fluorescent dye Calcein-AM (1:500; Invitrogen). Imaging was performed with an Olympus IX71 inverted microscope fitted with an Olympus DP70 camera and images were analysed using ImageJ software. For neurite growth and cluster area measurements, ≥ 180 cells from three experiments were analysed and data are presented as mean ± SEM.

2.3.4 Transfection of cell cultures.

2 x 10^5 of SK-N-BE(2) per well were transfected with a pSK^+ mBMPR-1B dominant negative (mBMPR1B-dn) plasmid (a gift from Lee Niswander & Peter ten Dijke; Addgene plasmid # 49530; http://n2t.net/addgene:49530; RRID:Addgene_49530) and a GFP-expressing plasmid, using TransIT-X2 Dynamic Delivery System (Mirus) according to the manufacturer's instructions. Images of GFP-positive cells were captured using an Olympus IX71 inverted microscope fitted with an Olympus DP70 camera, and neurite length was analysed using ImageJ. For neurite growth measurements, ≥ 240 cells from three independent experiments were analysed and data are presented as mean ± SEM.

2.3.5 Statistical analysis of cell culture data.

Statistical analysis was performed using GraphPad Prism 6 (©2018 GraphPad Software, CA USA). Statistical differences were analysed using a Student's *t*-test or one-way ANOVA as appropriate, with *post-hoc* tests as indicated in the Figure legends.

2.4 Results

2.4.1 Alterations in expression of specific BMPRs based on stage and *MYCN*-status of NB.

We first sought to examine the expression levels of *BMPR2*, *BMPR1A* and *BMPR1B* in NB tumour samples. To do this, we probed NB transcriptome data derived from the publicly-available data sets, GSE45547 and GSE62564, using the R2 genomics and analysis visualisation platform (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi) with a rank based approach. We first examined the mean ranked expression (hereafter referred to as expression) of the type 2 BMP receptor, BMPR2, and found a significantly lower level of expression of *BMPR2* in stage 4 NB compared to stage 1 in both data sets (Figure. 2.1A, B). We also examined *BMPR2* expression by *MYCN* status, since *MYCN* amplification is the primary and most important prognostic marker of poor survival in NB (Huang and Weiss 2013). We also found that *BMPR2* expression was lower in *MYCN*-amplified NB than in non-*MYCN*-amplified NB in both data sets (Figure. 2.1C). We next examined the expression of the two type 1 BMPRs, BMPR1A and BMPR1B, which are required for signal transduction following ligand binding to BMPR2. In contrast to *BMPR2*, there was a significant increase in the expression of *BMPR1A* in stage 4 NB compared to stage 1 in both data sets (Figure. 2.1D, E). There was also an increase in *BMPR1A* expression in *MYCN*-amplified NB when compared to non-*MYCN*-amplified NB in both data sets (Figure. 2.1F). In contrast to *BMPR1A*, we found that the expression of *BMPR1B* was significantly decreased in stage 4 NB (Figure. 2.1G, H) and in *MYCN*-amplified NB samples (Figure. 2.1I). These data suggest that the expression of distinct BMPRs may be associated with different survival outcomes in NB patients.

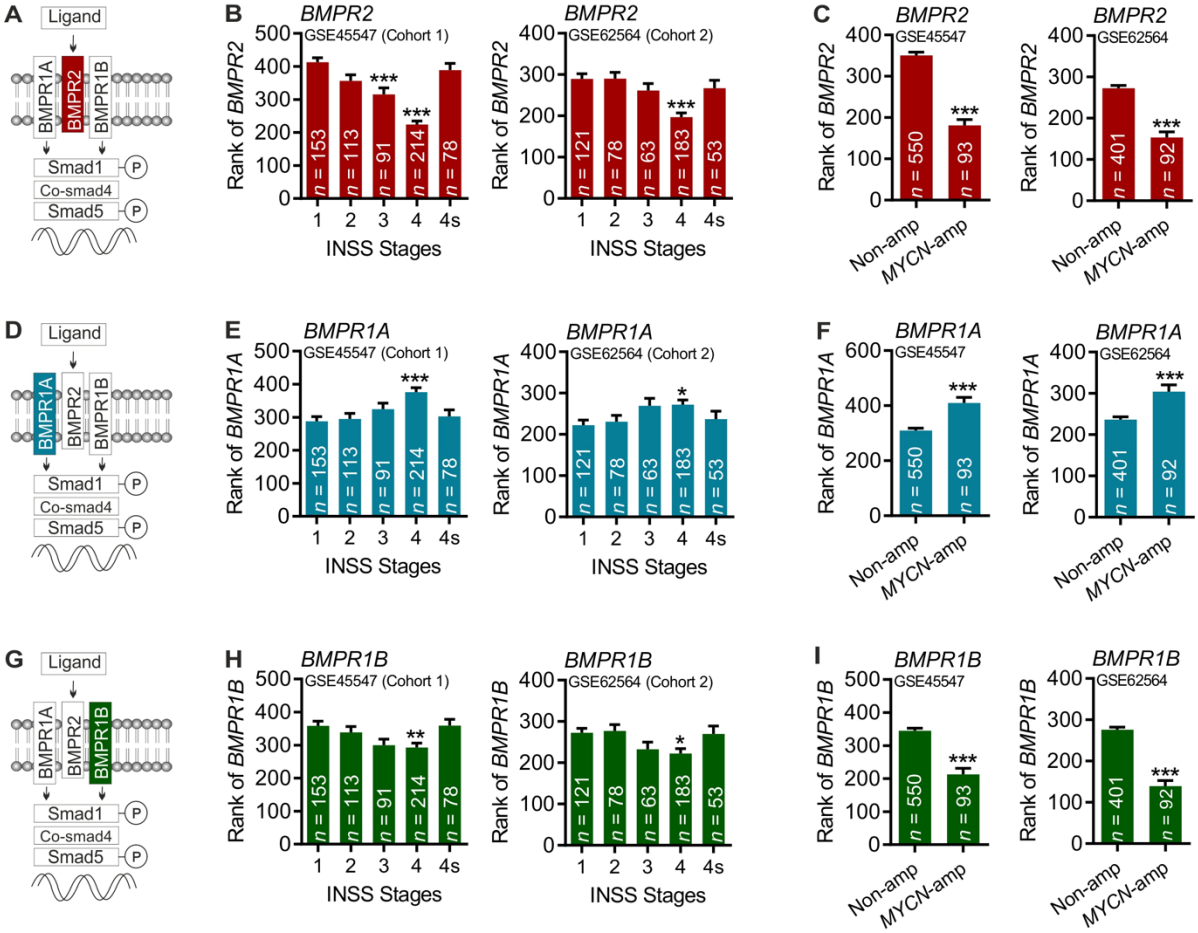


Figure 2.1 Alterations in expression of specific BMPRs based on stage and *MYCN*-status of NB.

Schema and graphs showing the mean ranked expression of (A-C) *BMPR2*, (D-F) *BMPR1A* and (G-I) *BMPR1B*, based on (B, E, H) INNS stage or (C, F, I) *MYCN* status, as indicated, in data set GSE45547 or GSE62564, as indicated (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to INNS stage 1 or non-*MYCN*-amplified ('Non-amp') NB; Kruskal-Wallis or Mann-Whitney test, as appropriate.

2.4.2 Associations between expression of BMPRs and survival probability in NB patients.

To investigate any associations between BMPR expression and survival probability, we constructed Kaplan-Meier survival plots of the *n* = 476 NB cases in the GSE45547 and the *n* = 498 NB cases in the GSE62564 data sets. To do this, the samples were divided into two groups that were classified as having 'high' or 'low' expression *of MYCN* (as a known control) or of *BMPR*, with the cut-off based on the median ranked gene expression value in each data set. As expected, patients with high *MYCN* expression had significantly lower probability of survival (Figure. 2.2A, B). Interestingly, patients with high *BMPR2* expression had a significantly greater survival probability than those with low *BMPR2* expression (Figure. 2.2C, D). In contrast, patients with high *BMPR1A* expression had a significantly lower survival probability than those with low *BMPR1A* expression (Figure. 2.2E, F), while those with high BMPR1B expression had a significantly higher survival probability than those with low BMPR1B expression (Figure. 2.2G, H). This suggests that BMPR1A and BMPR1B are associated with distinct sets of genes in NB cells.

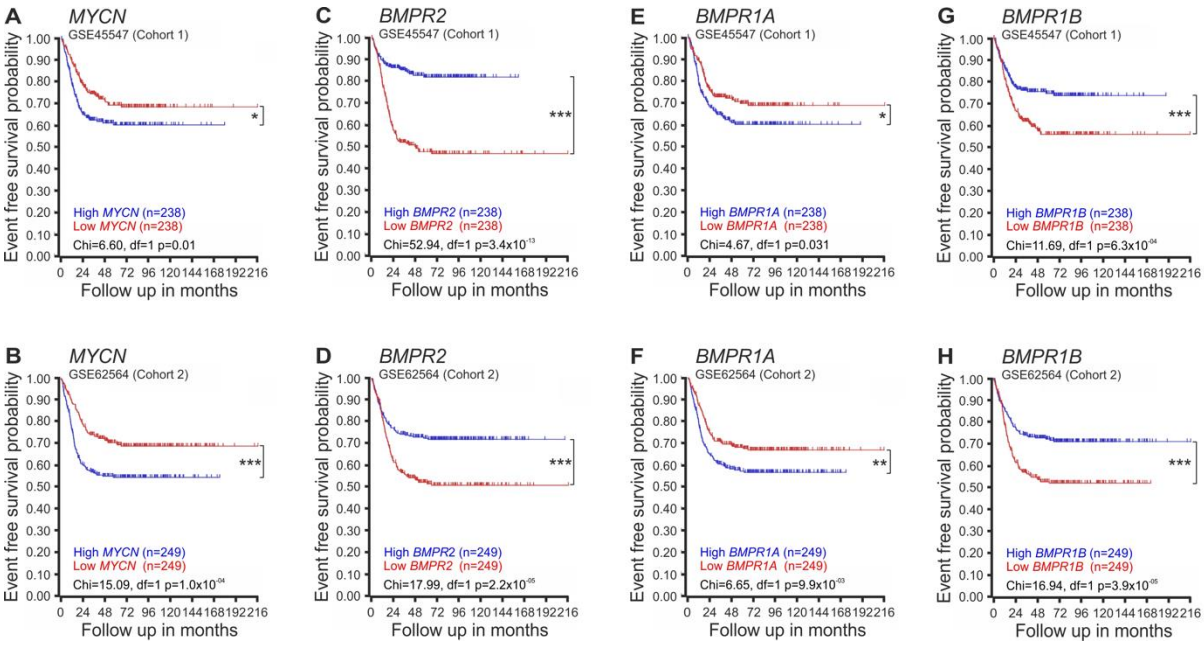


Figure 2.2 Associations between expression of BMPRs and survival probability in NB patients.

Kaplan-Meier plots showing the event-free survival probability of patients with 'high' or 'low' (A, B) *MYCN*, (C, D) *BMPR2*, (E, F) *BMPR1A*, or (G, H) *BMPR1B* expression divided

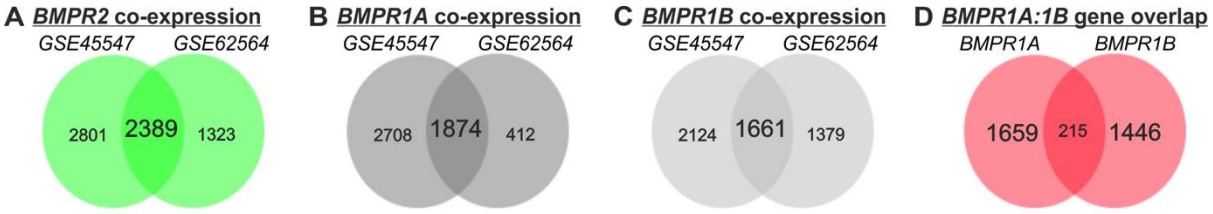
based on the median expression value of each gene in data set (A, C, E, G) GSE45547 (n = 476) or (B, D, F, H) GSE62564 (n = 498). *p < 0.05, **p < 0.01, *** p < 0.001; exact p values are indicated in black just above the x-axis on each graph.

2.4.3 Specific BMPRs display distinct gene co-expression profiles that are enriched in genes associated with molecular pathways linked to NB proliferation or differentiation.

To investigate the signalling pathways that may underlie the distinct survival probabilities associated with expression of different BMPRs, we used gene co-expression analysis to identify all genes that displayed positively correlated pattern of expression with distinct BMPRs in NB samples. The rationale for this approach is that correlated patterns of gene expression can reflect a functional association (Eisen, Spellman et al. 1998, Homouz and Kudlicki 2013). We identified from among all genes, those that had a significant positive correlation with the expression of *BMPR2* (n=2389; Figure. 2.3A), *BMPR1A* (n=1874; Figure. 2.3B) and *BMPR1B* (n=1661; Figure. 2.3C) in both data sets (GSE45547 and GSE62564). We also compared the genes that were co-expressed with BMPR1A and with BMPR1B, and found that these two receptors were associated with largely distinct groups of genes (Figure. 2.3D). We next performed a gene set enrichment analysis on these distinct gene lists for *BMPR2* (n=2389), *BMPR1A* (n=1659) and *BMPR1B* (n=1446). For those genes that were co-expressed with *BMPR2* or *BMPR1B*, GO (gene ontology) enrichment analysis revealed a statistically significant overrepresentation of genes within the multiple GO categories associated with neuritogenesis, including "dendrite development" (GO:0016358) and 'regulation of axogenesis' (GO:0050770) (Figure. 2.3E). These categories were not represented among BMPR1A co-expressed genes; in this case, GO enrichment analysis revealed a statistically significant overrepresentation of genes within the multiple GO categories associated with cell division and cell proliferation, including "Cell cycle DNA replication initiation" (GO:1902292) (Figure. 2.3E). Selected GO categories are shown in Figure. 2.3E, with the full list available in (Appendix Tables A8.1-8.3). We next sought to determine whether there was an overrepresentation in the list of BMPR co-expressed genes, of those belonging to specific Panther pathways. For *BMPR2* and *BMPR1B* co-expressed genes, there was a statistically significant overrepresentation of genes within Panther pathways associated with NB differentiation (Figure. 2.3F). In contrast, in BMPR1A co-expressed genes there was a statistically significant

overrepresentation of genes within Panther pathways linked to NB proliferation, such as 'DNA replication' and the 'p53 pathway' (Figure. 2.3F). Selected GO categories are shown in (Figure. 2.3E), with the full list available in (Appendix Tables A8.4-8.6). These data suggest that the reduction in *BMPR1B* and increase in *BMPR1A* in stage 4 NB, and in *MYCN*-amplified NB, may lead to a failure of differentiation and an increase in proliferation, respectively.

We next examined the coexpression patterns of *BMPR2* with *BMPR1A* and with *BMPR1B*, in stage 1 and stage 4 NB, and in non-*MYCN*-amplified or *MYCN*-amplified NB cases in the GSE45547 and GSE62564 data sets. To allow statistical analysis of any changes, we also added an additional data set, GSE120572 (Ackermann, Cartolano et al. 2018). The rationale for doing this was that in a range of diseases, normal coexpression patterns tend to change, and broken or strengthened correlations can be used as an index of functional misregulation (Southworth, Owen et al. 2009, Torkamani, Dean et al. 2010, Zhang, Gaiteri et al. 2013). We found that the correlation between *BMPR2* and *BMPR1A* was strengthened in *MYCN*-amplified NB (Figure. 2.3G, H). However, the correlation between *BMPR2* and *BMPR1B* was no longer significant in *MYCN*-amplified NB (Figure. 2.3G) and was significant reduced compared to non-*MYCN*-amplified NB (Figure. 2.3I). Collectively, these data suggest a BMPR2:BMPR1B functional misregulation in *MYCN*-amplified NB, and that this may contribute to alterations in neuritogenesis that have been reported in NB cells (Molenaar, Koster et al. 2012).



E

BMPR (# genes)	GO Term ID	Category name	E.R.	Raw *p value*	FDR *p value*
BMPR2 (*n*=2389)	GO: 0048813	Dendrite morphogenesis	3.59	1.56×10^{-06}	1.17×10^{-04}
	GO: 0048814	Regulation of dendrite morphogenesis	3.09	4.87×10^{-07}	4.14×10^{-05}
	GO: 0016358	Dendrite development	2.96	1.02×10^{-07}	1.00×10^{-05}
	GO: 0030516	Regulation of axon extension	2.26	7.42×10^{-04}	2.64×10^{-02}
	GO: 0050770	Regulation of axogenesis	2.08	4.10×10^{-05}	2.28×10^{-03}
BMPR1A (*n*=1659)	GO: 1902292	Cell cycle DNA replication initiation	12.83	1.50×10^{-03}	3.61×10^{-02}
	GO: 1902975	Mitotic DNA replication initation	12.83	1.50×10^{-03}	3.60×10^{-02}
	GO: 1902969	Mitotic DNA replication	8.17	1.59×10^{-04}	5.34×10^{-03}
	GO: 0030516	DNA strand elongation	7.58	4.59×10^{-07}	2.86×10^{-05}
	GO: 0000088	Mitotic prophase	6.84	1.34×10^{-04}	4.60×10^{-03}
BMPR1B (*n*=1446)	GO: 0048813	Sympathetic nervous system development	15.08	8.51×10^{-04}	4.90×10^{-02}
	GO: 0048813	Dendrite morphogenesis	3.59	1.56×10^{-06}	1.17×10^{-04}
	GO: 0016358	Dendrite development	2.75	1.01×10^{-04}	8.29×10^{-03}
	GO: 0061564	Axon development	2.33	7.68×10^{-09}	2.49×10^{-06}
	GO: 0050770	Regulation of axogenesis	2.22	3.11×10^{-04}	2.19×10^{-02}

F

BMPR (# genes)	Panther ID	Pathway name	E.R.	Raw *p value*	FDR *p value*
BMPR2 (*n*=2389)	P00025	Hedgehog signalling pathway	3.84	2.05×10^{-03}	2.40×10^{-02}
	P04385	Histamine H1 receptor mediated signalling	3.23	2.04×10^{-04}	3.55×10^{-03}
BMPR1A (*n*=1659)	P00017	DNA replication	4.88	4.85×10^{-05}	7.96×10^{-03}
	P00059	p53 pathway	2.62	4.44×10^{-04}	3.64×10^{-02}
BMPR1B (*n*=1446)	P00001	Adrenaline and Noradrenaline biosynthesis	4.08	1.09×10^{-03}	3.56×10^{-02}
	P05912	Dopamine receptor mediated signalling	2.99	1.13×10^{-03}	3.09×10^{-02}

G

Gene pair	Data set	non-Amp (*r*, *p* value)	Amp (*r*, *p* value)
BMPR2:BMPR1A	GSE45547	$0.280; 2.37 \times 10^{-11}$	$0.490; 6.19 \times 10^{-07}$
	GSE62564	$0.388; 7.60 \times 10^{-16}$	$0.529; 5.84 \times 10^{-08}$
	GSE120572	$0.183; 1.20 \times 10^{-03}$	$0.335; 1.96 \times 10^{-03}$
BMPR2:BMPR1B	GSE45547	$0.440; 1.78 \times 10^{-27}$	**0.151; 0.149**
	GSE62564	$0.332; 9.21 \times 10^{-12}$	**0.141; 0.181**
	GSE120572	$0.321; 7.21 \times 10^{-19}$	**0.155; 0.163**

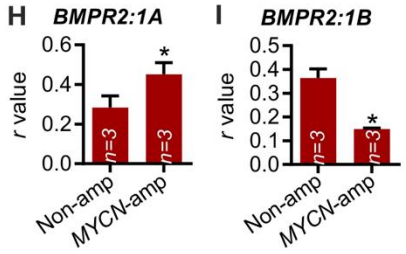


Figure 2.3 Specific BMPRs display distinct gene co-expression profiles enriched in genes associated with molecular pathways linked to NB proliferation or differentiation.

Venn diagrams generated using GeneVenn, showing the overlap in genes that display a Bonferroni-corrected significant positive co-expression pattern with (A) *BMPR2*, (B) *BMPR1A* or (C) *BMPR1B* in GSE45547 and GSE62564, as indicated. (D) Venn diagram of the overlap in *BMPR1A* and *BMPR1B* co-expressed genes, showing that the two receptors are associated with largely distinct groups of co-expressed genes. (E) Gene ontology (GO) and (F) Panther pathway enrichment analysis of the gene lists that were co-expressed with *BMPR2* (*n*=2389), *BMPR1A* (*n*=1659) and *BMPR1B* (*n*=1446) in both GSE45547 and

GSE62564 data sets. (G) Table showing the correlation (*r*) and associated *p* values between *BMPR2* and *BMPR1A* or *BMPR1A* in non-*MYCN*-amplified (non-Amp) or in *MYCN*-amplified (Amp) NB cases in GSE45547, GSE62564 and GSE85047, as indicated. Graphs showing the mean ± SEM of the *r* value for (H) *BMPR2:BMPR1A* and (I) *BMPR2:BMPR1B* * $p < 0.05$, vs. non-Amp; Student's *t*-test; GO and Panther pathway analysis was carried out using tools available at www.geneontology.org. Gene correlation analysis was carried out using the R2 genomics analysis and visualisation platform.

2.4.4 BMPR1B signalling promotes neurite growth in *MYCN*-amplified NB cells.

BMPR1A co-expressed genes were found to be enriched in pathways linked to proliferation, and *BMPR1B* co-expressed genes to be enriched in those linked to neurite growth. This suggests a predicted model in which the expression of BMPR1A may be decreased, while the expression of BMPR1B may be increased during sympathetic neurite growth (Figure. 2.4A). We tested this model by treating cultured P0 mouse SCG sympathetic neurons with NGF, a potent inducer of neurite growth in sympathetic neurons (O'Keeffe, Gutierrez et al. 2008). Cultures were also treated with 25 μM of pan-caspase inhibitor Boc–D–FMK, a well-established approach to prevent apoptosis at low concentrations of NGF (Kisiswa, Osorio et al. 2013, Howard, McWilliams et al. 2019). Treatment with NGF at concentrations of 1 or 10 ng/ml resulted in a significant decrease in the expression of *Bmpr1a* (Figure. 2.4B). In contrast, these concentrations of NGF resulted in significant increases in *Bmpr1b* expression (Figure. 2.4C). This supports the hypothesis that BMPRs are required for neuritogenesis in NB cells. To test this directly we use three complementary approaches. Firstly, *MYCN*-amplified SK-N-BE(2) were transfected with a plasmid expressing a dominant negative (dn) form of BMPR1B (Figure. 2.4D). Secondly, these cells were treated with K02288, a BMP signaling inhibitor (Figure. 2.4E), and neurite growth was measured at 72 h. Both dnBMPR1B (Figure. 2.4E) and K02288 (Figure. 2.4F) treatment led to significant reductions in neurite growth. Thirdly, treatment of SK-N-BE(2) cells with the BMP ligand GDF5, which signals via BMPR1B but not BMPR1A (Nishitoh, Ichijo et al. 1996, Nickel, Kotzsch et al. 2005), led to significant increases in neurite growth at 72 h (Figure. 2.4F). Moreover, GDF5 treatment for 72h also reduced the number of colonies (Figure. 2.4G) and the colony area (Figure. 2.4H) in SK-N-BE(2) cultures. Collectively, these data show that BMPR1B signalling is required to promote neuritogenesis in *MYCN*-amplified NB cells.

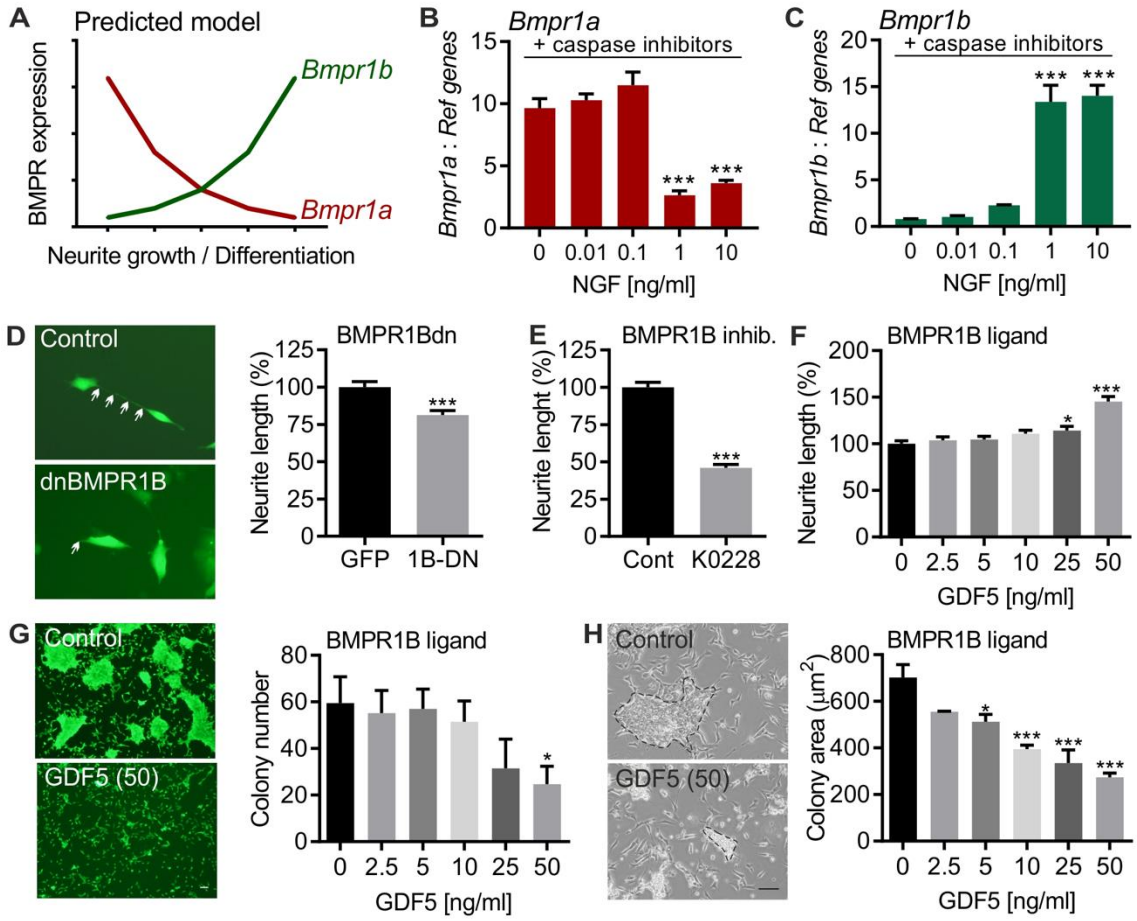


Figure 2.4 BMPR1B signalling promotes neurite growth in *MYCN*-amplified NB cells.

(A) Schema of a predicted model based on the NB data showing the predicted changes in type1 BMP receptor expression during sympathetic differentiation. Expression of transcripts for (B) *Bmpr1a* and (C) *Bmpr1b* in P1 mouse sympathetic neurons cultured with NGF at the indicated concentrations for 24 h to promote differentiation. Data are expressed relative to the levels of the geometric mean of three reference mRNAs: *Gapdh, Sdha* and *Hprt1*. Cultures were also treated with 25 µM of the pan-caspase inhibitor Boc-D-FMK. Data are mean ± SEM from $n = 4$ separate cultures. *** $p < 0.001$ vs. control (0 ng/ml NGF); one-way ANOVA with *post-hoc* Dunnett's test. Representative images and graph of neurite length of SK-N-BE(2) at 72 h post transfection with (D) a control plasmid (GFP) or a plasmid expressing dominant negative BMPR1B (dnBMPR1B), or (E) following treatment with 0.5 µM K02288, a BMPR1B inhibitor. (F) Graph of neurite length of SK-N-BE(2) at 72 h post treatment with GDF5 at the indicated concentrations. * $p < 0.05$, *** $p < 0.001$ vs. control (0 ng/ml GDF5);

Student's *t*-test for two groups or alternatively one-way ANOVA with *post-hoc* Dunnett's test, as appropriate. Data are mean ± SEM of (E) 240 or (F) 180 individual cells per group from $n = 3$ separate experiments. Representative images and graphs showing (G) the number of colonies and (H) colony area in SK-N-BE(2) cultures at 72 h post treatment GDF5 at the indicated concentrations. Data are mean ± SEM from $n = 3$ separate experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control (0 ng/ml GDF5); one-way ANOVA with *post-hoc* Dunnett's test.

2.5 Discussion

Our analysis of the expression patterns of specific BMPRs in NB patient data sets showed strong correlations with disease stage and type. Specifically, the expression levels of both BMPR1B and BMPR2 were significantly lower at stage 4 NB, which is associated with a poor survival outcome, than at stage 1 NB, which has a better prognosis. The expression of BMPR1B and BMPR2 was also found to be lower in *MYCN*-amplified than in non-*MYCN*-amplified NB. *MYCN* amplification is associated with poor survival in NB (Huang and Weiss 2013), suggesting that reduced BMPR1B and BMPR2 expression may be a factor underlying poor survival outcomes for NB patients. In contrast, the expression of BMPR1A was significantly higher in stage 4 than in stage 1 NB, and was also higher in *MYCN*-amplified than non-*MYCN*-amplified NB, implying that this BMP receptor plays an opposing role to that of BMPR1B and BMPR2 on NB cell fate. In agreement, our analysis of associations between the expression of distinct BMPRs and survival probability in patient data sets showed that there are strong correlations between low BMPR2, low BMPR1B, high BMPR1A, as well as high *MYCN*, with poor survival probability in NB patients.

Reduced expression of BMPR1B has previously been found to be correlated with poor prognosis in breast cancer (Bokobza, Ye et al. 2009). That study reported lower levels of BMPR1B expression in breast tumour samples compared to normal breast tissue samples, as well as reduced BMPR1B mRNA in samples taken from patients with predicted poor prognosis. Furthermore, downregulation of BMPR1B resulted in increased proliferation of breast cancer cells *in vitro* (Bokobza et al., 2009). Our finding that reduced BMPR2 expression was correlated with poor survival outcome is also consistent with a previous study, which reported a link between reduced BMPR2 expression and NB (Cui, Yang et al.

2017). BMPR2 expression was significantly downregulated in NB tissue samples, particularly in high-grade NB, and was inversely related to the expression of markers of NB differentiation (Cui, Yang et al. 2017). Disruption of BMPR2 has also been found to promote metastasis of mammary tumours, suggesting that BMPR2 has tumour-suppressive function in mammary epithelial cells (Owens, Pickup et al. 2012). A study on mammary cell tumours in mice found that conditional knockout of the BMPR1A receptor delayed the onset of tumour formation, and extended cell survival (Pickup, Hover et al. 2015). That study also analysed human data sets and found a correlation between high BMPR1A gene expression and decreased survival, regardless of the molecular breast cancer subtype, and concluded that BMPR1A is a tumour promoter in human breast cancer (Pickup, Hover et al. 2015). Interestingly, a recent paper identified parallels between mammary cell tumorigenesis and the NC lineage, by showing that SOX10 expression correlates with invasiveness and can elicit NC-like features in mammary tumours (Dravis, Chung et al. 2018). Along with our data, all of these findings suggest that reduced expression of BMPR1B and BMPR2, and / or increased expression of BMPR1A, may play a role in the determination of a proliferative cell fate, at least in some types of tumours.

Our analysis of the NB patient data sets showed that the expression of distinct BMPRs are associated with different survival outcomes in NB patients and suggest that the signalling pathways activated by these receptors may be involved in determining the fate of NB cells. We then proceeded to interrogate the signalling pathways that are associated with survival probability in these cells. Our gene co-expression analysis showed that high expression of BMPR1A was associated with proliferative cellular events, such as DNA replication and the p53 pathway. In contrast, high expression of BMPR1B and BMPR2 was associated with developmental cellular events, such as dendrite morphogenesis, axon development and sympathetic nervous system development. These data suggest that the reductions in BMPR1B and BMPR2, and the increase in BMPR1A, that are associated with both stage 4 NB and *MYCN*-amplified NB may combine to drive cells towards a fate involving failure of differentiation and promotion of proliferation. The balance between the expression profiles of BMPR1B and BMPR2 on one side, and BMPR1A and *MYCN* on the other, may be a important factor in deciding cell fate and state in NB.

Our analysis of the co-expression patterns of BMPR2 with BMPR1A and with BMPR1B, in three patient data sets, showed that in *MYCN*-amplified NB the correlation between BMPR2 and BMPR1A was strengthened, whereas the correlation between BMPR2 and BMPR1B expression was lost. This suggests a functional misregulation of BMPR2:BMPR1B binding in *MYCN*-amplified NB cells. It is possible that *MYCN* amplification may lead to defects in the formation of the BMPR2:BMPR1B receptor complex, which may in turn result in dysfunction of the downstream signalling pathways that are normally activated when BMP ligands bind to these receptors. It is known that there are defects in neuritogenesis in NB cells (Molenaar, Koster et al. 2012). Since BMP signalling through the BMPRs plays a critical role in neuritogenesis in the developing nervous system (for review see Hegarty et al 2013a), it is possible that cell autonomous alterations in BMPR subtype expression within NB cells cause these cells to undergo oncogenic signalling responses to the BMP morphogens that would normally induce their neuronal differentiation.

To test the hypothesis that BMPR1B signalling promotes neurite growth in *MYCN*-amplified NB cells, we used two experimental approaches to block BMP-BMPR1B signalling, both of which resulted in significant reductions in neurite growth. In support of this, treatment of the *MYCN*-amplified SK-N-BE(2) NB cell line with the BMP family member GDF5, which signals through BMPR1B but not BMPR1a, promoted neurite growth. Thus our data support the hypothesis that BMPR1B signalling is required to promote neuritogenesis in *MYCN*-amplified NB cells.

In summary, this study has shown that individual BMPRs have distinct expression patterns that are associated with different signalling pathways and survival outcomes in NB cells. This suggests that BMP ligands acting at distinct BMPRs play important roles in NB cell development and differentiation. In these cells, BMPR1A expression was associated with cell proliferation signalling pathways, whereas expression of BMPR1B and BMPR2 was associated with cellular differentiation and neurite growth. Furthermore, in NB patient data sets, poor survival outcomes were strongly correlated with reductions in BMPR1B and BMPR2 expression, and with high levels of BMPR1A expression. Finally, *MYCN*-amplification appears to be associated with BMPR2 binding preferentially to BMPR1A, rather than to BMPR1B, resulting in a shift in intracellular signalling responses towards cell proliferation. By examining changes in signalling pathway components in different tumor cell types and stages, the oncogenic mechanisms underlying cancer progression can be elucidated.

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Chapter 3

Chapter 3. Small molecule activators of BMP-Smad signaling in the differentiation of human neuroblastoma cells

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3.1 Abstract

NB is a developmental paediatric cancer that arise from peripheral sympathetic nervous system and it is the most common extracranial tumor in childhood. In 20 % of high-risk cases *MYCN* amplification was detected as a hallmark. Relapse survival rate of stage 4 patients with *MYCN* amplification is only 4%, based on INRG. Chromothripsis, a local shredding of chromosomes, and neuritogenesis gene alterations, were reported in primary tumor of high-risk NB. Recently, a screen of 5,600 small molecules identified the first small molecule activators of BMP-Smad signaling. Two chalcones, ISLQ and 4'-hydroxychalcone were shown to increase Smad1 and Smad5 phosphorylation in a dose-dependent manner in a human cervical carcinoma cell line. Therefore, herein we investigated the ability of these chalcones, to promote neurite growth, and their mechanism of action, in two NB cell lines: SH-SY5Y and the *MYCN*-amplified SK-N-BE(2). We found that ISLQ and 4HC are non-toxic at low concentrations for NB cells at 3DIV. Systematic treatment of these chalcones significantly promotes neurite growth in tested NB cells. To further examine this neurite growth mechanism, cells were transfected with a reporter construct in which Smad transcriptional activity is accompanied with GFP expression. ISLQ and 4HC significantly activate BMP-Smad signaling in different time points. These results were comparable to GDF5 treatment which induced a significant increase in Smad signaling activation in both transfected cells and cell lysates measured with ELISA. Furthermore, blocking BMP-Smad signaling by K02288, a small molecule inhibitor of BMP signaling, showed significant reduction in neurite growth in cells treated with ISLQ or 4HC compared to the control (untreated) cells. In our previous work we demonstrated that treatment of GDF5 reduces cell mass in SK-N-BE(2) cells. Similarly, ISLQ and 4HC treatment showed similar effect to that of GDF5 in shrinking cell mass in the SK-N-BE(2) *MYCN*-amplified NB cell line. Collectively, these findings demonstrate that ISLQ and 4HC can promote neuronal differentiation in NB cells by activation of BMP-Smad signaling.

3.2 Introduction

NB is a paediatric cancer that arises from peripheral sympathetic nervous system originating from NC development (Hoehner, Gestblom et al. 1996). Depending on age, extent of disease, histology and cytogenetic abnormalities NB heterogeneous tumors are classified into three risk groups (low, intermediate and high). Around 20 % of the high risk group metastatic tumors with amplification of the *MYCN* oncogene (Molenaar, Koster et al. 2012). Overall survival rate of high risk NB is < 50 % at 5 years with most relapses (80%) occurring within 2 years of diagnosis (Basta, Halliday et al. 2016). *MYCN* amplification is the hallmark of high-risk NB that features earlier tumor relapse and development of chemotherapy resistance (Kushner, Modak et al. 2014). Survival after relapses was reported as a rare condition as relapsed stage 4 patients in the INRG database is only 4% for *MYCN* amplified NB (Herd, Basta et al. 2019) (Garaventa, Parodi et al. 2009, London, Castel et al. 2011).

Mainly, high risk NB therapy full into three phases, induction, consolidation, and post-consolidation therapy (maintenance phase) (Armideo, Callahan et al. 2017). One main known strategies cancer cells use to overcome treatments is the persistence of cancer stem cells (CSCs). NB CSCs are a small cell population of multipotent undifferentiated cells that survive therapeutic toxicity of all chemotherapy phases and facilitating the disease progression (Bahmad, Chamaa et al. 2019).

A recent study that performed a whole-genome paired-end sequencing of 87 untreated primary NB tumors of all stages has revealed that chromothripsis, a local shredding of chromosomes, and neuritogenesis gene alterations, frequently occurs in high-risk tumors (Molenaar, Koster et al. 2012).

High-risk NB maintenance therapy includes; differentiation inducers such as retinoic acid (Kohler, Imeson et al. 2000), that are used to clear any minimal residual that may survive the induction therapy (Armideo, Callahan et al. 2017). Nowadays, the retinoid drug 13-cis-retinoic acid (13-cisRA; isotretinoin) is a well-known agent of high-risk NB treatment, which is used as maintenance treatment compinged with antibody therapy in the US and Europe (Veal, Errington et al. 2013). However, it has been reported that 13-cisRA standard intake doses do not reflect its real concentrations in the plasma in different patients (Veal, Cole et al. 2007). In addition, 4-oxo-13-cisRA the metabolite of 13-cisRA has been shown to accumulate beyond the original RA concentrations of treatment in approximately 70% of patients studied. Furthermore, it also have been shown to be less active than the parent RA

in various tumour cell lines which lead to a diminished efficacy of 13-cisRA (van der Leede, van den Brink et al. 1997) (Kim, Yoo et al. 2002). These findings all confirm the critical need for finding novel therapies that not only induces cytotoxicity by selectively targeting *MYCN* amplification but also promote and maintain neuritogenesis in survival cells.

In our recent work we reported that BMPRs are associated with different survival outcomes in NB. Lower BMPR2 and BMPR1B, and higher BMPR1A, expression is observed in stage 4 and in *MYCN*-amplified NB. In addition, inducing differentiation with GDF5 which signals via BMPR1B, showed that BMPR1B signaling is required to promote neuritogenesis in *MYCN*-amplified SK-N-BE(2) NB cells (Alshangiti, Wyatt et al. 2020).

Recently, a screen of 5,600 small molecules identified the first small molecules activators of BMP-Smad signaling (Vrijens, Lin et al. 2013). The two chalcones, ISLQ and 4HC were shown to increase Smad1 and Smad5 phosphorylation in a dose-dependent manner in a human cervical carcinoma cell line (Vrijens, Lin et al. 2013). Chalcones a subclass of flavonoids, have been studied and reported to possess many beneficial biological effects including anti-inflammatory, antimicrobial, antifungal, antioxidant, cytotoxic, antitumor and chemopreventive activities (Rozmer and Perjési 2016). Induction of differentiation has been reported among the functions of chalcones and of their derivatives in lymphoblastic leukemia and human breast cancer cells (dos Santos, Bertholin Anselmo et al. 2019). However, chalcones have not been shown to promote human NB differentiation. This work sheds light on the potential anticancer properties of chalcones, a relatively novel approach for NB therapy. We found that the dietary flavonoid ISLQ induces cell death in IMR-32 and SK-NBE(2), *MYCN* amplified NB cells, through an elevation of reactive oxygen species (ROS) that was blocked by necrostatin-1, a necroptosis inhibitor (Alshangiti, Togher et al. 2019). Moreover, we also found that *MYCN*-amplified NB cells SK-N-BE(2) and IMR-32 are more sensitive to another chalcone, 4HC than are other cell types (Alshangiti, Tuboly et al. 2019). Here we investigate the effects of low doses of ISLQ and 4HC on NB cells morphological differentiation and neurite growth, and the molecular mechanisms mediating the actions of these chalcone small molecules.

3.3 Materials and Methods

3.3.1 SH-SY5Y and SK-N-BE(2) cell culture.

SH-SY5Y cells (ATTC) were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham's supplemented with 100 nM L-glutamine, 1% penicillin-streptomycin, and 10% FBS. SK-N-BE(2) cells (ATTC) were cultured in Minimum Essential Medium supplemented with 100n M L-glutamine, 1% penicillin-streptomycin, 1% (1:1) non-essential amino acid solution: Ham's F-12, and 15% fetal bovine serum (FBS). All cells were cultured at 37°C in a humidified atmosphere with 5% CO2.

3.3.2 Immunocytochemistry.

SH-SY5Y and SK-N-BE(2) were plated overnight and fixed with 4% PFA (PFA; Sigma) for 15 min at RT. Then, they were washed for 5 min in 0.02% TritonX-100 (Sigma) in 10X phosphate buffered saline (PBS-T) for three times. Fixed cells were blocked in 5% of bovine serum albumin (BSA) for 1h at TR. Cells were incubated overnight at 4Cº in (1:200) primary antibodies of BMPR1A, BMPR1B, BMPR2, (R&D) or P-Smad1/5/8 (Cell signaling). Next, cells were washed again with PBS-T three times for 5 min each before they were incubated in Alex Flour (488 or 594) conjugated secondary antibody (1:1000) in the dark at RT for 2 h. All antibodies used were diluted in 1% BSA in 0.02% PBS-T. BMPs signaling component expression were imaged with an Olympus IX71 inverted microscope fitted with an Olympus DP70 camera.

3.3.3 Cell viability and neurite length.

2 X 10^4 of SH-SY5Y, and 1 X 10^5 of SK-N-BE(2) cells per well were plated overnight in 24 well plate and then treated with indicated concentration of ISLQ and 4HC or their equivalent vehicle, DMSO and MEOH respectively for 3DIV. Cell viability was determined using 1mg/ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 3.5 h at 37°C MTT solution was removed and the crystals were lysed in DMSO. Spectrophotometric absorbance was measured at 595 nm (with a reference wavelength of 620 nm) and raw absorbances were expressed as a percentage of the control. For neurites length, colony measurements and counting, cells were treated by the indicated doses of ISLQ and 4HC, or pre-treated for 30min with 0.5µM of K02288, a highly selective 2-aminopyridine-based BMP signaling inhibitor, for three days before there were labelled with the vital fluorescent dye Calcein-AM (1:500; Invitrogen) for cell skeleton. Four random, non-overlapping fields were captured per well each experiment (in three wells per group for each n) using an Olympus IX71 inverted microscope fitted with an Olympus DP70 camera.

Images were blinded before being measured using ImageJ software and normalised to untreated cells. These measurements have been done in both drugs treated groups and the vehicles. Colony area and number were presented directly with micrometer measurements.

3.3.4 Transfection

For Smad activation GFP reporter assay kit has been used (Cignal) according to the manufacturer's instructions. This Kit consist of a reporter, which is an inducible transcription factor responsive GFP reporter i.e. Smad activation is accompanied with GFP expression. Cells were plated and incubated 24 h before they were transfected with SBE-GFP reporter (R). After another 24h cells were treated with indicated concentrations of ISLQ, 4HC for 3DIV. Cells were imaged using an Olympus IX71 inverted microscope every day before Smad activation intensity was measured using ImageJ and presented as a percentage of the control (untreated).

3.3.5 Phospho-SMAD 1/5/8 measured by enzyme-linked immunosorbent assay (ELISA)

2 X 10^4 of SK-N-BE(2) cells per well were plated in 96 well plate overnight. Then, they were treated with 50ng/ml of GDF5 for (30- 60 min or 24h). BMP-Smad activation of GDF5 in SH-SY5Y cells have been confirmed before in our lab (Hegarty, Sullivan et al. 2013). At the end point phospho-Smad1/5/8 was measured in cells using SMAD1 (Phospho) [pS463/pS465] Multispecies Instant One™ ELISA Kit (Invitrogen). The media was removed and cells were washed quickly with 10mM sterilised PBS before 100µl of 1 X cells lysis mix was added. Then the plate was shacked on plate shaker at 300 rpm for 10 min at RT before 50 µl per well of each of cell lysate and pre-prepared antibodies cocktail were moved into special plate provided with the kit. The plate was sealed and incubated in the dark for 1h at RT on an microplate shaker (300rpm) before they were washed three times with washing buffer. Then, 100µl for detecting reagent was added before plate was incubated on microplate shaker for 10-30min. When the colour developed stop solution was added before it was read at spectrophotometric plate reader at 450 nm. All results were presented as a percentage of the control (untreated cells).

3.3.6 Statistical Analysis

To analyse significant differences, one-way ANOVA with a post-hoc Tuckey's test, Dunnett, Fisher's LSD test, or Student T-test was carried out where indicated. All data are presented as mean ± SEM and all experiments were repeated three times. Differences were deemed significant when $p < 0.05$.

3.4 Results

3.4.1. SH-SY5Y and SK-N-BE(2) express BMP signaling components.

Induction of cell differentiation is being well established of therapy for high-risk NB patients. This therapy is the third phase with the first induction chemotherapy with multiple agents and autologous bone morrow transplantation, and the second being consolidation chemotherapy and/or subsequent surgical resection and radiation (Armideo, Callahan et al. 2017). To apply such an approach in NB cells we first examined the expression of BMP signaling pathway components in both cell lines. We found that both SH-SY5Y (Figure 3.1A) and SK-N-BE(2) (Figure 3.1B) are expressing BMPR and Smad 1/5/8 compared to the negative control (Figure 3.1).

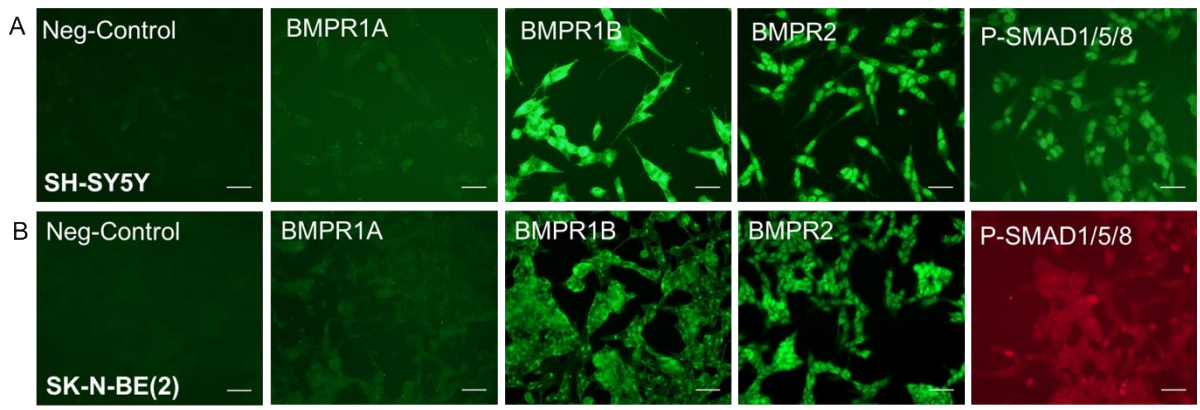


Figure 3.1 SH-SY5Y and SK-N-BE(2) expressing BMPs signaling component.

photomicrographs of (A) SH-SY5Y and (B) SK-N-BE(2) cells immunocytochemically stained for the BMPRs; BMPR1A, BMPR1B, and BMPR2, or Smad 1/5/8. Scale bar = 50µm.

3.4.2 ISLQ and 4HC are non-toxic in low concentrations for NB cells at 3DIV.

In our previous work we demonstrated cell differentiation in NB SH-SY5Y cells through the activation of BMP-Smad signaling by BMP2 and GDF5 ligands (Hegarty, Sullivan et al. 2013). However, the therapeutic application of recombinant BMP ligands in NB is limited by the fact that systemic administration of supra-physiological levels of BMPs induces severe side effects including heterotopic bone formation (Gottfried and Dailey 2008). For this reason, small molecules that activate BMP-Smad signaling activators may be potential therapeutic agents particular if they can be used at lower levels to mitigate the undesirable side effects of systemic BMP administration. Recently, a screen of 5,600 small molecules identified the first small molecules activators of BMP-Smad signaling (Vrijens, Lin et al. 2013). The two chalcones, ISLQ and 4HC were shown to increase Smad1 and Smad5 phosphorylation in a dose-dependent manner in a human cervical carcinoma cell line (Vrijens, Lin et al. 2013). As SH-SY5Y cells express wild-type p53 and are not *MYCN* amplified, we carried out additional experiments to compare the effects of ISLQ and 4HC in SK-N-BE(2) cells (*MYCN* amplified, p53 mutant), given that *MYCN* amplification is the main and most important prognostic marker of poor survival in NB (Huang and Weiss 2013). To do this we first treated NB cells with relatively low concentrations of ISLQ or 4HC (0-5µM for SH-SY5Y and 0-2.5 µM for SK-N-BE(2)) for 3 days in vitro (DIV) (Figure. 3.2) to identify any dose-dependent effects. This range of doses was limited based on our previous work that reported ISLQ (Alshangiti, Togher et al. 2019) and 4HC (Alshangiti, Tuboly et al. 2019) cell toxicity for NB cells. We found that ISLQ causes a reduction in cell proliferation rate at 5µM ($p < 0.01$) on SH-SY5Y as they start to show some cell death (Figure. 3.2A, E), and at 2.5µM ($p < 0.001$) on SK-N-BE(2), (Figure. 3.2C, E). However, NB were more sensitive to 4HC as significant reduction in cell proliferation at 2.5 µM ($p < 0.001$) for SH-SY5Y (Figure 3.2B, E), and at 0.5 µM ($p < 0.01$) for SK-N-BE(2). Of note, when we examined cell morphology that cell death was mainly appear on cell mass like aggregations (colonies) on SK-N-BE(2) rather than polar individual cells (Figure. 3.2E). These data suggesting that ISLQ and 4HC have significantly reduced NB cell proliferation at low doses.

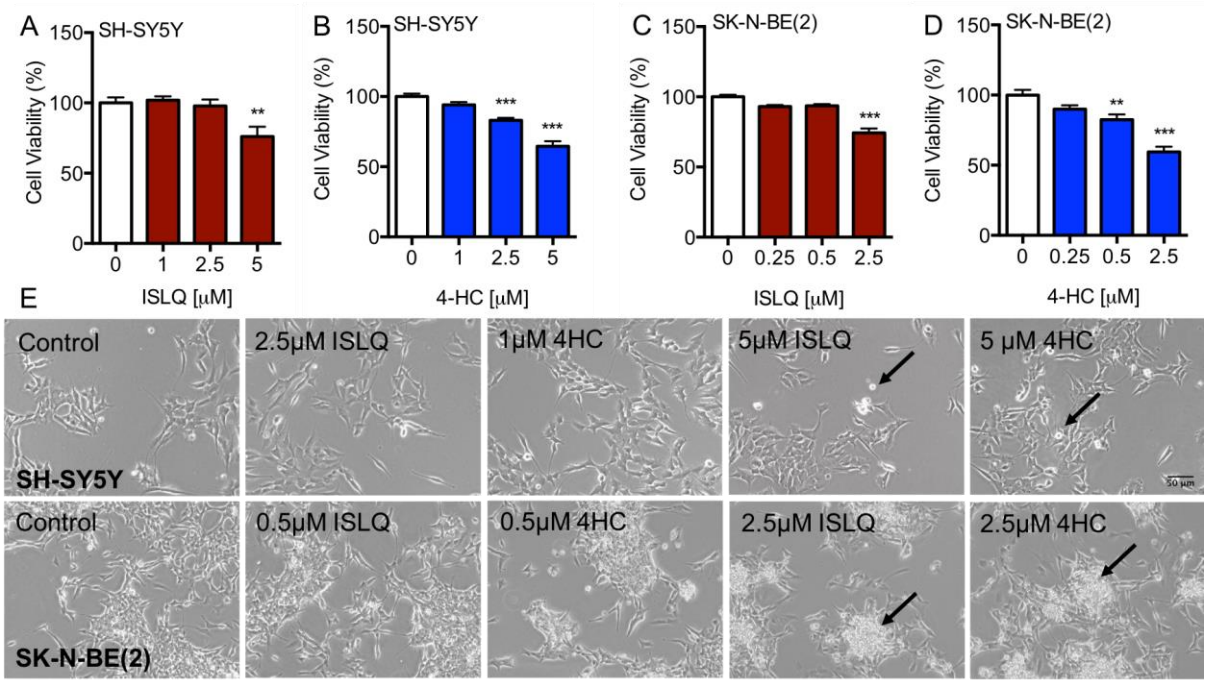


Figure 3.2 : ISLQ and 4HC are non-toxic at low concentration for NB cells in 3DIV. Cell viability measured by MTT assay on (A, B) SH-SY5Y (C, D) SK-N-BE(2), treated with low concentrations of ISLQ or 4HC for 3DIV. (E) Representative phase-contrast micrographs. Black arrows indicate dead cells. Scale bar = 50µm All data are mean ± SEM; *n*=3 independent experiments; ** ***P*** < 0.01, *** ***P*** < 0.001 vs control; one-way ANOVA with Tukey's test.

3.4.3 ISLQ and 4HC promote neurite growth in NB cells in 3DIV.

To further investigate and show whether the reduction of cell proliferation levels is due to cells differentiation or not we treated NB with the same concentration of chalcones used in the MTT experiment, and then we measured neurite growth compared to the control. We found that 2.5 µM ISLQ significantly promoted neurite growth ($p < 0.001$) on SH-SY5Y (Figure. 3.3A, E), and at both 0.25 µM ($p < 0.05$), and 0.5 µM ($p < 0.001$) on SK-N-B(2) (Figure. 3.3C, E). Moreover, 1 µM 4HC significantly promoted neurite growth as well ($p < 0.001$) on SH-SY5Y (Figure. 3B, E), and at both 0.25 µM ($p < 0.01$), and 0.5 µM ($p < 0.001$) on SK-N-B(2) (Figure. 3.3D, E). These data suggest the observed ISLQ and 4HC induced reduction in cell proliferation in NB is due to cell differentiation rather than cell death. Equivalent vehicles used were DMSO for ISLQ and MEOH for 4HC. We found no effect on any of either on neurite growth at the concentrations used (Figure 3.7).

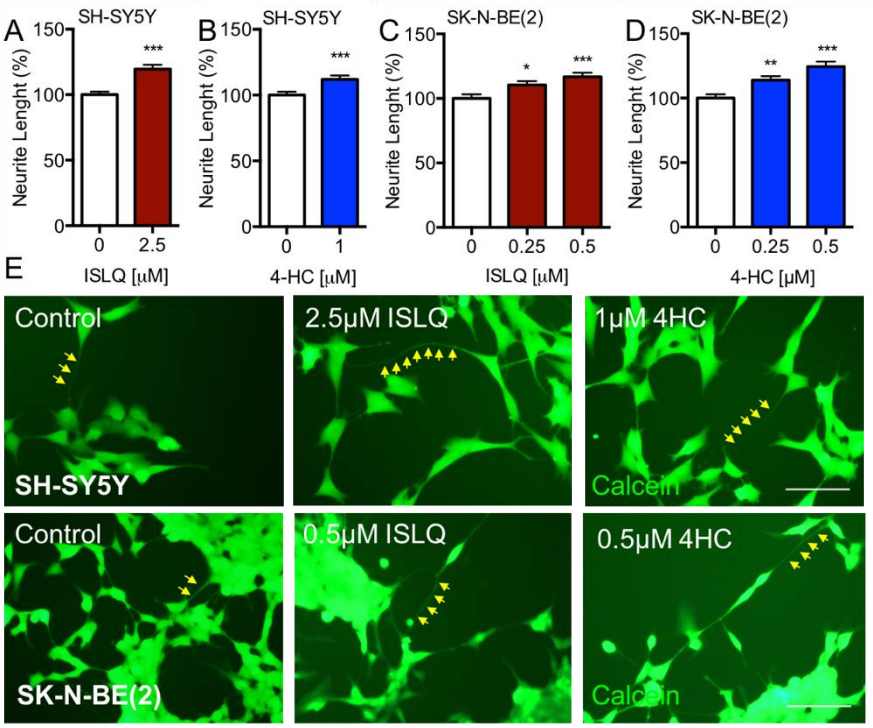


Figure 3.3 : ISLQ and 4HC promote neurites growth in NB cells in 3DIV.

Neurite growth graphs of (A, B) SH-SY5Y treated with 2.5 µM of ISLQ or 1 µM 4HC for 3DIV. All data are mean ± SEM; of 180 cells each group from *n*=3 independent experiments; ***P< 0.001 vs control; Student's t-test. (C, D) SK-N-BE(2), treated with 0.25, 0.5 µM of ISLQ and 4HC for 3DIV. (E) Representative micrographs stained with Calcein. Yellow arrows indicating cell neurites. Scale bar = 50µm. All data are mean ± SEM; of 180 cells each group from *n*=3 independent experiments; ***P*** < 0.05, ****P*** < 0.01, ***** ***P*** < 0.001 vs control; one-way ANOVA with Tukey's test.

3.4.4 ISLQ and 4HC activate BMP-Smad signaling in NB cells.

To determine the precise mechanism of ISLQ and 4HC induced neurite growth, and whether it is through BMP-Smad signaling we measured this signaling activation on NB cells treated with ISLQ and 4HC. For this purpose, we transfected SH-SY5Y and SK-NBE(2) with small Smad binding elements plasmids with GFP Reporter before they were treated with 2.5 µM ISLQ or 1 µM 4HC for SH-SY5Y, and 0.5 µM of either ISLQ or 4HC, and 50 ng /ml GDF5

for SK-N-BE(2). Continuous monitoring for cells every 24 h shows significant activation of Smad signaling at different time points 24, 48, and 72 hours. Specifically, 4HC increased Smad1/5/8 activation after 24 h in SH-SY5Y cells ($p< 0.01$) (Figure. 3.4A, C), while ISLQ activation of Smad1/5/8 was significant after 2 h ($p< 0.05$) (Figure. 3.4B, C). Moreover, there was a significant activation of Smad1/5/8 signaling in SK-N-BE(2) after 72h by all tested drugs: ISLQ ($p < 0.05$), 4HC ($p < 0.01$), and GDF5 ($P < 0.05$) (Figure. 3.4D, E). To further confirm Smad activation in SK-N-BE(2), cells were treated with 50 ng/ml GDF5 for 0.5, 1 , 24 h and Smad1/5/8 activation were measured with ELISA test. GDF5 was found to increase Smad1/5/8 phosphorylation after 1h ($P < 0.001$), while this activation still increased after 24h ($P < 0.01$) (Figure 3.4F). All of these data suggest that ISLQ and 4HC small molecule Smad activators can activate BMP-Smad signaling in NB cells. However, SK-N-BE(2) is more sensitive to those chalcones than SH-SY5Y.

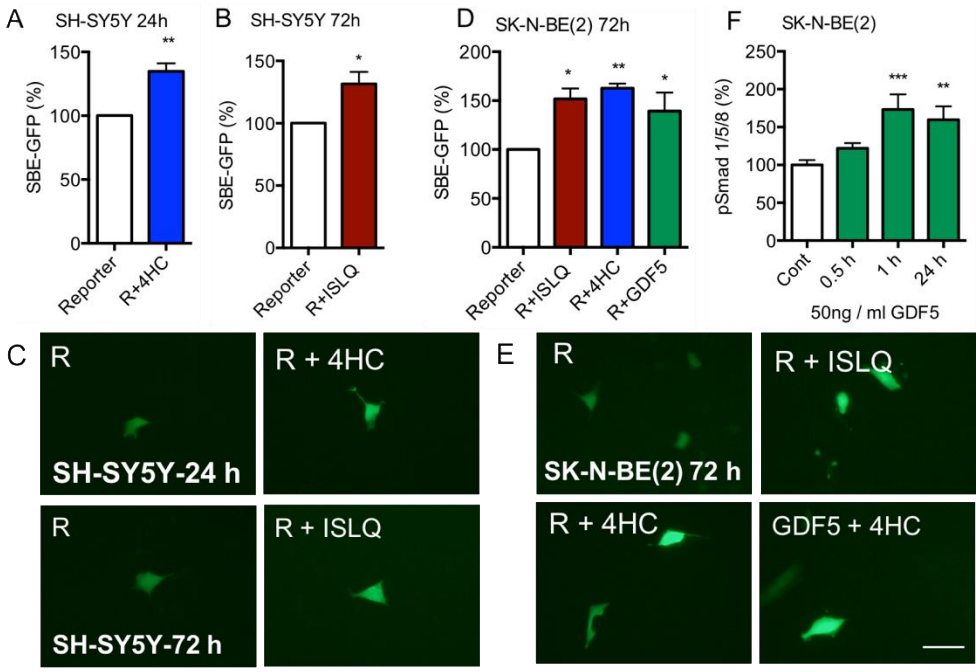


Figure 3.4: ISLQ and 4HC activate BMP-Smad signalling in NB cells.

Graphs and representative micrographs of BMP-Smad activation in (A-C) SH-SY5Y, transfected with Smad Binding Element with GFP Reporter (SBE-GFP) and treated with or without 2.5µM ISLQ, 1 µM 4HC for (A) 24 h, and (B) 72 h. All data are mean ± SEM;

n=3 independent experiments; ***P*< 0.05, ****P*< 0.01 vs control; Student's t-test. (D-E) Graphs and representative micrographs of BMP-Smad activation in SK-N-BE(2) transfected cells with SBE-GFP and treated with or without 0.5µM ISLQ and 4HC, or 50ng / ml GDF5 for 72 h. (F) graph of SK-N-BE(2) cells time-points measuring pSmad1/5/8 at 0.5, 1, 24 h with ELISA. Scale bar = 50µm. All data are mean ± SEM; *n*=3 independent experiments; ****P*** < 0.05, ***** P*** < 0.01, ****** P*** < 0.001 vs control; one-way ANOVA with Fisher's LSD test.

3.4.5 ISLQ and 4HC induced neurite growth is significantly blocked by K02288 a small molecule inhibitor of BMP signaling in NB cells.

BMP ligands signal by binding a heteromeric complex of bone morphogenetic protein receptor BMPR2, and BMPR1A and BMPR1B, which are transmembrane serine/threonine kinase receptors (Weiss and Attisano 2013). BMP2, BMP7 and GDF5 (BMP14) all bind to BMPR2 (Weber, Kotzsch et al. 2007, Yin, Yeh et al. 2008). In the canonical BMP pathway BMP receptors activate transcription factors known as the R-Smads (Smad1/5/8), which form a complex with Smad4, then translocate to the nucleus and regulate gene expression (Hegarty, O'Keeffe et al. 2013). To further confirm the neurite growth mechanism, we have used the small molecule BMP-signaling inhibitor K02288. K02288 was confirmed to selectively inhibit BMP-Smad1/5/8 signaling (Sanvitale, Kerr et al. 2013). Therefore, SH-SY5Y and SK-N-BE (2) were pretreated with 0.5 µM K02288 for 30 min before they were treated with 2.5 µM ISLQ and 1 µM 4HC for SH-SHY5Y and 0.5 µM of ISLQ or 4HC for SK-N-BE(2) for 3DIV and neurites length have been measured. We found that K02288 blocked the neurite growth promoted by ISLQ and 4HC in both SH-SY5Y ($P < 0.001$) for both ISLQ and 4HC (Figure. 3.5A, B), and SK-N-BE(2) ($P<0.001$) for both ISLQ and for 4HC (Figure. 3.5C, D). K02288 caused cells to form clumps and lead to significant alteration in cells morphology that was still present with ISLQ and 4HC in both NB cell lines (Figure. 3.5E).

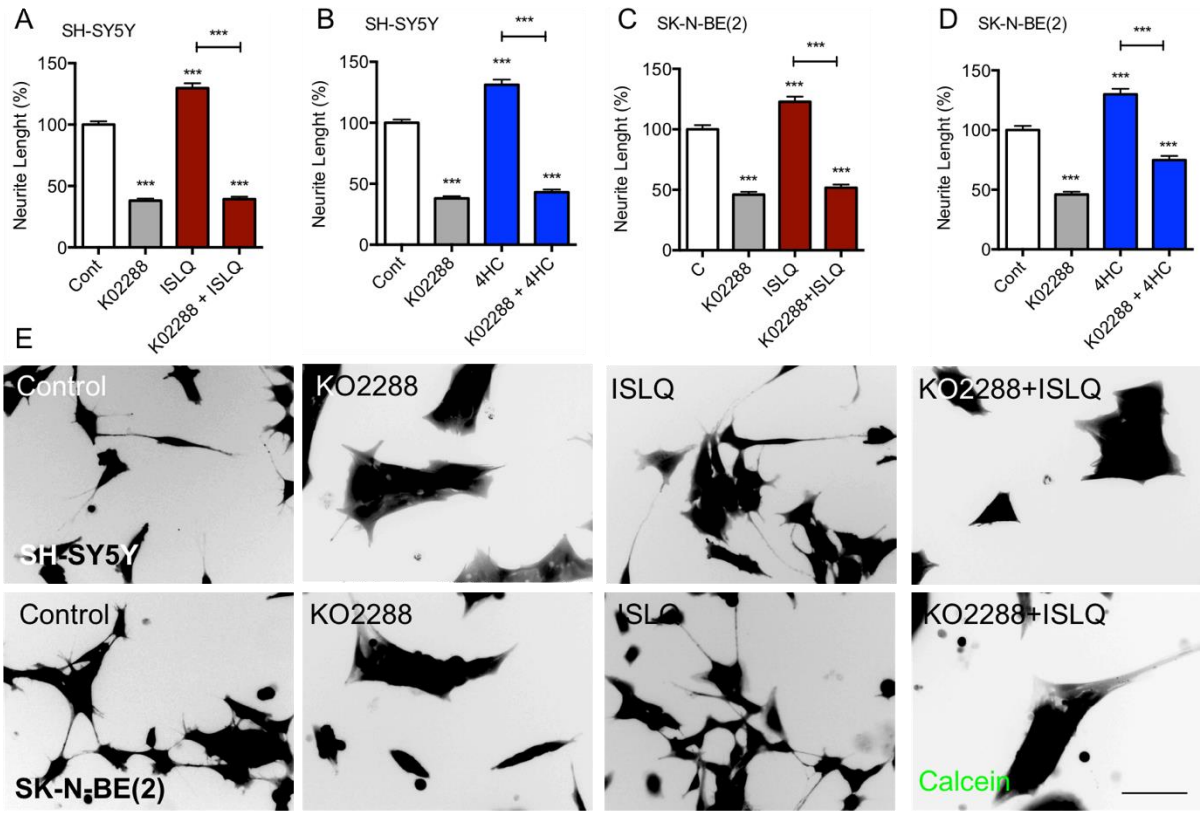


Figure 3.5: ISLQ and 4HC promoted neurite growth is significantly blocked by K02288 a small molecule inhibitor of BMP signalling in NB cells.

(A, D) Neurite growth graphs and (E) representative photomicrographs stained with Calcein of (A, B) SH-SY5Y and (C, D) SK-N-BE(2) cells pretreated with 0.5 µM K02288, for 30 min, before being cultured with or without 2.5 µM ISLQ, and 1 µM 4HC for SH-SY5Y and 0.5 µM ISLQ, and 0.5 4HC for SK-N-BE(2) for 3DIV. Scale bar = 50µm All data are mean ± SEM; from 180 cells each group from n=3 independent experiments; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control; one-way ANOVA with Tukey's test.

3.4.6 BMP-Smad signaling activation decrease cell mass area in SK-N-BE(2) cells.

From the cell viability experiment it was noticed that SK-N-BE(2) treated with 2.5 µM of ISLQ and 4HC for 3DIV lead to cell death in the tumor sphere area (Colonies). To further examine this, we have treated those cells with the same concentrations of ISLQ and 4HC, while cells were loaded with the vital cytoskeletal stain Calcein for 1 h before they were imaged at low magnification (4X) to examine wider fields. We found that 2.5 µM ISLQ led to a significant increase in colony number accompanied with a decrease in their area ($p < 0.001$) (Figure. 3.6A, B). Moreover, 4HC seemed to have the same effect ($p < 0.001$)

(Figure. 3.6C, D). This was consistent with the SK-N-BE(2) cells population morphology, as colonies in the control group consist of larger cells aggregation while in the treated groups they looked far smaller and we noticed more individual cells in the field (Figure 3.6E). It is worth noting that those individual cells seems more in ISLQ which indicating SK-N-BE(2) sensitivity to 4HC. This was seen in the MTT results as 4HC decreases cell proliferation more than the same concentration of ISLQ ($P < 0.01$) (Figure 3.6F). These data collectively suggesting that 2.5 µM of ISLQ and 4HC targeting SK-N-BE(2) colonies and cause them to detach and that lead to a reduction in their size.

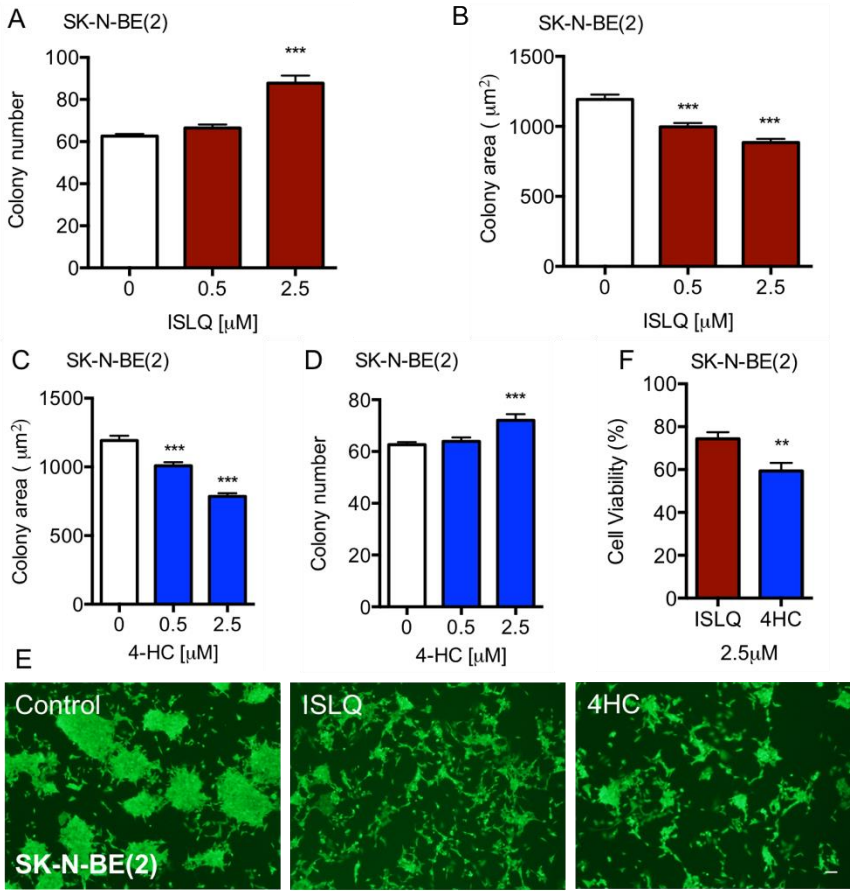


Figure 3.6: ISLQ and 4HC treatments decrease cell mass area after 3DIV in SK-N-BE(2) cells.

Graphs of colonies number and area and of SK-N-BE(2) cells treated with (A-B) 0.5, 2.5 µM ISLQ, and (C, D) 0.5, 2.5 µM of 4HC for 3DIV. (E) the representative micrographs stained with Calcein. Scale bar = 50µm. All data are mean ± SEM; from 180 colony each group from *n*=3 independent experiments; ***$p < 0.001$, vs control; one-way ANOVA with post-hoc Dunnett's test. (F) Graph comparison of cell viability measured by MTT of

2.5µM of ISLQ or 4HC in SK-N-BE(2) cells. All data are mean ± SEM; *n*=3 independent experiments; ****P*< 0.01 vs control; Student's t-test.

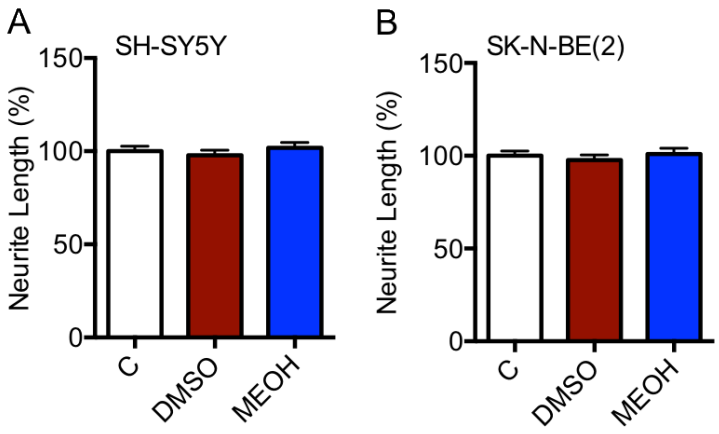


Figure 3.7 Treatment with vehicle control does not affect neurite growth.

Neurite growth measurements (A) SH-SY5Y, and (B) SK-N-BE(2), treated with equivalent to the ones used in the main experiment. For SH-SY5Y 2.5µM DMSO and 1 µM of MeOH, for SK-N-BE(2) 0.5 µM of either DMSO or MeOH, for 3DIV. All data are mean ± SEM; ANOVA with *post-hoc* Tukey's test.

3.5 Discussion

NB is characterized by tumor heterogeneity that is caused by the wide diversity of gene alteration and/or mutations pre- and post-chemotherapy (Ramaswamy and Taylor 2015). Among these genes defects are those responsible for cell growth and neuritogenesis (Molenaar, Koster et al. 2012). Bone morphogenetic proteins (BMPs) have been confirmed to initiate cell growth and differentiation in NC cells (Rohrer 2003, Gajavelli, Wood et al. 2004). Alterations in expression of the BMPRs may contribute to the defects in neuritogenesis seen in NB (Molenaar, Koster et al. 2012). Down-regulation of BMPR2 expression in NB has been reported, and its knockdown increased cell growth and tumourigenicity in mice, also patients with high BMPR2 expression have better overall survival (Cui, Yang et al. 2017). Moreover, our previous work demonstrates the distinctive expression patterns of each of the BMPRs that are associated with different signaling pathways and subsequent NB outcomes (Alshangiti, Wyatt et al. 2020). That is to say,

BMPR1A expression was associated with cell proliferation signaling pathways, while the expression of BMPR1B and BMPR2 was associated with cellular differentiation and neurite growth.

ISLQ and 4HC were among a drug screen library that identified them as small molecule activators of the BMP-Smad signaling pathway on the human cervical carcinoma cell line C33A (Vrijens, Lin et al. 2013). Here we are investigating those natural chalcones as differentiation inducers on two NB cells types: SHSY5Y and the *MYCN* amplified SK-N-BE(2). First, we confirmed the functionality of BMPs pathway component in SH-SY5Y and SK-N-BE(2) cells by specific immunostaining of BMPR1A, BMPR1B, BMPR2, and Smad1/5/8 protein. We found that low concentrations of ISLQ and 4HC are non-toxic to NB cells. From MTT results, that the lowest non-toxic doses were used to investigate neurite growth effects and their molecular mechanism. Chalcones concentrations showing a significant reduction in cell proliferation were furthered tested on SK-N-BE(2) colonies morphology alterations. ISLQ and 4HC significantly promote neurite growth in SH-SY5Y and SK-N-BE(2), and this was through the canonical BMP-Smad activation similar to BMP ligand GDF5. Moreover, the blockage of BMP-Smad signaling with K02288, blocked the neurite growth induced by ISLQ and 4HC in NB cells. Finally, ISLQ and 4HC were found to significantly decrease colonies area and increasing their number by detaching and shirking them along 3DIV.

Here we found that low concentrations of both chalcones induce neurite growth in NB cells. Flavonoids have been tested for their ability to induce neurite outgrowth in PC12 cells by an extracellular signal-regulated kinase-dependent process (Sagara, Vanhnasy et al. 2004). In another study, citrus flavonoids were found to induce the differentiation of human promyelocytic leukaemia cells (HL-60) into mature monocytes/macrophages, in a dose-dependent manner (Kawaii, Tomono et al. 1999). Moreover, synthesized chalcone derivatives have been shown to cause osteogenic differentiation through BMP2, the BMP signaling ligand, activity and also inhibit the activity of noggin, a BMP antagonist in mouse myoblast cell line C2C12 (Choi, Park et al. 2018). ISLQ has been demonstrated to inhibit the proliferation and induce the differentiation of human glioma stem cells (Lin, Sun et al. 2018).

The SK-N-BE(2) cell line is established from patients with recurrent NB, and are characterized by *MYCN* amplification and mutant P53, which cause their aggressive proliferation phenotype (Stafman and Beierle 2016). ISLQ and 4HC showed a double effect

on SK-N-BE(2) cells, as 0.5 µM of each chalcone significantly promoted neurite growth, whereas 2.5 µM showed a significant reduction in colony area, suggesting an anti-proliferative activity.

Chalcone's anti-proliferative properties have been reported previously. For example, ISLQ has been reported recently to inhibit cellular proliferation and migration in the human NB cell line SH-SY5Y, through ATP depletion, cell cycle arrest, and necroptotic cell death. (Escobar, Fong et al. 2019). ISLQ and 4HC's anti-proliferative effect were also confirmed in our previous work that demonstrated chalcones cytotoxicity to both *MYCN* non-amplified and amplified NB cells in a dose-dependent manner, that illustrates their potential properties to be used along with high-risk NB chemotherapy agents (Alshangiti, Togher et al. 2019, Alshangiti, Tuboly et al. 2019). Moreover, chalcone have also been reported to have anti-proliferative and P53 upregulation effects on human breast cancer cells (MCF-7 line) (dos Santos, Bertholin Anselmo et al. 2019).

It is noteworthy that *MYCN* amplified NB cells SK-N-BE(2) show more sensitivity to 4HC than ISLQ. This was also observed in our previous work (Alshangiti, Tuboly et al. 2019). This could be due to chalcones different special structure. Substitutions of chalcones (hydroxyl and methoxy group) on A and B rings result in different toxicity and proliferation potentials (Choi, Park et al. 2018). In addition, this differentiation mechanism of citrus flavonoids was found to be linked to their structure, as the presence of an ortho-catechol moiety in ring B and C2-C3 double bond both had important impacts on the ability of the flavonoid to induce differentiation of HL-60 cells (Kawaii, Tomono et al. 1999).

Collectively, ISLQ and 4HC promote cell differentiation on both *MYCN* non-amplified and amplified NB cells. The effect is mediated through canonical BMP-Smad signaling. Moreover, ISLQ and 4HC have also anti-proliferative effect on SK-N-BE(2) cells that form aggregation of undifferentiated colonies which could contribute to overcoming relapses and drug resistance caused by stem cells population inside NB tumors that survive chemotherapy and initiate secondary tumors.

3.6 Conclusion

Activation of BMP-Smad signaling has promising potentials in NB neuritogenesis. Activation of BMPR1B and BMPR2 have a profound impact in enhancing NB patients' outcomes. The use of BMP neurotrophic ligands for this purpose has unfavorable side

effects, not to mention their high cost and delivery difficulties due to their large molecular weight. However, natural compound, such as the small molecules chalcones that activate BMP-Smad signaling have a promising potential to induce cell differentiation in NB cells. This study proved a proof-of-principle in using ISLQ and 4HC as NB differentiation agents that could contribute in elimination of drug resistance tumor minimal residual populations and subsequently preventing tumor relapses.

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Chapter 4

Chapter 4 The dietary flavonoid isoliquiritigenin is a potent cytotoxin for human neuroblastoma cells

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4.1 Abstract

NB is the most common extracranial solid tumor of early childhood; it accounts for approximately 8-10% of all childhood cancers and is the most common cancer in children in the first year of life. Patients in the high-risk group have a poor prognosis, with relapses being common and often refractory to drug treatment in those that survive. Moreover, the drug treatment itself can lead of a range of long-term sequelae. Therefore, there is a critical need to identify new therapeutic targets for NB. ISLQ is a naturally occurring, dietary chalcone-type flavonoid with a range of biological effects depending on the cell-type and cellular context that has potential as an anti-cancer agent. Here we show that ISLQ has potent cytotoxic effects on the SK-N-BE(2) human NB cell line, which carries an amplification of the *MYCN* gene, the main prognostic marker of poor survival. This effect of ISLQ was found to be independent of both Bax and caspase, and to be mediated through reactive oxygen species, since it was blocked small molecule inhibitors of oxidative stress-induced cell death, and by the anti-oxidant N-acetyl-L-cysteine. We confirmed these cytotoxic effects of ISLQ in *MYCN*-amplified IMR32 NB cells. This study provides proof-of-principle that ISLQ is a potent cytotoxin for *MYCN*-amplified human NB cells. This is an important first step in rationalizing the further study of ISLQ as a potential adjunct therapy for high-risk NB.

Keywords

Isoliquiritigenin; Neuroblastoma; Cancer; Small molecule; Chemotherapy; Cell death.

4.2 Introduction

NB is the most common extracranial solid tumor of early childhood. It accounts for approximately 8-10% of all childhood cancers and is responsible for 15% of childhood deaths as a result of cancer (Modak and Cheung 2010). NB arises from cells of sympathoadrenal lineage of the NC during development and often presents as tumors in the neck, chest, abdomen and pelvis (Maris 2010, Cheung and Dyer 2013, Matthay, Maris et al. 2016). Age, stage and tumour phenotypes are important prognostic factors in NB, and they guide treatment strategies (Maris 2010). In particular, amplification of the *MYCN* oncogene (Huang and Weiss 2013) and/or inactivation of p53 (Cattelani, Ferrari-Amorotti et al. 2012) are associated with increased disease risk and poorer outcomes. *MYCN* amplification is associated with rapid NB progression and a poor prognosis (Huang and Weiss 2013, Campbell, Gastier-Foster et al. 2017), while p53 stabilisation has been linked to multi-drug resistance in NB (Xue, Haber et al. 2007). For those diagnosed with low- to intermediate-risk NB, the prognosis is relatively good, however many patients have high-risk metastatic disease that is often resistant to multimodal therapy and frequently relapses, resulting in a 5-year overall survival rate of approximately 50% (Matthay, Maris et al. 2016). Those patients that survive often have long-term sequelae as a result of toxicity associated with current chemotherapy regimens (Perwein, Lackner et al. 2011, Ducassou, Gambart et al. 2015). Therefore, the need for novel therapies with fewer off-target effects is essential for improving prognosis and quality-of-life among NB patients.

The use of diet-derived pharmacological agents to prevent, inhibit and delay carcinogenesis is a growing field of research, particularly in the area of cancer chemoprevention (Steward and Brown 2013, Landis-Piwowar and Iyer 2014, Benetou, Lagiou et al. 2015). This strategy has been derived from epidemiological studies which suggest that diets rich in fruit and vegetables can reduce the risk of cancer (Key 2011, Bradbury, Appleby et al. 2014). Flavonoids have received significant attention in this regard, not only as preventative strategies, but also as potential chemotherapeutic agents (Chahar, Sharma et al. 2011, Batra and Sharma 2013). In particular, ISLQ, a chalcone-derived flavonoid naturally found in liquorice and shallots (Peng, Du et al. 2015), has been investigated for its anti-cancer properties due to its potent inhibition of cell proliferation and viability in a range of cancer cell types (Ma, Fu et al. 2001, Ii, Satomi et al. 2004, Hsu, Kuo et al. 2005, Jung, Chung et al. 2006, Kim, Ramachandran et al. 2008, Kwon, Cho et al. 2009, Zhang, Yeung et al. 2010, Zhou, Song et al. 2013, Wu, Chen et al. 2016, Chen, Huang et al. 2017, Si, Yang et al. 2017).

The anti-cancer effects of ISLQ on NB has yet to be studied, however ISLQ has recently been found to induce cytotoxicity in the pheochromocytoma cell line (PC-12), which, like NB, has an embryonic origin of the NC (Yang, Neale et al. 2013), suggesting that ISLQ may have anti-cancer effects in NB cells.

The anti-cancer properties of ISLQ have been attributed to its ability to inhibit cell cycle progression, induce oxidative stress, apoptosis, autophagy and/or necrosis. However, the precise mechanism of cytotoxicity is dependent on cell type and usually involves a combination of cell death pathways (Jung, Chung et al. 2006, Wu, Chen et al. 2016, Si, Yang et al. 2017, Yang, Zhang et al. 2017). Understanding the mechanisms of cytotoxicity of ISLQ in NB cells will be important in determining its translational potential by improving knowledge of its potential off-target effects, efficacy in tumor subtypes and/or usefulness as a possible combination therapy (Cattley and Radinsky 2004). Here we sought to examine whether ISLQ treatment can exerted anti-tumorigenic effects in *MYCN*-amplified NB cells, as the first step in rationalising the study of ISLQ as a potential therapeutic agent for high-risk pediatric NB.

4.3 Materials and Methods

4.3.1 Cell Culture

Cells were purchased from the American Type Culture Collection (through Sigma). SK-N-BE(2) cells were maintained in Minimum Essential Medium (MEM) medium with 100nM L-glutamine, 1% penicillin-streptomycin, 1% non-essential amino acid solution (100×), 1% F-12 Hams with 15% fetal bovine serum (FBS). IMR-32 cells were maintained in MEM with 100nM L-glutamine, 1% penicillin-streptomycin, 1% non-essential amino acid Solution (100×) and 10% FBS. All reagents were from Sigma. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37˚C. After 2 days in vitro, when the cells had optimally adhered, cultures were treated with 5-100 µM ISLQ or a vehicle (equivalent percentage of dimethyl sulfoxide (DMSO); Sigma). For experiments involving inhibitors, cells were pre-treated with either: 50 µM Boc-D-FMK (Calbiochem), a cell-permeable, irreversible, broad-spectrum caspase inhibitor (37); 10 µM necrostatin-1 (NEC-1, Bio-techne), an ATP-competitive, allosteric inhibitor of receptor-interacting protein kinase 1 (RIPK1) that blocks non-apoptotic cell death via inhibition of a specific cellular pathway, necroptosis, which leads to necrosis (39); 3 µM IM-54 (Calbiochem), a small molecule

inhibitor that selectively blocks oxidative stress-induced necrotic cell death; or 100 µM Bax Inhibiting Peptide V5 ('Bax Inh'; Calbiochem), a membrane-permeable pentapeptide Bax-mediated apoptosis inhibitor (Yoshida, Tomioka et al. 2004). As a control for the latter experiment, 100 µM of a corresponding cell-permeable control for the Bax-Inhibiting Peptide, that does not suppress Bax-mediated apoptosis ('Bax Cont'; Calbiochem) was used. The antioxidant N-acetyl-L-cysteine (NAC; Sigma) was used at 1 mM. Cultures were pre-treated with the inhibitors for 1 or 2 h before treatment with 25 µM of ISLQ for 24h. In other experiments, cultures were treated with 200 µM 6-OHDA for 4, 6 or 24h, or with at 10-100 µM cisplatin, a chemotherapeutic drug (Dasari and Tchounwou 2014) (AdooQ Bioscience) for 24h.

4.3.2 Cell viability and death assays

For cell viability assays, cells were plated at a density of 1 x 10^5 cells per well on 24-well plates. Phase contrast images were captured using an Olympus IX71 inverted microscope fitted with an Olympus DP70 camera. At each experimental end-point, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells at a concentration of 1 mg/ml in culture medium for 4h at 37°C. The culture medium was then removed and the formazon crystals were solubilized in DMSO. Absorbance was measured at 540 nm with a reference wavelength of 690 nm. Lactate dehydrogenase (LDH) activity was measured in 50µl of cell culture medium of each treatment group using an LDH Activity Assay Kit (Sigma), according to the manufacturer's instructions.

4.3.3 Oxidative stress assessment

To analyse oxidative stress, cultures were treated with 25 µM of ISLQ for 24 h, after which the cell-permeable dye CellRox® (ThermoFisher) was added at a final concentration of 5 µM, then cells were incubated for 30 min at 37°C. This dye exhibits bright green photostable fluorescence upon oxidation by reactive oxygen species (ROS) in living cells. The cells were washed in sterilised 10 mM PBS and four microscopic fields were randomly captured per well for each experiment, using an Olympus IX70 inverted microscope. The fluorescence intensity of individual cells was measured using Image J.

4.3.4 RNA isolation, cDNA synthesis and real-time PCR

For analysis of gene expression, cultures were treated with 5 µM or 25 µM of ISLQ for 24h. The media was removed and the cells washed in 10 mM PBS, before total RNA was extracted using Trizol reagent as per the manufacturer's instructions (Invitrogen). The quantity and quality of the mRNA was assessed using a NanoDrop 1000. Reverse transcription was carried out using the high capacity cDNA Reverse Transcription Kit (Applied Biosystems) under the following parameters: 25 °C for 10 min; 37 °C for 120 min; 85 °C for 5 min; 4 °C for 10 min. For real-time PCR, a reaction mix consisting of 0.5 µl 20× Gene Expression Assays (*gapdh, bax, bcl2, caspase3*; TaqMan® Applied Biosystems), 0.5 µl of cDNA (20 ng/ml), 5 µl TaqMan® Gene Expression Master Mix (Applied Biosystems) and 4 µl of RNase-free H_2O (Applied Biosystems) was used. Each sample was run in duplicate under the following cycling parameters: 50 °C for 2 min; 95 °C for 10 min; 40 repetitions of 95 °C for 15 s and 60 °C for 1 min. Gene expression levels were calculated using the 2-δCT method, normalizing to ACTB (Schmittgen and Livak 2008).

4.3.5 Immunocytochemistry

For immunocytochemistry, cells were fixed in 4% paraformaldehyde in 10 mM phosphate buffered saline (PBS) for 15 min. The paraformaldehyde was removed and the cells were washed three times for 5 min per wash in 10 mM PBS with 0.02% Triton (PBS-T). Non-specific binding was blocked by incubation in 5% BSA in PBS-T for 1 h at room temperature. The blocking solution was removed and primary antibodies (Cell Signaling) to either cleaved caspase-3 or Bax, each diluted 1:400 in 1% BSA in PBS-T, were added to the culture wells, which were then incubated overnight at 4°C. The primary antibody solution was removed, cells were washed in PBS-T then incubated in AlexaFluor488-conjugated secondary antibody (1:1000; Invitrogen) diluted in 1% BSA in PBS-T, in the dark at room temperature for 2h, then counterstained with 4'-6-Diamidino-2-phenylindole (DAPI). Five microscopic fields were randomly captured per well per experiment, using under an Olympus IX71 inverted microscope and analysed using Image J.

4.3.6 Statistical Analysis

A one-way ANOVA with *post-hoc* Tukey's or Dunnett's test was performed to measure any significant differences between groups. Results were expressed as mean ± SEM and deemed significant when $p<0.05$.

4.4 Results

4.4.1 Effects of ISLQ treatment on cell viability in SK-N-BE cultures

We first carried out a dose-response experiment on the effects of ISLQ treatment on cell viability in SK-N-BE(2) cultures. This cell line is *MYCN*-amplified (Veas-Perez de Tudela, Delgado-Esteban et al. 2010) and therefore a suitable model of aggressive NB, given that *MYCN* amplification is the main prognostic marker of poor survival in NB (Huang and Weiss 2013). Treatment with ISLQ at concentrations equal to or greater than 5 µM resulted in a dose-dependent decrease ($p<0.01$) in cell viability in SK-N-BE(2), as measured by MTT assays (Figure. 4.1A). This decrease in cell viability was not found in the corresponding control (DMSO vehicle only)-treated cultures (Figure. 4.1C). To confirm these findings, LDH activity was examined in culture media from ISLQ-treated SK-N-BE(2) cells. ISLQ treatment led to a significant increase ($p<0.001$) in LDH activity in SK-N-BE(2) cells, at concentrations greater than 50 µM (Figure. 4.1B). There were no changes in LDH activity in DMSO vehicle-treated cultures (data not shown). These results were confirmed by phase-contrast microscopy which showed that ISLQ-treated cells displayed morphological features consistent with cell death (Figure. 4.1D). Collectively, these data demonstrate that ISLQ treatment exerts dose-dependent effects on viability in SK-N-BE(2) cells.

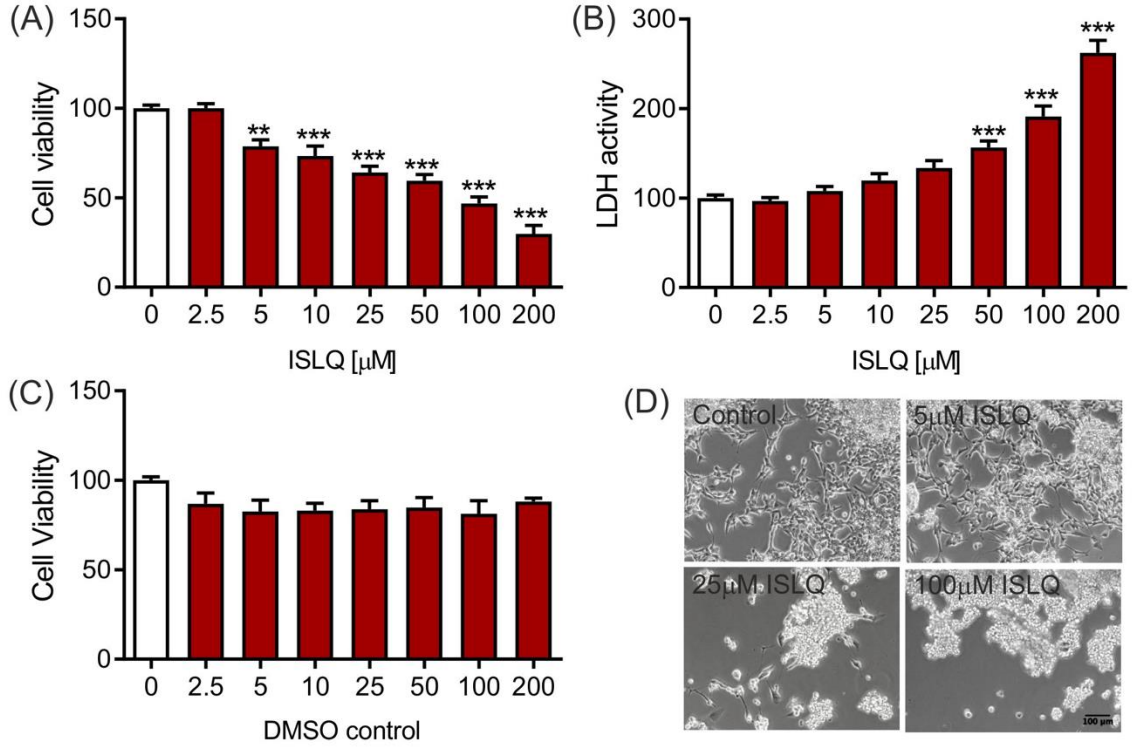


Figure 4.1: ISLQ induces cell death in SK-N-BE(2) neuroblastoma cells.

Graphs of (A) MTT and (B) LDH assays on SH-N-BE(2) cells treated with isoliquiritigenin (ISLQ) for 24 h. (C) Graph of an MTT assay on SK-N-BE(2) cells treated with vehicle (DMSO) for 24 h. (D) Representative phase-contrast photomicrographs of ISLQ-treated cultures at 24 h. (******$p < 0.01$, ***$p < 0.001$ vs control; ANOVA with *post-hoc* Tukey's test). Scale bar = 100μm. All data are mean ± sem.

4.4.2 The cytotoxic effects of ISLQ on SK-N-BE cells are Bax- and caspase-independent

Since the biological mechanisms mediating the effects of ISLQ are varied and cell-type-specific (Peng, Du et al. 2015), we sought to determine the mode of cell death induced by ISLQ in SK-N-BE(2) cells. As ISLQ has been shown to increase the expression of Bax, decrease the expression of Bcl-2 and lead to caspase-dependent cell death in human carcinoma and hepatoma cells (Hsu, Kuo et al. 2005, Hirchaud, Hermetet et al. 2013), we examined the involvement of Bax, Bcl-2 and caspases in ISLQ-induced cell death in SK-N-BE(2) cells. Firstly, SK-N-BE(2) cells were pre-treated with 100 μM of a cell-permeable peptide ('Bax inhibitor') which is an effective inhibitor of Bax-mediated apoptosis, or with 100 μM of a control peptide ('Bax control'), for 1h prior to the addition of 25 μM ISLQ for

24 h. An MTT assay revealed that ISLQ treatment led to a significant decrease in cell viability in SK-N-BE(2) cells ($p<0.001$), which was not affected by pre-treatment with the Bax inhibitor or the Bax control peptide (Figure. 4.2A). Secondly, SK-N-BE(2) cells were pre-treated with 50µM of a broad-spectrum small molecule caspase inhibitor (Boc-D-FMK) (D'Mello, Aglieco et al. 1998) for 2h prior to the addition of 25 µM ISLQ for 24 h. An MTT assay showed that ISLQ-induced cell death in SK-N-BE(2) cells was not prevented by caspase inhibition (Figure. 4.2B). Furthermore, treatment with 200 µM 6-hydroxydopamine (6-OHDA), which has been reported to induce caspase-dependent cell death in this cell line (Kim, Woo et al. 2010), resulted in a significant decrease ($p<0.05$) in cell viability, which was prevented by pre-treatment with Boc-D-FMK (Figure. 4.2B). Phase-contrast microscopy verified that pre-treatment with Boc-D-FMK did not prevent the cell death induced by ISLQ (Figure. 4.2C). This lack of rescue of ISLQ-induced cell death by inhibition of either Bax or caspase was further supported by real-time PCR experiments, which showed that ISLQ treatment did not significantly alter the levels of expression of *bax* (Figure. 4.2D), *bcl-2* (Figure. 4.2E) or *caspase-3* (Figure. 4.2F) mRNA in SN-N-BE(2) cells. In addition, and to further confirm these findings, we performed immunocytochemistry on SK-N-BE(2) cells using antibodies for Bax and for cleaved caspase-3. Following treatment of SK-N-BE(2) cultures for 4, 6 or 24 h with 200 µM 6-OHDA, significant numbers of cells stained positively for Bax and for cleaved caspase-3 (6h time-point shown in Figure 4.2G). In contrast, there were no Bax- or cleaved caspase-3-positive cells in cultures treated with 25 µM ISLQ (Figure 4.2G). Collectively these data suggest that the cytotoxic effects of ISLQ in SK-N-BE(2) cells are Bax- and caspase-independent, meaning that ISLQ induces cell death through other mechanisms in NB cells.

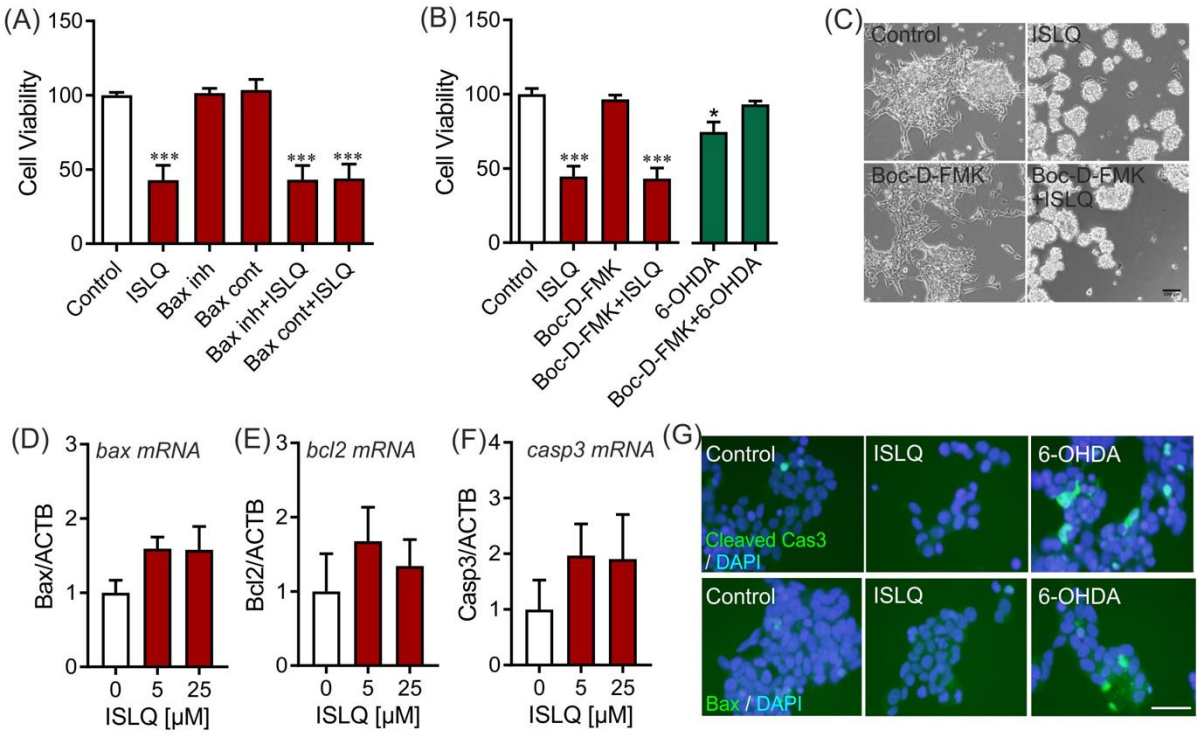


Figure 4.2: Inhibition of Bax or of caspase does not prevent ISLQ-induced death of SK-N-BE(2) cells.

(A, B) Graphs of MTT assays and (C) representative phase-contrast photomicrographs of SK-N-BE(2) cells treated with 25 μM ISLQ for 24 h with or without pre-treatment with (A) 100 μM of a Bax inhibitor peptide ('Bax inh') or of a control peptide ('Bax cont'), or (B) 50 μM of Boc-D-FMK. Treatment with 200 μM 6-OHDA (green bars) for 24 h was used as a positive control. Scale bar = 100μm. (D-F) Graphs of real-time PCR data showing the relative expression of (D) *bax* mRNA, (E) *bcl2* mRNA and (F) *caspase (casp)3* mRNA, normalised to that of β-actin (ACTB), in SK-N-BE(2) cells treated with non-cytotoxic (5μM) and cytotoxic (25 μM) concentrations of ISLQ for 24 h. (G) Representative photomicrographs of SK-N-BE(2) cells treated with 25 μM ISLQ for 6 h, immunostained for cleaved caspase-3 or Bax. Treatment with 200 μM 6-OHDA was used as a positive control. Scale bar = 50μm (***p* < 0.05, ****p* < 0.001 vs control; ANOVA with *post-hoc* Tukey's test). All data are mean ± sem.

4.4.3 The cytotoxic effects of ISLQ on SK-N-BE cells are mediated by ROS-induced necrosis

Given that inhibition of either Bax or caspase did not block ISLQ-induced cell death, we next sought to determine if ISLQ induced cell death through necroptosis or necrosis. Firstly, pre-treatment of SK-N-BE(2) cells for 2 h with Necrostatin-1 (Nec-1), a cell-permeable, potent and selective blocker of necroptosis (39), was used. MTT assays showed that 25 µM ISLQ led to a significant decrease in SK-N-BE(2) cell viability ($p<0.001$) at 24 h, which was partially prevented by pre-treatment with 10µM nec-1 (Figure. 3A, D). Pre-treatment for 1 h with 3 µM IM-54, a small molecule inhibitor that selectively blocks oxidative stress-induced necrotic cell death (Dodo, Katoh et al. 2005), fully blocked ISLQ-induced loss of cell viability in SK-N-BE(2) cells ($p<0.001$) (Figure. 4.3B, D). To further investigate the involvement of oxidative stress in ISLQ-induced cell death, hypothesis, pre-treatment with the anti-oxidant N-acetyl-cysteine (NAC) was used. An MTT assay showed that pre-treatment with 1 mM NAC fully prevented ($p<0.001$) ISLQ-induced cell death in SK-N-BE(2) cells (Figure. 4.3C, D). To confirm the role of oxidative stress in ISLQ-induced cell death, the CellRox® system was used to evaluate levels of reactive oxygen species (ROS), which are increased in conditions of cellular oxidative stress. SK-N-BE(2) cells were treated with ISLQ (25, 50, 100µM) for 2, 4 and 6 h. Cultures were loaded with the CellRox® fluorogenic probe and fluorescence intensity was measured as a readout of cellular ROS. ISLQ treatment for 24 h was found to increase ROS levels, to a similar extent to those in cultures treated with a positive control (0.6 mM H_2O_2), and significantly greater than those in untreated cultures (Figure. 4.3E). The ISLQ-induced elevation of ROS levels was blocked by pre-treatment for 1 h with IM-54 (Figure 4.3F, G). These data suggest that ISLQ-induced cell death is mediated by ROS-mediated cell death.

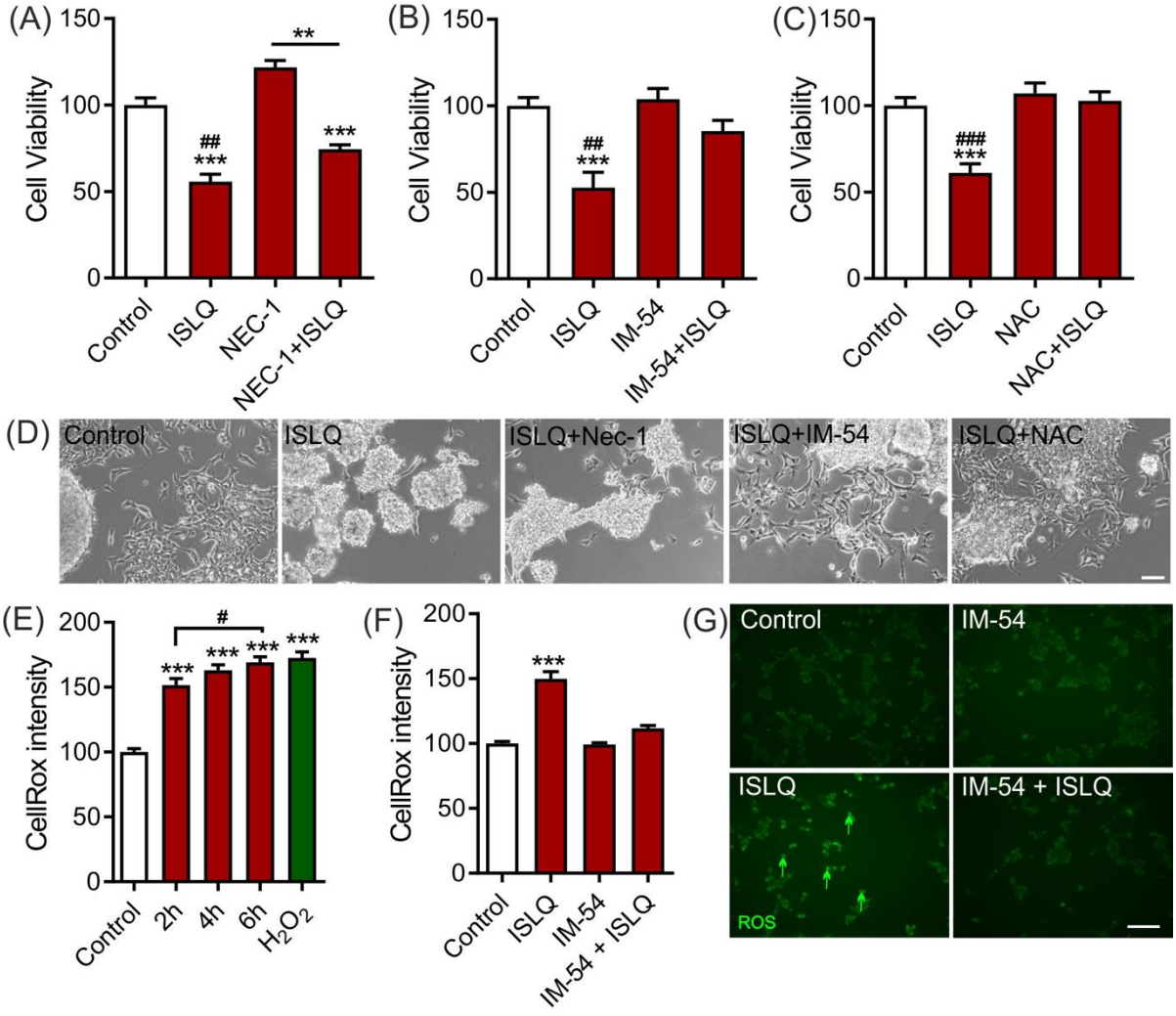


Figure 4.3: Inhibition of oxidative stress prevents ISLQ-induced death of SK-N-BE(2) cells.

(A-C) Graphs of MTT assays and (D) representative photomicrographs of SK-N-BE(2) cells treated with 25 µM ISLQ for 24 h with or without pre-treatment with (A) 10 µM Nec-1 necroptosis inhibitor), (B) 3 µM IM-54 (inhibitor of oxidative stress-induced necrosis) or (C) 1 mM NAC (anti-oxidant). (E, F) Graphs and (G) representative photomicrographs showing relative fluorescence intensity of SK-N-BE(2) cells loaded with the CellRox fluorogenic probe that measures cellular oxidative stress (green fluorescence). (E) Cells were treated with 25 µM ISLQ for 2, 4 or 6 h, or with 0.6 mM H_2O_2 for 30 min. (F) Cells were pre-treated with 3 µM IM-54 then cultured with or without 25 µM ISLQ for 24 h. (**$p < 0.01$, ***$p < 0.001$ versus control or as indicated; # $p < 0.05$, ## $p < 0.01$, ###

$p < 0.001$ ISLQ versus ISLQ + inhibitor; ANOVA with *post-hoc* Tukey's test). Scale bar = 100µm. All data are mean ± sem.

4.4.4 Effects of ISLQ treatment on cell viability in IMR-32 cultures

We next performed experiments to investigate the cytotoxic effects of ISLQ treatment in IMR-32 NB cells, which is another *MYCN*-amplified cell line. In agreement with our findings in SK-N-BE(2) cells, ISLQ treatment significantly reduced ($p<0.001$) IMR-32 cell viability at concentrations greater than 5 µM, as measured using at MTT assay (Figure. 4.4A, D), whereas there were no significant alterations in cell viability in cultures treated with DMSO vehicle (Figure. 4.4C). These results were confirmed using an LDH assay which showed that ISLQ treatment led to a significant increase ($p<0.05$) in LDH activity in IMR-32 cells, at concentrations greater than 5 µM (Figure. 4.4B). Moreover, in agreement with our data on SK-N-BE(2) cells, pre-treatment with nec-1 (Figure. 4.5A, D), IM-54 (Figure. 4.5B, D) or NAC (Figure. 4.5C, D) significantly blocked ISLQ-induced cell death in IMR-32 cells.

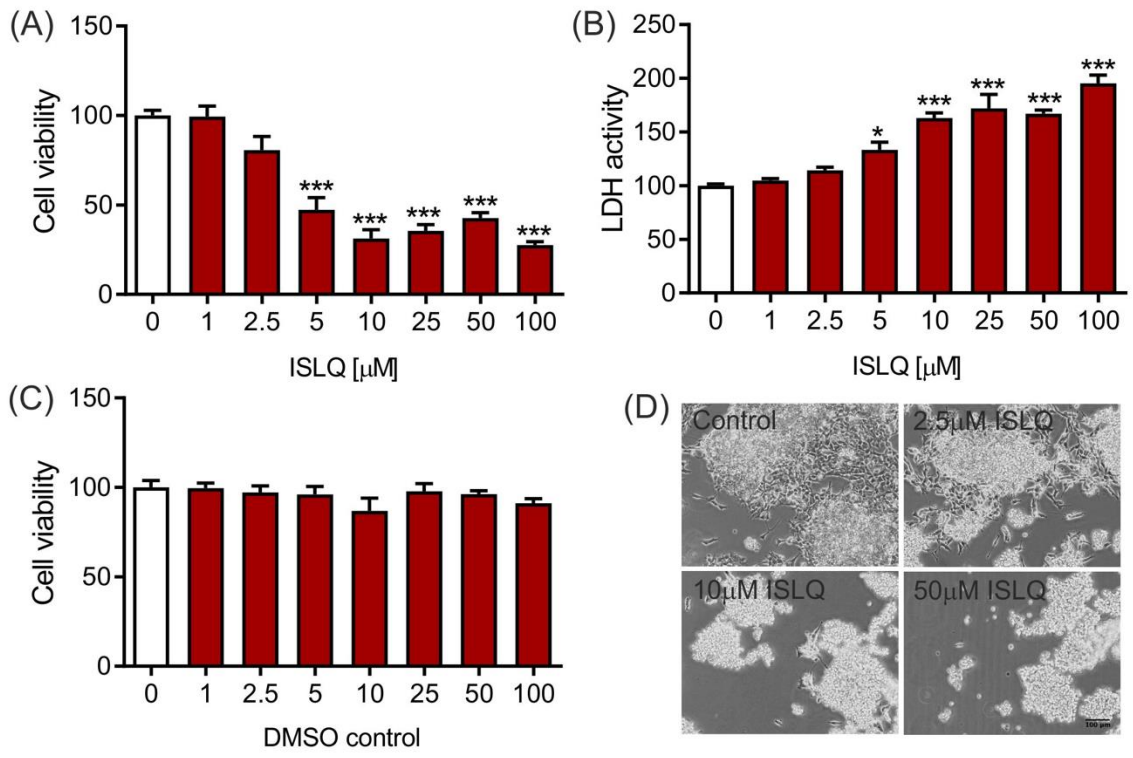


Figure 4.4: ISLQ induces cell death in IMR-32 neuroblastoma cells.

Graphs of (A) MTT and (B) LDH assays on IMR-32 cells treated with ISLQ for 24 h. (C) Graph of an MTT assay on IMR-32 cells treated with vehicle (DMSO) for 24 h. (D) Representative phase-contrast photomicrographs of ISLQ-treated IMR-32 cells at 24 h. (*****$p < 0.05$, ***$p < 0.001$ vs control; ANOVA with *post-hoc* Tukey's test). Scale bar = 100μm. All data are mean ± sem.

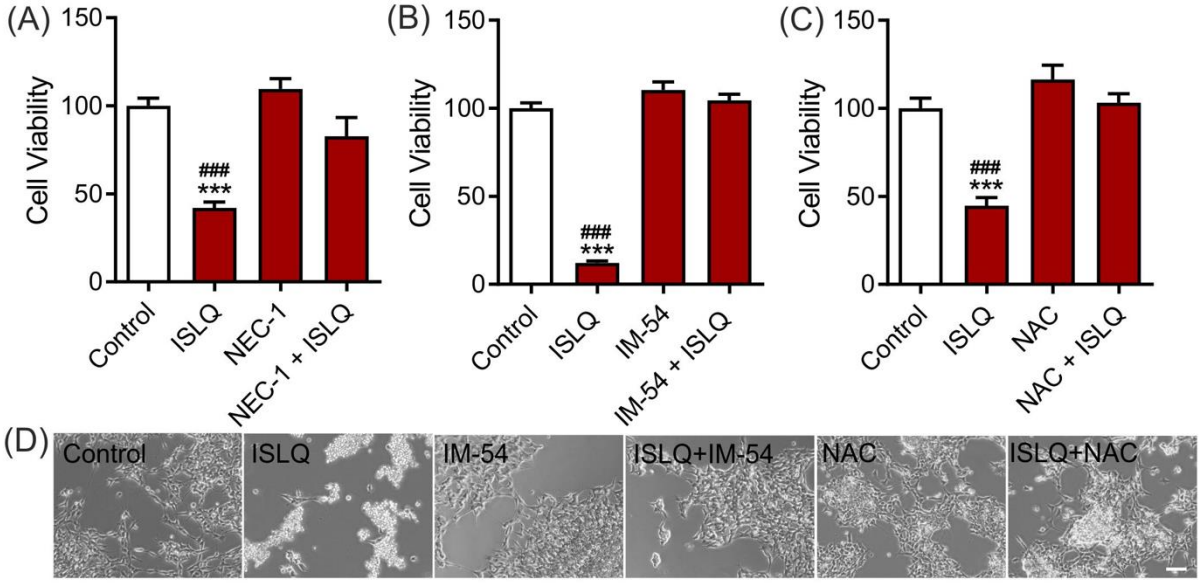


Figure 4.5: Inhibition of oxidative stress prevents ISLQ-induced cell death of IMR-32 cells.

(A-C) Graphs of MTT assay and (D) representative photomicrographs of IMR-32 cells treated with 25 µM ISLQ for 24 h with or without pre-treatment with (A) 10 µM Nec-1, (B) 3 µM IM-54 or (C) 1 mM of NAC. (***$p < 0.001$ versus control; ### $p < 0.001$ ISLQ versus ISLQ + inhibitor; ANOVA with *post-hoc* Tukey's test). Scale bar = 100µm. All data are mean ± sem.

4.4.5 Effects of combined treatment with ISLQ and cisplatin in MYC-amplified NB cells

To further explore the anticancer actions of ISQL, we compared the cytotoxic effects of ISLQ treatment of both SK-N-B-E(2) and IMR-32 cells with those of a commonly-used anti-cancer drug, cisplatin. Firstly, we conducted a dose-response experiment using cisplatin alone and found that both cell lines were resistant to treatment with this agent at concentrations less than 100 µM (Figure 4.6A, B), while 100 µM cisplatin induced significant loss of cell viability. Combined treatment of either SK-N-B-E(2) and IMR-32 cells with 10 µM ISLQ and 100 µM cisplatin for 24h resulted in loss of cell viability that was significantly greater ($p < 0.001$) than that induced by either drug alone (Figure. 4.6C, D).

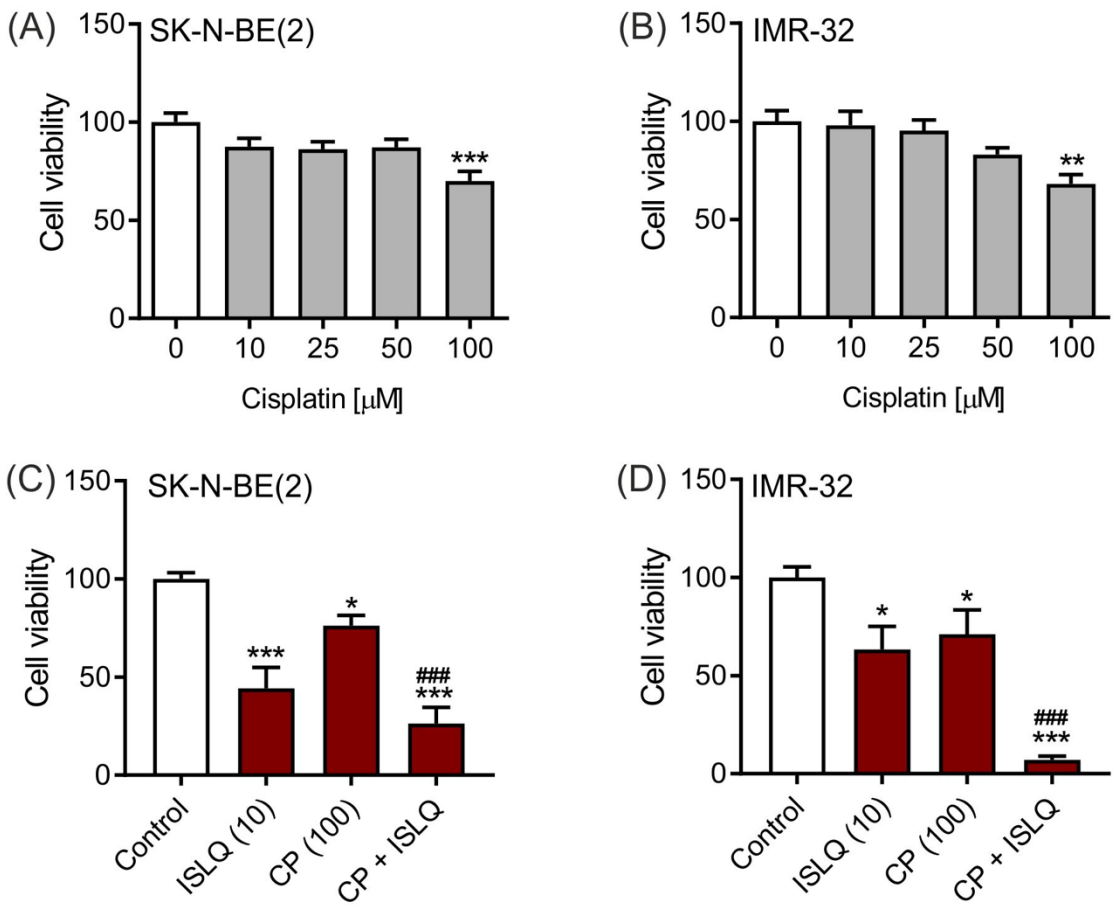


Figure 4.6: Effects of combined treatment with ISLQ and cisplatin on NB cells.
(A, B) Graphs of MTT assays on (A) SK-N-BE(2) cells and (B) IMR-32 cells treated with cisplatin (CP) for 24 h. (C, D) Graphs of MTT assays on (C) SK-N-BE(2) cells and (D) IMR-32 cells treated with 25 µM ISLQ for 24 h with or without 100µM CP. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control; ### $p < 0.001$ versus both ISLQ and CP in C, versus CP in D. ANOVA with *post-hoc* Tukey's test. ANOVA with *post-hoc* Tukey's test). Scale bar = 100µm. All data are mean ± sem.

4.5 Discussion

ISLQ is a naturally-occurring bioactive compound isolated from the roots of plants belonging to the liquorice family, with a range of biological effects depending on the cell type and context (Peng, Du et al. 2015). Although the effects of ISLQ in a range of different

cancers have been reported (Yamamoto, Aizu et al. 1991, Lee, Son et al. 2008, Lee, Lim et al. 2009, Chen, Zhang et al. 2012, Jung, Lee et al. 2014, Chen, Huang et al. 2017, Peng, Tang et al. 2017, Si, Yang et al. 2017, Zhang, Lai et al. 2018), to our knowledge there has been no study that has investigated the potential of ISLQ as a cytotoxic agent for NB. This is particularly important given the poor prognosis of NB, and the fact that many NB relapses are drug-resistant, while current therapies can have a range of long-term sequelae (Perwein, Lackner et al. 2011, Ducassou, Gambart et al. 2015). Thus, the identification of new and/or adjunct therapeutic approaches for NB is essential. To address this, we used SK-N-BE(2) (p53 mutant) and IMR-32 cells to demonstrate that ISLQ exerts potent cytotoxic effects in both of these *MYCN*-amplified NB cell lines. SK-N-BE(2) cells, due to *MYCN* amplification and mutant p53, become highly resistant to apoptosis. One of the most important functions of p53 is its ability to activate apoptosis, and disruption of this process can promote tumor progression and chemoresistance (Fridman and Lowe 2003). We found that the cytotoxic effects of ISLQ in NB cells were mediated through oxidative stress-induced cell death, as evidenced by increases in cellular ROS levels, rather than through apoptosis or necroptosis. As a result, these data provide proof-of-principle that further study of ISLQ may be useful in developing new therapeutic approaches to high-risk NB.

In this study, we found that ISLQ decreased cell viability in SK-N-BE(2) and IMR-32 when applied in the lower micromolar range, at concentrations of 5 μM or greater, for 24h. This is similar to data reported in a recent study on the PC-12 neuronal cell line, which used a similar approach and found that ISLQ had a cytotoxic effect on these cells, with an IC50 of 17.8 μM (Yang, Neale et al. 2013). Similarly, another study reported a cytotoxic effect of ISLQ on U87 glioma cells, with an IC50 6.3 μM, while it displayed minimal toxicity against normal brain cells (Zhao, Chang et al. 2015). A cytotoxic effect of ISLQ on SH-SY5Y cells has also been reported in a published abstract which stated that treatment with 10-200 μM ISLQ for 24 h decreased cell viability (Escobar, Munoz et al. 2016). This is fully consistent with our data on IMR-32 and SK-N-BE(2) NB cells, where ISLQ treatment for 24h significantly reduced cell viability at concentrations of 5 μM or greater.

The precise mechanism of ISLQ-induced cytotoxicity in cancer cells is dependent on cell type and usually involves a combination of cell death pathways, including oxidative stress, apoptosis, autophagy and/or necrosis (Jung, Chung et al. 2006, Wu, Chen et al. 2016, Si, Yang et al. 2017, Yang, Zhang et al. 2017). Given that previous studies had reported that ISLQ treatment of human bladder cancer T24 cells led to the increase in the expression of

bax and *caspase 3* transcripts (Si, Yang et al. 2017), we hypothesised that ISLQ may have a similar effect in NB cells. In contrast, we found no change in the expression of these genes in SK-N-BE(2) cells after treatment with cytotoxic concentrations of ISLQ. In agreement with this, a published abstract reported no significant effect on capase3/7 activation in SH-SY5Y cells after treatment with 10-200 µM ISLQ for 24h (Escobar, Munoz et al. 2016). This is fully consistent with our real-time PCR and immunocytochemistry results in SK-N-BE(2) cells, and is also further supported by our finding that treatment with a broad spectrum caspase inhibitor or with a Bax inhibitor did not prevent ISLQ-induced cell death. Also in support of this, a previous report demonstrated that ISLQ induced cell death and caspase activation in U87 glioma cells (Zhou, Song et al. 2013). However, in that study, while treatment of U87 glioma cells with the pan-caspase inhibitor Z-VAD-FMK blocked ISLQ-induced caspase activation, it did not eliminate ISLQ-induced cell death (Zhou, Song et al. 2013). In agreement, we also report that ISLQ-induced cell death was not prevented by a broad-spectrum caspase inhibitor or by a Bax inhibitor, suggesting that at least in SK-N-BE(2) cells, ISLQ-induced cell death is independent of both Bax and caspase.

Necrosis has been considered as an accidental and non-regulated cell death process. However, recent studies have shed light on a new concept of regulated necrosis called receptor-interacting protein kinase (RIPK)-dependent necrosis (or necroptosis). The most prominent characteristics of this type of cell death are RIPK1 kinase activation, which can be assessed by monitoring RIPK1 phosphorylation, and suppression of the cell death by several RIPK1 inhibitors, including necrostatin-1 (Nec-1) (Galluzzi, Vitale et al. 2012). Currently, the primary method for confirming necroptosis involves detection of the key molecules that control this process (i.e. RIP1, RIP3, and MLKL) (Wang, Jin et al. 2017, Weber, Roelandt et al. 2018). RIP1/3 has been found to promote anti-metastatic outcomes by regulating oxidative stress to kill metastatic tumor cells (Cho, Challa et al. 2009, Philipp, Sosna et al. 2016). In the present study, we found that ISLQ-induced death in SK-N-BE(2) and IMR-32 cells was partially prevented by pre-treatment with nec-1, an inhibitor of RIPK1. The fact that the inhibition was partial may be explained by the fact that some necrosis inducers can act by directly activating RIPK3 or MLKL, by-passing RIPK1 activation (Dillon, Weinlich et al. 2014). In addition, we found that cell death induced by ISLQ was fully blocked by pre-treatment with IM-54, an inhibitor of oxidative stress-induced cell death (Dodo, Katoh et al. 2005). This is supported by the facts that ISLQ treatment was found to increase cellular ROS, and that the antioxidant NAC prevented

ISLQ-induced cell death, in both cell lines tested in our study. The high concentration of (1 mM) of NAC used in the present study is due to the need for compensation of the depleted cellular antioxidant GSH (van Zandwijk 1995). An involvement of ROS in necroptosis has been reported (Lin, Choksi et al. 2004). Moreover, an elevation of oxidative stress as a potential mechanism underlying cell death triggered by necroptosis, might explain the fact that several studies have shown that necroptosis is accompanied by increased ROS production in human (Ma, Peng et al. 2016) and murine (Wang, Wu et al. 2015) cancer cell types. These findings are supported by studies showing that ISLQ treatment induced redox imbalance and increased oxidative stress in HepG2 hepatocellular carcinoma cells (Sun, Wang et al. 2015) and in HeLa cells (Yuan, Zhang et al. 2013). Moreover, dietary flavonoids including ISLQ have been shown to be metabolised to pro-oxidant radicals (Galati, Sabzevari et al. 2002). Similar findings have been reported in neuronal-like cells; for example, PC-12 cells treated with 10 or 20 µM ISLQ for 24 h display an increase in intracellular ROS (Yang, Neale et al. 2013). This is very similar to the present study, in which SK-N-BE(2) treated with 25 µM for 24 h showed an increase in intracellular ROS. This elevation of ROS levels in SK-N-BE(2) cells peaked quite early after ISLQ treatment, that is, between 2 and 4h. This suggests that oxidative stress plays a substantial role in the initiation of cell death induced by ISLQ. Elevated rates of ROS have been detected in almost all cancers, where they promote many aspects of tumor development and progression. However, tumor cells also express increased levels of antioxidant proteins which can detoxify ROS, suggesting that a delicate balance of intracellular ROS levels is required for cancer cell function. The dependence of tumor cells and cancer stem cells on their antioxidant capacity makes them vulnerable to agents that dampen antioxidant systems. There is a realistic prospect for treatments aimed at dramatically increasing intracellular ROS to kill cancer cells by decreasing their antioxidant capacity (Storz 2005, Liou and Storz 2010). The advantage of such a strategy is that normal cells are not significantly affected, since they have lower basal ROS levels and therefore are less dependent on antioxidants. However, it is worth noting that there are numerous instances where anti-oxidant effects of ISLQ have been reported. For example, treatment of human arterial smooth muscle cells with a concentration of 10 µM of ISLQ, which is similar to that used in the present study, has been reported to reduce ROS levels, despite the fact that it also significantly decreased cell viability (Chen, Deng et al. 2017). These data suggest that ISLQ can have both pro- and anti-oxidant activities, depending on the cell type. However, in the present study we report that ISLQ treatment increases cellular ROS, and that this is involved in mediating the

cytotoxic effects of ISLQ on human NB cells. It is also worth noting that a recent study aimed at identifying natural mimetics of rapamycin, which is an FDA-approved mTOR inhibitor that has anti-carcinogenic effects in NB cells (Misawa, Hosoi et al. 2003), has identified ISLQ as a rapamycin mimetic (Aliper, Jellen et al. 2017). Treatment of NB cells with rapamycin for 24 h has been shown to increase cellular levels of ROS (Huang, Chen et al. 2017), which is consistent with the increases in cellular oxidative stress in *MYCN*-amplified SK-N-BE(2) cells induced by ISLQ treatment for 24h, reported in the present study. It is also worth noting that *MYCN* amplification has recently been shown to increase ROS, and to confer sensitivity to an ROS-augmenting agent in NB cells (Wang, Liu et al. 2018). Our present study is consistent with this finding, as it found that ISLQ had cytotoxic effects at concentrations of 5 µM or greater, on *MYCN*-IMR-32 and *MYCN*-amplified SK-N-BE(2) cells. This suggests that MCYN-amplified NB may be particularly sensitive to ISLQ-induced ROS.

Both the effectiveness and limitation of the many mainstream chemotherapy agents are due to the generation of ROS and consequent increases in oxidative stress, which exceed the reduction capacity of cancerous tissue, leading ultimately to apoptosis and necrosis (Castaldo, Freitas et al. 2016). Unfortunately, this mechanism could lead to the damage of healthy tissues also, resulting in numerous deleterious effects. For example, cardiotoxicity mediated by anthracycline and nephrotoxicity triggered by platinum compounds are long-term sequelae of these commonly-used chemotherapy agents (Angsutararux, Luanpitpong et al. 2015, Karasawa and Steyger 2015). Moreover, paediatric NB has been reported to be resistant to the chemotherapeutic agent, cisplatin (D'Aguanno, D'Alessandro et al. 2011). In the present study, we found that both SK-N-BE(2) and IMR-32 NB cells lines were both resistant to treatment with cisplatin at concentrations less than 100 µM. Furthermore, we report synergistic effects of combined treatment with ISLQ and cisplatin, at relatively low doses. The use of such low doses in combination could minimise the advent of deleterious side-effects on other healthy tissues, which may occur following treatment with larger doses of cisplatin.

This study has shown potent cytotoxic effects of the dietary flavonoid, ISLQ, on *MYCN*-amplified SK-N-BE(2) and IMR-32 NB cell lines. Given the aggressive nature of *MYCN*-amplified NB, and the fact that NB relapses can be refractory to treatment, these data are an important first step in rationalizing the further study of ISLQ as a potential therapeutic agent for high-risk pediatric NB. Additionally, combination therapy of ISLQ with standard

chemotherapeutic drugs such as cisplatin could allow the use of lower doses of these agents, which has potential to enhance NB patients' life expectancies, by reducing the incidence of serious long-term side-effects.

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Chapter 5

Chapter 5 4-hydroxychalcone induces cell death via oxidative stress in *MYCN*-amplified human neuroblastoma cells.

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5.1 Abstract

NB is an embryonal malignancy that arises from cells of sympathoadrenal lineage during the development of the nervous system. It is the most common pediatric extracranial solid tumor, and is responsible for 15% of childhood deaths from cancer. Fifty-percent of cases are diagnosed as high-risk metastatic disease with a low overall 5-year survival rate. More than half of patients experience disease recurrence that can be refractory to treatment. Amplification of the *MYCN* gene is an important prognostic indicator that is associated with rapid disease progression and a poor prognosis, highlighting the need for new therapeutic approaches. In recent years, there has been an increasing focus on identifying anti-cancer properties of naturally-occurring chalcones, which are secondary metabolites with variable phenolic structures. Here we report that 4HC is a potent cytotoxin for *MYCN*-amplified IMR-32 and SK-N-BE(2) NB cells, when compared to non-*MYCN*-amplified SH-SY5Y NB cells and to the non-NB human embryonic kidney cell line, HEK293t. Moreover, 4HCtreatment significantly decreased cellular levels of the anti-oxidant, GSH, and increased cellular reactive oxygen species. In addition, 4HC treatment led to mitochondrial respiratory functional impairment, compared to the controls. In support of this, the cytotoxic effect of 4HCwas prevented by co-treatment with either the anti-oxidant N-acetyl-L-cysteine, the pharmacological inhibitor of oxidative stress-induced cell death IM-54, or the mitochondrial reactive oxygen species scavenger Mito-TEMPO. When combined with the anti-cancer drugs cisplatin or doxorubicin, 4HCled to greater reductions in cell viability than was induced by either anti-cancer agent alone. In summary, this study identifies a cytotoxic effect of 4HCin *MYCN*-amplified human NB cells, which rationalises its further study in the development of new therapies for pediatric NB.

Keywords

4-hydroxychalcone; Neuroblastoma; Cancer; Small molecule; Chemotherapy; Cell death.

5.2 Introduction

NB is the most common extracranial solid pediatric cancer and the most frequent cancer diagnosed in the first year of life (Kaatsch 2010). The incidence of NB is 10 - 20 cases per million children (Kaatsch 2010, Nakata, Ito et al. 2018), yet NB is responsible for 15% of childhood deaths from cancer (Maris, Hogarty et al. 2007, Matthay, Maris et al. 2016). NB is an embryonal malignancy that arises from cells of the sympathoadrenal lineage, and can develop anywhere in the developing sympathetic nervous system (Cheung and Dyer 2013, Marshall, Carter et al. 2014, Tsubota and Kadomatsu 2018). Amplification of the *MYCN* gene is an important prognostic indicator that is associated with rapid disease progression and poor prognosis, irrespective of patient age or disease stage (Maris 2010, Huang and Weiss 2013, Zimmerman, Liu et al. 2018). *MYCN* is amplified in about 20% of NB cases, and these tumors display an undifferentiated and aggressive phenotype (Molenaar, Koster et al. 2012, Dzieran, Rodriguez Garcia et al. 2018). Moreover, in high-risk NB without *MYCN* amplification, there is often high Myc pathway activity, highlighting the importance of Myc as a driver of high-risk metastatic disease (Fredlund, Ringner et al. 2008). Indeed, *MYCN* amplification has been associated with the lowest response rate of NB after chemotherapy (Campbell, Gastier-Foster et al. 2017). Half of affected children are diagnosed with high-risk metastatic disease and, despite intensive multi-modal therapy (Matthay, Villablanca et al. 1999, Matthay, Reynolds et al. 2009), the overall 5-year survival rate is just 40-50% (Matthay, Reynolds et al. 2009). Furthermore, over half of patients experience disease recurrence, and this can be refractory to treatment (Maris 2010, Yalcin, Kremer et al. 2015). There is thus a continuing need to identify new compounds that are potential cytotoxins for high-risk, *MYCN*-amplified NB.

While the molecular basis of chemoresistance in *MYCN*-amplified NB cells is multifactorial, the resistance of *MYCN*-amplified human NB cells to oxidative stress through transcriptional up-regulation of glutamate cysteine ligase (GCL) plays an important role (Veas-Perez de Tudela, Delgado-Esteban et al. 2010). GCL is the rate-limiting enzyme in the production of the most abundant cellular anti-oxidant, GSH. GSH is a target for chemotherapeutic reagents and it is elevated in cancer cells, at levels which correlate with metastatic potential and with drug resistance (Bansal and Simon 2018, Pljesa-Ercegovac, Savic-Radojevic et al. 2018). GSH has also been targeted for the induction of cell death in NB cells, through both *MYCN* and P53 (Mlakar, Jurkovic Mlakar et al. 2019). For this

reason, compounds that induce oxidative stress or that deplete GSH levels may have promising potential as therapies for NB.

In recent years, there has been increasing interest in the anti-cancer effects of naturally-occurring compounds (Mishra and Tiwari 2011, Robles-Fernandez, Rodriguez-Serrano et al. 2013, Dai, Li et al. 2016, de Oliveira Junior, Ferraz et al. 2018). One group of compounds that has received significant attention is the flavonoids, which is a broad class of secondary metabolites with variable phenolic structures (Panche, Diwan et al. 2016). Chalcones are a subclass of flavonoids that have an open-chain structure in which two aromatic rings, known as the A and B rings, are joined by a three-carbon α,β-unsaturated carbonyl system (Rozmer and Perjési 2016, Zhuang, Zhang et al. 2017). As the chalcone structure can be modified with relative ease (Liu, Ge et al. 2013, Jandial, Blair et al. 2014), and since chalcones have been reported to be the most potent flavonoid subclass in terms of anti-tumour activity (Cabrera, Simoens et al. 2007, Loa, Chow et al. 2009, Liu, Ge et al. 2013), there has been significant interest in chalcone-based anti-cancer drug development (Mahapatra, Bharti et al. 2015, Sharma, Kumar et al. 2015). Previous reports suggest that the anti-cancer effects of chalcones are associated with the presence of hydroxyl groups, and consequently, hydroxylated chalcone derivatives have the greatest anti-cancer effects (De Vincenzo, Scambia et al. 1995, Cabrera, Simoens et al. 2007, Loa, Chow et al. 2009). The position of the hydroxyl group is important in regulating the biological activity of chalcones (Calliste, Le Bail et al. 2001, Nerya, Musa et al. 2004). Hydroxychalcones have been reported to deplete GSH levels in hepatocytes (Sabzevari, Galati et al. 2004), indicating that they have chemotherapeutic properties. A study that compared the cytotoxic effects of thirteen hydroxychalcones on melanoma cells confirmed that the number of hydroxyl groups in the molecule affects the strength of their cytotoxic activity (Navarini, Chiaradia et al. 2009). Our previous work found that the chalcone ISLQ had cytotoxic effects on NB cells, that were induced through elevation of reactive oxygen species (ROS), leading to activation of necroptotic cell death (Alshangiti, Togher et al. 2019).

Structurally, 4HC is the simplest hydroxylated chalcone, and it has been shown to have an anti-angiogenic effect that may be useful in halting cancer spread (Varinska, van Wijhe et al. 2012). There is some evidence for cytotoxic effects of other hydroxylated chalcones in melanoma (Navarini, Chiaradia et al. 2009), colon (Pande, Biswas et al. 2017), glioma (Ku, Ryu et al. 2010), breast (Padhye, Ahmad et al. 2010) and leukaemia (Orlikova, Tasdemir et al. 2011) cancer cells. In addition, 4′-hydroxychalcone, an isomer of 4HC, has been reported

to induce cytotoxic effects, via mitochondrial dysfunction, in human SH-SY5Y NB cells (de Moura Escobar, Simone et al. 2019). However, to our knowledge, no studies have examined the direct anti-cancer effects of 4HC in *MYCN*-amplified NB cancer. In the present study, we investigated whether 4HC is cytotoxic to *MYCN*-amplified NB cells and we studied its mode of cell death, its effects on oxidative stress and its effects when combined with the anti-cancer drugs, cisplatin and doxorubicin.

5.3 Materials and Methods

5.3.1 Culture of cell lines

SK-N-BE(2) cells (ATCC) were cultured in Minimum Essential Medium supplemented with 100 nM L-glutamine, 1% penicillin-streptomycin, 1% 1:1 non-essential amino acid solution: Ham's F-12, and 15% fetal bovine serum (FBS). IMR-32 cells (ATCC) were cultured in Minimum Essential Medium supplemented with 100 nM L-glutamine, 1% penicillin-streptomycin, 1% non-essential amino acid solution and 10% FBS. SH-SY5Y cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F-12 Ham's supplemented with 100 nM L-glutamine, 1% penicillin-streptomycin, and 10% FBS. HEK293T cells were cultured in high glucose DMEM supplemented with 100 nM L-glutamine, 1% penicillin-streptomycin and 10% FBS (all from Sigma). All cells were cultured at 37°C in a humidified atmosphere with 5% CO2.

5.3.2 Primary culture of E14 rat VM

Embryonic day (E) 14 rat embryos were removed by laparotomy from time-mated pregnant Sprague-Dawley rats after terminal anesthesia followed by decapitation. Embryos were removed and kept on ice in cold (4°C) Hank's balanced salts solution (HBSS). The ventral midbrain (VM) was micro-dissected from each embryo and transferred to a tube containing HBSS at 4°C. HBSS was removed and the tissue pieces were incubated in 0.1 % trypsin solution for 20 min at 37 °C. Trypsin was inactivated by the addition of FBS, and the tissue pieces were pelleted by centrifugation at 1,100 rpm for 5 min, after which the trypsin/FBS supernatant was discarded. The tissue pieces were resuspended in culture medium consisting DMEM/F12, 33 mM D-glucose, 1 % L-glutamine, 1 % FBS (all from Sigma) supplemented with 2 % B27 (Invitrogen). Tissue pieces were dissociated to a single cell suspension. Cells

were plated on poly-D-lysine (Sigma)-coated 24-well tissue culture plates at a density of 105 of cells per well in 500µl of differentiation medium and cultured at 37 °C in a humidified atmosphere with 5 % CO2.

5.3.3 Pharmacological treatments

Cultures were treated with 4HC (Sigma) (0 to 100 µM) or vehicle control (methanol (MeOH); Sigma), with or without Cisplatin (CP) or Doxorubicin (Dox) where indicated. Cells were pretreated for 1 h with 3 µM IM-54 (Calbiochem), or 150 µM Mito-TEMPO, or with N-acetyl-L-cysteine (NAC; Sigma; 300 µM for IMR-32, 1mM for SK-N-BE(2)), prior to the addition of 4HC. All of these concentrations were empirically determined prior to use (data not shown).

5.3.4 Cell viability and cell death assays

Cell viability was determined at 24 h using an 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At the end of each experiment, cultures were incubated in 1mg/ml MTT solution for 3.5 h at 37°C. MTT solution was removed and the crystals were dissolved in DMSO. Spectrophotometric absorbance was measured at 595 nm (with a reference wavelength of 620 nm) and raw absorbances were expressed as a percentage of the control. Cell death was measured using a lactate dehydrogenase (LDH) assay. For this assay, 150 µL of culture medium was removed and centrifuged at 1000 rpm for 5 min to remove cellular debris. LDH activity was measured using an LDH assay kit (ThermoFisher), according to the manufacturer's instructions. Phase-contrast images were captured using an Olympus IX71 inverted microscope fitted with an Olympus DP70 camera.

5.3.5 Measurement of cellular reactive oxygen species (ROS)

CellRox®, a cell-permeable oxidative stress indicator dye (ThermoFisher), was added to culture medium at a final concentration of 5 µM for 30 min at 37 °C. For time-course and dose-response experiments, SK-N-BE(2) were plated and treated with 25, 50 or 100 µM of 4HC for 2, 4 or 6 h, before 5 µM CellRox was added to the medium. For somal and nuclear area measurements, cells were plated and treated for 6 h with 4HC at 10 µM for IMR-32

and 25 µM for SK-N-BE(2), before being labelled with the vital fluorescent dye Calcein-AM (1:500; Invitrogen) and the nuclear stain Hoechst (1µg/ml) then incubated at 37 C for 30 min. Four random, non-overlapping fields were captured per well in each experiment (in three wells per group for each n) using an Olympus IX71 inverted microscope fitted with an Olympus DP70 camera. CellRox fluorescence intensity, somal area and nuclear size were measured in a blinded fashion using Image J in ten random individual cells per field for each well of each group. The intensity/area results were normalized to the control and expressed as a percentage of the control group.

5.3.6 GSH recycling assay

GSH levels were measured using a spectrophotometric assay involving oxidation of GSH to GSSG by the sulfhydryl reagent 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5'-thio-2-nitrobenzoic acid (TNB). The GSSG formed can be recycled to GSH by glutathione reductase in the presence of ß-NADPH (Rahman, Kode et al. 2007). SK-N-BE(2) and IMR-32 were plated in 6-well plates at the density of 106 per well for 72 h and then treated with 25, 50 µM 4HC for 4 h. At the end of the incubation time, cells and medium were removed by mechanical scraping using a sterilized rubber cell scraper in 1.5 ml Eppendorf tubes and centrifuged for 5 min at 1000g at 4C°. The media was discarded and the pellet was washed in 0.5 ml cold PBS before being centrifuged again. The supernatant was removed and the pellet was resuspended in extraction buffer (0.1% Triton X-100 and 0.6% sulfosalicylic acid in 0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5 (KPE buffer). The cells were sonicated and vortexed repeatedly, before two cycles of freezing and defrosting to ensure complete cell lysis. Cell lysates were centrifuged for 4 min at 3000g at 4 C° then the supernatant was removed. 20 µl of those supernatant samples, or GSH standards, were added into 96-well plate with 120 µl of 1:1 DTNB : glutathione reductase. 60 µl of ß-NADPH was added and absorbance measured at a wavelength of 405 nm, at 0, 30, 60, 90, and 120 s timepoints. Total GSH was calculated from the linear regression obtained from the standard curve of each timepoint, as previously described (Rahman, Kode et al. 2007). All samples were normalised for total amounts of protein using a BCA Bradford assay kit according to the manufacturer's instructions (Thermofisher).

5.3.7 Analysis of mitochondrial function using Agilent Seahorse XF 96

For analysis of mitochondrial function, IMR-32 and SK-N-BE(2) were plated in 96-well plates at 4 x 105 cells per well. On reaching 90% confluence, cells were treated with 50 μM 4HC for 4 h before mitochondrial respiration was measured using the Seahorse XF 96 Extracellular Flux Analyser and XF Wave Analysis software version 1.4. (Agilent Technologies, Santa Clara, CA, USA), and Seahorse XF 96 Extracellular Flux assay kits. All chemicals were purchased from Agilent Technologies (Santa Clara, USA). Following the 4 h drug treatment, cell culture media was replaced with 200 μl/well DMEM-based XF modified media (free of bicarbonate and phenol red, containing 25 mM glucose and 1 mM pyruvate) and cell plates were incubated for 60 min in a non-CO2 incubator before sensor calibration was performed and mitochondrial respiration stress test experiments were initiated at a consistent temperature of 37 °C. The instrument was programmed for 3 cycles of drug injection, followed by 3 mixing steps and 3 measuring periods (3 min each). The overall experimental time was 110 min. All oxygen consumption rate (OCR) analyses were carried out at least 3 times, with a minimal of 6-12 technical replicates for each treatment. Four different compounds were used to assess mitochondrial OCR: 1.5 μM oligomycin, an inhibitor of mitochondrial membrane adenosine triphosphate (ATP) synthase (F1F0 ATP synthase), 1.0 μM carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP), an ionophore uncoupler of oxidative phosphorylation, 0.3 μM antimycin A, which inhibits the mitochondrial electron transport chain from cytochrome b to cytochrome C1, and 1.0 μM rotenone, an inhibitor of mitochondrial electron transport at nicotinamide adenine dinucleotide:ubiquinone oxidoreductase in Complex I.

5.3.8 Statistical analysis

To detect significant differences, T-test, or one-way ANOVA with post-hoc Fisher's or Tukey's test, was carried out where indicated. All data are presented as mean ± SEM and all experiments were repeated at least three times. Differences were deemed significant when $p < 0.05$.

5.4 Results

5.4.1 4HC has potent cytotoxic effects on several *MYCN*-amplified and non-*MYCN*-amplified cell lines.

We first sought to determine whether 4HC had cytotoxic effects on *MYCN*-amplified NB cells. SK-N-BE(2) and IMR-32 cells were treated for 24 h with increasing concentrations of 4HC, ranging from 0 to 100 μM. MTT and LDH assays were used to assess cell viability and cell death. These analyses revealed a concentration-dependent effect of 4HC on NB cell viability (Figure. 5.1). Specifically, MTT assays showed that treatment with ≥ 5μM 4HC in SK-N-BE(2) cells (Figure. 5.1A), or ≥ 2.5μM 4HC in IMR-32 cells (Figure. 5.1C), led to a significant reduction in cell viability. SK-N-BE(2) and IMR-32 cells treated with the equivalent amount of vehicle control (MeOH) displayed no significant reductions in cell viability (Figure. 5.8A, B), indicating a cytotoxic effect of 4HC on *MYCN*-amplified NB cells. To confirm these findings, LDH assays were used and revealed increases in LDH activity in both IMR-32 and SK-N-BE(2) cells after 4HC treatment, that complemented the results of the MTT assays. Specifically, increasing concentrations of 4HC led to significant increases in LDH activity in both SK-N-BE(2) cells (Figure. 5.1B) and IMR-32 cells (Figure. 5.1D). To determine if this was a general cytotoxic effect of 4HC, we examined the effects of 4HC on SH-SY5Y cells, a non-*MYCN*-amplified NB cell line, and on a non-NB human embryonic kidney cell line, HEK293t. MTT and LDH assays revealed that 4HC treatment affected both SH-SY5Y (Figure. 5.1E, F) and HEK293t (Figure. 5.1G, H) cell viability only at concentrations ≥ 25μM. SH-SY5Y and HEK293t cells treated with the vehicle control (MeOH) displayed no significant reductions in cell viability (Figure. 5.8C, D). To further confirm this, we examined the effects of 4HC on primary cultures of E14 rat midbrain, and found that 4HC treatment for 3 DIV was not toxic to these cells at concentrations of ≥ 25 μM (Figure. 5.1I, J). Comparison of the effects of 4HC treatment on cell viability in SK-N-BE(2) and IMR-32 cells with our previous study on the chalcone ISLQ in these cell lines [41] showed that 10 μM 4HC treatment for 24 h induced a significantly greater reduction in cell viability than the same dose of ISLQ, in both of these cell types (Figure 5.9). Moreover, treatment with (MEOH vehicle only) dose not result in any effect cell viability on tested cells (Figure 5.8). This suggests that *MYCN*-amplified NB cells have differential sensitivity to distinct chalcones.

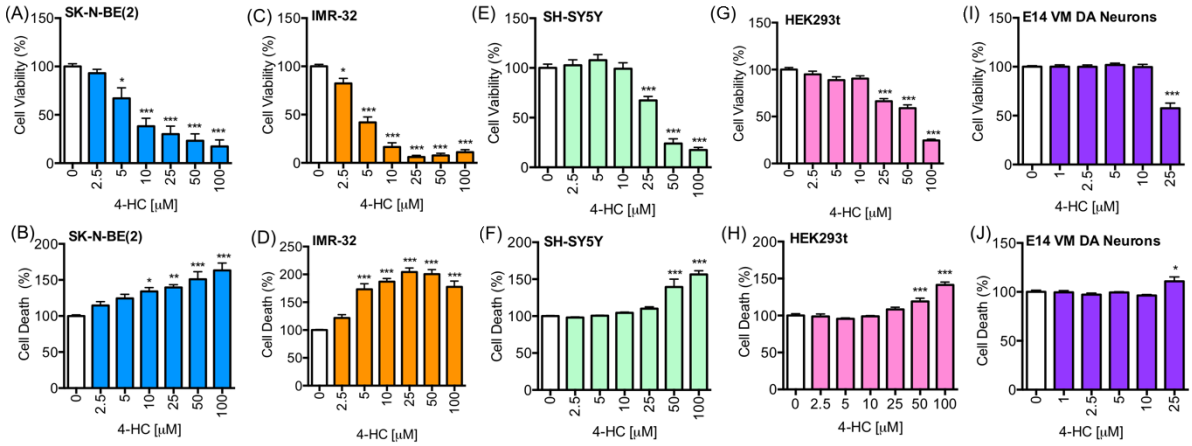


Figure 5.1: 4HC has potent cytotoxic effects on several *MYCN*-amplified and non-*MYCN*-amplified cell lines.

Cell viability measured by MTT assay (upper panels) and cell death measured by LDH assay (lower panels) on (A, B) SK-N-BE(2), (C, D) IMR-32, (E, F) SH-SY5Y, (G, H) HEK293t cells and (I, J) primary cultures of E14 rat midbrain, treated with the indicated concentrations 4HC for 24 h or 3DIV for E14 cells. All data are mean ± sem; *n*=3 independent experiments for A-H, *n*=6 independent experiments for I and J; ***p* < 0.05, ****p* < 0.01, *****p* < 0.001 vs control; one-way ANOVA with Tukey's test.

5.4.2 *MYCN*-amplified NB cells are more sensitive to the effects of 4HC than non-*MYCN*-amplified cell lines.

To specifically examine the sensitivity of *MYCN*-amplified NB cells, we compared the percentage changes on the MTT and LDH assays in each of the four cell lines after treatment with 25 μM 4HC. This showed that 4HC had a significantly greater cytotoxic effect on SK-N-BE(2) and IMR-32 cells, than on SH-SY5Y and HEK293t cells (Figure. 5.2A, B). From this point on, we focused on studying the role of 4HC in the *MYCN*-amplified NB cell lines, SK-N-BE(2) and IMR-32.

To determine whether 4HC treatment led to morphological changes consistent with cell death, we examined cell morphology in SK-N-BE(2) (Figure. 5.2C) and IMR-32 (Figure. 5.2D) cells, using Calcein-AM and Hoechst staining. Significant reductions in both cell area and nuclear area were induced by treatment with 25 μM 4HC in SK-N-BE(2) cells (Figure.

5.2E) and 10 µM 4HC in IMR-32 cells for 6h (Figure. 5.2F). Collectively, these data show that 4HC can induce cell shrinkage and are consistent with the data showing reductions in cell viability and increases in cell death.

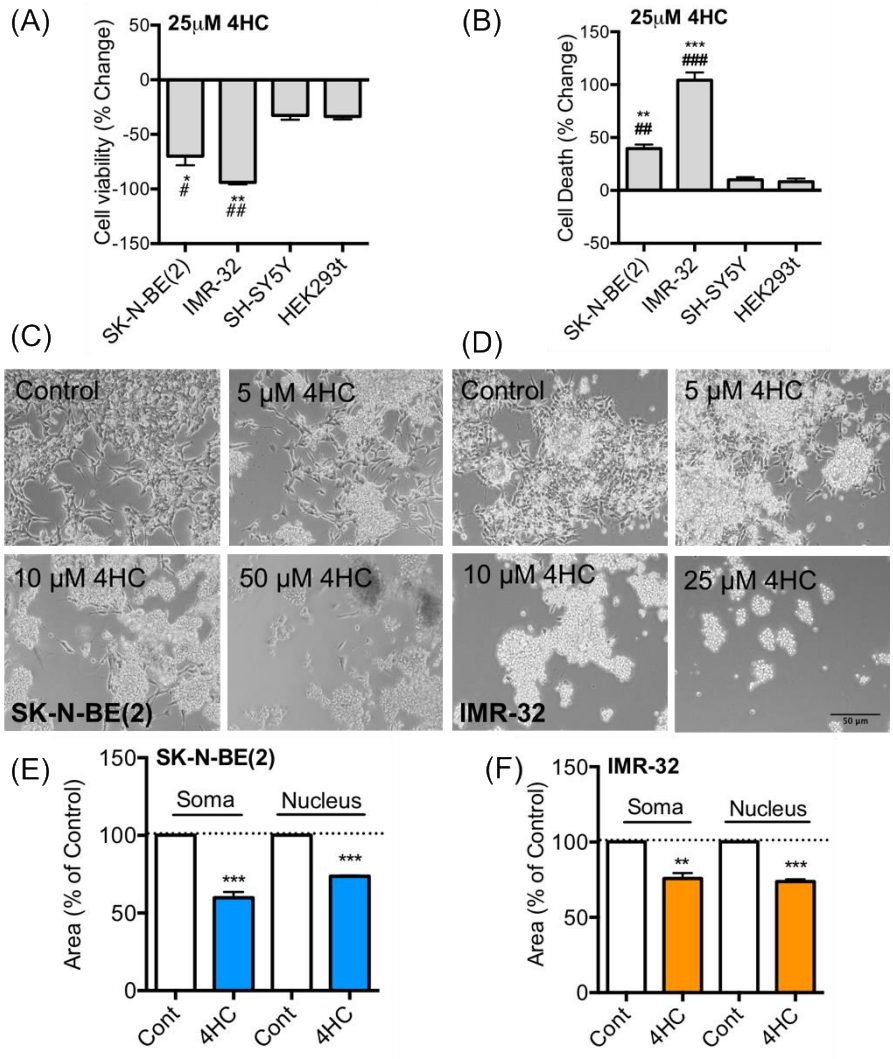


Figure 5.2: *MYCN*-amplified NB cells are more sensitive to the effects of 4HC than non-*MYCN*-amplified cell lines.

Percentage change in (A) cell viability measured by MTT assay and (B) cell death measured by LDH assay on the four indicated cell lines after treatment with 25 µM 4HC for 24 h. All data are mean ± sem; n=3 independent experiments. ***$p < 0.05$, ****$p < 0.01$, *****$p < 0.01$ compared to SH-SY5Y cells and #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ compared to HEK283t cells; one-way ANOVA with Fisher's LSD test. Representative phase contrast micrographs of (C) SK-N-BE(2) cells and (D) IMR-32 cells treated with the indicated

concentrations of 4HC for 24 h. Scale bar = 50 µm. Somal and nuclear areas as percentages of controls in (E) SK-N-BE(2) cells treated with 25 µM 4HC for 6 h or (F) IMR-32 treated with 10 µM 4HC for 6 h. All data are mean ± sem; *n*=3 independent experiments. ****p* < 0.01, *****p* < 0.01 compared to controls (Cont) for each parameter; Students t-test for each parameter in each cell type.

5.4.3 4HC depletes the anti-oxidant glutathione and increases oxidative stress in NB cells.

We next examined the pathways involved in 4HC-induced cell death in NB cells. Chalcones have been shown to regulate cellular levels of ROS, depending on the cellular type and context (Guzy, Vašková-Kubálková et al. 2010). Chalcones also have been reported to induce depletion of the antioxidant GSH, which is considered a potential target for cancer treatment (Kachadourian and Day 2006). We first examined whether 4HC treatment induced oxidative stress in *MYCN*-amplified NB cells. We used the enzymatic recycling method of measuring the reduced GSH form of the antioxidant GSH (Rahman, Kode et al. 2007). GSH is tripeptide thiol (γ-glutamyl cysteinyl glycine) a cellular antioxidant that acts as a free radical scavenger, and its intracellular concentration is an indicator of oxidative stress. In cells undergoing oxidative stress there is a reduction in cellular GSH levels. Cells were treated with higher doses of 4HC for a shorter time, to examine the early effect of 4HC treatment on ROS levels in living cells. SK-N-BE(2) and IMR-32 cells were treated with 25 or 50µM 4HC for 4 h before GSH levels were measured. 4HC treatment led to significantly lower GSH levels in both SK-N-BE(2) cells (Figure. 5.3A) and IMR-32 cells (Figure. 5.3B). To gather further evidence to support that finding that 4HC induces oxidative stress, we loaded SK-N-BE(2) with CellRox, which is a compound that fluorescence with an intensity that correlates with intracellular levels of ROS, a measure of cellular oxidative stress (Figure. 5.3C). Treatment with 25 µM 4HC led to a significant increase in cellular ROS after 4 and 6h (Figure. 5.3D). Treatment with 25 µM, 50 µM or 100 µM 4HC for 2 h induced a concentration-dependent increase in cellular ROS (Figure. 5.3E). We also examined the ROS level in the presence of IM-54 oxidative stress inhibitor and we found that 4HC-induced oxidative stress is prevented by IM-54 (Figure 5.10). Collectively, these data show that 4HC increases cellular oxidative stress in SK-N-BE(2) and IMR-32 cells.

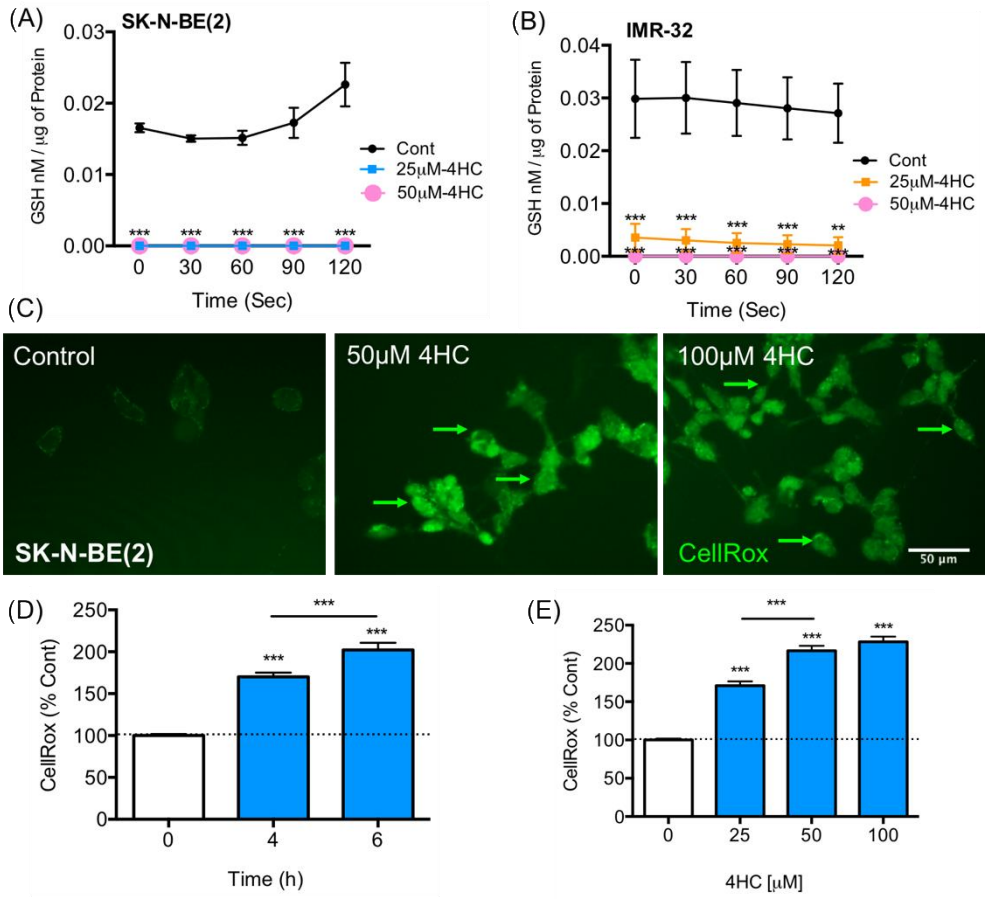


Figure 5.3: 4HC depletes the anti-oxidant glutathione and increases oxidative stress in NB cells.

GSH concentration in (A) SK-N-BE(2) cells and (B) IMR-32 cells treated with 50 and 25 µM 4HC for 4 h. All data are mean ± sem; *n*=3 independent experiments. ***p* < 0.05, ****p* < 0.01 vs. Control (Cont) at each timepoint; two-way ANOVA with Sidak's post hoc test. (C) Representative photomicrographs of CellRox fluorescence intensity in SK-N-BE(2) cells treated 50 or 100 4HC for 6 h. Arrows indicate elevated ROS levels in individual cells. Scale bar = 50 µm. CellRox fluorescence intensity as percentages of control in SK-N-BE(2) cells treated with (D) 25µM 4HC for 2, 4 or 6 h and (E) 25, 50 or 100 µM 4HC for 2 h. All data are mean ± sem of 360 cells from *n*=3 independent experiments. ****p* < 0.001 vs. Control; ANOVA with Fisher's LSD test.

5.4.4 4HC-induced cell death affects oxygen consumption rate in NB cells.

To further investigate cell death induced by 4HC in NB cells, we analysed bioenergetic state by measuring the rate of oxygen consumption in cells treated with 50 µM 4HC for 4h, using the Seahorse XF 96 Extracellular Flux Analyser. 4HC-treated cells showed impairment in

respiratory function throughout the experiment, compared to the vehicle (Figure 5.4A-B). Specifically, there was a significant decrease in basal respiration levels in both IMR-32 and SK-N-BE(2) cells after treatment with 4HC (Figure. 5.4C, G). Addition of oligomycin, an inhibitor of mitochondrial ATP-synthase, caused a decrease in ATP production. Consistent with the changes in basal respiration, there was also a reduction in mitochondrial ATP synthase in 4HC-treated cells (Figure. 5.4D, H). Subsequent addition of FCCP caused uncoupling of mitochondrial oxidative phosphorylation (OX-PHOS) to induce maximal respiration. Treatment with 4HC led to a significant decrease in maximal respiration capacity, indicative of uncoupling (Figure. 5.4E, H). Finally, a combination of rotenone and antimycin A was added to inhibit complex I of the mitochondrial respiration chain. Following these treatments, there was no notable effect of 4HC on the spare capacity of either of the two cell lines (Figure. 5.4F, J). These data suggest that 4HC treatments cause impairment in mitochondrial oxygen consumption rate in NB cells that lead to cell death.

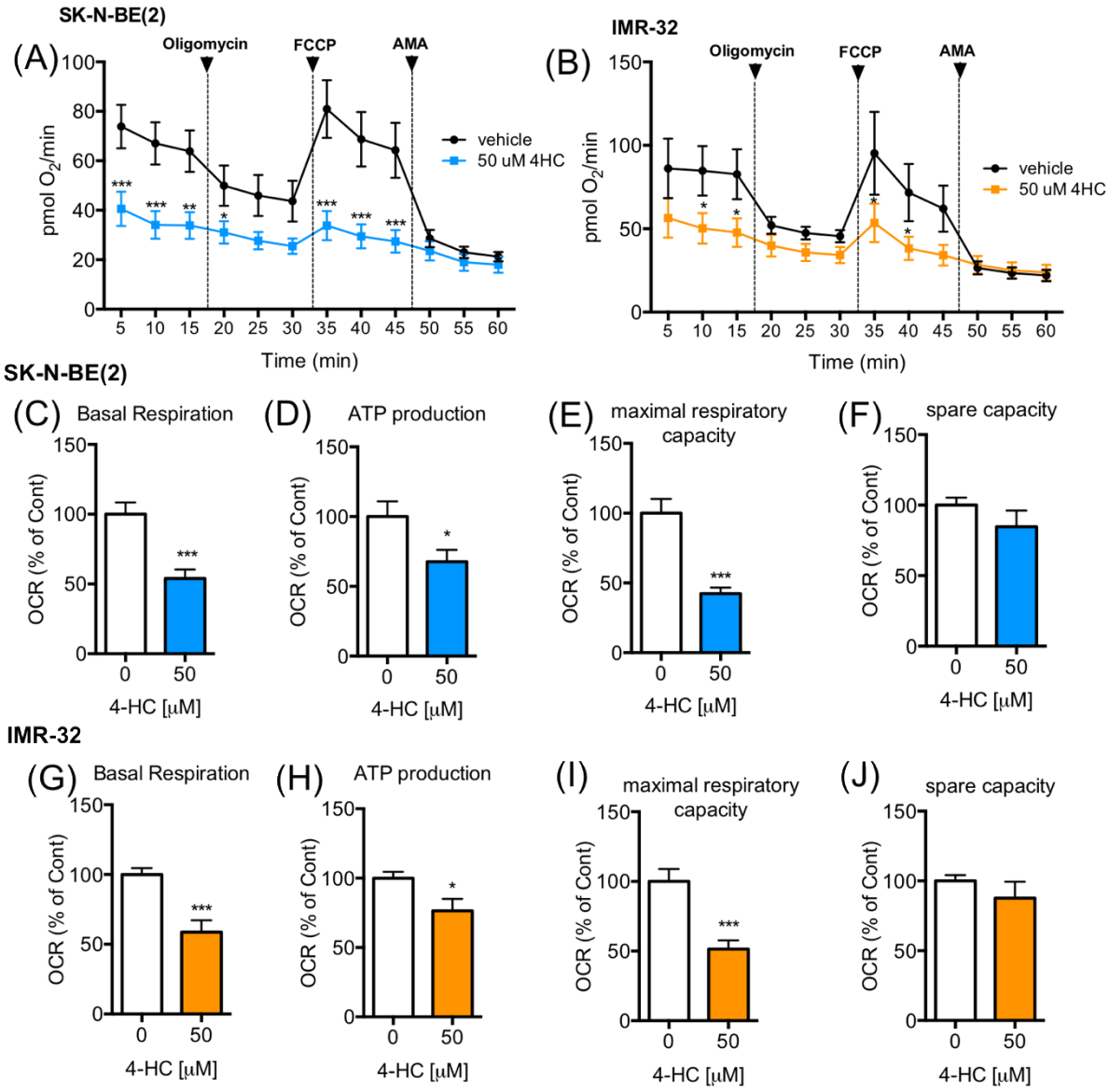


Figure 5.4: 4HC-induced cell death affects oxygen consumption rate in NB cells.

Mitochondrial oxygen consumption rate (OCR) of (A) SK-N-BE(2) and (B) IMR-32 cells treated with 50µM 4HC or vehicle (MeOH) for 4 h, at various stages: initially, 'basal respiration' cellular OCR, then OCR after treatment with oligomycin, a complex V inhibitor ('ATP production'), then OCR after treatment with FCCP, a protonophore ('maximal respiratory capacity'), finally OCR after treatment with AMA ('spare capacity'). OCR at each of the four individual stages in (C-F) SK-N-BE(2) and (G-J) IMR-32 cells from the same experiment, presented as a percentage of vehicle. All data are mean ± sem; n=3 independent experiments. *$p < 0.05$, **$p < 0.01$,***$p < 0.001$ vs untreated; ANOVA with Fisher's LSD test, unpaired T-test.

5.4.5 4HC-induced NB cell death is prevented by inhibition of oxidative stress and by scavenging of mitochondrial superoxide.

To determine whether increases in oxidative stress were involved in 4HC-induced cell death, we used two complementary approaches. Firstly, SK-N-BE(2) and IMR-32 cells were pretreated for 2 h with the anti-oxidant NAC prior to the addition of 4HC at 25µM for SK-N-BE(2) and 10 µM for IMR-32, before cell viability was examined after 24 h using an MTT assay. These analyses revealed that 4HC treatment led to a significant reduction in cell viability in both SK-N-BE(2) (Figure. 5.5A) and IMR-32 cells (Figure. 5.5B), which was fully prevented by NAC pretreatment in both cell lines (Figure. 5.5A-C).

To confirm these findings, we examined whether a small molecule inhibitor (IM-54), that selectively blocks oxidative stress-induced cell death (Dodo, Katoh et al. 2005), could prevent the cell death induced by 4HC. To do this, SK-N-BE(2) cells and IMR-32 were pretreated for 1 h with 3 µM IM-54, before 4HC was added for 24 h. In agreement with the NAC experiment, 4HC treatment for 24 h led to a significant reduction in cell viability in SK-N-BE(2) (Figure. 5.5D), and in IMR-32 cells (Figure. 5.5E), which was fully prevented by IM-54 pretreatment in both cell lines. In agreement with this, IM-54 prevented the increase in cellular ROS following 4HC treatment in SK-N-BE(2) cells (Figure. 5.10). To further study the role of mitochondrial ROS in 4HC-induced cell death, we pretreated with Mito-TEMPO, a compound that scavenges superoxide by mimicking superoxide dismutase from mitochondria (Yang, Park et al. 2018). Mito-TEMPO significantly protected SK-N-BE(2) and IMR-32 cells against 4HC-induced cytotoxicity (Figure 5.6). Collectively, these data show that 4HC-induced oxidative stress plays a functional role in 4HC-induced cell death in SK-N-BE(2) and IMR-32 cells.

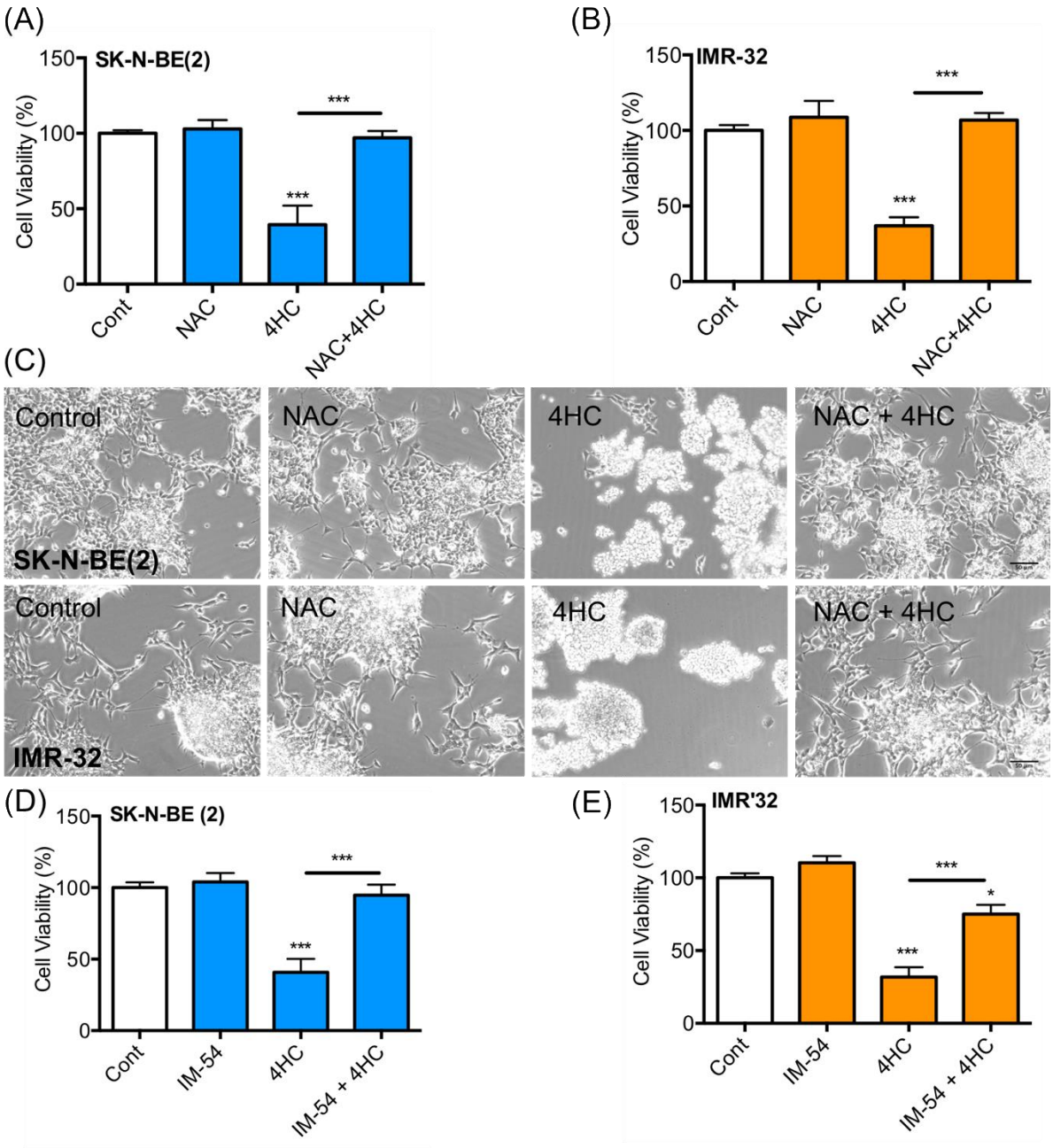


Figure 5.5: 4HC-induced NB cell death is prevented by inhibition of oxidative stress. (A, B) Cell viability measured by MTT assay and (C) representative photomicrographs of (A, C) SK-N-BE(2) and (B, C) IMR-32 cells pretreated with NAC before being cultured with or without 25 µM 4H for SK-N-BE(2) and 10 µM 4H for IMR-32 C for 24 h. Scale bar = 50µm. Cell viability measured by MTT assay on (D) SK-N-BE(2) and (E) IMR-32 cells pretreated with IM-54 before being cultured with or without 25 µM 4HC for SK-N-BE(2) and 10 µM 4HC for IMR-32 for 24 h. All data are mean ± sem; *n*=3 independent experiments. *p < 0.05, ***p < 0.001 versus control or as indicated; ANOVA with Fisher's LSD test).

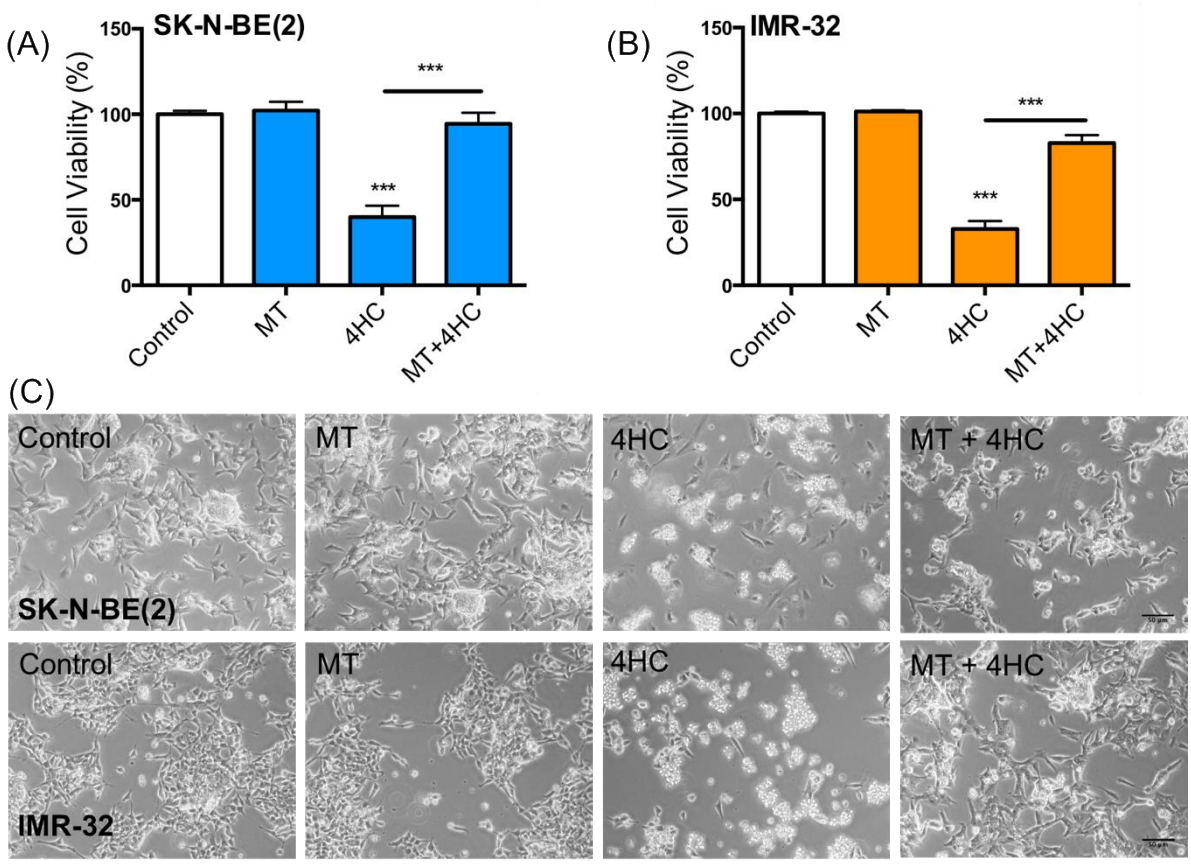


Figure 5.6: 4HC-induced NB cell death is prevented by scavenging of mitochondrial superoxide.

(A, B) Cell viability measured by MTT assay and (C) representative photomicrographs of (A, C) SK-N-BE(2) and (B, C) IMR-32 cells pretreated with mito-TEMPO (MT) before being cultured with or without 25 µM of 4HC or SK-N-BE(2) and 10 µM 4HC for IMR-32 for 24 h. Scale bar = 50µm. All data are mean ± sem; *n*=3 independent experiments. ***p < 0.001 versus control or as indicated; ANOVA with Fisher's LSD test.

5.4.6 4HC enhances cytotoxicity induced by doxorubicin and cisplatin in SK-N-BE(2) and IMR-32 cells.

Given the toxicity associated with high-dose chemotherapy, it is important to identify agents that enhance the cytotoxic effects of commonly-used anti-cancer drugs such as cisplatin and doxorubicin. To this end, we performed dose-response experiments with doxorubicin in both SK-N-BE(2) and IMR-32 cells and assessed cell viability at 24 h using an MTT assay. Treatment with 10 µM doxorubicin resulted in approximately 50% decrease in cell viability at 24 h in both cell lines (Figure. 5.11). Both cell lines, as well as the non-*MYCN*-amplified

SH-SY5Y cell line, were treated with 5µM doxorubicin and 100µM cisplatin for 24 h, with or without co-treatment with 4HC. The addition of 4HC resulted in a significantly greater cytotoxic effect than treatment with either of the anti-cancer drugs alone, in all three cell lines, SK-N-BE(2) (Figure. 5.7A, D), IMR-32 (Figure. 5.7B, D) and SH-SY5Y (Figure. 5.7C, D).

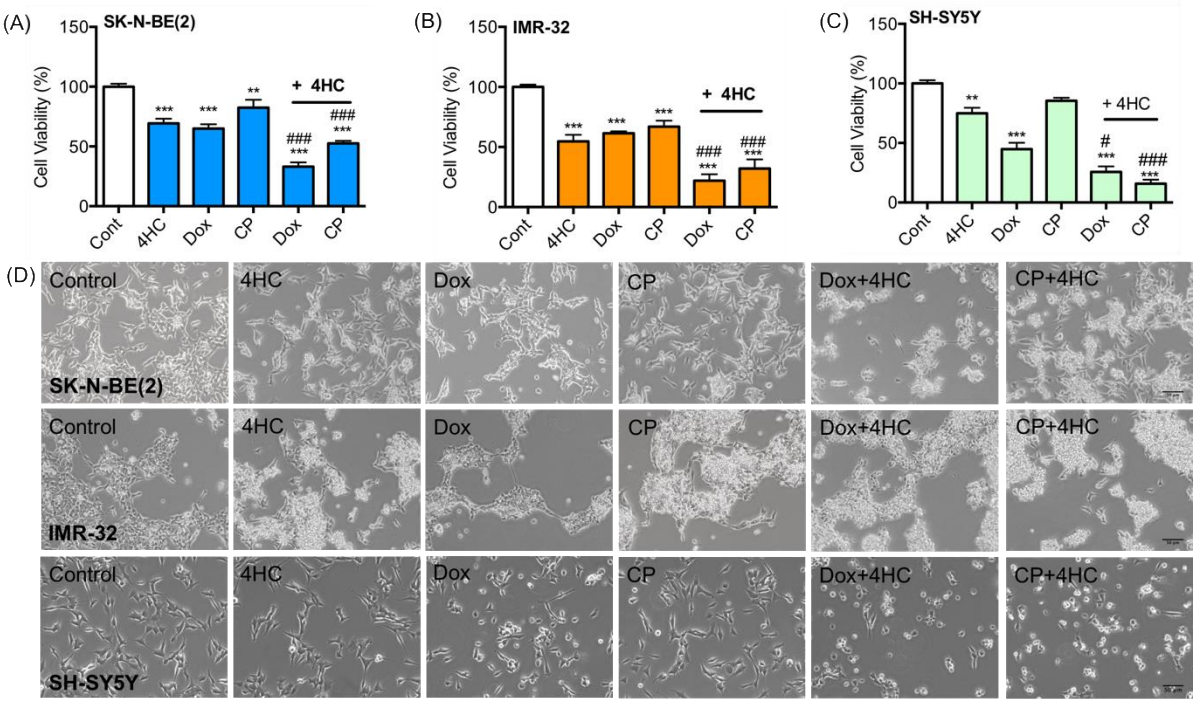


Figure 5.7: 4HC enhances cytotoxicity induced by doxorubicin and cisplatin in SK-N-BE(2) and IMR-32 cells.

(A-C) Cell viability measured by MTT assay and (D) representative photomicrographs of (A) SK-N-BE(2) and (B) IMR-32 treated with 5µM 4HC, and (C) SH-SY5Y cells treated with 25 µM 4HC, or with 5µM doxorubicin (Dox) or 100µM cisplatin (CP) with or without 4HC for 24 h. All data are mean ± sem. *p < 0.05, **p < 0.01, ***p < 0.001 versus control. ### Combined treatment versus Dox or CP alone; ANOVA with Fisher's LSD test. Scale bar = 50µm.

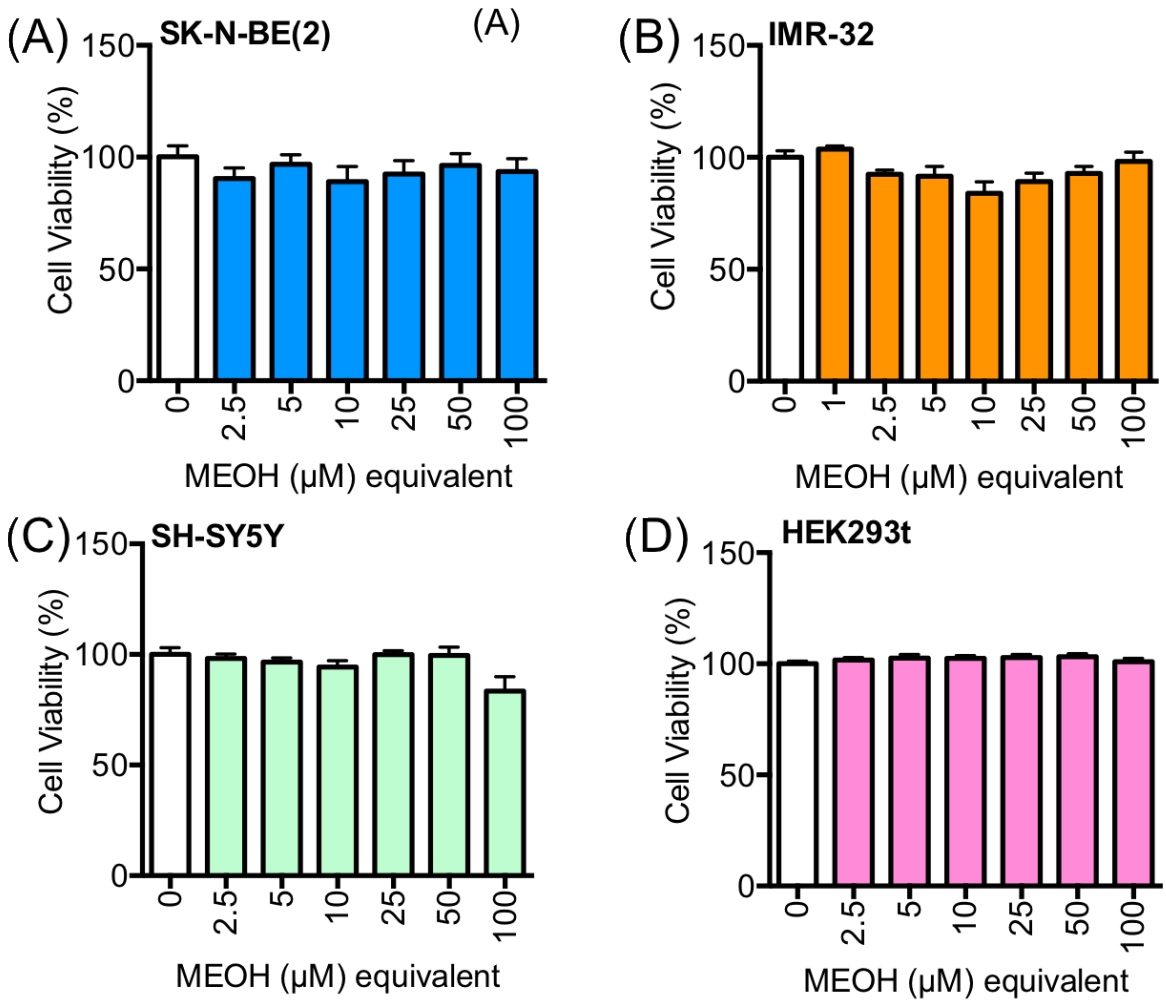


Figure 5.8 Treatment with vehicle control does not affect cell viability.

Cell viability as measured using MTT assay on (A) SK-N-BE(2), (B) IMR-32, (C) SH-SY5Y and (D) HEK293t cell lines, treated with the indicated concentrations of vehicle (MeOH) control for 24 h. All data are mean ± sem; ANOVA with post-hoc Tukey's test.

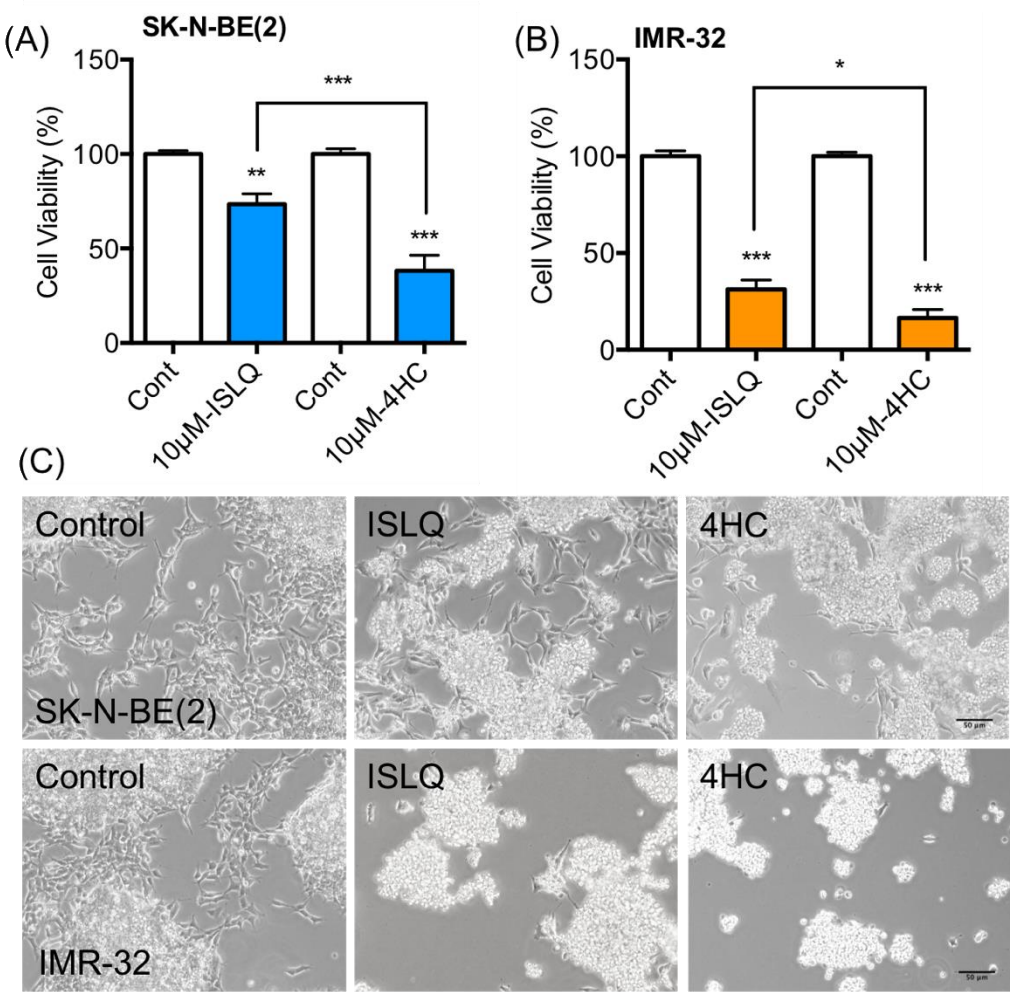


Figure 5.9 NB cells are more sensitive to 4HC than to ISLQ.

(A, B) Cell viability as measured using MTT assay and (C) representative photomicrographs of SK-N-BE(2) and IMR-32 cell lines, as indicated, treated with 10μM of ISLQ or 4HC for 24 h. The ISLQ data have been previously published (Alshangiti, Togher et al. 2019) All data are mean ± sem; n =3 independent experiments. *$p < 0.05$, ***$p < 0.001$ versus control; ANOVA with post-hoc Tukey's test.

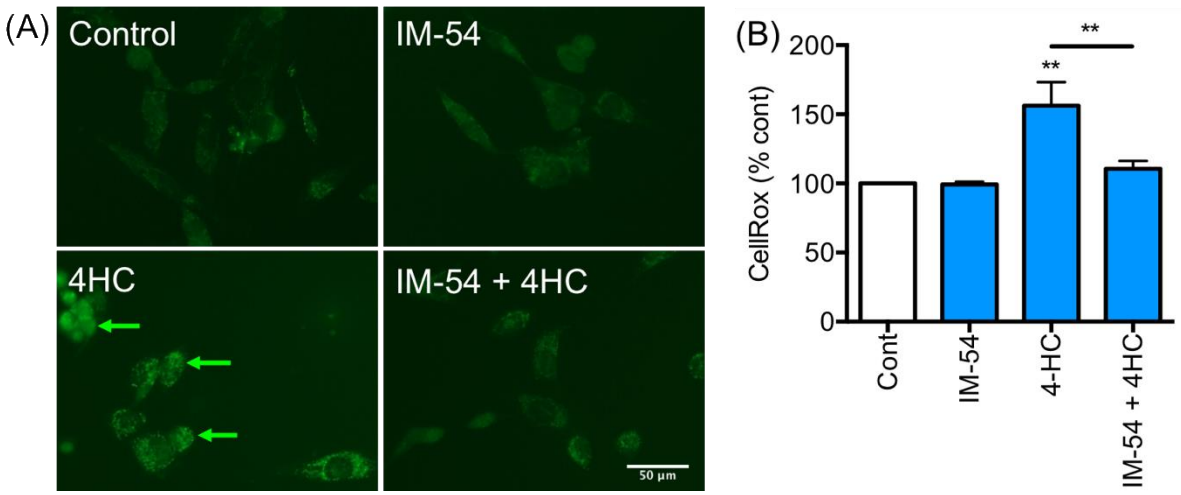


Figure 5.10 4HC-induced oxidative stress is prevented by IM-54.

(A) Representative photomicrographs of and (B) relative CellRox fluorescence intensity in SK-N-BE(2) cells treated with 25 µM of 4HC for 24 h with or without pretreatment with 3 µM IM-54. Arrows show elevated ROS. Scale bar = 50 µm. All data are mean ± sem; n=3 independent experiments. ** $p < 0.01$, ***$p < 0.001$ vs. Control or as indicated; ANOVA with Tukey's post-hoc test.

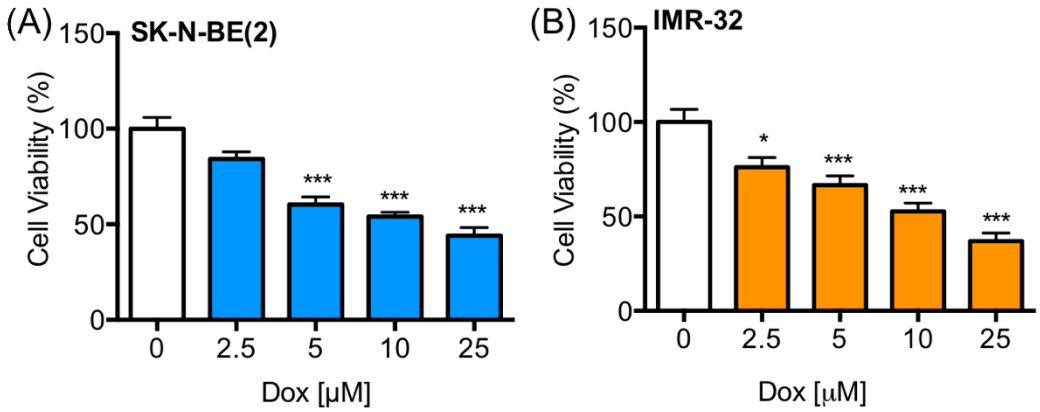


Figure 5.11 Effects of increasing Dox concentrations on cell viability.

(A, B) Cell viability as measured using MTT assay on (A) SK-N-BE(2), (B) IMR-32 cell lines, treated with the indicated concentrations of doxorubicin (Dox) for 24 h. All data are mean ± sem; n =3 independent experiments. *$p < 0.05$, ***$p < 0.001$ versus control; ANOVA with post-hoc Tukey's test.

5.5 Discussion

The use of chalcones as potential anti-cancer agents is relatively novel. To date, there have been no reports on the potential of 4HC as an anti-cancer agent in human *MYCN*-amplified NB. In the current study, we found that 4HC exerted potent cytotoxic effects on *MYCN*-amplified SK-N-BE(2) and IMR-32 human NB cells at low concentrations, in comparison to other non-*MYCN*-amplified NB cells, kidney embryonic cells, or healthy murine primary neuronal cultures. We also found that the cytotoxicity induced in *MYCN*-amplified NB cell lines by 4HC was higher than that induced by ISLQ, another chalcone which we had investigated in a previous study (Alshangiti, Togher et al. 2019). The cytotoxic effect of 4HC treatment was found to be both time- and concentration-dependent and to by accompanied by decreases in both cell somal and nuclear areas. The mechanism of the cell death induced by 4HC involved elevation of intracellular ROS combined with significant depletion of the antioxidant GSH. Furthermore, we found that replenishing the depleted antioxidants by treatment with NAC, or blocking oxidative stress signaling by treatment with the small molecule IM-54, or protecting the mitochondria using Mito-TEMPO, a mitochondria-specific superoxide scavenger, could significantly protect cells from 4HC-induced cytotoxicity. In addition, 4HC treatment caused a severe impairment of mitochondrial oxygen consumption in both of the *MYCN*-amplified cell lines. Co-treatment with 4HC was also found to increase the cytotoxic effects of cisplatin and doxorubicin on SK-N-BE(2), IMR-32 and SH-SY5Y cells.

In agreement with our current findings, previous studies have documented the anti-cancer activity of other chalcones on a wide variety of cancer cells. Isobavachalcone, a chalcone constituent of Angelica keiskei, was found to induce caspase-dependent cell death in IMR-32 and NB-39 NB cells, when applied at a concentration 10 µM for 24 h (Nishimura, Tabata et al. 2007). ISLQ and phloretin, similar chalcones to 4HC, have also been found to exert cytotoxic effects on B16 melanoma cells (Iwashita, Kobori et al. 2000), while the dihydrochalcone phloretin can induce cell death via apoptosis in HL60 human leukaemia cells (Kobori, Iwashita et al. 1999). In addition, the cytotoxic effect of the synthetic naphthylchalcone, (2E)-1-(2,5-dimethoxy-phenyl)-3-(1-naphthyl)-2-propene-1-one, was reported to be both concentration- and time-dependent, with IC50 values ranging between 1.5 µM and 40 µM, in K562, Jurkat, Kasumi, U937, CEM and NB4 cells (Sabzevari, Galati et al. 2004, Maioral, Gaspar et al. 2013). Moreover, another chalcone, trans-chalcone, has

been shown to suppress the growth of U2OS osteosarcoma cells in a concentration- and time-dependent manner, with significant inhibition observed after treatment with for 48 h (Silva, Marins et al. 2016). Recently, de Moura Escobar and colleagues have shown cytotoxic effects of 10-60 μM 4′-hydroxychalcone, an isomer of 4HC, on SH-SY5Y cells (de Moura Escobar, Simone et al. 2019). Collectively, these data are consistent with our current findings, which show that 4HC has cytotoxic effects on *MYCN*-amplified NB cells at concentrations ≥ 5 μM in SK-N-BE(2) and ≥ 2.5 μM in IMR-32 cells. These cytotoxic effects of 4HC on NB cells are also consistent with our previous work on the chalcone-derived flavonoid, ISLQ. In that study, we found that treatment with ≥ 5 μM ISLQ for 24 h had potent cytotoxic effects on both SK-N-BE(2) and IMR-32 cells (Alshangiti, Togher et al. 2019). 4HC had a greater cytotoxic effect on *MYCN*-amplified NB cells than the related chalcone, ISLQ. The reasons for these different effects of similar chalcones require further investigation. However, it is known that the number of hydroxyl groups and their positioning can regulate the ability of chalcones to uncouple mitochondria by collapsing the mitochondrial membrane (Sabzevari, Galati et al. 2004), suggesting that structural differences between chalcones may account for the differences in the magnitude of their cytotoxic effects on NB cells. Another chalcone, cardamonin, has been found to selectively induce death of human melanoma cells, and not of healthy human melanocytes or dermal fibroblasts (Berning, Scharf et al. 2019). This is consistent with the selectivity cytotoxicity of low doses of 4HC on *MYCN*-amplified NB but not on cultured midbrain neurons, found here in our study. Collectively, these data support the growing body of evidence that chalcone molecules can induce time- and concentration-dependent cytotoxic effects on NB cells.

A substantial issue facing cancer treatments, particularly high-risk metastatic cancers, is resistance to apoptosis. This mechanism has been reported to occur in *MYCN*-amplified tumours, and it confers cell resistance to apoptosis induced by the tumour necrosis factor-related apoptosis-inducing ligand system (Adhikary and Eilers 2005). The genetic profiles of both of the *MYCN*-amplified cell lines used in the present study may promote cell proliferation, rather than differentiation or death via apoptosis (Kang, Rychahou et al. 2006, Agarwal, Milazzo et al. 2018). *MYCN* amplification has been also shown to be associated with enriched glutaminolysis (Wang, Liu et al. 2018). Glutamine is a critical amino acid for many fundamental functions of cancer cells, including synthesis of metabolites that maintain

mitochondrial metabolism and generation of antioxidants, such as GSH, to control ROS levels (Wang, Liu et al. 2018). In cancer cells, the constant proliferation is a highly metabolic process that leads to an enormous accumulation of ROS. Therefore, as an adaptive response, cancer cells harbour elevated levels of ROS-scavenging molecules such as GSH (Bansal and Simon 2018). *MYCN*-amplified NB have been shown to have a two-fold enrichment of GSH levels in comparison to cells without *MYCN* amplification (SH-SY5Y) (Veas-Perez de Tudela, Delgado-Esteban et al. 2010). Moreover, glutaminolysis has been reported to result in excessive amounts of ROS in *MYCN*-amplified NB cells, rendering them even more sensitive to ROS augmentation and increasing their sensitivity to treatment with pro-oxidants such as dimethyl fumarate (Wang, Liu et al. 2018). These findings are consistent with those of our study, where there was a higher sensitivity to 4HC of *MYCN*-amplified cells, compared to non-*MYCN*-amplified cells. That is, treatment with 5 µM 4HC for 24 h was significantly toxic to IMR-32 and SK-N-BE(2) cells, while 4HC was only toxic at 25 µM for SH-SY5Y and HEK293t cells after the same treatment time.

To confirm whether 4HC-induced cell death targets the delicate balance between extensive GSH production and high levels of ROS in SK-N-BE(2) and IMR-32 cells, we measured GSH levels in 4HC-treated cells, using the GSH recycling assay. We found that treatment of IMR-32 and SK-N-BE(2) with 25-50 µM 4HC caused severe depletion of cellular levels of the antioxidant GSH after 4 h. This was consistent with the findings of a previous study, which found that thirteen distinct hydroxychalcones induced apoptosis in B16-F10 melanoma cells via GSH and ATP depletion (Navarini, Chiaradia et al. 2009). We used CellRox fluorogenic probe to measure oxidative stress in live SK-N-BE(2) cells to determine the level of ROS at various treatment times and concentrations of 4HC. Treatment with 4HC induced an elevation in ROS in a dose- and time-dependant manner. This confirms that 4HC cell toxicity is caused by GSH depletion that is combined with an elevation of ROS levels. Targeting of GSH is a promising approach in the anticancer effect of 4HC, given the role of GSH in promoting tumour progression and metastasis.

We then explored the potential role of mitochondria in 4HC-induced cell death. Cancer cells have been previously reported to rely on glycolysis, due to their impaired mitochondria (Kim and Dang 2006). However, other studies have shown that the mitochondria of some cancer cells are functional and not solely dependent on glycolysis for energy requirement, but that they also derive energy from mitochondrial respiration (Rodriguez-Enriquez, Carreno-Fuentes et al. 2010) (Dar, Chhina et al. 2017). For example, an isomer of 4-HC has been

reported to induce mitochondrial dysfunctional, depletion of intracellular ATP levels, and increases in ROS, in SH-SY5Y cells (de Moura Escobar, Simone et al. 2019). In the current study, we detected significant impairment of cellular OCR in 4HC-treated *MYCN*-amplified NB cells, including decreases in ATP-linked respiration, proton leak respiration and the maximal rate of the electron transport chain, which is reflective of uncoupling. In contrast, non-mitochondrial respiration was not affected by 4HC treatment. Mitochondria are known to maintain a precise balance between bioenergetic and biosynthetic molecular pathways, under normal conditions. In cancer cells, a mismatch in this balance promotes increased ROS generation, favouring metabolic flexibility and mitigating oxidative stress via additional protective mechanisms, such as upregulation of un-coupling proteins (Baffy 2017). We found that 4HC treatment induced mitochondrial dysfunction in SK-N-BE(2) and IMR-32 cells, by interrupting respiration at several stages. Targeting mitochondrial function in cancer cells has been recently reported as a novel antitumour activity that may counteract drug resistance, inhibit cell proliferation, or decrease cancer metastasis (Baffy, Derdak et al. 2011) (Wang, He et al. 2018) (Alasadi, Chen et al. 2018, Zhu, Dean et al. 2018).

To further investigate the mechanisms involved in 4HC-induced cell death and to confirm the pro-oxidant effect of 4HC, we used IM-54, a small molecule inhibitor of oxidative stress-induced cell death. Pretreatment with IM-54 fully prevented 4HC-induced cell death. Pretreatment with NAC, the precursor of the antioxidant GSH, also protected cells from 4HC cytotoxicity; this is likely due to compensation of 4HC-induced depletion of GSH (van Zandwijk 1995). Moreover, pretreatment with Mito-TEMPO, a mitochondria-specific superoxide scavenger, significantly protected cells from 4HC-induced cytotoxicity. All of these findings confirm that 4HC treatment leads to ROS-induced cell death in *MYCN*-amplified NB cells. This is consistent with our previous study showing that another chalcone, ISLQ, induced cell death in *MYCN*-amplified NB cells through elevation of ROS levels, which was fully prevented by pretreatment with NAC (Alshangiti, Togher et al. 2019). This is an important finding, as the ability of ROS to chemosensitise cancer cells depends on the basal ROS levels in these cells. ROS levels could represent a double-edged sword, since their activation below a specific threshold promotes cell survival, but activation above this threshold can lead to cell death. This may be the reason for the limited success of antioxidant therapies in clinical trials (Sosa, Moliné et al. 2013), and may support the use of pro-oxidants, including chalcones, in approaches to selectively target malignant cells by

exploiting their abnormal levels of ROS (Wang, Luo et al. 2017). Moreover, selectively targeting the delicate balance of ROS levels in cancer cells is emerging as a potential new strategy (Martin-Cordero, Leon-Gonzalez et al. 2012). For instance, a novel chalcone derivative, S17, has been found recently to induce apoptosis through ROS-dependent upregulation of DR5 in MGC803 gastric cancer cells; similar to our findings, this death was completely prevented by NAC pretreatment (Zhang, Li et al. 2017).

In this study, we also found that the cytotoxic effects of 4HC increased when this agent was applied in combination with doxorubicin or cisplatin, two of the most commonly-used drugs in chemotherapeutic regimens in high-risk NB treatment (Ganeshan and Schor 2011, Hara 2012, Smith and Foster 2018). This is important since high-dose and multi-agent chemotherapy regimens can lead to significant side-effects. For example, the main dose-limiting side-effect of treatment with anthracyclines such as doxorubicin is cardiotoxicity, leading to heart failure in the most severe cases (Cavaletti 2007). One in every ten children treated with a cumulative anthracycline dose of 300 mg/m2 body surface area or more eventually develops anthracycline-induced clinical heart failure (van Dalen, van der Pal et al. 2006). Doxorubicin has been reported to lead to cardiomyopathy, resulting in a form of congestive heart failure that is usually refractory to common treatment (Takemura and Fujiwara 2007). Moreover, cisplatin has dose-dependent side-effects including nephrotoxicity, ototoxicity and neurotoxicity (Oh, Kim et al. 2014). For this reason, the use of adjunct agents that allows the application of lower doses of doxorubicin and cisplatin may mitigate against the most severe long-term side-effects. Our findings show that 4HC is worthy of further study in this regard.

In addition, GSH has been reported to regulate drug resistance mechanism to cisplatin chemotherapy (Chen and Kuo 2010) and doxorubicin (Gottesman, Fojo et al. 2002). Therefore, cotreatment with 4HC could overcome this problem in chemo-resistant NB paediatric cancer. Agents which can modify GSH metabolism, leading to its depletion, could possibly be used to regulate the cellular response to various anticancer agents (Mena, Benlloch et al. 2007, Ortega, Mena et al. 2011). For example, GSH depletion has been reported to increase glioma cancer cell sensitivity to both cisplatin and temozolomide (Rocha, Garcia et al. 2014), and leukaemia cell sensitivity to doxorubicin and etoposide (Mahbub, Le Maitre et al. 2015). Therefore, depletion of GSH induced by 4HC could enhance NB cell sensitivity to treatment with anticancer agents. Moreover, the presence of

a high uncoupling rate in malignant tumours, rather than an indicator of oxidative stress, could be an anti-apoptotic mechanism that results in increased survival and chemoresistance ability (Valle, Oliver et al. 2010). Therefore, the use of 4HC as a supplement or co-treatment could enhance NB cells' responses to mainstream chemotherapy agents via several methods.

In this study we show that 4HC is a dietary compound that has a higher selective cytotoxicity on *MYCN*-amplified NB cells than on other cell types. This cytotoxic effect involved depletion of GSH and elevation of ROS levels. Application of 4HC in combination with well-known chemotherapy agents led to a greater toxicity on SK-N-BE(2), IMR-32 in relatively low doses; this combined application could potentially be used to overcome drug resistance issues associated with the use of high doses of chemotherapy agents. Therefore, the use of 4HC alone, or in combination with mainstream anti-tumour drugs may have potential for NB treatment, in particular if it allowed the use of lower doses of chemotherapeutic agents, reducing the incidence of toxic long-term side-effects.

5.6 Conclusion

MYCN amplification occurs in ~30% of high-risk NB cases, which influences drug resistance (Harvey, Piskareva et al. 2015). Therefore, the search for novel drugs with fewer side-effects and/or with greater therapeutic efficiency is a key priority for NB therapy, due to the rapid development of resistance against chemotherapeutic drugs and their undesirable side-effects (Demain and Vaishnav 2011). This study shows that 4HC exerts potent cytotoxic effects on *MYCN*-amplified NB cells, and that it can be combined with clinically-approved anti-cancer drugs, doxorubicin or cisplatin, to potentiate NB cell death. In summary, this provides important proof-of-principle that rationalises further study of 4HC as a therapeutic agent of interest for NB.

5.7 References

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Chapter 6: Discussion

6.1 Final discussion and future work

The present thesis focuses on the potential therapeutic role of dietary chalcones ISLQ and 4HC, as a treatments and/or supplements for NB sympathetic neurons pediatric cancer. Regarding the link that has been demonstrated between NC cells developmental errors and the development of NB cancer, it was hypothesized that alterations in BMP-Smad signaling may be a pathogenic element, that could be targeted as therapeutic factors by these naturally existing compounds. This potential therapeutic role has been investigated through two approaches, taking into consideration the genetic alterations of neuritogenesis genes in high risk *MYCN* amplified NB tumors including the impairment of BMP-Smad pathway. The first approach is the examination of BMP-Smad signaling component specific expression patterns and their impact on NB tumors in different risk stages and *MYCN* states. In addition, testing the effect of low nontoxic concentrations of chalcones on the activation of BMP-Smad signaling in NB cells to induce cell differentiation, and the confirmation of the molecular mechanism. This include the two first studies titled; "Association of distinct type 1 bone morphogenetic protein receptors with different molecular pathways and survival outcomes in neuroblastoma", and "Small molecule activators of BMP-Smad signaling in the differentiation of human neuroblastoma cells". However, the sensitivity of NB cells to relatively low doses of tested chalcones treatments provoked the second approach, which is the examination of chalcones cytotoxicity in toxic concentrations on NB cells and the determination of cell death mode, in light of *MYCN* oncogene state. This include the third and the fourth studies titled; "The dietary flavonoid isoliquiritigenin is a potent cytotoxin for human neuroblastoma cells", and "4-hydroxychalcone induces cell death via oxidative stress in MYCN-amplified human neuroblastoma cells".

NB is the most common extracranial solid tumor of early childhood; it accounts for approximately 8–10% of all childhood cancers and is the most common cancer in children in the first year of life (Kaatsch 2010). Despite the massive efforts of researchers of NB treatments, and the advanced therapy methods that are used, the vast majority of children die from recurrent and/or metastatic disease (Ramaswamy and Taylor 2015). Around 20% of the high-risk NB tumours present with amplification of *MYCN* (Molenaar, Koster et al. 2012). While the molecular drivers of NB progression are multi-factorial, a whole-genome analysis of 87 NB samples identified frequent alterations in the expression of genes associated with neuritogenesis, in high-risk tumours (Molenaar, Koster et al. 2012). This

suggests that alterations in the molecular mechanisms that control normal neuritogenesis may occur in cells of the sympathetic lineage in NB, and we aimed to test this hypothesis.

NC induction is thought to occur in two stages from gastrulation to neurulation. The first phase where FGF and Wnt signaling induce NC progenitors at the border of the neural plate. The second phase BMP, Wnt, and Notch signaling maintain these progenitors and bring about the expression of definitive NC markers (Stuhlmiller and Garcia-Castro 2012). Given that BMP signaling controls neuritogenesis in the developing sympathetic nervous system, alterations in expression of the BMPRs may contribute to the defects in neuritogenesis seen in NB (Molenaar, Koster et al. 2012). In support of this, a previous study has shown that there is a significant down-regulation of BMPR2 expression in NB, and that BMPR2 knockdown increased cell growth and tumorigenicity in mice, also that patients with high BMPR2 expression have better overall survival (Cui, Yang et al. 2017). It has also been shown that Smad4 is required for growth inhibition, and the suppression of invasion and metastasis in NB cells, and that patients with high Smad4 expression have greater survival probability (Qu, Zheng et al. 2016). Moreover, it has been tested that BMP ligands promote the neurite growth and differentiation of human NB cells, such as BMP4 (Ferlemann, Menon et al. 2017), BMP9 (Lauzon, Drevelle et al. 2017), BMP2 ((Du and Yip 2010), BMP6 (Sumantran, Brederlau et al. 2003) and GDF5 (Hegarty, Sullivan et al. 2013).

Therefore, in study (1) (Alshangiti, Wyatt et al. 2020) we examined BMPRs component expression in two different databases of NB patients based on International Neuroblastoma Staging System (INSS) and *MYCN* state. We examined the difference between NB patient's survival probability based on INSS stages and *MYCN* state. We also investigated the signaling pathways that may underlie the specific survival probabilities associated with expression of different BMPRs using gene co-expression analysis in NB samples. Then we focused on BMPR1B effect on *MYCN*-amplified NB cells differentiation and neurite growth. We found that each individual BMPR has a specific expression pattern that are associated with different signaling pathways and survival outcomes in NB cells. BMPR1A expression was associated with cell proliferation signaling pathways, whereas expression of BMPR1B and BMPR2 was associated with cellular differentiation and neurite growth. Poor survival outcomes were strongly correlated with reductions in BMPR1B and BMPR2 expression, and with high levels of BMPR1A expression. Interstenglly, *MYCN*-amplification appears to be associated with BMPR2 binding preferentially to BMPR1A,

rather than to BMPR1B, resulting in a shift in intracellular signaling responses towards cell proliferation. These results demonstrate BMP involvement in the mechanism underlying NB tumor progression and therefore contribute to an increased understanding of NB cancer nature and the development of new therapeutic strategies.

Future plans:

A comprehensive evaluation of overexpression or knockdown of BMPR1A effect on both *MYCN*-amplified and non-amplified NB cells would reveal an interesting result. It also would be interesting to examine the molecular mechanism of BMP ligands, such as GDF5, toxicity on SK-N-BE(2) cell mass that led to tumor size reduction. Moreover, an in vivo model of nude mice with a xenograft of *MYCN*-amplified NB SK-N-BE(2) cells to investigated BMPRs expression effect on tumor size and/or cells differentiation. Growth of NB cells in the subcutaneous space of immunocompromised mice is a common method for assessing the effects of therapeutic interventions on NB cells as an in vivo model of solid NB tumor formation and growth (Braekeveldt, Wigerup et al. 2015, Stewart, Shelat et al. 2015, Nolo, Herbrich et al. 2017, Swift, Zhang et al. 2019). This model that use human culture cells closer mimics the clinical condition in terms of the bulky intra-abdominal tumors and other aspects of metastatic disease, that TH-MYCN mice model (mentioned in section 1.1.12) cannot provide. However, a most recent mouse-human NC NB model has been developed known as Chimeric mice. Chimeric mice were produced by injecting human NC cells carrying NB relevant oncogenes in utero into gastrulating mouse embryos. Then, those mice developed tumors that composed of a heterogenous population, which is resembled those seen in primary NB tumors. This model overcome the xenograft homogeneous tumors and the most important is that it provide a study of NB human tumor initiation, progression, manifestation, and tumor-immune-system interactions in an animal model system (Cohen, Zhang et al. 2020).

In study (2) we examined Small molecule activators of BMP-Smad signaling in the differentiation of human NB cells. Recently, ISLQ and 4HC were among a screen of 5,600 small molecules identified as the first small molecules activators of BMP-Smad signaling (Vrijens, Lin et al. 2013). They were extracted from the dried roots and rhizomes of Glycyrrhiza species (Ramalingam, Kim et al. 2018). ISLQ has been reported to promote

differentiation in mouse melanoma B16F0 cells in a dose-dependent manner (Chen, Zhang et al. 2012). In addition, anti-angiogenic effects of 4HC have been reported in endothelial cells (Varinska, van Wijhe et al. 2012). Therefore, we examined ISLQ and 4HC on *MYCN* amplified SK-N-BE(2) and non-amplified SH-SY5Y NB cells differentiation.

First, we determined nontoxic concentrations of chalcones in 3DIV. Then, we examined chalcones effect on neurite growth, BMP-Smad signaling activation or blocking, and we also examined SK-N-BE(2) cell mass morphology changes. We found that ISLQ and 4HC promote cell differentiation on both *MYCN* non-amplified and amplified NB cells. The effect was confirmed to be through canonical BMP-Smad signaling activation, as inhibition of this pathway by K02288, specific BMP inhibitor, blocked the chalcones differentiating effect. Moreover, ISLQ and 4HC have also anti-proliferative effects on SK-N-BE(2) cells aggregation of undifferentiated colonies, which could aid in treating relapses and drug resistance in neuroblastoma. This study clearly illustrated that targeting BMP-Smad activation could promote cell differentiation in both *MYCN*-amplified and non-amplified NB cells. In addition, the combined toxic effect of cell mass in SK-N-BE(2) is promising results that provoke the theory of potential anticancer effect of those chalcones against *MYCN*-amplified NB cells and especially NB cells. Using chalcones in NB maintenance treatment as alternative agents to RA for differentiation induction has a very promising future. This particularly due to the successful clinical trials of chalones that approved their reasonable plasma concentrations, reviewed in (Gomes, Muratov et al. 2017), unlike the accumulations that appear in RA treatments (Veal, Errington et al. 2013). All of these features along with the ease of chalcones synthesis, making chalcones fascinating biological therapies, not only for NB but maybe for other type of cancers.

Future plans:

A comprehensive study of identification of the lead candidate genes mediating ISLQ and 4HC-induced cell differentiation by performing RNA-sequencing following ISLQ and 4HC treatment in these cells. Also, knockdown of the expression of these candidate genes to show that they play a functional role in ISLQ and 4HC-promoted neurite growth and differentiation in NB cells would help in developing those chalcones therapeutic properties against NB tumors.

In study (3) to further examine chalcones therapeutic effect on high-risk MYCN amplified NB, we examined the dietary flavonoid ISLQ as a potent cytotoxin for human NB cells (Alshangiti, Togher et al. 2019). First, we carried out a dose-response experiment on the effects of ISLQ treatment on cell viability in *MYCN*-amplified NB cultures. Then, we determine the mode of cell death induced by ISLQ in *MYCN*-amplified NB cells and the involvement of oxidative stress. Additionally, we combined ISLQ with the anticancer drug Cisplatin (CP), and showed that their cytotoxic effects are additive. We found that, dietary flavonoid ISLQ shows potent cytotoxic effects on *MYCN*-amplified SK-N-BE(2) and IMR-32 NB cell lines in does depending manner. This effect was through an elevation of reactive oxygen species (ROS) -induced necroptosis caspase-independent cell death. The key molecules involved in necroptosis cell death pathway are RIP1, RIP3, and MLKL (Wang, Jin et al. 2017, Weber, Roelandt et al. 2018). The block of RIPK1 by Necrostatin1 (NEC1) prevented ISLQ cell death induced in NB cells (Figure 6.1). Finally, combination therapy of ISLQ with standard chemotherapeutic drugs such as CP cause loss of cell viability that was significantly greater than that induced by either drug alone.

ISLQ anticancer role is well-established, however, the induction of necroptosis cell death in *MYCN*-amplified NB cells, that resistance to CP and to apoptosis, is very important role in NB treatment field.

Future plans:

A comparison of ISLQ and other chemotherapy agents used in NB treatments, such as epipodophyllotoxins (etoposide), or camptothecins (topotecan/irinotecan), and then examine cell death mechanism and the potential synergetic effect of both drugs on NB cells. An in vivo study of the therapeutic potential effect of ISLQ on nude mice with SK-N-BE(2) xenograft, and examination ISLQ treatment on mice general health, tumor growth and metastasis, and cell death mode. Similar studies have demonstrated ISLQ anticancer effect in vivo using HEC-1A-LUC mouse xenograft model through induction of apoptosis and autophagy and inhibition of endometrial cancer growth in mice (Wu, Chen et al. 2016). Examination of ISLQ treatment in sensitize chemotherapy resistance cells to currently used anticancer agents, and the mechanism underlying this effect.

Since this study has been published on January 2019, a number of new papers have reported the anticancer effect of ISLQ. For example, ISLQ was reported to inhibit ovarian cancer metastasis by reversing EMT. The precise mechanism included reduced levels of mesenchymal markers accompanied by the gain of E-cadherin expression, decrease in cell migration and in vitro invasion upon ISLQ treatment. Moreover, they show that ISLQ effectively blocks the intraperitoneal xenograft development of the SKOV3 cell line and prolonged the survival of tumor-bearing mice (Chen, Huang et al. 2019). As it was mentioned in section 1.1.5, the epithelial marker E-Cadherin expression is decreased (Shao, Gao et al. 2017) during EMT process caused by the high levels of TGF-beta in NB (Zhen, Guo et al. 2016). However, in NC migration, E-Cadherin shifts to N-Cadherin during EMT, as the former was found to inhibit NC migration in vivo this shift is essential for normal NC migration (Scarpa, Szabó et al. 2015). ISLQ has also found to inhibit the proliferation of Hep3B cells by causing G1/S cell cycle arrest in vitro (Huang, Liu et al. 2020). These results along with current study ones supporting the multiple potential therapeutic properties that ISLQ possess. which could be effective not only against NB but other type of cancer with related profiles. For example, the diversity of anticancer mode of action of ISLQ could be profoundly effective against the heterogenetic microenvironment of different tumors.

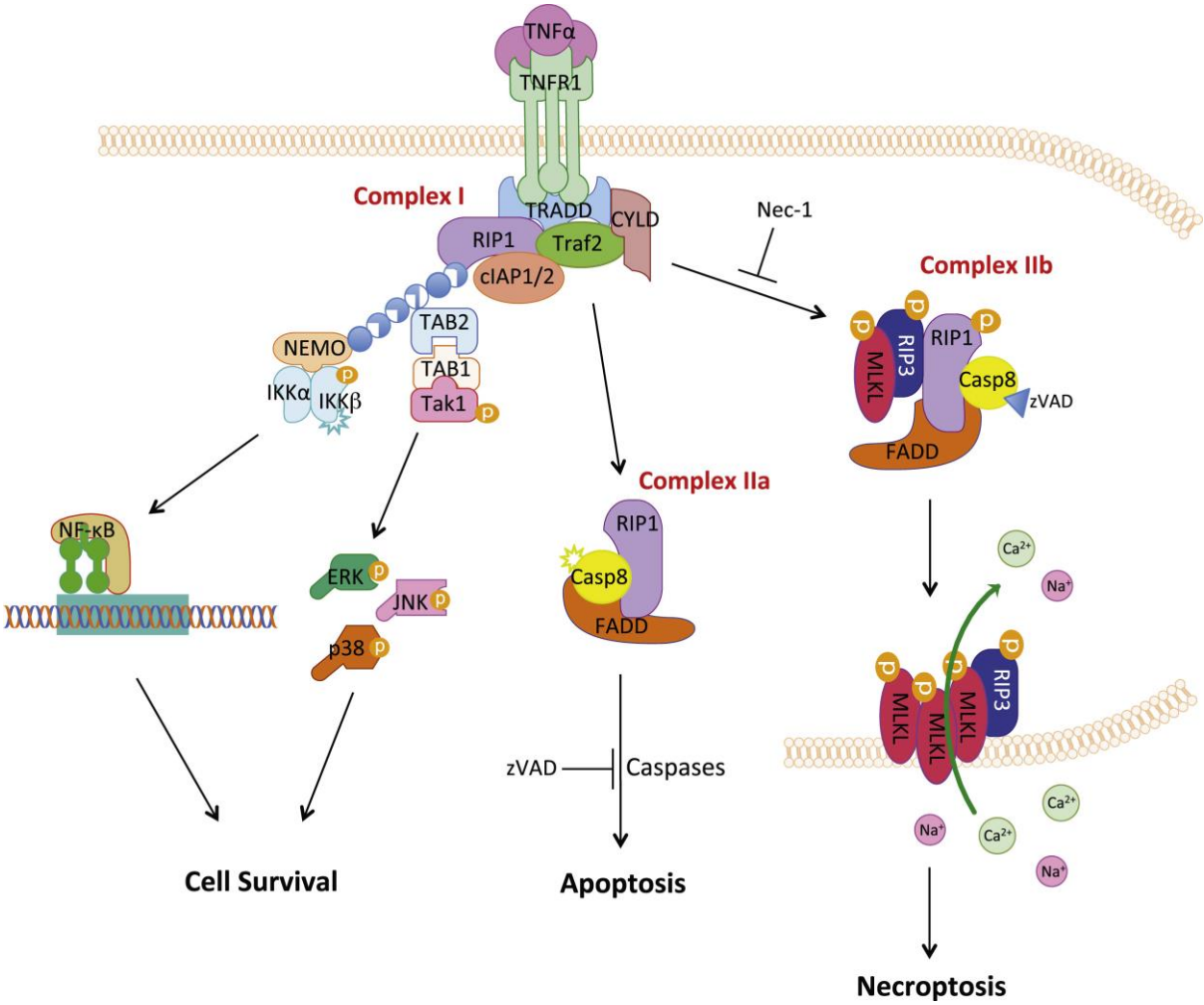


Figure 6.1 TNF-alpha receptor – mediated cell death signaling pathway of each of cell survival, apoptosis, and necroptosis. When caspase-8 suppressed under specific conditions, RIPK1 interacts with RIPK3 and MLKL to form complex IIb that involved in regulating cell death by necroptosis. Nec-1 block this pathway by inhibiting RIPK1. RIPK3 and MLKL are phosphorylated in complex IIb, then translocate to the plasma membrane, to mediates membrane permeabilization. necroptosis specific mechanism is reviewed here (Zhou and Yuan 2014).

Study (4) is a comprehensive examination of how 4HC cytotoxicity is mediated via oxidative stress in *MYCN*-amplified human NB Cells (Alshangiti, Tuboly et al. 2019). 4HC is the most simple hydroxylated chalcone, and it has been shown to have an anti-angiogenic effect that may be useful in halting cancer spread (Varinska, van Wijhe et al. 2012). First we examined the cytotoxic effect of 4HC on each of; *MYCN* amplified, non-amplified NB

cells, HEK293t cells, and primary neurons, to highlight *MYCN* amplification role in cell sensitivity to 4HC. Then we examined cell death mechanism through several methods including: measuring the intercellular levels of the antioxidant Glutathione (GSH), ROS levels, mitochondrial oxygen consumption, cell death inhibitors. Finally, we performed dose-response experiments of 4HC with two of NB chemotherapy agents; doxorubicin (DOX) and CP on *MYCN* amplified, non-amplified NB cells.

We found that 4HC the dietary compound has a higher selective cytotoxicity on *MYCN*-amplified NB cells than on other cell types. This cytotoxic effect involved depletion of GSH and an elevation of ROS levels. Application of 4HC in combination with well-known chemotherapy agents led to a greater toxicity on *MYCN*-amplified NB cells in relatively low doses. This study revealed the anticancer activity 4HC has that similar to well-known chemotherapy agents, such as oxidative stress elevation, yet with lesser toxicity for non-*MYCN*-amplified NB then to normal cells. GSH depletion is already used approach to overcome drug resistance and sensitize cancer cells to treatment, whereas, here 4HC act both roles by GSH depletion and inducing cell death directly in DOX and CP resistance NB cells

In addition, the slightly higher toxicity of 4HC than ISLQ demonstrated the variation in chalcones antitumor function on same cells, which could help in finding suitable drug for each patient condition. For example, ISLQ could be used in high-risk NB induction treatment, and in the case of tumor relapses, where more aggressive chemotherapy regimens are needed, 4HC would be recommended.

Future plans:

MYCN-amplified NB cells show more sensitivity to same concentrations of 4HC than ISLQ. Previous reports suggested that the anti-cancer effects of chalcones are associated with the presence of hydroxyl groups (De Vincenzo, Scambia et al. 1995, Cabrera, Simoens et al. 2007, Loa, Chow et al. 2009). In addition, the position of the hydroxyl group is important in regulating the biological activity of chalcones (Calliste, Le Bail et al. 2001, Nerya, Musa et al. 2004). Therefore, the precise mechanism of *MYCN*-amplified NB to 4HC would need further comprehensive investigation. Examination of 4HC potential effect on sympathetic superior cervical ganglia primary culture differentiation and in vivo study. Such study could demonstrate the therapeutic effect of 4HC on human tumor xenograft on mice, and the

specific impact of 4HC on different cancer and non-cancer tissues. 4HC is relatively a novel drug and studies investigating its anticancer properties are still limited.

Collectively, this thesis shed light on specific molecular impairments in NB tumor and how these alterations could be targeted as therapeutic factors by chalcones the natural compounds. The use of chalcones as an anticancer agent is still relatively novel, however, this thesis investigated a very important mode of action. The elevation of ROS and the mitochondrial impairment are an example of the familiar strategies of current chemotherapy agents. Glutathione depletion is a method of increase cancer cells sensitivity to the chemotherapy and to overcome drug resistance. Moreover, the selective effect of tested chalcones on cancer cells rather than normal cells along with all of their anti-tumor mechanisms makes them an exciting solution for most of the NB tumor complications.

6.2 Flavonoids anticancer clinical trials

The selective anticancer potential of flavonoids make them a promising candidate for chemotherapy. Whether used as a single agent or in a companion with other chemotherapy regimens, flavonoids have been used in human cancer clinical trials. Despite the extensive preclinical studies of the anti-cancer effects of chalcones and their derivatives, they have been far fewer clinical trials that are not as anticancer drugs.

A systematic review conducted on the use of flavonoids compounds in human cancer clinical trials identified 22 (Phase II) and 1 (Phase III) clinical trial studies related to testing flavonoids in the treatment of solid tumors (12 trials) and hematopoietic/lymphoid tumors (11 trials) up to January 2019 (Bisol, de Campos et al. 2020). According to the FDA, Phase II clinical trials are conducted to measure the efficacy of a drug against a disease, and Phase III trials are conducted to verify that the potential drugs are better than the standard treatment agent. Flavopiridol was tested as single agent for 64 chronic lymphocytic leukaemia patients, and 34 patients (53%) reported high clinical activity as the median progression-free survival among responders patients was 10 to 12 months across all risk groups. (Lin, Ruppert et al. 2009). Moreover, Flavopiridol treatment in that study has reduced the number of weekly treatments per cycle from four to three, which resulted in improved tolerability.

However, some previous studies have demonstrated an increase in binding of flavopiridol to human serum proteins that cause a reduction in its clinical activity (Byrd, Peterson et al. 2005, Flinn, Byrd et al. 2005). Moreover, the use of flavopiridol as an adjuvant (given in addition to the primary therapy) with current chemotherapy drugs appeared to have better clinical effect (Zeidner, Foster et al. 2015) over it use as a single agent against chronic or acute leukaemia (Lin, Ruppert et al. 2009). Another example of flavonoids used in human cancer clinical trials is phenoxodiol, which is a laboratory-modified version of the naturally occurring plant isoflavone. Phenoxodiol has shown synergistic interaction with cisplatin and carboplatin, combined or not with gold standard drugs, in Phase II studies of 32 patients, with more than 55% of patients showing good tolerance for the cancer treatment (Kelly, Mor et al. 2011).

However, the main problem facing flavonoids future research is to fully understand how they can promote positive outcomes for cancer patients is the high variability in how these compounds are delivered and the dosing regimens used, therefore, more studies are still needed (Bisol, de Campos et al. 2020). The investigation conducted on ISLQ and 4HC anticancer properties in this thesis has shed light on the profound role of those chalcones as treatment of NB cancer.

6.3 Conclusion

This thesis has shown that the dietary flavonoids ISLQ and 4HC have potential to exert a promising double-edged therapeutic effect on NB pediatric cancer tumors with *MYCN*-amplification. These studies have clearly demonstrated that ISLQ and 4HC exert dose-dependent effects on these cells. Specifically, low doses of these two chalcones promote differentiation of NB cells by the activation of the BMP-Smad signaling pathway. Modulation of altered BMP signaling in high-risk NB by ISLQ and 4HC could be applied to therapeutic approaches for enhancing survival rate in NB patients. However, some of these chalcones have shown selective cytotoxicity on *MYCN*-amplified NB cells. These chalcones have shown multiple effects on cancer cells, such as the selective effect of ISLQ and 4HC on those cancer cells with elevated oxidative stress, GSH depletion, impairment of mitochondrial function, or induction of cell death via necroptosis. Furthermore, the chalcones tested in this project have been shown to induce shrinking of tumor mass in *MYCN*-amplified NB cells SK-N-BE(2). For instance, induction of NB cell differentiation by chalcones via activation of BMP-Smad signaling in drug-resistant tumour cells could also contribute to prevent tumor relapse, and subsequently prolong a patient's life span. However, the combination of these chalcones in with the chemotherapeutic agents, DOX and CP, has been shown to result in greater levels of cell death than induced by the chemotherapy agent alone. The ability to use reduced doses of chemotherapy agents, has a promising role in reducing some of the serious side-effects and long-term complications that occur with these agents, and that affect patients' quality of life. The anticancer properties of chalcones hold great promise, and further investigation of their molecular mechanisms of action will contribute to the development of new regimens for the treatment of NB patients.

Chapter 7: Bibliography

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Chapter 8: Appendices

8. Appendices

Table A8.1 GO-BMPR2

BMPR2 co-expressed genes

Analysis Type:	PANTHER Overrepresentation Test (Released 20190711)
Annotation Version and Release Date:	GO Ontology database Released 2019-10-08
Analyzed List:	upload_1 (Homo sapiens)
Reference List:	Homo sapiens (all genes in database)
Test Type:	FISHER
Correction:	FDR

GO biological process complete	**#**	**#**	**expected**	**Plus/minus**	**Fold enrichment**	**Raw P value**	**FDR**
microtubule anchoring at microtubule organizing center (GO:0072393)	13	8	1.45	+	5.52	6.21E-04	2.28E-02
phosphatidylinositol-3-phosphate biosynthetic process (GO:0036092)	14	8	1.56	+	5.13	8.91E-04	3.03E-02
Golgi to endosome transport (GO:0006895)	18	10	2.01	+	4.98	2.43E-04	1.09E-02
venous blood vessel development (GO:0060841)	15	8	1.67	+	4.78	1.25E-03	4.01E-02
calcium ion-regulated exocytosis of neurotransmitter (GO:0048791)	18	9	2.01	+	4.48	8.85E-04	3.03E-02
retrograde axonal transport (GO:0008090)	18	9	2.01	+	4.48	8.85E-04	3.02E-02
mitochondrion transport along microtubule (GO:0047497)	20	10	2.23	+	4.48	4.61E-04	1.79E-02
establishment of mitochondrion localization, microtubule-mediated (GO:0034643)	20	10	2.23	+	4.48	4.61E-04	1.79E-02
protein localization to Golgi apparatus (GO:0034067)	28	13	3.12	+	4.16	1.19E-04	5.75E-03
establishment of mitochondrion localization (GO:0051654)	24	11	2.68	+	4.11	4.31E-04	1.71E-02
axonal transport (GO:0098930)	53	24	5.91	+	4.06	2.67E-07	2.44E-05
vesicle cytoskeletal trafficking (GO:0099518)	51	23	5.69	+	4.04	4.98E-07	4.21E-05

myelin assembly (GO:0032288)	20	9	2.23	+	4.04	1.57E-03	4.86E-02
microtubule anchoring (GO:0034453)	27	12	3.01	+	3.99	2.97E-04	1.29E-02
vesicle transport along microtubule (GO:0047496)	45	20	5.02	+	3.99	3.25E-06	2.33E-04
calcium-ion regulated exocytosis (GO:0017156)	43	19	4.79	+	3.96	6.07E-06	4.11E-04
production of miRNAs involved in gene silencing by miRNA (GO:0035196)	28	12	3.12	+	3.84	3.87E-04	1.58E-02
clathrin-dependent endocytosis (GO:0072583)	28	12	3.12	+	3.84	3.87E-04	1.58E-02
cerebellar cortex formation (GO:0021697)	24	10	2.68	+	3.74	1.39E-03	4.35E-02
organelle transport along microtubule (GO:0072384)	78	32	8.7	+	3.68	1.89E-08	2.08E-06
production of small RNA involved in gene silencing by RNA (GO:0070918)	32	13	3.57	+	3.64	3.38E-04	1.43E-02
dsRNA processing (GO:0031050)	32	13	3.57	+	3.64	3.38E-04	1.43E-02
Golgi to plasma membrane protein transport (GO:0043001)	32	13	3.57	+	3.64	3.38E-04	1.42E-02
intraciliary transport (GO:0042073)	52	21	5.8	+	3.62	6.13E-06	4.13E-04
dendrite morphogenesis (GO:0048813)	60	24	6.69	+	3.59	1.56E-06	1.17E-04
positive regulation of microtubule polymerization or depolymerization (GO:0031112)	35	14	3.9	+	3.59	2.29E-04	1.04E-02
intraciliary transport involved in cilium assembly (GO:0035735)	40	16	4.46	+	3.59	8.37E-05	4.24E-03
axo-dendritic transport (GO:0008088)	63	25	7.02	+	3.56	1.07E-06	8.23E-05
anterograde axonal transport (GO:0008089)	43	17	4.79	+	3.55	5.70E-05	3.04E-03
positive regulation of dendritic spine development (GO:0060999)	46	18	5.13	+	3.51	3.88E-05	2.17E-03
Golgi to plasma membrane transport (GO:0006893)	54	21	6.02	+	3.49	9.77E-06	6.31E-04
platelet-derived growth factor receptor signaling pathway (GO:0048008)	36	14	4.01	+	3.49	2.89E-04	1.26E-02
protein transport along microtubule (GO:0098840)	67	26	7.47	+	3.48	9.22E-07	7.26E-05
microtubule-based protein transport (GO:0099118)	67	26	7.47	+	3.48	9.22E-07	7.23E-05
positive regulation of microtubule polymerization (GO:0031116)	31	12	3.46	+	3.47	7.99E-04	2.79E-02
Wnt signaling pathway, calcium modulating pathway (GO:0007223)	37	14	4.13	+	3.39	3.62E-04	1.50E-02
cellular response to nerve growth factor stimulus (GO:1990090)	53	20	5.91	+	3.38	2.24E-05	1.34E-03
response to nerve growth factor (GO:1990089)	56	21	6.24	+	3.36	1.52E-05	9.39E-04
post-Golgi vesicle-mediated transport (GO:0006892)	133	49	14.83	+	3.3	7.56E-11	1.32E-08
cytosolic transport (GO:0016482)	145	53	16.17	+	3.28	1.64E-11	3.30E-09

cytoskeleton-dependent intracellular transport (GO:0030705)	175	63	19.51	+	3.23	3.56E-13	1.01E-10
Dendrite morphogenesisDendri	154	55	17.17	+	3.2	1.42E-11	2.89E-09
synaptic vesicle localization (GO:0097479)	45	16	5.02	+	3.19	2.51E-04	1.12E-02
cytoplasmic microtubule organization (GO:0031122)	54	19	6.02	+	3.16	7.69E-05	3.93E-03
regulation of autophagosome assembly (GO:2000785)	37	13	4.13	+	3.15	1.02E-03	3.37E-02
regulation of dendrite morphogenesis (GO:0048814)	93	32	10.37	+	3.09	4.87E-07	4.14E-05
regulation of dendritic spine development (GO:0060998)	76	26	8.47	+	3.07	6.06E-06	4.12E-04
regulation of synaptic vesicle cycle (GO:0098693)	117	40	13.05	+	3.07	2.21E-08	2.38E-06
vesicle docking involved in exocytosis (GO:0006904)	44	15	4.91	+	3.06	5.58E-04	2.09E-02
mitochondrion localization (GO:0051646)	44	15	4.91	+	3.06	5.58E-04	2.08E-02
autophagosome assembly (GO:0000045)	63	21	7.02	+	2.99	6.27E-05	3.30E-03
synaptic vesicle transport (GO:0048489)	39	13	4.35	+	2.99	1.51E-03	4.70E-02
regulation of dendritic spine morphogenesis (GO:0061001)	45	15	5.02	+	2.99	6.75E-04	2.46E-02
dendrite development (GO:0016358)	115	38	12.82	+	2.96	1.02E-07	1.00E-05
regulation of synaptic vesicle exocytosis (GO:2000300)	82	27	9.14	+	2.95	7.23E-06	4.77E-04
positive regulation of dendrite development (GO:1900006)	76	25	8.47	+	2.95	1.58E-05	9.60E-04
microtubule polymerization or depolymerization (GO:0031109)	49	16	5.46	+	2.93	5.46E-04	2.06E-02
regulation of dendrite development (GO:0050773)	154	50	17.17	+	2.91	2.10E-09	3.09E-07
regulation of microtubule polymerization (GO:0031113)	53	17	5.91	+	2.88	4.39E-04	1.73E-02
microtubule-based transport (GO:0099111)	182	58	20.29	+	2.86	1.68E-10	2.84E-08
autophagosome organization (GO:1905037)	66	21	7.36	+	2.85	1.08E-04	5.28E-03
presynaptic endocytosis (GO:0140238)	51	16	5.69	+	2.81	7.81E-04	2.76E-02
synaptic vesicle endocytosis (GO:0048488)	51	16	5.69	+	2.81	7.81E-04	2.76E-02
regulation of postsynapse organization (GO:0099175)	102	32	11.37	+	2.81	3.20E-06	2.30E-04
establishment of protein localization to plasma membrane (GO:0061951)	51	16	5.69	+	2.81	7.81E-04	2.75E-02
positive regulation of macroautophagy (GO:0016239)	67	21	7.47	+	2.81	1.29E-04	6.15E-03
endosome to lysosome transport (GO:0008333)	52	16	5.8	+	2.76	9.27E-04	3.13E-02
epidermal growth factor receptor signaling pathway (GO:0007173)	49	15	5.46	+	2.75	1.38E-03	4.36E-02
acidic amino acid transport (GO:0015800)	49	15	5.46	+	2.75	1.38E-03	4.35E-02

synaptic vesicle recycling (GO:0036465)	59	18	6.58	+	2.74	7.85E-04	2.76E-02
regulation of potassium ion transmembrane transporter activity (GO:1901016)	59	18	6.58	+	2.74	7.85E-04	2.76E-02
ERBB signaling pathway (GO:0038127)	69	21	7.69	+	2.73	2.53E-04	1.13E-02
vesicle docking (GO:0048278)	66	20	7.36	+	2.72	3.78E-04	1.55E-02
regulation of neurotransmitter secretion (GO:0046928)	110	33	12.26	+	2.69	4.20E-06	2.95E-04
vesicle-mediated transport to the plasma membrane (GO:0098876)	87	26	9.7	+	2.68	4.86E-05	2.64E-03
vesicle-mediated transport in synapse (GO:0099003)	134	40	14.94	+	2.68	6.33E-07	5.19E-05
synaptic vesicle cycle (GO:0099504)	124	37	13.83	+	2.68	1.90E-06	1.40E-04
establishment of vesicle localization (GO:0051650)	181	54	20.18	+	2.68	5.03E-09	6.61E-07
vesicle localization (GO:0051648)	192	57	21.41	+	2.66	3.38E-09	4.55E-07
retrograde transport, endosome to Golgi (GO:0042147)	91	27	10.15	+	2.66	3.74E-05	2.10E-03
peptidyl-threonine modification (GO:0018210)	81	24	9.03	+	2.66	1.06E-04	5.22E-03
regulation of focal adhesion assembly (GO:0051893)	61	18	6.8	+	2.65	9.22E-04	3.13E-02
regulation of cell-substrate junction assembly (GO:0090109)	61	18	6.8	+	2.65	9.22E-04	3.12E-02
lysosomal transport (GO:0007041)	102	30	11.37	+	2.64	1.54E-05	9.45E-04
posttranscriptional gene silencing (GO:0016441)	58	17	6.47	+	2.63	1.38E-03	4.35E-02
regulation of macroautophagy (GO:0016241)	173	50	19.29	+	2.59	5.70E-08	5.67E-06
insulin receptor signaling pathway (GO:0008286)	88	25	9.81	+	2.55	1.22E-04	5.87E-03
positive regulation of neuron projection development (GO:0010976)	290	82	32.33	+	2.54	6.01E-12	1.37E-09
negative regulation of protein depolymerization (GO:1901880)	64	18	7.14	+	2.52	1.27E-03	4.05E-02
regulation of adherens junction organization (GO:1903391)	68	19	7.58	+	2.51	9.63E-04	3.23E-02
negative regulation of ion transmembrane transport (GO:0034766)	102	28	11.37	+	2.46	9.29E-05	4.63E-03
peptidyl-threonine phosphorylation (GO:0018107)	73	20	8.14	+	2.46	8.39E-04	2.91E-02
regulation of neuron projection development (GO:0010975)	506	137	56.42	+	2.43	8.74E-18	6.04E-15
regulation of microtubule polymerization or depolymerization (GO:0031110)	85	23	9.48	+	2.43	5.24E-04	1.98E-02
microtubule-based movement (GO:0007018)	268	72	29.88	+	2.41	8.19E-10	1.31E-07
regulation of potassium ion transmembrane transport (GO:1901379)	82	22	9.14	+	2.41	7.67E-04	2.72E-02
synapse assembly (GO:0007416)	97	26	10.82	+	2.4	2.20E-04	1.01E-02

positive regulation of exocytosis (GO:0045921)	90	24	10.03	+	2.39	4.25E-04	1.69E-02
endosomal transport (GO:0016197)	229	61	25.53	+	2.39	1.94E-08	2.13E-06
positive regulation of protein localization to membrane (GO:1905477)	124	33	13.83	+	2.39	4.13E-05	2.29E-03
regulation of microtubule cytoskeleton organization (GO:0070507)	196	52	21.85	+	2.38	3.14E-07	2.81E-05
vacuole organization (GO:0007033)	132	35	14.72	+	2.38	2.34E-05	1.39E-03
action potential (GO:0001508)	95	25	10.59	+	2.36	3.56E-04	1.49E-02
Golgi vesicle transport (GO:0048193)	399	105	44.49	+	2.36	3.32E-13	9.59E-11
negative regulation of ion transmembrane transporter activity (GO:0032413)	80	21	8.92	+	2.35	1.21E-03	3.92E-02
protein localization to plasma membrane (GO:0072659)	191	50	21.3	+	2.35	6.97E-07	5.68E-05
regulation of receptor-mediated endocytosis (GO:0048259)	107	28	11.93	+	2.35	2.32E-04	1.05E-02
negative regulation of transporter activity (GO:0032410)	88	23	9.81	+	2.34	6.82E-04	2.47E-02
regulation of exocytosis (GO:0017157)	222	58	24.75	+	2.34	1.02E-07	1.00E-05
postsynapse organization (GO:0099173)	92	24	10.26	+	2.34	5.18E-04	1.97E-02
vacuolar transport (GO:0007034)	142	37	15.83	+	2.34	2.46E-05	1.44E-03
negative regulation of transmembrane transport (GO:0034763)	123	32	13.71	+	2.33	7.12E-05	3.69E-03
positive regulation of cell projection organization (GO:0031346)	390	101	43.48	+	2.32	1.87E-12	4.51E-10
regulation of cell junction assembly (GO:1901888)	93	24	10.37	+	2.31	5.76E-04	2.14E-02
protein dephosphorylation (GO:0006470)	214	55	23.86	+	2.31	3.22E-07	2.86E-05
positive regulation of neuron differentiation (GO:0045666)	379	97	42.26	+	2.3	1.33E-11	2.74E-09
cytokinesis (GO:0000910)	110	28	12.26	+	2.28	2.85E-04	1.25E-02
localization within membrane (GO:0051668)	87	22	9.7	+	2.27	1.21E-03	3.92E-02
regulation of axon extension (GO:0030516)	99	25	11.04	+	2.26	7.42E-04	2.64E-02
regulation of cell-matrix adhesion (GO:0001952)	115	29	12.82	+	2.26	2.35E-04	1.06E-02
regulation of microtubule-based process (GO:0032886)	230	58	25.64	+	2.26	2.80E-07	2.53E-05
regulation of neurotransmitter transport (GO:0051588)	147	37	16.39	+	2.26	3.55E-05	2.01E-03
regulation of regulated secretory pathway (GO:1903305)	163	41	18.17	+	2.26	1.69E-05	1.02E-03
phosphatidylinositol biosynthetic process (GO:0006661)	108	27	12.04	+	2.24	4.47E-04	1.75E-02
regulation of cell morphogenesis involved in differentiation (GO:0010769)	308	77	34.34	+	2.24	3.26E-09	4.47E-07
macroautophagy (GO:0016236)	160	40	17.84	+	2.24	2.41E-05	1.42E-03

regulation of plasma membrane bounded cell projection organization (GO:0120035)	678	169	75.6	+	2.24	1.53E-18	1.21E-15
neurotransmitter secretion (GO:0007269)	101	25	11.26	+	2.22	8.54E-04	2.94E-02
signal release from synapse (GO:0099643)	101	25	11.26	+	2.22	8.54E-04	2.93E-02
regulation of cell projection organization (GO:0031344)	687	170	76.6	+	2.22	1.57E-18	1.19E-15
positive regulation of autophagy (GO:0010508)	122	30	13.6	+	2.21	3.26E-04	1.39E-02
protein localization to cell periphery (GO:1990778)	234	57	26.09	+	2.18	9.59E-07	7.48E-05
establishment of organelle localization (GO:0051656)	368	89	41.03	+	2.17	7.89E-10	1.28E-07
cell morphogenesis involved in neuron differentiation (GO:0048667)	443	107	49.39	+	2.17	1.81E-11	3.59E-09
peptidyl-tyrosine dephosphorylation (GO:0035335)	104	25	11.6	+	2.16	1.10E-03	3.62E-02
memory (GO:0007613)	125	30	13.94	+	2.15	4.00E-04	1.61E-02
organelle localization by membrane tethering (GO:0140056)	171	41	19.07	+	2.15	4.28E-05	2.35E-03
regulation of protein localization to membrane (GO:1905475)	192	46	21.41	+	2.15	1.67E-05	1.02E-03
axonogenesis (GO:0007409)	381	91	42.48	+	2.14	8.91E-10	1.40E-07
axon development (GO:0061564)	415	99	46.27	+	2.14	2.31E-10	3.83E-08
regulation of extent of cell growth (GO:0061387)	114	27	12.71	+	2.12	1.00E-03	3.33E-02
neuron projection morphogenesis (GO:0048812)	494	117	55.08	+	2.12	6.64E-12	1.47E-09
regulation of neuron differentiation (GO:0045664)	663	157	73.92	+	2.12	1.26E-15	5.57E-13
organelle localization (GO:0051640)	584	138	65.11	+	2.12	7.50E-14	2.25E-11
synapse organization (GO:0050808)	280	66	31.22	+	2.11	2.98E-07	2.68E-05
regulation of protein polymerization (GO:0032271)	225	53	25.09	+	2.11	4.49E-06	3.12E-04
neuron projection development (GO:0031175)	680	160	75.82	+	2.11	1.04E-15	4.85E-13
cell projection morphogenesis (GO:0048858)	502	118	55.97	+	2.11	6.34E-12	1.42E-09
plasma membrane bounded cell projection morphogenesis (GO:0120039)	498	117	55.53	+	2.11	8.31E-12	1.81E-09
cell part morphogenesis (GO:0032990)	524	123	58.42	+	2.11	2.50E-12	5.83E-10
regulation of organelle assembly (GO:1902115)	196	46	21.85	+	2.1	2.08E-05	1.25E-03
regulation of vesicle-mediated transport (GO:0060627)	552	129	61.55	+	2.1	1.26E-12	3.28E-10
membrane docking (GO:0022406)	180	42	20.07	+	2.09	6.49E-05	3.41E-03
regulation of GTPase activity (GO:0043087)	481	112	53.63	+	2.09	3.98E-11	7.36E-09

positive regulation of GTPase activity (GO:0043547)	408	95	45.49	+	2.09	1.17E-09	1.83E-07
positive regulation of cell morphogenesis involved in differentiation (GO:0010770)	159	37	17.73	+	2.09	1.69E-04	7.87E-03
regulation of cell morphogenesis (GO:0022604)	499	116	55.64	+	2.08	2.11E-11	4.14E-09
regulation of axonogenesis (GO:0050770)	190	44	21.18	+	2.08	4.10E-05	2.28E-03
vesicle organization (GO:0016050)	304	70	33.9	+	2.07	3.25E-07	2.87E-05
plasma membrane bounded cell projection organization (GO:0120036)	1114	255	124.21	+	2.05	2.10E-23	8.34E-20
membrane fusion (GO:0061025)	127	29	14.16	+	2.05	1.12E-03	3.66E-02
neuron migration (GO:0001764)	123	28	13.71	+	2.04	1.49E-03	4.64E-02
learning (GO:0007612)	150	34	16.72	+	2.03	4.65E-04	1.79E-02
cell projection organization (GO:0030030)	1159	262	129.23	+	2.03	2.87E-23	9.13E-20
Golgi organization (GO:0007030)	133	30	14.83	+	2.02	9.56E-04	3.21E-02
negative regulation of cytoskeleton organization (GO:0051494)	142	32	15.83	+	2.02	8.24E-04	2.87E-02
regulation of gene expression, epigenetic (GO:0040029)	231	52	25.76	+	2.02	1.54E-05	9.46E-04
positive regulation of protein polymerization (GO:0032273)	129	29	14.38	+	2.02	1.25E-03	4.02E-02
plasma membrane bounded cell projection assembly (GO:0120031)	432	97	48.17	+	2.01	4.60E-09	6.09E-07
negative regulation of ion transport (GO:0043271)	156	35	17.39	+	2.01	4.00E-04	1.61E-02
cellular response to insulin stimulus (GO:0032869)	165	37	18.4	+	2.01	3.42E-04	1.44E-02
regulation of autophagy (GO:0010506)	330	74	36.79	+	2.01	3.75E-07	3.26E-05
regulation of dephosphorylation (GO:0035303)	210	47	23.41	+	2.01	5.71E-05	3.04E-03
microtubule-based process (GO:0007017)	654	146	72.92	+	2	5.59E-13	1.56E-10
learning or memory (GO:0007611)	266	59	29.66	+	1.99	6.44E-06	4.31E-04
neuron development (GO:0048666)	821	182	91.54	+	1.99	1.25E-15	5.69E-13
positive regulation of neurogenesis (GO:0050769)	483	107	53.85	+	1.99	1.48E-09	2.24E-07
cell projection assembly (GO:0030031)	447	99	49.84	+	1.99	5.33E-09	6.95E-07
axon guidance (GO:0007411)	267	59	29.77	+	1.98	6.87E-06	4.57E-04
establishment of localization in cell (GO:0051649)	394	87	43.93	+	1.98	5.47E-08	5.47E-06
cell morphogenesis involved in differentiation (GO:0000904)	562	124	62.66	+	1.98	6.79E-11	1.20E-08
endomembrane system organization (GO:0010256)	423	93	47.16	+	1.97	1.97E-08	2.15E-06

small GTPase mediated signal transduction (GO:0007264)	319	70	35.57	+	1.97	1.40E-06	1.05E-04
regulation of endocytosis (GO:0030100)	210	46	23.41	+	1.96	9.08E-05	4.55E-03
neuron projection guidance (GO:0097485)	270	59	30.1	+	1.96	1.09E-05	6.94E-04
modulation of chemical synaptic transmission (GO:0050804)	450	98	50.17	+	1.95	1.36E-08	1.56E-06
cilium assembly (GO:0060271)	340	74	37.91	+	1.95	8.47E-07	6.73E-05
regulation of trans-synaptic signaling (GO:0099177)	451	98	50.29	+	1.95	1.44E-08	1.62E-06
cilium organization (GO:0044782)	359	78	40.03	+	1.95	4.64E-07	3.96E-05
regulation of actin filament polymerization (GO:0030833)	166	36	18.51	+	1.95	5.85E-04	2.17E-02
signal release (GO:0023061)	171	37	19.07	+	1.94	6.57E-04	2.40E-02
regulation of cell size (GO:0008361)	185	40	20.63	+	1.94	3.17E-04	1.36E-02
regulation of phosphatase activity (GO:0010921)	176	38	19.62	+	1.94	5.16E-04	1.96E-02
regulation of Ras protein signal transduction (GO:0046578)	246	53	27.43	+	1.93	3.93E-05	2.19E-03
dephosphorylation (GO:0016311)	311	67	34.68	+	1.93	3.79E-06	2.69E-04
cognition (GO:0050890)	307	66	34.23	+	1.93	4.93E-06	3.38E-04
regulation of neurogenesis (GO:0050767)	827	177	92.21	+	1.92	4.58E-14	1.40E-11
Ras protein signal transduction (GO:0007265)	248	53	27.65	+	1.92	6.17E-05	3.26E-03
modification-dependent macromolecule catabolic process (GO:0043632)	545	116	60.77	+	1.91	2.27E-09	3.25E-07
regulation of plasma membrane bounded cell projection assembly (GO:0120032)	174	37	19.4	+	1.91	7.42E-04	2.64E-02
regulation of small GTPase mediated signal transduction (GO:0051056)	348	74	38.8	+	1.91	1.71E-06	1.26E-04
positive regulation of nervous system development (GO:0051962)	549	116	61.21	+	1.9	2.65E-09	3.72E-07
regulation of cellular component size (GO:0032535)	379	80	42.26	+	1.89	8.92E-07	7.06E-05
positive regulation of supramolecular fiber organization (GO:1902905)	199	42	22.19	+	1.89	3.43E-04	1.44E-02
homophilic cell adhesion via plasma membrane adhesion molecules (GO:0007156)	166	35	18.51	+	1.89	1.27E-03	4.07E-02
phosphatidylinositol metabolic process (GO:0046488)	171	36	19.07	+	1.89	1.02E-03	3.37E-02
regulation of cell projection assembly (GO:0060491)	176	37	19.62	+	1.89	8.24E-04	2.87E-02
negative regulation of cell projection organization (GO:0031345)	181	38	20.18	+	1.88	9.11E-04	3.10E-02
regulation of synapse organization (GO:0050807)	224	47	24.98	+	1.88	2.13E-04	9.81E-03
regulation of nervous system development (GO:0051960)	935	196	104.25	+	1.88	1.02E-14	3.60E-12

neuron differentiation (GO:0030182)	1012	212	112.84	+	1.88	8.32E-16	4.13E-13
cell morphogenesis (GO:0000902)	724	151	80.72	+	1.87	2.66E-11	5.03E-09
regulation of intracellular transport (GO:0032386)	356	74	39.69	+	1.86	4.54E-06	3.13E-04
modification-dependent protein catabolic process (GO:0019941)	534	111	59.54	+	1.86	1.22E-08	1.45E-06
proteasomal protein catabolic process (GO:0010498)	347	72	38.69	+	1.86	5.27E-06	3.60E-04
positive regulation of cytoskeleton organization (GO:0051495)	222	46	24.75	+	1.86	2.99E-04	1.29E-02
endoplasmic reticulum to Golgi vesicle-mediated transport (GO:0006888)	203	42	22.63	+	1.86	5.51E-04	2.07E-02
proteasome-mediated ubiquitin-dependent protein catabolic process (GO:0043161)	324	67	36.13	+	1.85	1.25E-05	7.83E-04
regulation of cytoskeleton organization (GO:0051493)	547	113	60.99	+	1.85	1.21E-08	1.45E-06
ubiquitin-dependent protein catabolic process (GO:0006511)	528	109	58.87	+	1.85	3.06E-08	3.20E-06
regulation of synaptic plasticity (GO:0048167)	189	39	21.07	+	1.85	8.27E-04	2.87E-02
regulation of protein complex assembly (GO:0043254)	441	91	49.17	+	1.85	3.71E-07	3.24E-05
positive regulation of protein catabolic process (GO:0045732)	228	47	25.42	+	1.85	2.53E-04	1.13E-02
response to insulin (GO:0032868)	234	48	26.09	+	1.84	2.86E-04	1.25E-02
positive regulation of cell development (GO:0010720)	557	114	62.1	+	1.84	2.20E-08	2.38E-06
generation of neurons (GO:0048699)	1550	317	172.82	+	1.83	4.63E-22	9.20E-19
regulation of supramolecular fiber organization (GO:1902903)	357	73	39.8	+	1.83	7.04E-06	4.66E-04
regulation of synapse structure or activity (GO:0050803)	235	48	26.2	+	1.83	2.97E-04	1.29E-02
microtubule cytoskeleton organization (GO:0000226)	467	95	52.07	+	1.82	3.59E-07	3.15E-05
cellular component morphogenesis (GO:0032989)	826	168	92.1	+	1.82	9.86E-12	2.06E-09
peptidyl-serine modification (GO:0018209)	197	40	21.96	+	1.82	1.01E-03	3.35E-02
proteolysis involved in cellular protein catabolic process (GO:0051603)	593	120	66.12	+	1.81	1.31E-08	1.53E-06
cellular protein catabolic process (GO:0044257)	623	125	69.46	+	1.8	9.57E-09	1.19E-06
regulation of intracellular protein transport (GO:0033157)	235	47	26.2	+	1.79	6.19E-04	2.27E-02
protein polyubiquitination (GO:0000209)	300	60	33.45	+	1.79	8.72E-05	4.40E-03
muscle cell differentiation (GO:0042692)	245	49	27.32	+	1.79	3.90E-04	1.58E-02
cellular response to peptide hormone stimulus (GO:0071375)	266	53	29.66	+	1.79	2.29E-04	1.04E-02
positive regulation of catabolic process (GO:0009896)	442	88	49.28	+	1.79	2.34E-06	1.70E-04

regulation of cell development (GO:0060284)	950	189	105.92	+	1.78	2.02E-12	4.79E-10
neurogenesis (GO:0022008)	1649	328	183.86	+	1.78	3.80E-21	5.49E-18
cell-cell signaling by wnt (GO:0198738)	347	69	38.69	+	1.78	3.26E-05	1.87E-03
Wnt signaling pathway (GO:0016055)	347	69	38.69	+	1.78	3.26E-05	1.86E-03
regulation of cellular component biogenesis (GO:0044087)	946	188	105.48	+	1.78	2.59E-12	5.97E-10
regulation of actin filament organization (GO:0110053)	263	52	29.32	+	1.77	3.06E-04	1.32E-02
intracellular transport (GO:0046907)	1529	302	170.48	+	1.77	3.66E-19	3.43E-16
regulation of developmental growth (GO:0048638)	338	66	37.69	+	1.75	7.55E-05	3.89E-03
regulation of cellular protein localization (GO:1903827)	548	107	61.1	+	1.75	4.32E-07	3.73E-05
regulation of ion transmembrane transporter activity (GO:0032412)	272	53	30.33	+	1.75	3.77E-04	1.55E-02
autophagy (GO:0006914)	278	54	31	+	1.74	4.21E-04	1.69E-02
process utilizing autophagic mechanism (GO:0061919)	278	54	31	+	1.74	4.21E-04	1.68E-02
positive regulation of cellular component organization (GO:0051130)	1226	238	136.7	+	1.74	2.01E-14	6.81E-12
transmembrane receptor protein tyrosine kinase signaling pathway (GO:0007169)	516	100	57.53	+	1.74	1.20E-06	9.06E-05
positive regulation of cellular catabolic process (GO:0031331)	372	72	41.48	+	1.74	4.22E-05	2.33E-03
divalent metal ion transport (GO:0070838)	305	59	34.01	+	1.73	2.04E-04	9.44E-03
regulation of cellular localization (GO:0060341)	813	157	90.65	+	1.73	1.44E-09	2.22E-07
negative regulation of cellular component organization (GO:0051129)	711	137	79.27	+	1.73	1.78E-08	1.98E-06
positive regulation of organelle organization (GO:0010638)	628	121	70.02	+	1.73	1.21E-07	1.15E-05
brain development (GO:0007420)	739	142	82.4	+	1.72	1.18E-08	1.43E-06
protein modification by small protein conjugation or removal (GO:0070647)	987	189	110.05	+	1.72	3.63E-11	6.79E-09
regulation of transporter activity (GO:0032409)	293	56	32.67	+	1.71	4.22E-04	1.68E-02
protein ubiquitination (GO:0016567)	698	133	77.83	+	1.71	5.11E-08	5.17E-06
regulation of anatomical structure size (GO:0090066)	511	97	56.98	+	1.7	3.99E-06	2.82E-04
membrane organization (GO:0061024)	855	162	95.33	+	1.7	2.43E-09	3.44E-07
regulation of membrane potential (GO:0042391)	433	82	48.28	+	1.7	2.86E-05	1.66E-03
negative regulation of organelle organization (GO:0010639)	386	73	43.04	+	1.7	7.78E-05	3.96E-03
divalent inorganic cation transport (GO:0072511)	312	59	34.79	+	1.7	4.47E-04	1.75E-02

cellular localization (GO:0051641)	2396	453	267.15	+	1.7	1.24E-25	1.96E-21
head development (GO:0060322)	784	148	87.41	+	1.69	1.52E-08	1.70E-06
regulation of transmembrane transporter activity (GO:0022898)	281	53	31.33	+	1.69	8.75E-04	3.00E-02
regulation of protein catabolic process (GO:0042176)	398	75	44.38	+	1.69	7.13E-05	3.68E-03
protein catabolic process (GO:0030163)	701	132	78.16	+	1.69	1.03E-07	1.01E-05
cytoskeleton organization (GO:0007010)	1095	206	122.09	+	1.69	2.16E-11	4.18E-09
cell surface receptor signaling pathway involved in cell-cell signaling (GO:1905114)	420	79	46.83	+	1.69	4.28E-05	2.35E-03
protein modification by small protein conjugation (GO:0032446)	777	146	86.63	+	1.69	2.51E-08	2.66E-06
protein transport (GO:0015031)	1528	287	170.37	+	1.68	1.35E-15	5.80E-13
regulation of neurotransmitter levels (GO:0001505)	357	67	39.8	+	1.68	1.64E-04	7.69E-03
protein localization to membrane (GO:0072657)	518	97	57.76	+	1.68	6.37E-06	4.28E-04
enzyme linked receptor protein signaling pathway (GO:0007167)	721	135	80.39	+	1.68	1.09E-07	1.05E-05
establishment of protein localization (GO:0045184)	1621	303	180.74	+	1.68	2.90E-16	1.71E-13
positive regulation of cellular protein localization (GO:1903829)	332	62	37.02	+	1.67	3.52E-04	1.47E-02
regulation of organelle organization (GO:0033043)	1298	242	144.72	+	1.67	5.96E-13	1.61E-10
nervous system development (GO:0007399)	2357	439	262.8	+	1.67	1.45E-23	7.68E-20
organelle assembly (GO:0070925)	725	135	80.84	+	1.67	1.21E-07	1.15E-05
positive regulation of cellular component biogenesis (GO:0044089)	523	97	58.31	+	1.66	9.44E-06	6.16E-04
peptide transport (GO:0015833)	1558	288	173.71	+	1.66	6.13E-15	2.38E-12
protein localization (GO:0008104)	2178	402	242.84	+	1.66	6.85E-21	9.08E-18
cellular response to peptide (GO:1901653)	327	60	36.46	+	1.65	6.13E-04	2.27E-02
amide transport (GO:0042886)	1592	292	177.5	+	1.65	8.24E-15	3.12E-12
chemical synaptic transmission (GO:0007268)	420	77	46.83	+	1.64	1.10E-04	5.35E-03
anterograde trans-synaptic signaling (GO:0098916)	420	77	46.83	+	1.64	1.10E-04	5.34E-03
forebrain development (GO:0030900)	393	72	43.82	+	1.64	2.26E-04	1.03E-02
regulation of catabolic process (GO:0009894)	999	183	111.39	+	1.64	1.98E-09	2.94E-07
synaptic signaling (GO:0099536)	465	85	51.85	+	1.64	5.30E-05	2.85E-03
regulation of cation transmembrane transport (GO:1904062)	351	64	39.14	+	1.64	5.00E-04	1.91E-02

regulation of actin cytoskeleton organization (GO:0032956)	346	63	38.58	+	1.63	6.14E-04	2.26E-02
regulation of transmembrane transport (GO:0034762)	573	104	63.89	+	1.63	1.21E-05	7.64E-04
regulation of cellular component organization (GO:0051128)	2491	451	277.74	+	1.62	3.67E-22	8.33E-19
histone modification (GO:0016570)	365	66	40.7	+	1.62	4.77E-04	1.83E-02
regulation of cellular catabolic process (GO:0031329)	836	151	93.21	+	1.62	1.28E-07	1.21E-05
trans-synaptic signaling (GO:0099537)	439	79	48.95	+	1.61	1.52E-04	7.18E-03
nitrogen compound transport (GO:0071705)	1861	333	207.5	+	1.6	1.71E-15	7.18E-13
response to peptide hormone (GO:0043434)	397	71	44.26	+	1.6	4.36E-04	1.72E-02
positive regulation of hydrolase activity (GO:0051345)	771	137	85.96	+	1.59	1.11E-06	8.48E-05
regulation of actin filament-based process (GO:0032970)	389	69	43.37	+	1.59	6.81E-04	2.47E-02
vesicle-mediated transport (GO:0016192)	1954	345	217.87	+	1.58	2.70E-15	1.10E-12
macromolecule localization (GO:0033036)	2517	444	280.64	+	1.58	7.49E-20	7.94E-17
cellular protein localization (GO:0034613)	1618	285	180.4	+	1.58	1.40E-12	3.60E-10
cellular macromolecule localization (GO:0070727)	1626	286	181.29	+	1.58	1.56E-12	3.88E-10
covalent chromatin modification (GO:0016569)	376	66	41.92	+	1.57	1.00E-03	3.34E-02
regulation of transport (GO:0051049)	1877	329	209.28	+	1.57	2.99E-14	9.69E-12
regulation of ion transmembrane transport (GO:0034765)	491	86	54.75	+	1.57	1.87E-04	8.68E-03
positive regulation of cell differentiation (GO:0045597)	978	171	109.04	+	1.57	1.09E-07	1.05E-05
developmental growth (GO:0048589)	396	69	44.15	+	1.56	9.97E-04	3.33E-02
actin cytoskeleton organization (GO:0030036)	496	86	55.3	+	1.56	2.62E-04	1.16E-02
cell development (GO:0048468)	1633	283	182.08	+	1.55	9.09E-12	1.93E-09
protein phosphorylation (GO:0006468)	964	167	107.48	+	1.55	2.65E-07	2.44E-05
actin filament-based process (GO:0030029)	573	99	63.89	+	1.55	8.82E-05	4.44E-03
mitotic cell cycle (GO:0000278)	696	120	77.6	+	1.55	1.80E-05	1.08E-03
behavior (GO:0007610)	581	100	64.78	+	1.54	9.67E-05	4.79E-03
regulation of establishment of protein localization (GO:0070201)	763	131	85.07	+	1.54	8.73E-06	5.71E-04
growth (GO:0040007)	402	69	44.82	+	1.54	1.42E-03	4.44E-02
cellular response to growth factor stimulus (GO:0071363)	502	86	55.97	+	1.54	3.69E-04	1.53E-02
central nervous system development (GO:0007417)	975	167	108.71	+	1.54	5.14E-07	4.32E-05

cellular protein modification process (GO:0006464)	3084	527	343.86	+	1.53	2.60E-21	4.59E-18
protein modification process (GO:0036211)	3084	527	343.86	+	1.53	2.60E-21	4.13E-18
regulation of protein localization (GO:0032880)	1038	174	115.73	+	1.5	1.06E-06	8.16E-05
response to growth factor (GO:0070848)	532	89	59.32	+	1.5	5.37E-04	2.03E-02
regulation of anatomical structure morphogenesis (GO:0022603)	1077	180	120.08	+	1.5	7.73E-07	6.24E-05
intracellular protein transport (GO:0006886)	1003	167	111.83	+	1.49	2.47E-06	1.78E-04
regulation of protein transport (GO:0051223)	716	119	79.83	+	1.49	8.04E-05	4.09E-03
organic substance transport (GO:0071702)	2224	369	247.97	+	1.49	5.80E-13	1.59E-10
organelle organization (GO:0006996)	3418	567	381.1	+	1.49	1.71E-20	1.95E-17
positive regulation of transferase activity (GO:0051347)	660	109	73.59	+	1.48	2.37E-04	1.07E-02
cellular macromolecule catabolic process (GO:0044265)	910	150	101.46	+	1.48	1.32E-05	8.21E-04
heart development (GO:0007507)	528	87	58.87	+	1.48	1.05E-03	3.46E-02
regulation of localization (GO:0032879)	2790	459	311.08	+	1.48	1.01E-15	4.89E-13
mitotic cell cycle process (GO:1903047)	610	100	68.01	+	1.47	4.68E-04	1.80E-02
regulation of cell migration (GO:0030334)	861	141	96	+	1.47	3.29E-05	1.87E-03
macromolecule modification (GO:0043412)	3304	541	368.39	+	1.47	1.98E-18	1.43E-15
regulation of peptide transport (GO:0090087)	748	122	83.4	+	1.46	1.40E-04	6.62E-03
negative regulation of phosphate metabolic process (GO:0045936)	558	91	62.22	+	1.46	1.12E-03	3.66E-02
negative regulation of phosphorus metabolic process (GO:0010563)	559	91	62.33	+	1.46	1.13E-03	3.69E-02
intracellular signal transduction (GO:0035556)	1680	273	187.32	+	1.46	6.55E-09	8.27E-07
endocytosis (GO:0006897)	554	90	61.77	+	1.46	1.35E-03	4.29E-02
phosphorylation (GO:0016310)	1306	212	145.62	+	1.46	4.40E-07	3.78E-05
positive regulation of protein kinase activity (GO:0045860)	536	87	59.76	+	1.46	1.45E-03	4.55E-02
cellular response to hormone stimulus (GO:0032870)	610	99	68.01	+	1.46	7.36E-04	2.64E-02
regulation of ion transport (GO:0043269)	704	114	78.49	+	1.45	2.90E-04	1.26E-02
positive regulation of catalytic activity (GO:0043085)	1440	233	160.56	+	1.45	1.48E-07	1.39E-05
positive regulation of kinase activity (GO:0033674)	583	94	65	+	1.45	1.14E-03	3.70E-02
regulation of cellular component movement (GO:0051270)	1009	162	112.5	+	1.44	2.10E-05	1.25E-03
positive regulation of developmental process (GO:0051094)	1373	220	153.09	+	1.44	6.28E-07	5.18E-05

positive regulation of transport (GO:0051050)	1007	161	112.28	+	1.43	2.59E-05	1.51E-03
cell cycle (GO:0007049)	1328	212	148.07	+	1.43	1.30E-06	9.77E-05
regulation of locomotion (GO:0040012)	998	159	111.27	+	1.43	3.67E-05	2.07E-03
regulation of cell differentiation (GO:0045595)	1824	290	203.37	+	1.43	1.41E-08	1.60E-06
regulation of cell motility (GO:2000145)	926	147	103.25	+	1.42	9.11E-05	4.55E-03
import into cell (GO:0098657)	675	107	75.26	+	1.42	8.52E-04	2.94E-02
regulation of secretion by cell (GO:1903530)	764	121	85.18	+	1.42	3.96E-04	1.60E-02
regulation of hydrolase activity (GO:0051336)	1290	204	143.83	+	1.42	4.27E-06	2.97E-04
cellular component assembly (GO:0022607)	2473	390	275.73	+	1.41	5.31E-11	9.60E-09
phosphorus metabolic process (GO:0006793)	2178	342	242.84	+	1.41	1.75E-09	2.62E-07
regulation of multicellular organismal development (GO:2000026)	2047	321	228.24	+	1.41	7.09E-09	8.88E-07
phosphate-containing compound metabolic process (GO:0006796)	2151	337	239.83	+	1.41	2.77E-09	3.86E-07
organonitrogen compound catabolic process (GO:1901565)	1073	168	119.64	+	1.4	5.32E-05	2.85E-03
cellular protein metabolic process (GO:0044267)	3713	579	413.99	+	1.4	7.13E-16	3.66E-13
positive regulation of molecular function (GO:0044093)	1797	280	200.36	+	1.4	1.47E-07	1.38E-05
macromolecule catabolic process (GO:0009057)	1066	166	118.86	+	1.4	7.59E-05	3.89E-03
cell-cell signaling (GO:0007267)	1111	173	123.87	+	1.4	4.71E-05	2.58E-03
regulation of signaling (GO:0023051)	3581	555	399.27	+	1.39	1.11E-14	3.83E-12
peptidyl-amino acid modification (GO:0018193)	879	136	98.01	+	1.39	4.15E-04	1.67E-02
regulation of secretion (GO:0051046)	821	127	91.54	+	1.39	7.37E-04	2.63E-02
anatomical structure morphogenesis (GO:0009653)	2153	333	240.05	+	1.39	1.21E-08	1.45E-06
regulation of cell communication (GO:0010646)	3540	547	394.7	+	1.39	3.20E-14	1.02E-11
cellular component organization (GO:0016043)	5642	869	629.07	+	1.38	1.66E-24	1.32E-20
regulation of developmental process (GO:0050793)	2592	396	289	+	1.37	1.47E-09	2.25E-07
response to organonitrogen compound (GO:0010243)	1005	153	112.05	+	1.37	3.59E-04	1.49E-02
cell cycle process (GO:0022402)	986	150	109.94	+	1.36	4.62E-04	1.79E-02
cellular response to endogenous stimulus (GO:0071495)	1221	185	136.14	+	1.36	9.72E-05	4.80E-03
response to nitrogen compound (GO:1901698)	1089	165	121.42	+	1.36	2.81E-04	1.24E-02
cellular component organization or biogenesis (GO:0071840)	5830	883	650.03	+	1.36	6.63E-23	1.76E-19

cellular component biogenesis (GO:0044085)	2699	408	300.93	+	1.36	2.26E-09	3.27E-07
movement of cell or subcellular component (GO:0006928)	1535	232	171.15	+	1.36	1.46E-05	9.09E-04
circulatory system development (GO:0072359)	872	131	97.23	+	1.35	1.51E-03	4.69E-02
regulation of signal transduction (GO:0009966)	3154	473	351.66	+	1.35	2.03E-10	3.40E-08
proteolysis (GO:0006508)	1283	192	143.05	+	1.34	1.38E-04	6.55E-03
positive regulation of multicellular organismal process (GO:0051240)	1762	262	196.46	+	1.33	9.85E-06	6.34E-04
transport (GO:0006810)	4550	674	507.31	+	1.33	2.44E-14	8.08E-12
establishment of localization (GO:0051234)	4681	693	521.92	+	1.33	8.72E-15	3.22E-12
regulation of intracellular signal transduction (GO:1902531)	1834	271	204.49	+	1.33	9.98E-06	6.40E-04
positive regulation of protein modification process (GO:0031401)	1240	183	138.26	+	1.32	3.76E-04	1.55E-02
positive regulation of signaling (GO:0023056)	1851	273	206.38	+	1.32	1.08E-05	6.89E-04
negative regulation of signaling (GO:0023057)	1371	202	152.86	+	1.32	2.15E-04	9.88E-03
negative regulation of cell communication (GO:0010648)	1367	201	152.42	+	1.32	2.50E-04	1.12E-02
regulation of protein modification process (GO:0031399)	1851	272	206.38	+	1.32	1.51E-05	9.36E-04
positive regulation of cell communication (GO:0010647)	1842	269	205.38	+	1.31	2.42E-05	1.43E-03
cellular catabolic process (GO:0044248)	1787	259	199.25	+	1.3	6.00E-05	3.18E-03
regulation of catalytic activity (GO:0050790)	2342	338	261.13	+	1.29	4.51E-06	3.12E-04
localization (GO:0051179)	5765	832	642.78	+	1.29	6.78E-16	3.59E-13
cellular macromolecule metabolic process (GO:0044260)	5083	732	566.74	+	1.29	2.48E-13	7.32E-11
protein metabolic process (GO:0019538)	4315	621	481.11	+	1.29	5.82E-11	1.04E-08
response to endogenous stimulus (GO:0009719)	1503	216	167.58	+	1.29	4.50E-04	1.76E-02
negative regulation of signal transduction (GO:0009968)	1268	182	141.38	+	1.29	1.33E-03	4.25E-02
system development (GO:0048731)	4424	633	493.26	+	1.28	8.56E-11	1.48E-08
multicellular organism development (GO:0007275)	5026	719	560.39	+	1.28	1.57E-12	3.84E-10
cellular developmental process (GO:0048869)	3806	543	424.36	+	1.28	5.60E-09	7.23E-07
regulation of multicellular organismal process (GO:0051239)	3147	448	350.88	+	1.28	2.78E-07	2.52E-05
positive regulation of signal transduction (GO:0009967)	1691	240	188.54	+	1.27	3.66E-04	1.52E-02
anatomical structure development (GO:0048856)	5422	767	604.54	+	1.27	1.54E-12	3.88E-10
cell differentiation (GO:0030154)	3710	523	413.66	+	1.26	5.46E-08	5.50E-06

regulation of molecular function (GO:0065009)	3022	426	336.95	+	1.26	1.62E-06	1.20E-04
regulation of biological quality (GO:0065008)	4073	573	454.13	+	1.26	1.13E-08	1.38E-06
positive regulation of metabolic process (GO:0009893)	3650	511	406.97	+	1.26	1.85E-07	1.72E-05
positive regulation of protein metabolic process (GO:0051247)	1722	241	192	+	1.26	7.28E-04	2.61E-02
positive regulation of cellular protein metabolic process (GO:0032270)	1616	226	180.18	+	1.25	1.21E-03	3.92E-02
negative regulation of response to stimulus (GO:0048585)	1640	229	182.86	+	1.25	1.14E-03	3.70E-02
cellular response to stress (GO:0033554)	1729	241	192.78	+	1.25	9.81E-04	3.28E-02
positive regulation of cellular process (GO:0048522)	5465	758	609.33	+	1.24	1.01E-10	1.73E-08
positive regulation of macromolecule metabolic process (GO:0010604)	3375	468	376.3	+	1.24	2.19E-06	1.60E-04
negative regulation of cellular process (GO:0048523)	4754	658	530.06	+	1.24	6.01E-09	7.65E-07
negative regulation of cellular metabolic process (GO:0031324)	2567	355	286.21	+	1.24	6.70E-05	3.51E-03
developmental process (GO:0032502)	5862	810	653.6	+	1.24	2.64E-11	5.06E-09
positive regulation of cellular metabolic process (GO:0031325)	3344	457	372.85	+	1.23	1.24E-05	7.77E-04
organonitrogen compound metabolic process (GO:1901564)	5331	727	594.39	+	1.22	5.80E-09	7.44E-07
catabolic process (GO:0009056)	2065	281	230.24	+	1.22	1.25E-03	4.01E-02
regulation of response to stimulus (GO:0048583)	4331	589	482.9	+	1.22	5.73E-07	4.80E-05
regulation of protein metabolic process (GO:0051246)	2804	381	312.64	+	1.22	1.38E-04	6.54E-03
negative regulation of biological process (GO:0048519)	5333	724	594.62	+	1.22	1.22E-08	1.44E-06
regulation of cellular protein metabolic process (GO:0032268)	2633	356	293.57	+	1.21	3.32E-04	1.41E-02
negative regulation of metabolic process (GO:0009892)	2918	394	325.35	+	1.21	1.57E-04	7.34E-03
macromolecule metabolic process (GO:0043170)	6193	836	690.5	+	1.21	8.59E-10	1.37E-07
positive regulation of biological process (GO:0048518)	6214	835	692.85	+	1.21	2.15E-09	3.13E-07
positive regulation of nitrogen compound metabolic process (GO:0051173)	3203	430	357.13	+	1.2	1.18E-04	5.71E-03
negative regulation of nitrogen compound metabolic process (GO:0051172)	2369	318	264.14	+	1.2	1.18E-03	3.82E-02
regulation of metabolic process (GO:0019222)	6690	896	745.92	+	1.2	5.83E-10	9.56E-08
regulation of cellular metabolic process (GO:0031323)	6216	828	693.07	+	1.19	1.36E-08	1.57E-06
cellular metabolic process (GO:0044237)	7671	1019	855.3	+	1.19	5.14E-11	9.40E-09
regulation of macromolecule metabolic process (GO:0060255)	6160	816	686.82	+	1.19	4.89E-08	5.02E-06
negative regulation of macromolecule metabolic process (GO:0010605)	2666	353	297.25	+	1.19	1.45E-03	4.54E-02

regulation of cellular macromolecule biosynthetic process (GO:2000112)	3908	515	435.73	+	1.18	9.84E-05	4.84E-03
regulation of biosynthetic process (GO:0009889)	4264	561	475.43	+	1.18	4.80E-05	2.61E-03
regulation of gene expression (GO:0010468)	4463	587	497.61	+	1.18	2.87E-05	1.65E-03
regulation of cellular biosynthetic process (GO:0031326)	4185	550	466.62	+	1.18	6.74E-05	3.51E-03
nitrogen compound metabolic process (GO:0006807)	7023	922	783.05	+	1.18	1.31E-08	1.52E-06
regulation of macromolecule biosynthetic process (GO:0010556)	4046	531	451.12	+	1.18	1.08E-04	5.29E-03
primary metabolic process (GO:0044238)	7501	983	836.34	+	1.18	3.27E-09	4.44E-07
regulation of cellular process (GO:0050794)	10981	1431	1224.35	+	1.17	4.03E-16	2.29E-13
regulation of RNA metabolic process (GO:0051252)	3785	493	422.02	+	1.17	3.87E-04	1.57E-02
regulation of primary metabolic process (GO:0080090)	6023	783	671.55	+	1.17	2.04E-06	1.50E-04
regulation of nitrogen compound metabolic process (GO:0051171)	5839	758	651.03	+	1.16	4.23E-06	2.96E-04
organic substance metabolic process (GO:0071704)	7874	1017	877.93	+	1.16	2.57E-08	2.70E-06
regulation of nucleobase-containing compound metabolic process (GO:0019219)	4040	520	450.45	+	1.15	7.21E-04	2.59E-02
metabolic process (GO:0008152)	8428	1084	939.7	+	1.15	1.04E-08	1.28E-06
signaling (GO:0023052)	5393	693	601.31	+	1.15	5.20E-05	2.80E-03
regulation of biological process (GO:0050789)	11688	1498	1303.18	+	1.15	9.28E-15	3.35E-12
cell communication (GO:0007154)	5500	704	613.24	+	1.15	7.11E-05	3.69E-03
biological regulation (GO:0065007)	12387	1582	1381.12	+	1.15	5.23E-16	2.87E-13
multicellular organismal process (GO:0032501)	6973	884	777.47	+	1.14	1.17E-05	7.44E-04
cellular response to stimulus (GO:0051716)	6703	831	747.37	+	1.11	5.14E-04	1.96E-02
cellular process (GO:0009987)	14575	1799	1625.08	+	1.11	3.27E-14	1.02E-11
biological_process (GO:0008150)	17840	2139	1989.11	+	1.08	8.29E-19	7.32E-16
immune system process (GO:0002376)	2806	254	312.86	-	0.81	5.47E-04	2.06E-02
defense response (GO:0006952)	1342	95	149.63	-	0.63	3.54E-06	2.52E-04
response to biotic stimulus (GO:0009607)	1353	93	150.86	-	0.62	7.84E-07	6.30E-05
immune response (GO:0006955)	1868	128	208.28	-	0.61	3.58E-09	4.78E-07
response to other organism (GO:0051707)	1327	90	147.96	-	0.61	6.06E-07	5.05E-05
response to external biotic stimulus (GO:0043207)	1329	90	148.18	-	0.61	6.12E-07	5.07E-05

peptide metabolic process (GO:0006518)	542	35	60.43	-	0.58	9.27E-04	3.12E-02
Unclassified (UNCLASSIFIED)	3156	202	351.89	-	0.57	8.29E-19	6.94E-16
innate immune response (GO:0045087)	766	49	85.41	-	0.57	5.06E-05	2.73E-03
G protein-coupled receptor signaling pathway (GO:0007186)	1315	84	146.62	-	0.57	4.24E-08	4.38E-06
defense response to other organism (GO:0098542)	952	58	106.15	-	0.55	9.77E-07	7.58E-05
sensory perception (GO:0007600)	966	54	107.71	-	0.5	3.39E-08	3.53E-06
response to bacterium (GO:0009617)	701	37	78.16	-	0.47	7.41E-07	6.01E-05
peptide biosynthetic process (GO:0043043)	419	22	46.72	-	0.47	1.54E-04	7.27E-03
translation (GO:0006412)	393	20	43.82	-	0.46	1.80E-04	8.36E-03
regulation of B cell activation (GO:0050864)	211	9	23.53	-	0.38	1.51E-03	4.70E-02
cellular response to biotic stimulus (GO:0071216)	227	9	25.31	-	0.36	6.43E-04	2.35E-02
adaptive immune response (GO:0002250)	599	23	66.79	-	0.34	2.79E-09	3.86E-07
cellular response to lipopolysaccharide (GO:0071222)	194	7	21.63	-	0.32	8.39E-04	2.90E-02
epidermal cell differentiation (GO:0009913)	313	11	34.9	-	0.32	9.53E-06	6.19E-04
detection of stimulus (GO:0051606)	693	24	77.27	-	0.31	8.31E-12	1.79E-09
cellular response to molecule of bacterial origin (GO:0071219)	203	7	22.63	-	0.31	4.55E-04	1.78E-02
keratinocyte differentiation (GO:0030216)	268	9	29.88	-	0.3	2.97E-05	1.71E-03
translational initiation (GO:0006413)	144	4	16.06	-	0.25	1.34E-03	4.24E-02
immunoglobulin production (GO:0002377)	155	4	17.28	-	0.23	4.73E-04	1.82E-02
immunoglobulin mediated immune response (GO:0016064)	205	5	22.86	-	0.22	2.55E-05	1.49E-03
B cell mediated immunity (GO:0019724)	208	5	23.19	-	0.22	2.66E-05	1.55E-03
defense response to bacterium (GO:0042742)	333	8	37.13	-	0.22	4.91E-08	5.01E-06
xenobiotic metabolic process (GO:0006805)	127	3	14.16	-	0.21	1.11E-03	3.66E-02
viral gene expression (GO:0019080)	132	3	14.72	-	0.2	7.91E-04	2.77E-02
protein targeting to ER (GO:0045047)	110	2	12.26	-	0.16	1.30E-03	4.15E-02
regulation of complement activation (GO:0030449)	117	2	13.05	-	0.15	6.15E-04	2.26E-02
detection of chemical stimulus (GO:0009593)	519	8	57.87	-	0.14	2.98E-15	1.18E-12
regulation of humoral immune response (GO:0002920)	136	2	15.16	-	0.13	9.59E-05	4.77E-03
sensory perception of chemical stimulus (GO:0007606)	534	7	59.54	-	0.12	8.79E-17	5.59E-14

detection of stimulus involved in sensory perception (GO:0050906)	543	7	60.54	-	0.12	4.13E-17	2.73E-14
humoral immune response (GO:0006959)	372	4	41.48	-	0.1	1.08E-12	2.85E-10
SRP-dependent cotranslational protein targeting to membrane (GO:0006614)	97	1	10.82	-	0.09	6.04E-04	2.24E-02
antimicrobial humoral immune response mediated by antimicrobial peptide (GO:0061844)	104	1	11.6	-	0.09	4.37E-04	1.72E-02
phagocytosis, recognition (GO:0006910)	106	1	11.82	-	0.08	2.80E-04	1.24E-02
cornification (GO:0070268)	113	1	12.6	-	0.08	1.28E-04	6.11E-03
keratinization (GO:0031424)	226	2	25.2	-	0.08	2.23E-08	2.38E-06
sensory perception of smell (GO:0007608)	462	4	51.51	-	0.08	1.37E-16	8.38E-14
antimicrobial humoral response (GO:0019730)	142	1	15.83	-	0.06	8.36E-06	5.49E-04
complement activation, classical pathway (GO:0006958)	161	1	17.95	-	0.06	1.15E-06	8.74E-05
humoral immune response mediated by circulating immunoglobulin (GO:0002455)	167	1	18.62	-	0.05	8.24E-07	6.58E-05
complement activation (GO:0006956)	176	1	19.62	-	0.05	2.34E-07	2.17E-05
detection of chemical stimulus involved in sensory perception (GO:0050907)	482	2	53.74	-	0.04	1.30E-19	1.29E-16
cytoplasmic translation (GO:0002181)	74	0	8.25	-	< 0.01	6.91E-04	2.49E-02
disruption of cells of other organism (GO:0044364)	83	0	9.25	-	< 0.01	3.13E-04	1.34E-02
detection of chemical stimulus involved in sensory perception of smell (GO:0050911)	433	0	48.28	-	< 0.01	1.38E-20	1.69E-17
killing of cells of other organism (GO:0031640)	83	0	9.25	-	< 0.01	3.13E-04	1.34E-02
animal organ regeneration (GO:0031100)	73	0	8.14	-	< 0.01	6.86E-04	2.48E-02

Table A8.2 GO-BMPR1A

BMPR1A co-expressed genes

Analysis Type:	PANTHER Overrepresentation Test (Released 20190711)
Annotation Version and Release Date:	GO Ontology database Released 2019-10-08
Analyzed List:	upload_1 (Homo sapiens)
Reference List:	Homo sapiens (all genes in database)
Test Type:	FISHER
Correction:	FDR

GO biological process complete	#	#	expected	Plus/minus	Fold enrichment	Raw P value	FDR
nuclear cell cycle DNA replication initiation (GO:1902315)	4	4	0.31	+	12.83	1.50E-03	3.61E-02
cell cycle DNA replication initiation (GO:1902292)	4	4	0.31	+	12.83	1.50E-03	3.61E-02
mitotic DNA replication initiation (GO:1902975)	4	4	0.31	+	12.83	1.50E-03	3.60E-02
nuclear pore organization (GO:0006999)	14	10	1.09	+	9.17	2.90E-06	1.51E-04
G-quadruplex DNA unwinding (GO:0044806)	7	5	0.55	+	9.17	1.01E-03	2.53E-02
pre-replicative complex assembly involved in cell cycle DNA replication (GO:1902299)	7	5	0.55	+	9.17	1.01E-03	2.53E-02
pre-replicative complex assembly involved in nuclear cell cycle DNA replication (GO:0006267)	7	5	0.55	+	9.17	1.01E-03	2.52E-02
pre-replicative complex assembly (GO:0036388)	7	5	0.55	+	9.17	1.01E-03	2.52E-02
kinetochore organization (GO:0051383)	18	12	1.4	+	8.56	4.96E-07	3.07E-05
positive regulation of protein localization to Cajal body (GO:1904871)	9	6	0.7	+	8.56	4.00E-04	1.13E-02
regulation of protein localization to Cajal body (GO:1904869)	9	6	0.7	+	8.56	4.00E-04	1.13E-02
mitotic DNA replication (GO:1902969)	11	7	0.86	+	8.17	1.59E-04	5.34E-03
chromatin remodeling at centromere (GO:0031055)	34	21	2.65	+	7.93	7.46E-11	8.73E-09
kinetochore assembly (GO:0051382)	13	8	1.01	+	7.9	6.35E-05	2.41E-03
CENP-A containing nucleosome assembly (GO:0034080)	31	19	2.42	+	7.87	6.58E-10	6.66E-08
CENP-A containing chromatin organization (GO:0061641)	31	19	2.42	+	7.87	6.58E-10	6.62E-08

centromere complex assembly (GO:0034508)	38	23	2.96	+	7.77	1.27E-11	1.71E-09
positive regulation of establishment of protein localization to telomere (GO:1904851)	10	6	0.78	+	7.7	6.01E-04	1.62E-02
DNA strand elongation (GO:0022616)	22	13	1.71	+	7.58	4.59E-07	2.86E-05
DNA strand elongation involved in DNA replication (GO:0006271)	17	10	1.32	+	7.55	1.02E-05	4.72E-04
replication fork processing (GO:0031297)	32	18	2.49	+	7.22	5.12E-09	4.40E-07
DNA replication-independent nucleosome organization (GO:0034724)	41	23	3.19	+	7.2	4.02E-11	4.87E-09
DNA replication-independent nucleosome assembly (GO:0006336)	40	22	3.12	+	7.06	1.42E-10	1.60E-08
DNA double-strand break processing (GO:0000729)	22	12	1.71	+	7	2.39E-06	1.27E-04
double-strand break repair via break-induced replication (GO:0000727)	11	6	0.86	+	7	8.73E-04	2.23E-02
attachment of mitotic spindle microtubules to kinetochore (GO:0051315)	11	6	0.86	+	7	8.73E-04	2.23E-02
regulation of establishment of protein localization to telomere (GO:0070203)	11	6	0.86	+	7	8.73E-04	2.22E-02
positive regulation of mRNA 3'-end processing (GO:0031442)	11	6	0.86	+	7	8.73E-04	2.22E-02
prophase (GO:0051324)	15	8	1.17	+	6.84	1.34E-04	4.61E-03
mitotic prophase (GO:0000088)	15	8	1.17	+	6.84	1.34E-04	4.60E-03
regulation of establishment of protein localization to chromosome (GO:0070202)	12	6	0.94	+	6.42	1.23E-03	3.02E-02
DNA-dependent DNA replication maintenance of fidelity (GO:0045005)	41	20	3.19	+	6.26	4.76E-09	4.14E-07
protein localization to chromosome, centromeric region (GO:0071459)	17	8	1.32	+	6.04	2.60E-04	8.03E-03
DNA unwinding involved in DNA replication (GO:0006268)	15	7	1.17	+	5.99	6.60E-04	1.76E-02
positive regulation of telomerase RNA localization to Cajal body (GO:1904874)	15	7	1.17	+	5.99	6.60E-04	1.75E-02
regulation of attachment of spindle microtubules to kinetochore (GO:0051988)	13	6	1.01	+	5.92	1.69E-03	3.96E-02
positive regulation of protein localization to chromosome, telomeric region (GO:1904816)	13	6	1.01	+	5.92	1.69E-03	3.95E-02
protein localization to kinetochore (GO:0034501)	13	6	1.01	+	5.92	1.69E-03	3.95E-02
histone exchange (GO:0043486)	49	22	3.82	+	5.76	2.73E-09	2.52E-07
mitotic recombination (GO:0006312)	18	8	1.4	+	5.7	3.52E-04	1.02E-02
regulation of telomerase RNA localization to Cajal body (GO:1904872)	18	8	1.4	+	5.7	3.52E-04	1.02E-02
nuclear DNA replication (GO:0033260)	43	19	3.35	+	5.67	4.00E-08	3.00E-06
response to X-ray (GO:0010165)	30	13	2.34	+	5.56	6.58E-06	3.21E-04
cell cycle DNA replication (GO:0044786)	44	19	3.43	+	5.54	5.34E-08	3.92E-06

negative regulation of DNA-dependent DNA replication (GO:2000104)	19	8	1.48	+	5.4	4.69E-04	1.31E-02
somatic recombination of immunoglobulin gene segments (GO:0016447)	24	10	1.87	+	5.35	9.95E-05	3.60E-03
mitotic chromosome condensation (GO:0007076)	17	7	1.32	+	5.28	1.18E-03	2.90E-02
regulation of sister chromatid cohesion (GO:0007063)	22	9	1.71	+	5.25	2.47E-04	7.66E-03
mitotic prometaphase (GO:0000236)	170	69	13.25	+	5.21	3.77E-24	1.71E-21
pore complex assembly (GO:0046931)	20	8	1.56	+	5.13	6.15E-04	1.65E-02
histone mRNA metabolic process (GO:0008334)	25	10	1.95	+	5.13	1.30E-04	4.52E-03
negative regulation of peptidyl-lysine acetylation (GO:2000757)	18	7	1.4	+	4.99	1.54E-03	3.66E-02
centriole replication (GO:0007099)	18	7	1.4	+	4.99	1.54E-03	3.66E-02
tRNA transport (GO:0051031)	36	14	2.81	+	4.99	7.93E-06	3.81E-04
protein localization to microtubule cytoskeleton (GO:0072698)	36	14	2.81	+	4.99	7.93E-06	3.80E-04
somatic diversification of immune receptors via germline recombination within a single locus (GO:0002562)	34	13	2.65	+	4.91	1.93E-05	8.46E-04
tRNA-containing ribonucleoprotein complex export from nucleus (GO:0071431)	34	13	2.65	+	4.91	1.93E-05	8.44E-04
tRNA export from nucleus (GO:0006409)	34	13	2.65	+	4.91	1.93E-05	8.42E-04
somatic cell DNA recombination (GO:0016444)	34	13	2.65	+	4.91	1.93E-05	8.40E-04
negative regulation of protein acetylation (GO:1901984)	21	8	1.64	+	4.89	7.96E-04	2.06E-02
centrosome duplication (GO:0051298)	29	11	2.26	+	4.87	8.88E-05	3.27E-03
sister chromatid segregation (GO:0000819)	138	52	10.75	+	4.84	2.01E-17	4.98E-15
ATP-dependent chromatin remodeling (GO:0043044)	78	29	6.08	+	4.77	3.11E-10	3.26E-08
positive regulation of G2/M transition of mitotic cell cycle (GO:0010971)	27	10	2.1	+	4.75	2.16E-04	6.89E-03
NLS-bearing protein import into nucleus (GO:0006607)	19	7	1.48	+	4.73	1.97E-03	4.51E-02
somatic diversification of immunoglobulins involved in immune response (GO:0002208)	19	7	1.48	+	4.73	1.97E-03	4.51E-02
somatic recombination of immunoglobulin genes involved in immune response (GO:0002204)	19	7	1.48	+	4.73	1.97E-03	4.50E-02
isotype switching (GO:0045190)	19	7	1.48	+	4.73	1.97E-03	4.49E-02
positive regulation of cell cycle G2/M phase transition (GO:1902751)	30	11	2.34	+	4.71	1.13E-04	4.02E-03
histone monoubiquitination (GO:0010390)	30	11	2.34	+	4.71	1.13E-04	4.01E-03
metaphase plate congression (GO:0051310)	52	19	4.05	+	4.69	4.32E-07	2.71E-05

regulation of centriole replication (GO:0046599)	22	8	1.71	+	4.67	1.02E-03	2.53E-02
double-strand break repair via nonhomologous end joining (GO:0006303)	58	21	4.52	+	4.65	1.23E-07	8.33E-06
DNA-dependent DNA replication (GO:0006261)	119	43	9.27	+	4.64	3.87E-14	6.68E-12
telomere maintenance via semi-conservative replication (GO:0032201)	25	9	1.95	+	4.62	5.28E-04	1.45E-02
non-recombinational repair (GO:0000726)	64	23	4.99	+	4.61	3.48E-08	2.67E-06
negative regulation of G0 to G1 transition (GO:0070317)	39	14	3.04	+	4.61	1.66E-05	7.36E-04
protein localization to cytoskeleton (GO:0044380)	42	15	3.27	+	4.58	8.75E-06	4.15E-04
mitotic sister chromatid segregation (GO:0000070)	105	37	8.18	+	4.52	4.22E-12	5.93E-10
mitotic spindle assembly (GO:0090307)	37	13	2.88	+	4.51	3.96E-05	1.60E-03
mitotic metaphase plate congression (GO:0007080)	43	15	3.35	+	4.48	1.10E-05	5.04E-04
sister chromatid cohesion (GO:0007062)	49	17	3.82	+	4.45	3.08E-06	1.59E-04
positive regulation of chromosome segregation (GO:0051984)	29	10	2.26	+	4.43	3.46E-04	1.01E-02
regulation of mRNA 3'-end processing (GO:0031440)	29	10	2.26	+	4.43	3.46E-04	1.01E-02
negative regulation of mitotic metaphase/anaphase transition (GO:0045841)	29	10	2.26	+	4.43	3.46E-04	1.00E-02
DNA replication (GO:0006260)	209	72	16.29	+	4.42	8.89E-22	3.45E-19
DNA replication initiation (GO:0006270)	32	11	2.49	+	4.41	1.81E-04	5.90E-03
positive regulation of mRNA processing (GO:0050685)	32	11	2.49	+	4.41	1.81E-04	5.89E-03
ncRNA export from nucleus (GO:0097064)	38	13	2.96	+	4.39	4.97E-05	1.96E-03
DNA duplex unwinding (GO:0032508)	106	36	8.26	+	4.36	1.94E-11	2.57E-09
regulation of mitotic metaphase/anaphase transition (GO:0030071)	56	19	4.36	+	4.35	1.08E-06	6.19E-05
DNA geometric change (GO:0032392)	115	39	8.96	+	4.35	2.95E-12	4.18E-10
double-strand break repair via homologous recombination (GO:0000724)	98	33	7.64	+	4.32	1.59E-10	1.73E-08
negative regulation of metaphase/anaphase transition of cell cycle (GO:1902100)	30	10	2.34	+	4.28	4.32E-04	1.22E-02
recombinational repair (GO:0000725)	99	33	7.71	+	4.28	1.98E-10	2.14E-08
chromosome separation (GO:0051304)	30	10	2.34	+	4.28	4.32E-04	1.21E-02
reciprocal meiotic recombination (GO:0007131)	51	17	3.97	+	4.28	4.80E-06	2.41E-04
biological phase (GO:0044848)	267	89	20.8	+	4.28	8.25E-26	4.53E-23
protein localization to chromatin (GO:0071168)	24	8	1.87	+	4.28	1.61E-03	3.79E-02
mitotic cell cycle phase (GO:0098763)	267	89	20.8	+	4.28	8.25E-26	4.38E-23

microtubule polymerization (GO:0046785)	33	11	2.57	+	4.28	2.25E-04	7.14E-03
cell cycle phase (GO:0022403)	267	89	20.8	+	4.28	8.25E-26	4.23E-23
M phase (GO:0000279)	170	56	13.25	+	4.23	1.58E-16	3.58E-14
mitotic M phase (GO:0000087)	170	56	13.25	+	4.23	1.58E-16	3.53E-14
regulation of mitotic sister chromatid segregation (GO:0033047)	73	24	5.69	+	4.22	7.06E-08	5.11E-06
regulation of metaphase/anaphase transition of cell cycle (GO:1902099)	58	19	4.52	+	4.2	1.67E-06	9.08E-05
homologous recombination (GO:0035825)	52	17	4.05	+	4.2	5.94E-06	2.93E-04
regulation of G0 to G1 transition (GO:0070316)	43	14	3.35	+	4.18	4.03E-05	1.62E-03
double-strand break repair (GO:0006302)	185	60	14.42	+	4.16	2.38E-17	5.57E-15
negative regulation of sister chromatid segregation (GO:0033046)	37	12	2.88	+	4.16	1.46E-04	4.95E-03
regulation of chromosome segregation (GO:0051983)	108	35	8.42	+	4.16	1.06E-10	1.21E-08
negative regulation of DNA replication (GO:0008156)	34	11	2.65	+	4.15	2.79E-04	8.48E-03
regulation of mitotic sister chromatid separation (GO:0010965)	62	20	4.83	+	4.14	1.09E-06	6.22E-05
regulation of transcription initiation from RNA polymerase II promoter (GO:0060260)	28	9	2.18	+	4.13	1.03E-03	2.55E-02
mitotic spindle organization (GO:0007052)	75	24	5.84	+	4.11	1.07E-07	7.42E-06
protein sumoylation (GO:0016925)	66	21	5.14	+	4.08	7.13E-07	4.29E-05
regulation of sister chromatid segregation (GO:0033045)	85	27	6.62	+	4.08	1.99E-08	1.57E-06
protein localization to chromosome (GO:0034502)	63	20	4.91	+	4.07	1.34E-06	7.43E-05
centrosome cycle (GO:0007098)	82	26	6.39	+	4.07	3.74E-08	2.84E-06
nuclear chromosome segregation (GO:0098813)	215	68	16.75	+	4.06	5.23E-19	1.41E-16
positive regulation of DNA replication (GO:0045740)	38	12	2.96	+	4.05	1.80E-04	5.89E-03
negative regulation of chromosome segregation (GO:0051985)	38	12	2.96	+	4.05	1.80E-04	5.88E-03
ciliary basal body-plasma membrane docking (GO:0097711)	95	30	7.4	+	4.05	3.70E-09	3.31E-07
regulation of DNA replication (GO:0006275)	108	34	8.42	+	4.04	3.68E-10	3.80E-08
negative regulation of mitotic sister chromatid segregation (GO:0033048)	35	11	2.73	+	4.03	3.43E-04	1.00E-02
regulation of chromosome separation (GO:1905818)	67	21	5.22	+	4.02	8.73E-07	5.10E-05
negative regulation of mitotic sister chromatid separation (GO:2000816)	32	10	2.49	+	4.01	6.56E-04	1.75E-02
mitotic nuclear division (GO:0140014)	142	44	11.06	+	3.98	1.55E-12	2.26E-10

anaphase (GO:0051322)	156	48	12.16	+	3.95	1.87E-13	3.13E-11
interstrand cross-link repair (GO:0036297)	52	16	4.05	+	3.95	2.07E-05	8.91E-04
mitotic anaphase (GO:0000090)	156	48	12.16	+	3.95	1.87E-13	3.10E-11
chromosome segregation (GO:0007059)	273	84	21.27	+	3.95	1.53E-22	6.23E-20
regulation of DNA-dependent DNA replication (GO:0090329)	49	15	3.82	+	3.93	3.92E-05	1.58E-03
cell cycle G1/S phase transition (GO:0044843)	122	37	9.51	+	3.89	1.50E-10	1.66E-08
negative regulation of chromosome separation (GO:1905819)	33	10	2.57	+	3.89	8.01E-04	2.07E-02
histone ubiquitination (GO:0016574)	40	12	3.12	+	3.85	2.67E-04	8.21E-03
positive regulation of telomere maintenance (GO:0032206)	50	15	3.9	+	3.85	4.75E-05	1.89E-03
non-motile cilium assembly (GO:1905515)	47	14	3.66	+	3.82	8.99E-05	3.29E-03
G1/S transition of mitotic cell cycle (GO:0000082)	121	36	9.43	+	3.82	4.11E-10	4.22E-08
positive regulation of telomere maintenance via telomere lengthening (GO:1904358)	37	11	2.88	+	3.82	5.08E-04	1.41E-02
DNA recombination (GO:0006310)	223	66	17.38	+	3.8	2.83E-17	6.52E-15
positive regulation of telomere maintenance via telomerase (GO:0032212)	34	10	2.65	+	3.77	9.70E-04	2.44E-02
protein monoubiquitination (GO:0006513)	65	19	5.06	+	3.75	6.67E-06	3.24E-04
mitotic cell cycle phase transition (GO:0044772)	278	81	21.66	+	3.74	1.46E-20	4.95E-18
regulation of DNA-templated transcription, initiation (GO:2000142)	38	11	2.96	+	3.72	6.13E-04	1.65E-02
regulation of telomere maintenance (GO:0032204)	83	24	6.47	+	3.71	5.05E-07	3.10E-05
spindle assembly (GO:0051225)	83	24	6.47	+	3.71	5.05E-07	3.09E-05
negative regulation of mitotic nuclear division (GO:0045839)	45	13	3.51	+	3.71	2.05E-04	6.54E-03
G2/M transition of mitotic cell cycle (GO:0000086)	139	40	10.83	+	3.69	1.03E-10	1.18E-08
cell cycle phase transition (GO:0044770)	285	82	22.21	+	3.69	1.62E-20	5.37E-18
mRNA polyadenylation (GO:0006378)	35	10	2.73	+	3.67	1.17E-03	2.88E-02
somatic diversification of immunoglobulins (GO:0016445)	35	10	2.73	+	3.67	1.17E-03	2.87E-02
RNA export from nucleus (GO:0006405)	130	37	10.13	+	3.65	6.63E-10	6.63E-08
telomere organization (GO:0032200)	102	29	7.95	+	3.65	4.63E-08	3.42E-06
cell cycle G2/M phase transition (GO:0044839)	141	40	10.99	+	3.64	1.48E-10	1.64E-08
postreplication repair (GO:0006301)	53	15	4.13	+	3.63	8.28E-05	3.07E-03

microtubule cytoskeleton organization involved in mitosis (GO:1902850)	106	30	8.26	+	3.63	2.97E-08	2.30E-06
telomere maintenance (GO:0000723)	99	28	7.71	+	3.63	8.61E-08	6.17E-06
somatic diversification of immune receptors (GO:0002200)	46	13	3.58	+	3.63	2.46E-04	7.63E-03
microtubule organizing center organization (GO:0031023)	92	26	7.17	+	3.63	2.49E-07	1.63E-05
establishment of chromosome localization (GO:0051303)	71	20	5.53	+	3.62	6.13E-06	3.01E-04
spindle organization (GO:0007051)	139	39	10.83	+	3.6	3.27E-10	3.40E-08
intracellular transport of virus (GO:0075733)	54	15	4.21	+	3.56	9.88E-05	3.58E-03
establishment of mitotic spindle localization (GO:0040001)	36	10	2.81	+	3.56	1.40E-03	3.39E-02
chromosome localization (GO:0050000)	72	20	5.61	+	3.56	7.31E-06	3.53E-04
ribonucleoprotein complex export from nucleus (GO:0071426)	127	35	9.9	+	3.54	3.86E-09	3.41E-07
DNA conformation change (GO:0071103)	291	80	22.67	+	3.53	4.70E-19	1.29E-16
DNA synthesis involved in DNA repair (GO:0000731)	51	14	3.97	+	3.52	1.85E-04	6.01E-03
ribonucleoprotein complex localization (GO:0071166)	128	35	9.97	+	3.51	4.57E-09	4.00E-07
regulation of telomere maintenance via telomerase (GO:0032210)	55	15	4.29	+	3.5	1.17E-04	4.12E-03
nuclear division (GO:0000280)	279	76	21.74	+	3.5	5.63E-18	1.44E-15
negative regulation of chromosome organization (GO:2001251)	140	38	10.91	+	3.48	1.20E-09	1.17E-07
RNA polyadenylation (GO:0043631)	37	10	2.88	+	3.47	1.66E-03	3.91E-02
regulation of telomere maintenance via telomere lengthening (GO:1904356)	63	17	4.91	+	3.46	4.73E-05	1.88E-03
mitotic cell cycle process (GO:1903047)	610	164	47.53	+	3.45	3.48E-37	3.46E-34
translesion synthesis (GO:0019985)	41	11	3.19	+	3.44	1.04E-03	2.58E-02
chromosome organization involved in meiotic cell cycle (GO:0070192)	68	18	5.3	+	3.4	3.55E-05	1.46E-03
mitotic cell cycle (GO:0000278)	696	184	54.23	+	3.39	6.58E-41	8.72E-38
transport of virus (GO:0046794)	57	15	4.44	+	3.38	1.64E-04	5.49E-03
negative regulation of DNA recombination (GO:0045910)	46	12	3.58	+	3.35	7.71E-04	2.01E-02
DNA repair (GO:0006281)	516	134	40.21	+	3.33	3.52E-29	2.54E-26
negative regulation of nuclear division (GO:0051784)	54	14	4.21	+	3.33	3.06E-04	9.17E-03
meiotic chromosome segregation (GO:0045132)	89	23	6.93	+	3.32	4.34E-06	2.19E-04
protein export from nucleus (GO:0006611)	151	39	11.77	+	3.31	2.41E-09	2.25E-07
organelle fission (GO:0048285)	308	79	24	+	3.29	2.31E-17	5.56E-15

RNA phosphodiester bond hydrolysis, exonucleolytic (GO:0090503)	43	11	3.35	+	3.28	1.44E-03	3.49E-02
regulation of centrosome duplication (GO:0010824)	43	11	3.35	+	3.28	1.44E-03	3.48E-02
chromosome condensation (GO:0030261)	43	11	3.35	+	3.28	1.44E-03	3.48E-02
regulation of double-strand break repair via homologous recombination (GO:0010569)	47	12	3.66	+	3.28	9.05E-04	2.29E-02
nuclear export (GO:0051168)	161	41	12.55	+	3.27	1.34E-09	1.30E-07
mRNA export from nucleus (GO:0006406)	110	28	8.57	+	3.27	5.34E-07	3.26E-05
mRNA-containing ribonucleoprotein complex export from nucleus (GO:0071427)	110	28	8.57	+	3.27	5.34E-07	3.24E-05
positive regulation of cell cycle phase transition (GO:1901989)	95	24	7.4	+	3.24	3.78E-06	1.91E-04
RNA transport (GO:0050658)	198	50	15.43	+	3.24	2.78E-11	3.59E-09
nucleic acid transport (GO:0050657)	198	50	15.43	+	3.24	2.78E-11	3.56E-09
meiotic nuclear division (GO:0140013)	151	38	11.77	+	3.23	7.02E-09	5.84E-07
regulation of G2/M transition of mitotic cell cycle (GO:0010389)	195	49	15.19	+	3.22	5.07E-11	6.06E-09
cell division (GO:0051301)	494	124	38.49	+	3.22	6.29E-26	3.57E-23
mRNA transport (GO:0051028)	160	40	12.47	+	3.21	3.33E-09	3.01E-07
establishment of RNA localization (GO:0051236)	200	50	15.58	+	3.21	3.77E-11	4.61E-09
meiotic cell cycle process (GO:1903046)	165	41	12.86	+	3.19	2.48E-09	2.30E-07
meiotic cell cycle (GO:0051321)	219	54	17.06	+	3.16	9.80E-12	1.34E-09
regulation of cell cycle G2/M phase transition (GO:1902749)	212	52	16.52	+	3.15	2.79E-11	3.55E-09
microtubule polymerization or depolymerization (GO:0031109)	49	12	3.82	+	3.14	1.23E-03	3.02E-02
RNA localization (GO:0006403)	221	54	17.22	+	3.14	1.32E-11	1.76E-09
positive regulation of mitotic cell cycle phase transition (GO:1901992)	78	19	6.08	+	3.13	5.72E-05	2.19E-03
DNA packaging (GO:0006323)	177	43	13.79	+	3.12	1.82E-09	1.72E-07
multi-organism localization (GO:1902579)	70	17	5.45	+	3.12	1.42E-04	4.85E-03
multi-organism transport (GO:0044766)	70	17	5.45	+	3.12	1.42E-04	4.84E-03
nucleosome assembly (GO:0006334)	116	28	9.04	+	3.1	1.31E-06	7.31E-05
regulation of histone acetylation (GO:0035065)	54	13	4.21	+	3.09	8.97E-04	2.28E-02
amino acid activation (GO:0043038)	50	12	3.9	+	3.08	1.43E-03	3.46E-02
regulation of centrosome cycle (GO:0046605)	59	14	4.6	+	3.05	6.53E-04	1.75E-02

positive regulation of DNA repair (GO:0045739)	68	16	5.3	+	3.02	3.00E-04	9.03E-03
organelle localization by membrane tethering (GO:0140056)	171	40	13.32	+	3	1.64E-08	1.30E-06
regulation of DNA repair (GO:0006282)	124	29	9.66	+	3	1.48E-06	8.17E-05
regulation of peptidyl-lysine acetylation (GO:2000756)	60	14	4.68	+	2.99	7.53E-04	1.97E-02
chromatin remodeling (GO:0006338)	163	38	12.7	+	2.99	4.00E-08	3.02E-06
meiosis I cell cycle process (GO:0061982)	112	26	8.73	+	2.98	5.68E-06	2.82E-04
regulation of alternative mRNA splicing, via spliceosome (GO:0000381)	69	16	5.38	+	2.98	3.46E-04	1.01E-02
regulation of mRNA processing (GO:0050684)	143	33	11.14	+	2.96	3.73E-07	2.38E-05
regulation of chromosome organization (GO:0033044)	358	82	27.9	+	2.94	1.48E-15	3.06E-13
RNA splicing, via transesterification reactions (GO:0000375)	298	68	23.22	+	2.93	4.65E-13	7.46E-11
regulation of double-strand break repair (GO:2000779)	79	18	6.16	+	2.92	1.83E-04	5.94E-03
positive regulation of mRNA metabolic process (GO:1903313)	79	18	6.16	+	2.92	1.83E-04	5.93E-03
DNA metabolic process (GO:0006259)	752	171	58.6	+	2.92	5.63E-31	4.48E-28
chromatin assembly or disassembly (GO:0006333)	154	35	12	+	2.92	2.29E-07	1.51E-05
mRNA 3'-end processing (GO:0031124)	88	20	6.86	+	2.92	8.46E-05	3.13E-03
meiosis I (GO:0007127)	110	25	8.57	+	2.92	1.17E-05	5.30E-04
mRNA splicing, via spliceosome (GO:0000398)	295	67	22.99	+	2.91	8.31E-13	1.26E-10
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile (GO:0000377)	295	67	22.99	+	2.91	8.31E-13	1.25E-10
protein K48-linked ubiquitination (GO:0070936)	53	12	4.13	+	2.91	2.18E-03	4.94E-02
regulation of ubiquitin-protein transferase activity (GO:0051438)	53	12	4.13	+	2.91	2.18E-03	4.93E-02
chromatin assembly (GO:0031497)	133	30	10.36	+	2.89	1.86E-06	1.01E-04
regulation of posttranscriptional gene silencing (GO:0060147)	89	20	6.93	+	2.88	9.67E-05	3.52E-03
regulation of gene silencing by RNA (GO:0060966)	89	20	6.93	+	2.88	9.67E-05	3.51E-03
cell cycle checkpoint (GO:0000075)	201	45	15.66	+	2.87	6.77E-09	5.69E-07
positive regulation of chromosome organization (GO:2001252)	179	40	13.95	+	2.87	4.79E-08	3.53E-06
cell cycle process (GO:0022402)	986	220	76.83	+	2.86	4.02E-39	4.92E-36
DNA integrity checkpoint (GO:0031570)	148	33	11.53	+	2.86	7.26E-07	4.35E-05
nucleosome organization (GO:0034728)	157	35	12.23	+	2.86	3.40E-07	2.18E-05

cellular response to leukemia inhibitory factor (GO:1990830)	99	22	7.71	+	2.85	5.11E-05	2.01E-03
response to leukemia inhibitory factor (GO:1990823)	99	22	7.71	+	2.85	5.11E-05	2.00E-03
regulation of protein acetylation (GO:1901983)	72	16	5.61	+	2.85	5.18E-04	1.43E-02
cellular response to DNA damage stimulus (GO:0006974)	783	174	61.01	+	2.85	1.17E-30	8.85E-28
regulation of mitotic nuclear division (GO:0007088)	171	38	13.32	+	2.85	1.16E-07	7.98E-06
membrane docking (GO:0022406)	180	40	14.03	+	2.85	5.46E-08	3.98E-06
nucleocytoplasmic transport (GO:0006913)	266	59	20.73	+	2.85	6.48E-11	7.64E-09
protein-DNA complex assembly (GO:0065004)	208	46	16.21	+	2.84	6.40E-09	5.41E-07
regulation of mRNA splicing, via spliceosome (GO:0048024)	104	23	8.1	+	2.84	3.72E-05	1.52E-03
regulation of DNA recombination (GO:0000018)	104	23	8.1	+	2.84	3.72E-05	1.51E-03
regulation of cyclin-dependent protein kinase activity (GO:1904029)	104	23	8.1	+	2.84	3.72E-05	1.51E-03
regulation of gene silencing by miRNA (GO:0060964)	86	19	6.7	+	2.84	1.73E-04	5.73E-03
mitotic interphase (GO:0051329)	77	17	6	+	2.83	3.75E-04	1.08E-02
interphase (GO:0051325)	77	17	6	+	2.83	3.75E-04	1.07E-02
positive regulation of DNA biosynthetic process (GO:2000573)	68	15	5.3	+	2.83	8.17E-04	2.11E-02
positive regulation of DNA metabolic process (GO:0051054)	195	43	15.19	+	2.83	2.12E-08	1.66E-06
import into nucleus (GO:0051170)	118	26	9.19	+	2.83	1.26E-05	5.68E-04
nuclear transport (GO:0051169)	269	59	20.96	+	2.81	8.07E-11	9.30E-09
mitotic cell cycle checkpoint (GO:0007093)	160	35	12.47	+	2.81	4.99E-07	3.08E-05
negative regulation of gene expression, epigenetic (GO:0045814)	78	17	6.08	+	2.8	4.26E-04	1.20E-02
cell cycle (GO:0007049)	1328	289	103.48	+	2.79	3.76E-50	1.19E-46
nucleobase-containing compound transport (GO:0015931)	244	53	19.01	+	2.79	9.77E-10	9.53E-08
chromosome organization (GO:0051276)	1055	229	82.21	+	2.79	4.25E-39	4.82E-36
DNA-templated transcription, termination (GO:0006353)	79	17	6.16	+	2.76	4.84E-04	1.35E-02
nucleus organization (GO:0006997)	131	28	10.21	+	2.74	1.60E-05	7.16E-04
regulation of mitotic cell cycle phase transition (GO:1901990)	418	89	32.57	+	2.73	4.65E-15	9.02E-13
regulation of gene silencing (GO:0060968)	127	27	9.9	+	2.73	2.43E-05	1.03E-03
protein polymerization (GO:0051258)	80	17	6.23	+	2.73	5.48E-04	1.50E-02
protein-DNA complex subunit organization (GO:0071824)	250	53	19.48	+	2.72	1.78E-09	1.70E-07

regulation of cell cycle phase transition (GO:1901987)	453	96	35.3	+	2.72	4.34E-16	9.20E-14
regulation of cyclin-dependent protein serine/threonine kinase activity (GO:0000079)	100	21	7.79	+	2.7	1.49E-04	5.04E-03
nucleotide-excision repair (GO:0006289)	110	23	8.57	+	2.68	1.23E-04	4.30E-03
regulation of DNA metabolic process (GO:0051052)	354	74	27.58	+	2.68	2.09E-12	3.00E-10
positive regulation of response to DNA damage stimulus (GO:2001022)	106	22	8.26	+	2.66	1.87E-04	6.06E-03
protein-containing complex localization (GO:0031503)	261	54	20.34	+	2.66	3.65E-09	3.28E-07
signal transduction involved in DNA damage checkpoint (GO:0072422)	73	15	5.69	+	2.64	1.52E-03	3.63E-02
signal transduction involved in DNA integrity checkpoint (GO:0072401)	73	15	5.69	+	2.64	1.52E-03	3.62E-02
regulation of cytokinesis (GO:0032465)	88	18	6.86	+	2.63	9.10E-04	2.30E-02
response to UV (GO:0009411)	142	29	11.06	+	2.62	1.69E-05	7.50E-04
signal transduction involved in cell cycle checkpoint (GO:0072395)	74	15	5.77	+	2.6	1.71E-03	3.98E-02
regulation of response to DNA damage stimulus (GO:2001020)	222	45	17.3	+	2.6	1.25E-07	8.49E-06
regulation of cellular response to heat (GO:1900034)	79	16	6.16	+	2.6	2.02E-03	4.58E-02
RNA splicing (GO:0008380)	396	80	30.86	+	2.59	1.20E-12	1.78E-10
blastocyst development (GO:0001824)	104	21	8.1	+	2.59	3.21E-04	9.57E-03
signal transduction in response to DNA damage (GO:0042770)	104	21	8.1	+	2.59	3.21E-04	9.56E-03
response to ionizing radiation (GO:0010212)	144	29	11.22	+	2.58	2.02E-05	8.74E-04
regulation of gene expression, epigenetic (GO:0040029)	231	46	18	+	2.56	1.17E-07	7.99E-06
regulation of nuclear division (GO:0051783)	196	39	15.27	+	2.55	1.31E-06	7.30E-05
mitotic DNA integrity checkpoint (GO:0044774)	106	21	8.26	+	2.54	3.69E-04	1.06E-02
positive regulation of mitotic cell cycle (GO:0045931)	152	30	11.84	+	2.53	1.94E-05	8.41E-04
negative regulation of translation (GO:0017148)	132	26	10.29	+	2.53	7.29E-05	2.76E-03
mRNA processing (GO:0006397)	474	93	36.93	+	2.52	1.01E-13	1.71E-11
tRNA metabolic process (GO:0006399)	189	37	14.73	+	2.51	3.15E-06	1.62E-04
rRNA processing (GO:0006364)	210	41	16.36	+	2.51	8.71E-07	5.11E-05
rRNA metabolic process (GO:0016072)	221	43	17.22	+	2.5	4.88E-07	3.03E-05
cilium assembly (GO:0060271)	340	66	26.49	+	2.49	7.03E-10	6.98E-08
protein import into nucleus (GO:0006606)	98	19	7.64	+	2.49	8.64E-04	2.21E-02

negative regulation of DNA metabolic process (GO:0051053)	124	24	9.66	+	2.48	1.71E-04	5.70E-03
ribonucleoprotein complex biogenesis (GO:0022613)	424	82	33.04	+	2.48	6.38E-12	8.83E-10
regulation of mitotic cell cycle (GO:0007346)	638	123	49.71	+	2.47	2.04E-17	5.00E-15
regulation of cell cycle process (GO:0010564)	763	147	59.45	+	2.47	1.06E-20	3.65E-18
cilium organization (GO:0044782)	359	69	27.97	+	2.47	3.11E-10	3.27E-08
DNA damage checkpoint (GO:0000077)	136	26	10.6	+	2.45	1.06E-04	3.80E-03
cytokinesis (GO:0000910)	110	21	8.57	+	2.45	5.12E-04	1.42E-02
regulation of DNA biosynthetic process (GO:2000278)	111	21	8.65	+	2.43	5.58E-04	1.52E-02
microtubule cytoskeleton organization (GO:0000226)	467	87	36.39	+	2.39	5.69E-12	7.93E-10
ribosome biogenesis (GO:0042254)	296	55	23.06	+	2.38	5.63E-08	4.09E-06
regulation of RNA splicing (GO:0043484)	141	26	10.99	+	2.37	2.17E-04	6.88E-03
negative regulation of cell cycle process (GO:0010948)	327	60	25.48	+	2.35	2.43E-08	1.89E-06
positive regulation of cell cycle process (GO:0090068)	289	53	22.52	+	2.35	1.34E-07	9.05E-06
nucleic acid phosphodiester bond hydrolysis (GO:0090305)	302	55	23.53	+	2.34	8.78E-08	6.26E-06
RNA processing (GO:0006396)	868	158	67.63	+	2.34	5.25E-20	1.55E-17
regulation of mRNA metabolic process (GO:1903311)	330	60	25.71	+	2.33	2.84E-08	2.20E-06
gene silencing (GO:0016458)	149	27	11.61	+	2.33	1.90E-04	6.11E-03
DNA biosynthetic process (GO:0071897)	111	20	8.65	+	2.31	1.75E-03	4.07E-02
protein import (GO:0017038)	156	28	12.16	+	2.3	1.59E-04	5.33E-03
nucleic acid metabolic process (GO:0090304)	2289	407	178.36	+	2.28	1.08E-51	8.59E-48
regulation of chromatin organization (GO:1902275)	197	35	15.35	+	2.28	3.21E-05	1.33E-03
regulation of histone modification (GO:0031056)	152	27	11.84	+	2.28	2.38E-04	7.43E-03
RNA 3'-end processing (GO:0031123)	142	25	11.06	+	2.26	4.59E-04	1.29E-02
chromatin organization (GO:0006325)	695	122	54.15	+	2.25	1.16E-14	2.10E-12
negative regulation of cellular amide metabolic process (GO:0034249)	149	26	11.61	+	2.24	5.63E-04	1.53E-02
RNA phosphodiester bond hydrolysis (GO:0090501)	162	28	12.62	+	2.22	3.26E-04	9.64E-03
regulation of cell cycle (GO:0051726)	1206	208	93.97	+	2.21	8.45E-24	3.73E-21
microtubule-based process (GO:0007017)	654	112	50.96	+	2.2	7.97E-13	1.22E-10
ribonucleoprotein complex assembly (GO:0022618)	187	32	14.57	+	2.2	1.78E-04	5.87E-03

ncRNA metabolic process (GO:0034660)	470	80	36.62	+	2.18	1.78E-09	1.70E-07
ribonucleoprotein complex subunit organization (GO:0071826)	194	33	15.12	+	2.18	1.37E-04	4.69E-03
organelle localization (GO:0051640)	584	99	45.51	+	2.18	2.96E-11	3.74E-09
positive regulation of cell cycle (GO:0045787)	386	65	30.08	+	2.16	9.85E-08	6.93E-06
negative regulation of organelle organization (GO:0010639)	386	65	30.08	+	2.16	9.85E-08	6.90E-06
regulation of microtubule cytoskeleton organization (GO:0070507)	196	33	15.27	+	2.16	1.50E-04	5.06E-03
regulation of signal transduction by p53 class mediator (GO:1901796)	185	31	14.42	+	2.15	2.77E-04	8.45E-03
regulation of RNA stability (GO:0043487)	185	31	14.42	+	2.15	2.77E-04	8.43E-03
regulation of mRNA stability (GO:0043488)	180	30	14.03	+	2.14	3.85E-04	1.10E-02
nucleobase-containing compound metabolic process (GO:0006139)	2804	465	218.49	+	2.13	1.57E-52	2.50E-48
establishment of organelle localization (GO:0051656)	368	61	28.67	+	2.13	3.92E-07	2.47E-05
regulation of ubiquitin-dependent protein catabolic process (GO:2000058)	157	26	12.23	+	2.13	8.39E-04	2.16E-02
posttranscriptional regulation of gene expression (GO:0010608)	529	87	41.22	+	2.11	1.80E-09	1.70E-07
regulation of translation (GO:0006417)	359	59	27.97	+	2.11	7.77E-07	4.63E-05
regulation of mRNA catabolic process (GO:0061013)	202	33	15.74	+	2.1	3.07E-04	9.20E-03
protein localization to nucleus (GO:0034504)	167	27	13.01	+	2.07	1.11E-03	2.75E-02
negative regulation of cell cycle phase transition (GO:1901988)	236	38	18.39	+	2.07	1.34E-04	4.60E-03
regulation of protein ubiquitination (GO:0031396)	205	33	15.97	+	2.07	3.38E-04	9.92E-03
heterocycle metabolic process (GO:0046483)	2983	479	232.43	+	2.06	1.00E-50	5.32E-47
mRNA metabolic process (GO:0016071)	693	111	54	+	2.06	3.67E-11	4.52E-09
plasma membrane bounded cell projection assembly (GO:0120031)	432	69	33.66	+	2.05	2.78E-07	1.81E-05
peptidyl-lysine modification (GO:0018205)	314	50	24.47	+	2.04	1.34E-05	6.01E-04
negative regulation of mitotic cell cycle phase transition (GO:1901991)	220	35	17.14	+	2.04	2.27E-04	7.19E-03
protein folding (GO:0006457)	220	35	17.14	+	2.04	2.27E-04	7.17E-03
regulation of organelle assembly (GO:1902115)	196	31	15.27	+	2.03	6.64E-04	1.76E-02
RNA metabolic process (GO:0016070)	1685	266	131.29	+	2.03	2.40E-25	1.19E-22
negative regulation of cell cycle (GO:0045786)	577	91	44.96	+	2.02	4.99E-09	4.31E-07
cellular aromatic compound metabolic process (GO:0006725)	3027	477	235.86	+	2.02	2.64E-48	7.00E-45
organelle assembly (GO:0070925)	725	114	56.49	+	2.02	5.88E-11	6.98E-09

ncRNA processing (GO:0034470)	382	60	29.77	+	2.02	2.53E-06	1.34E-04
negative regulation of mitotic cell cycle (GO:0045930)	312	49	24.31	+	2.02	2.05E-05	8.83E-04
protein modification by small protein conjugation or removal (GO:0070647)	987	155	76.91	+	2.02	1.15E-14	2.11E-12
covalent chromatin modification (GO:0016569)	376	59	29.3	+	2.01	3.35E-06	1.71E-04
histone modification (GO:0016570)	365	57	28.44	+	2	6.06E-06	2.98E-04
protein modification by small protein removal (GO:0070646)	295	46	22.99	+	2	4.83E-05	1.91E-03
protein deubiquitination (GO:0016579)	278	43	21.66	+	1.99	8.05E-05	3.00E-03
cell projection assembly (GO:0030031)	447	69	34.83	+	1.98	6.97E-07	4.21E-05
cellular protein-containing complex assembly (GO:0034622)	793	122	61.79	+	1.97	4.02E-11	4.84E-09
gene expression (GO:0010467)	2016	309	157.09	+	1.97	1.20E-27	7.60E-25
cellular nitrogen compound metabolic process (GO:0034641)	3454	527	269.13	+	1.96	1.43E-50	5.68E-47
regulation of microtubule-based process (GO:0032886)	230	35	17.92	+	1.95	4.85E-04	1.35E-02
protein modification by small protein conjugation (GO:0032446)	777	118	60.54	+	1.95	1.56E-10	1.72E-08
regulation of protein modification by small protein conjugation or removal (GO:1903320)	232	35	18.08	+	1.94	7.37E-04	1.94E-02
regulation of cellular amide metabolic process (GO:0034248)	412	62	32.1	+	1.93	5.74E-06	2.84E-04
translation (GO:0006412)	393	59	30.62	+	1.93	1.27E-05	5.69E-04
cellular macromolecule biosynthetic process (GO:0034645)	1715	257	133.63	+	1.92	1.39E-21	5.24E-19
organic cyclic compound metabolic process (GO:1901360)	3261	485	254.1	+	1.91	5.78E-43	1.02E-39
regulation of proteolysis involved in cellular protein catabolic process (GO:1903050)	220	32	17.14	+	1.87	1.96E-03	4.50E-02
macromolecule biosynthetic process (GO:0009059)	1774	258	138.23	+	1.87	4.49E-20	1.37E-17
cellular response to stress (GO:0033554)	1729	251	134.72	+	1.86	2.13E-19	5.93E-17
RNA biosynthetic process (GO:0032774)	665	96	51.82	+	1.85	1.01E-07	7.03E-06
peptide biosynthetic process (GO:0043043)	419	60	32.65	+	1.84	3.39E-05	1.40E-03
ubiquitin-dependent protein catabolic process (GO:0006511)	528	74	41.14	+	1.8	6.74E-06	3.27E-04
nucleobase-containing compound biosynthetic process (GO:0034654)	1103	154	85.95	+	1.79	7.79E-11	9.04E-09
modification-dependent macromolecule catabolic process (GO:0043632)	545	76	42.47	+	1.79	6.27E-06	3.07E-04
heterocycle biosynthetic process (GO:0018130)	1174	163	91.48	+	1.78	2.18E-11	2.86E-09
modification-dependent protein catabolic process (GO:0019941)	534	74	41.61	+	1.78	1.06E-05	4.87E-04

organelle organization (GO:0006996)	3418	473	266.33	+	1.78	2.53E-34	2.24E-31
establishment of localization in cell (GO:0051649)	394	54	30.7	+	1.76	2.06E-04	6.58E-03
cellular nitrogen compound biosynthetic process (GO:0044271)	1658	226	129.19	+	1.75	1.04E-14	1.95E-12
transcription, DNA-templated (GO:0006351)	653	89	50.88	+	1.75	2.33E-06	1.25E-04
nucleic acid-templated transcription (GO:0097659)	654	89	50.96	+	1.75	2.39E-06	1.27E-04
proteasome-mediated ubiquitin-dependent protein catabolic process (GO:0043161)	324	44	25.25	+	1.74	1.05E-03	2.61E-02
proteasomal protein catabolic process (GO:0010498)	347	47	27.04	+	1.74	7.62E-04	1.99E-02
aromatic compound biosynthetic process (GO:0019438)	1182	160	92.1	+	1.74	2.10E-10	2.24E-08
viral process (GO:0016032)	702	95	54.7	+	1.74	1.31E-06	7.31E-05
proteolysis involved in cellular protein catabolic process (GO:0051603)	593	80	46.21	+	1.73	1.03E-05	4.73E-04
protein localization to organelle (GO:0033365)	757	102	58.99	+	1.73	7.70E-07	4.60E-05
amide biosynthetic process (GO:0043604)	542	73	42.23	+	1.73	2.58E-05	1.09E-03
transcription by RNA polymerase II (GO:0006366)	495	66	38.57	+	1.71	9.28E-05	3.39E-03
protein ubiquitination (GO:0016567)	698	93	54.39	+	1.71	3.36E-06	1.71E-04
protein-containing complex subunit organization (GO:0043933)	1707	227	133.01	+	1.71	9.27E-14	1.58E-11
cellular macromolecule catabolic process (GO:0044265)	910	121	70.91	+	1.71	9.87E-08	6.88E-06
regulation of organelle organization (GO:0033043)	1298	172	101.14	+	1.7	2.16E-10	2.29E-08
protein-containing complex assembly (GO:0065003)	1466	194	114.23	+	1.7	1.09E-11	1.49E-09
cellular protein catabolic process (GO:0044257)	623	81	48.54	+	1.67	3.11E-05	1.30E-03
cellular component biogenesis (GO:0044085)	2699	350	210.31	+	1.66	4.97E-20	1.49E-17
response to radiation (GO:0009314)	435	56	33.9	+	1.65	7.48E-04	1.96E-02
cellular macromolecule metabolic process (GO:0044260)	5083	654	396.07	+	1.65	2.07E-41	2.99E-38
organic cyclic compound biosynthetic process (GO:1901362)	1325	168	103.24	+	1.63	5.71E-09	4.85E-07
symbiotic process (GO:0044403)	783	99	61.01	+	1.62	1.09E-05	5.01E-04
regulation of catabolic process (GO:0009894)	999	126	77.84	+	1.62	8.17E-07	4.83E-05
cellular biosynthetic process (GO:0044249)	2773	349	216.07	+	1.62	4.85E-18	1.26E-15
positive regulation of organelle organization (GO:0010638)	628	79	48.93	+	1.61	1.14E-04	4.02E-03
protein catabolic process (GO:0030163)	701	88	54.62	+	1.61	4.54E-05	1.81E-03

negative regulation of nucleobase-containing compound metabolic process (GO:0045934)	1456	182	113.45	+	1.6	3.10E-09	2.84E-07
macromolecule metabolic process (GO:0043170)	6193	771	482.56	+	1.6	5.94E-47	1.35E-43
negative regulation of cellular macromolecule biosynthetic process (GO:2000113)	1399	174	109.01	+	1.6	9.96E-09	8.00E-07
negative regulation of macromolecule biosynthetic process (GO:0010558)	1481	184	115.4	+	1.59	4.03E-09	3.54E-07
macromolecule catabolic process (GO:0009057)	1066	132	83.06	+	1.59	9.72E-07	5.66E-05
regulation of nucleobase-containing compound metabolic process (GO:0019219)	4040	497	314.8	+	1.58	5.15E-25	2.48E-22
cellular component assembly (GO:0022607)	2473	304	192.7	+	1.58	2.29E-14	4.04E-12
negative regulation of cellular component organization (GO:0051129)	711	87	55.4	+	1.57	1.20E-04	4.19E-03
negative regulation of cellular biosynthetic process (GO:0031327)	1539	188	119.92	+	1.57	9.75E-09	7.87E-07
organic substance biosynthetic process (GO:1901576)	2878	351	224.25	+	1.57	3.37E-16	7.25E-14
cellular localization (GO:0051641)	2396	290	186.7	+	1.55	6.84E-13	1.07E-10
regulation of cellular catabolic process (GO:0031329)	836	101	65.14	+	1.55	5.95E-05	2.28E-03
regulation of cellular macromolecule biosynthetic process (GO:2000112)	3908	472	304.51	+	1.55	6.99E-22	2.78E-19
negative regulation of biosynthetic process (GO:0009890)	1566	189	122.02	+	1.55	1.70E-08	1.35E-06
intracellular protein transport (GO:0006886)	1003	121	78.15	+	1.55	8.68E-06	4.13E-04
biosynthetic process (GO:0009058)	2954	356	230.17	+	1.55	9.24E-16	1.93E-13
peptide metabolic process (GO:0006518)	542	65	42.23	+	1.54	1.42E-03	3.45E-02
negative regulation of RNA metabolic process (GO:0051253)	1354	162	105.5	+	1.54	3.37E-07	2.17E-05
peptidyl-amino acid modification (GO:0018193)	879	105	68.49	+	1.53	5.28E-05	2.05E-03
nitrogen compound metabolic process (GO:0006807)	7023	835	547.23	+	1.53	8.21E-45	1.63E-41
regulation of macromolecule biosynthetic process (GO:0010556)	4046	481	315.26	+	1.53	4.27E-21	1.54E-18
cellular macromolecule localization (GO:0070727)	1626	193	126.7	+	1.52	3.80E-08	2.87E-06
cellular protein localization (GO:0034613)	1618	192	126.07	+	1.52	4.59E-08	3.41E-06
negative regulation of gene expression (GO:0010629)	1738	206	135.42	+	1.52	1.51E-08	1.21E-06
positive regulation of nucleobase-containing compound metabolic process (GO:0045935)	1897	223	147.81	+	1.51	5.29E-09	4.53E-07
regulation of cellular biosynthetic process (GO:0031326)	4185	490	326.09	+	1.5	2.39E-20	7.44E-18
interspecies interaction between organisms (GO:0044419)	855	100	66.62	+	1.5	2.00E-04	6.40E-03

regulation of biosynthetic process (GO:0009889)	4264	498	332.25	+	1.5	1.72E-20	5.48E-18
regulation of gene expression (GO:0010468)	4463	519	347.76	+	1.49	3.75E-21	1.39E-18
regulation of RNA metabolic process (GO:0051252)	3785	440	294.93	+	1.49	2.33E-17	5.54E-15
intracellular transport (GO:0046907)	1529	176	119.14	+	1.48	1.06E-06	6.08E-05
negative regulation of nucleic acid-templated transcription (GO:1903507)	1261	145	98.26	+	1.48	1.21E-05	5.46E-04
negative regulation of RNA biosynthetic process (GO:1902679)	1263	145	98.41	+	1.47	1.23E-05	5.56E-04
cellular metabolic process (GO:0044237)	7671	878	597.72	+	1.47	9.65E-42	1.53E-38
primary metabolic process (GO:0044238)	7501	853	584.47	+	1.46	1.18E-38	1.25E-35
regulation of cellular response to stress (GO:0080135)	730	83	56.88	+	1.46	1.45E-03	3.50E-02
cellular protein metabolic process (GO:0044267)	3713	422	289.32	+	1.46	4.52E-15	8.88E-13
negative regulation of transcription by RNA polymerase II (GO:0000122)	880	100	68.57	+	1.46	4.99E-04	1.39E-02
macromolecule modification (GO:0043412)	3304	375	257.45	+	1.46	3.73E-13	6.11E-11
cellular component organization or biogenesis (GO:0071840)	5830	661	454.27	+	1.46	4.55E-26	2.78E-23
cytoskeleton organization (GO:0007010)	1095	124	85.32	+	1.45	1.05E-04	3.77E-03
regulation of macromolecule metabolic process (GO:0060255)	6160	697	479.98	+	1.45	8.23E-28	5.45E-25
negative regulation of transcription, DNA-templated (GO:0045892)	1208	136	94.13	+	1.44	5.57E-05	2.15E-03
negative regulation of macromolecule metabolic process (GO:0010605)	2666	299	207.73	+	1.44	7.90E-10	7.76E-08
positive regulation of macromolecule biosynthetic process (GO:0010557)	1919	215	149.53	+	1.44	3.25E-07	2.10E-05
cellular protein modification process (GO:0006464)	3084	345	240.3	+	1.44	2.98E-11	3.73E-09
protein modification process (GO:0036211)	3084	345	240.3	+	1.44	2.98E-11	3.70E-09
regulation of cellular metabolic process (GO:0031323)	6216	694	484.35	+	1.43	5.72E-26	3.37E-23
regulation of RNA biosynthetic process (GO:2001141)	3539	395	275.76	+	1.43	5.80E-13	9.23E-11
regulation of nucleic acid-templated transcription (GO:1903506)	3534	394	275.37	+	1.43	7.24E-13	1.12E-10
regulation of transcription, DNA-templated (GO:0006355)	3467	386	270.15	+	1.43	1.68E-12	2.43E-10
regulation of metabolic process (GO:0019222)	6690	744	521.28	+	1.43	2.93E-28	2.03E-25
regulation of nitrogen compound metabolic process (GO:0051171)	5839	648	454.97	+	1.42	5.55E-23	2.32E-20
organic substance metabolic process (GO:0071704)	7874	872	613.54	+	1.42	1.39E-35	1.30E-32
cellular component organization (GO:0016043)	5642	622	439.62	+	1.41	4.32E-21	1.53E-18
regulation of primary metabolic process (GO:0080090)	6023	664	469.31	+	1.41	4.47E-23	1.92E-20

positive regulation of cellular biosynthetic process (GO:0031328)	2007	220	156.38	+	1.41	1.05E-06	6.07E-05
positive regulation of RNA metabolic process (GO:0051254)	1736	190	135.27	+	1.4	7.68E-06	3.70E-04
regulation of transcription by RNA polymerase II (GO:0006357)	2294	251	178.75	+	1.4	1.83E-07	1.21E-05
negative regulation of nitrogen compound metabolic process (GO:0051172)	2369	259	184.59	+	1.4	1.14E-07	7.82E-06
plasma membrane bounded cell projection organization (GO:0120036)	1114	121	86.8	+	1.39	5.54E-04	1.51E-02
positive regulation of biosynthetic process (GO:0009891)	2039	220	158.88	+	1.38	2.92E-06	1.52E-04
cell projection organization (GO:0030030)	1159	125	90.31	+	1.38	5.70E-04	1.54E-02
negative regulation of cellular metabolic process (GO:0031324)	2567	276	200.02	+	1.38	1.58E-07	1.05E-05
protein localization (GO:0008104)	2178	234	169.71	+	1.38	1.99E-06	1.07E-04
metabolic process (GO:0008152)	8428	899	656.71	+	1.37	4.34E-31	3.63E-28
negative regulation of metabolic process (GO:0009892)	2918	311	227.37	+	1.37	4.20E-08	3.13E-06
protein transport (GO:0015031)	1528	162	119.06	+	1.36	1.99E-04	6.39E-03
positive regulation of RNA biosynthetic process (GO:1902680)	1652	175	128.72	+	1.36	1.08E-04	3.84E-03
organonitrogen compound biosynthetic process (GO:1901566)	1416	150	110.33	+	1.36	3.23E-04	9.60E-03
positive regulation of nucleic acid-templated transcription (GO:1903508)	1651	174	128.65	+	1.35	1.30E-04	4.52E-03
positive regulation of transcription, DNA-templated (GO:0045893)	1553	163	121.01	+	1.35	2.69E-04	8.26E-03
reproduction (GO:0000003)	1426	149	111.11	+	1.34	5.94E-04	1.61E-02
establishment of protein localization (GO:0045184)	1621	169	126.31	+	1.34	2.92E-04	8.85E-03
reproductive process (GO:0022414)	1423	148	110.88	+	1.33	8.37E-04	2.16E-02
peptide transport (GO:0015833)	1558	162	121.4	+	1.33	4.01E-04	1.13E-02
protein metabolic process (GO:0019538)	4315	445	336.22	+	1.32	7.44E-10	7.35E-08
amide transport (GO:0042886)	1592	163	124.05	+	1.31	7.69E-04	2.00E-02
regulation of cellular component organization (GO:0051128)	2491	253	194.1	+	1.3	3.00E-05	1.26E-03
positive regulation of gene expression (GO:0010628)	2011	204	156.7	+	1.3	2.36E-04	7.38E-03
macromolecule localization (GO:0033036)	2517	253	196.12	+	1.29	6.31E-05	2.40E-03
positive regulation of cellular metabolic process (GO:0031325)	3344	335	260.56	+	1.29	3.03E-06	1.56E-04
positive regulation of macromolecule metabolic process (GO:0010604)	3375	338	262.98	+	1.29	2.78E-06	1.46E-04
positive regulation of nitrogen compound metabolic process (GO:0051173)	3203	319	249.58	+	1.28	9.31E-06	4.38E-04
positive regulation of metabolic process (GO:0009893)	3650	361	284.41	+	1.27	3.39E-06	1.72E-04

organonitrogen compound metabolic process (GO:1901564)	5331	524	415.39	+	1.26	7.32E-09	6.07E-07
negative regulation of cellular process (GO:0048523)	4754	445	370.43	+	1.2	3.29E-05	1.36E-03
negative regulation of biological process (GO:0048519)	5333	489	415.55	+	1.18	8.23E-05	3.06E-03
regulation of cellular process (GO:0050794)	10981	990	855.64	+	1.16	1.42E-10	1.59E-08
positive regulation of cellular process (GO:0048522)	5465	490	425.83	+	1.15	6.44E-04	1.72E-02
cellular process (GO:0009987)	14575	1298	1135.68	+	1.14	3.35E-18	8.87E-16
regulation of biological process (GO:0050789)	11688	1030	910.72	+	1.13	8.92E-09	7.27E-07
biological regulation (GO:0065007)	12387	1083	965.19	+	1.12	8.43E-09	6.91E-07
biological_process (GO:0008150)	17840	1515	1390.09	+	1.09	8.35E-20	2.37E-17
response to stimulus (GO:0050896)	8495	585	661.93	-	0.88	1.80E-04	5.90E-03
system development (GO:0048731)	4424	293	344.72	-	0.85	2.17E-03	4.93E-02
regulation of localization (GO:0032879)	2790	173	217.4	-	0.8	1.57E-03	3.72E-02
response to chemical (GO:0042221)	4485	278	349.47	-	0.8	2.10E-05	9.00E-04
multicellular organismal process (GO:0032501)	6973	428	543.33	-	0.79	2.76E-09	2.53E-07
positive regulation of signaling (GO:0023056)	1851	108	144.23	-	0.75	1.88E-03	4.33E-02
positive regulation of cell communication (GO:0010647)	1842	107	143.53	-	0.75	1.57E-03	3.72E-02
anatomical structure morphogenesis (GO:0009653)	2153	125	167.76	-	0.75	5.41E-04	1.49E-02
vesicle-mediated transport (GO:0016192)	1954	113	152.25	-	0.74	9.60E-04	2.42E-02
regulation of multicellular organismal process (GO:0051239)	3147	181	245.21	-	0.74	9.50E-06	4.44E-04
positive regulation of signal transduction (GO:0009967)	1691	97	131.76	-	0.74	1.67E-03	3.93E-02
positive regulation of multicellular organismal process (GO:0051240)	1762	100	137.29	-	0.73	9.02E-04	2.28E-02
positive regulation of response to stimulus (GO:0048584)	2461	137	191.76	-	0.71	2.72E-05	1.14E-03
regulation of transport (GO:0051049)	1877	102	146.26	-	0.7	1.31E-04	4.52E-03
signal transduction (GO:0007165)	5061	261	394.35	-	0.66	7.65E-15	1.45E-12
regulation of cellular component movement (GO:0051270)	1009	52	78.62	-	0.66	1.93E-03	4.43E-02
cell communication (GO:0007154)	5500	283	428.56	-	0.66	1.73E-16	3.82E-14
transmembrane transport (GO:0055085)	1296	66	100.98	-	0.65	2.89E-04	8.77E-03
immune system process (GO:0002376)	2806	141	218.64	-	0.64	9.60E-09	7.79E-07
signaling (GO:0023052)	5393	270	420.22	-	0.64	1.03E-17	2.61E-15

negative regulation of multicellular organismal process (GO:0051241)	1207	60	94.05	-	0.64	2.72E-04	8.35E-03
cell-cell signaling (GO:0007267)	1111	55	86.57	-	0.64	3.89E-04	1.11E-02
tissue development (GO:0009888)	1762	87	137.29	-	0.63	5.14E-06	2.58E-04
ion transport (GO:0006811)	1341	66	104.49	-	0.63	7.97E-05	2.98E-03
regulation of response to external stimulus (GO:0032101)	1091	53	85.01	-	0.62	2.74E-04	8.36E-03
animal organ morphogenesis (GO:0009887)	972	47	75.74	-	0.62	6.37E-04	1.71E-02
response to external stimulus (GO:0009605)	2443	116	190.36	-	0.61	3.75E-09	3.33E-07
cell surface receptor signaling pathway (GO:0007166)	2417	114	188.33	-	0.61	3.14E-09	2.85E-07
epithelium development (GO:0060429)	1103	52	85.95	-	0.61	1.14E-04	4.02E-03
locomotion (GO:0040011)	1304	61	101.61	-	0.6	1.94E-05	8.41E-04
localization of cell (GO:0051674)	1061	49	82.67	-	0.59	1.06E-04	3.80E-03
cell motility (GO:0048870)	1061	49	82.67	-	0.59	1.06E-04	3.79E-03
ion transmembrane transport (GO:0034220)	913	42	71.14	-	0.59	3.32E-04	9.76E-03
positive regulation of intracellular signal transduction (GO:1902533)	1026	47	79.95	-	0.59	1.06E-04	3.78E-03
regulation of immune system process (GO:0002682)	1667	76	129.89	-	0.59	3.89E-07	2.46E-05
positive regulation of immune response (GO:0050778)	847	38	66	-	0.58	3.31E-04	9.75E-03
regulation of secretion by cell (GO:1903530)	764	34	59.53	-	0.57	6.16E-04	1.65E-02
response to biotic stimulus (GO:0009607)	1353	60	105.43	-	0.57	2.16E-06	1.16E-04
cation transmembrane transport (GO:0098655)	633	28	49.32	-	0.57	1.71E-03	3.98E-02
inorganic ion transmembrane transport (GO:0098660)	660	29	51.43	-	0.56	1.25E-03	3.06E-02
regulation of immune response (GO:0050776)	1139	50	88.75	-	0.56	1.15E-05	5.26E-04
regulation of secretion (GO:0051046)	821	36	63.97	-	0.56	2.65E-04	8.18E-03
cell migration (GO:0016477)	949	41	73.95	-	0.55	5.47E-05	2.12E-03
leukocyte activation (GO:0045321)	916	39	71.37	-	0.55	5.33E-05	2.07E-03
leukocyte activation involved in immune response (GO:0002366)	619	26	48.23	-	0.54	8.63E-04	2.21E-02
anion transport (GO:0006820)	574	24	44.73	-	0.54	1.30E-03	3.17E-02
positive regulation of immune system process (GO:0002684)	1150	48	89.61	-	0.54	2.36E-06	1.26E-04
cell activation involved in immune response (GO:0002263)	623	26	48.54	-	0.54	6.86E-04	1.82E-02
response to other organism (GO:0051707)	1327	55	103.4	-	0.53	3.08E-07	2.00E-05

cell activation (GO:0001775)	1063	44	82.83	-	0.53	5.48E-06	2.74E-04
response to external biotic stimulus (GO:0043207)	1329	55	103.56	-	0.53	2.42E-07	1.59E-05
regulation of hormone levels (GO:0010817)	536	22	41.76	-	0.53	1.57E-03	3.73E-02
exocytosis (GO:0006887)	786	31	61.24	-	0.51	4.44E-05	1.77E-03
immune effector process (GO:0002252)	1079	42	84.08	-	0.5	7.86E-07	4.67E-05
negative regulation of transport (GO:0051051)	493	19	38.41	-	0.49	9.89E-04	2.48E-02
Unclassified (UNCLASSIFIED)	3156	121	245.91	-	0.49	8.35E-20	2.42E-17
cellular calcium ion homeostasis (GO:0006874)	446	17	34.75	-	0.49	1.92E-03	4.41E-02
response to wounding (GO:0009611)	578	22	45.04	-	0.49	2.99E-04	9.02E-03
defense response to other organism (GO:0098542)	952	36	74.18	-	0.49	1.60E-06	8.79E-05
cytokine-mediated signaling pathway (GO:0019221)	692	26	53.92	-	0.48	5.10E-05	2.01E-03
organic anion transport (GO:0015711)	453	17	35.3	-	0.48	1.15E-03	2.84E-02
epithelial cell differentiation (GO:0030855)	674	25	52.52	-	0.48	5.52E-05	2.13E-03
secretion by cell (GO:0032940)	1002	37	78.08	-	0.47	4.28E-07	2.69E-05
synaptic signaling (GO:0099536)	465	17	36.23	-	0.47	6.97E-04	1.84E-02
regulation of lymphocyte activation (GO:0051249)	521	19	40.6	-	0.47	3.75E-04	1.07E-02
regulation of MAPK cascade (GO:0043408)	752	27	58.6	-	0.46	8.19E-06	3.91E-04
secretion (GO:0046903)	1119	40	87.19	-	0.46	3.54E-08	2.71E-06
regulation of system process (GO:0044057)	590	21	45.97	-	0.46	9.30E-05	3.39E-03
import into cell (GO:0098657)	675	24	52.6	-	0.46	2.12E-05	9.07E-04
export from cell (GO:0140352)	1043	37	81.27	-	0.46	9.44E-08	6.70E-06
activation of immune response (GO:0002253)	622	22	48.47	-	0.45	5.27E-05	2.05E-03
negative regulation of response to external stimulus (GO:0032102)	369	13	28.75	-	0.45	1.93E-03	4.43E-02
neutrophil degranulation (GO:0043312)	483	17	37.64	-	0.45	3.24E-04	9.60E-03
wound healing (GO:0042060)	485	17	37.79	-	0.45	3.24E-04	9.60E-03
neutrophil activation involved in immune response (GO:0002283)	487	17	37.95	-	0.45	3.28E-04	9.67E-03
neutrophil mediated immunity (GO:0002446)	494	17	38.49	-	0.44	2.55E-04	7.89E-03
defense response (GO:0006952)	1342	46	104.57	-	0.44	2.06E-10	2.22E-08
neutrophil activation (GO:0042119)	496	17	38.65	-	0.44	1.92E-04	6.19E-03

granulocyte activation (GO:0036230)	500	17	38.96	-	0.44	1.97E-04	6.31E-03
leukocyte degranulation (GO:0043299)	504	17	39.27	-	0.43	1.49E-04	5.03E-03
regulation of transmembrane transport (GO:0034762)	573	19	44.65	-	0.43	3.60E-05	1.47E-03
myeloid leukocyte mediated immunity (GO:0002444)	514	17	40.05	-	0.42	8.67E-05	3.20E-03
leukocyte mediated immunity (GO:0002443)	760	25	59.22	-	0.42	1.11E-06	6.31E-05
myeloid cell activation involved in immune response (GO:0002275)	518	17	40.36	-	0.42	8.89E-05	3.26E-03
immune response (GO:0006955)	1868	61	145.55	-	0.42	2.25E-15	4.52E-13
innate immune response (GO:0045087)	766	25	59.69	-	0.42	8.47E-07	4.99E-05
regulation of inflammatory response (GO:0050727)	341	11	26.57	-	0.41	1.69E-03	3.94E-02
regulation of cell adhesion (GO:0030155)	684	22	53.3	-	0.41	2.80E-06	1.47E-04
immune response-regulating signaling pathway (GO:0002764)	592	19	46.13	-	0.41	1.62E-05	7.19E-04
positive regulation of leukocyte activation (GO:0002696)	406	13	31.64	-	0.41	3.90E-04	1.11E-02
regulated exocytosis (GO:0045055)	696	22	54.23	-	0.41	1.62E-06	8.86E-05
regulation of leukocyte activation (GO:0002694)	603	19	46.99	-	0.4	9.29E-06	4.38E-04
response to bacterium (GO:0009617)	701	22	54.62	-	0.4	1.23E-06	6.93E-05
myeloid leukocyte activation (GO:0002274)	581	18	45.27	-	0.4	9.45E-06	4.43E-04
regulation of peptidase activity (GO:0052547)	452	14	35.22	-	0.4	1.28E-04	4.46E-03
positive regulation of cell activation (GO:0050867)	420	13	32.73	-	0.4	2.28E-04	7.17E-03
endocytosis (GO:0006897)	554	17	43.17	-	0.39	1.74E-05	7.68E-04
circulatory system process (GO:0003013)	401	12	31.25	-	0.38	2.34E-04	7.33E-03
regulation of cell activation (GO:0050865)	643	19	50.1	-	0.38	1.30E-06	7.30E-05
immune response-regulating cell surface receptor signaling pathway (GO:0002768)	481	14	37.48	-	0.37	3.13E-05	1.30E-03
immune response-activating signal transduction (GO:0002757)	555	16	43.25	-	0.37	5.54E-06	2.76E-04
regulation of ion transmembrane transport (GO:0034765)	491	14	38.26	-	0.37	1.74E-05	7.67E-04
regulation of cation transmembrane transport (GO:1904062)	351	10	27.35	-	0.37	3.10E-04	9.27E-03
regulation of endopeptidase activity (GO:0052548)	423	12	32.96	-	0.36	7.39E-05	2.78E-03
blood circulation (GO:0008015)	392	11	30.54	-	0.36	1.32E-04	4.55E-03
regulation of T cell activation (GO:0050863)	321	9	25.01	-	0.36	5.53E-04	1.51E-02

system process (GO:0003008)	1967	54	153.27	-	0.35	1.64E-20	5.31E-18
blood coagulation (GO:0007596)	292	8	22.75	-	0.35	1.00E-03	2.52E-02
monovalent inorganic cation transport (GO:0015672)	404	11	31.48	-	0.35	7.38E-05	2.78E-03
antigen receptor-mediated signaling pathway (GO:0050851)	294	8	22.91	-	0.35	7.34E-04	1.94E-02
coagulation (GO:0050817)	294	8	22.91	-	0.35	7.34E-04	1.93E-02
hemostasis (GO:0007599)	297	8	23.14	-	0.35	7.42E-04	1.95E-02
steroid metabolic process (GO:0008202)	260	7	20.26	-	0.35	1.79E-03	4.15E-02
nervous system process (GO:0050877)	1388	37	108.15	-	0.34	5.90E-15	1.13E-12
epidermis development (GO:0008544)	414	11	32.26	-	0.34	4.03E-05	1.62E-03
regulation of leukocyte cell-cell adhesion (GO:1903037)	302	8	23.53	-	0.34	5.48E-04	1.50E-02
regulation of cytosolic calcium ion concentration (GO:0051480)	340	9	26.49	-	0.34	2.30E-04	7.23E-03
positive regulation of MAPK cascade (GO:0043410)	544	14	42.39	-	0.33	1.22E-06	6.92E-05
epidermal cell differentiation (GO:0009913)	313	8	24.39	-	0.33	2.99E-04	9.02E-03
regulation of metal ion transport (GO:0010959)	401	10	31.25	-	0.32	2.91E-05	1.22E-03
regulation of heart contraction (GO:0008016)	243	6	18.93	-	0.32	1.27E-03	3.09E-02
immune response-activating cell surface receptor signaling pathway (GO:0002429)	446	11	34.75	-	0.32	9.22E-06	4.37E-04
regulation of cell-cell adhesion (GO:0022407)	406	10	31.64	-	0.32	2.13E-05	9.07E-04
regulation of ion transport (GO:0043269)	704	17	54.86	-	0.31	7.88E-09	6.49E-07
regulation of body fluid levels (GO:0050878)	497	12	38.73	-	0.31	1.50E-06	8.23E-05
regulation of blood circulation (GO:1903522)	290	7	22.6	-	0.31	3.99E-04	1.13E-02
positive regulation of cytosolic calcium ion concentration (GO:0007204)	296	7	23.06	-	0.3	2.94E-04	8.89E-03
positive regulation of leukocyte cell-cell adhesion (GO:1903039)	219	5	17.06	-	0.29	1.65E-03	3.89E-02
regulation of calcium ion transport (GO:0051924)	264	6	20.57	-	0.29	5.12E-04	1.42E-02
negative regulation of secretion (GO:0051048)	227	5	17.69	-	0.28	1.23E-03	3.02E-02
biological adhesion (GO:0022610)	926	20	72.15	-	0.28	1.35E-12	1.98E-10
phagocytosis (GO:0006909)	325	7	25.32	-	0.28	6.15E-05	2.34E-03
positive regulation of ion transport (GO:0043270)	283	6	22.05	-	0.27	1.40E-04	4.76E-03
skin development (GO:0043588)	379	8	29.53	-	0.27	9.92E-06	4.60E-04

receptor-mediated endocytosis (GO:0006898)	240	5	18.7	-	0.27	4.56E-04	1.28E-02
second-messenger-mediated signaling (GO:0019932)	338	7	26.34	-	0.27	2.26E-05	9.60E-04
cell adhesion (GO:0007155)	920	19	71.69	-	0.27	4.46E-13	7.24E-11
adaptive immune response (GO:0002250)	599	12	46.67	-	0.26	7.01E-09	5.86E-07
inflammatory response (GO:0006954)	500	10	38.96	-	0.26	1.38E-07	9.19E-06
cell-cell adhesion (GO:0098609)	502	10	39.12	-	0.26	9.59E-08	6.78E-06
negative regulation of secretion by cell (GO:1903531)	201	4	15.66	-	0.26	1.55E-03	3.69E-02
positive regulation of cell adhesion (GO:0045785)	406	8	31.64	-	0.25	1.93E-06	1.04E-04
positive regulation of cell-cell adhesion (GO:0022409)	260	5	20.26	-	0.25	1.74E-04	5.74E-03
positive regulation of ERK1 and ERK2 cascade (GO:0070374)	209	4	16.29	-	0.25	7.77E-04	2.02E-02
sensory perception (GO:0007600)	966	17	75.27	-	0.23	2.03E-15	4.14E-13
detection of stimulus (GO:0051606)	693	12	54	-	0.22	2.27E-11	2.96E-09
negative regulation of endopeptidase activity (GO:0010951)	251	4	19.56	-	0.2	7.50E-05	2.81E-03
negative regulation of peptidase activity (GO:0010466)	261	4	20.34	-	0.2	3.71E-05	1.52E-03
keratinocyte differentiation (GO:0030216)	268	4	20.88	-	0.19	2.68E-05	1.13E-03
extracellular matrix organization (GO:0030198)	342	5	26.65	-	0.19	1.05E-06	6.08E-05
extracellular structure organization (GO:0043062)	343	5	26.73	-	0.19	1.05E-06	6.08E-05
hormone metabolic process (GO:0042445)	206	3	16.05	-	0.19	2.33E-04	7.30E-03
cell chemotaxis (GO:0060326)	212	3	16.52	-	0.18	1.64E-04	5.50E-03
regulation of chemotaxis (GO:0050920)	215	3	16.75	-	0.18	1.73E-04	5.73E-03
membrane invagination (GO:0010324)	145	2	11.3	-	0.18	2.12E-03	4.81E-02
G protein-coupled receptor signaling pathway (GO:0007186)	1315	18	102.46	-	0.18	2.30E-24	1.08E-21
leukocyte migration (GO:0050900)	380	5	29.61	-	0.17	1.36E-07	9.09E-06
defense response to bacterium (GO:0042742)	333	4	25.95	-	0.15	3.78E-07	2.40E-05
cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742)	257	3	20.03	-	0.15	9.64E-06	4.49E-04
regulation of response to wounding (GO:1903034)	173	2	13.48	-	0.15	3.53E-04	1.02E-02
regulation of blood pressure (GO:0008217)	185	2	14.42	-	0.14	1.72E-04	5.72E-03
regulation of leukocyte migration (GO:0002685)	201	2	15.66	-	0.13	5.72E-05	2.20E-03

adenylate cyclase-modulating G protein-coupled receptor signaling pathway (GO:0007188)	208	2	16.21	-	0.12	4.02E-05	1.62E-03
G protein-coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger (GO:0007187)	244	2	19.01	-	0.11	2.95E-06	1.53E-04
cell-matrix adhesion (GO:0007160)	127	1	9.9	-	0.1	1.59E-03	3.75E-02
regulation of humoral immune response (GO:0002920)	136	1	10.6	-	0.09	7.23E-04	1.91E-02
leukocyte chemotaxis (GO:0030595)	147	1	11.45	-	0.09	3.39E-04	9.93E-03
homophilic cell adhesion via plasma membrane adhesion molecules (GO:0007156)	166	1	12.93	-	0.08	1.15E-04	4.03E-03
humoral immune response mediated by circulating immunoglobulin (GO:0002455)	167	1	13.01	-	0.08	7.32E-05	2.76E-03
cyclic-nucleotide-mediated signaling (GO:0019935)	171	1	13.32	-	0.08	7.73E-05	2.89E-03
detection of stimulus involved in sensory perception (GO:0050906)	543	3	42.31	-	0.07	3.13E-14	5.47E-12
humoral immune response (GO:0006959)	372	2	28.99	-	0.07	4.85E-10	4.94E-08
sensory perception of smell (GO:0007608)	462	2	36	-	0.06	6.37E-13	1.00E-10
detection of chemical stimulus (GO:0009593)	519	2	40.44	-	0.05	1.32E-14	2.36E-12
sensory perception of chemical stimulus (GO:0007606)	534	2	41.61	-	0.05	3.91E-15	7.78E-13
regulation of systemic arterial blood pressure (GO:0003073)	100	0	7.79	-	< 0.01	1.33E-03	3.23E-02
complement activation, classical pathway (GO:0006958)	161	0	12.55	-	< 0.01	9.71E-06	4.52E-04
complement activation (GO:0006956)	176	0	13.71	-	< 0.01	2.88E-06	1.50E-04
phagocytosis, recognition (GO:0006910)	106	0	8.26	-	< 0.01	5.41E-04	1.49E-02
neuropeptide signaling pathway (GO:0007218)	106	0	8.26	-	< 0.01	5.41E-04	1.48E-02
phospholipase C-activating G protein-coupled receptor signaling pathway (GO:0007200)	105	0	8.18	-	< 0.01	8.75E-04	2.22E-02
adenylate cyclase-activating G protein-coupled receptor signaling pathway (GO:0007189)	128	0	9.97	-	< 0.01	1.08E-04	3.84E-03
granulocyte chemotaxis (GO:0071621)	90	0	7.01	-	< 0.01	1.82E-03	4.22E-02
cellular response to chemokine (GO:1990869)	90	0	7.01	-	< 0.01	1.82E-03	4.22E-02
response to chemokine (GO:1990868)	90	0	7.01	-	< 0.01	1.82E-03	4.21E-02
detection of chemical stimulus involved in sensory perception of smell (GO:0050911)	433	0	33.74	-	< 0.01	1.10E-14	2.04E-12
detection of chemical stimulus involved in sensory perception (GO:0050907)	482	0	37.56	-	< 0.01	2.92E-16	6.36E-14

neutrophil migration (GO:1990266)	91	0	7.09	-	< 0.01	1.83E-03	4.22E-02
B cell receptor signaling pathway (GO:0050853)	126	0	9.82	-	< 0.01	1.72E-04	5.71E-03
cornification (GO:0070268)	113	0	8.8	-	< 0.01	3.65E-04	1.05E-02
keratinization (GO:0031424)	226	0	17.61	-	< 0.01	7.94E-08	5.71E-06
antimicrobial humoral immune response mediated by antimicrobial peptide (GO:0061844)	104	0	8.1	-	< 0.01	8.47E-04	2.18E-02
granulocyte migration (GO:0097530)	101	0	7.87	-	< 0.01	8.11E-04	2.10E-02
myeloid leukocyte migration (GO:0097529)	129	0	10.05	-	< 0.01	1.09E-04	3.86E-03
regulation of complement activation (GO:0030449)	117	0	9.12	-	< 0.01	2.41E-04	7.51E-03
cAMP-mediated signaling (GO:0019933)	147	0	11.45	-	< 0.01	3.40E-05	1.40E-03
antimicrobial humoral response (GO:0019730)	142	0	11.06	-	< 0.01	5.14E-05	2.01E-03

Table A8.3 GO-BMPR1B

BMPR1B co-expressed genes

Analysis Type: PANTHER Overrepresentation Test (Released 20190711)
Annotation Version and Release Date: GO Ontology database Released 2019-10-08
Analyzed List: upload_1 (Homo sapiens)
Reference List: Homo sapiens (all genes in database)
Test Type: FISHER
Correction: FDR

GO biological process complete	#	#	expected	Plus/minus	Fold enrichment	Raw P value	FDR
aminergic neurotransmitter loading into synaptic vesicle (GO:0015842)	4	4	0.27	+	15.08	8.51E-04	4.90E-02
sympathetic nervous system development (GO:0048485)	21	10	1.39	+	7.18	1.11E-05	1.24E-03
regulation of Rac protein signal transduction (GO:0035020)	15	7	0.99	+	7.04	2.63E-04	1.90E-02
regulation of cAMP-dependent protein kinase activity (GO:2000479)	19	7	1.26	+	5.56	8.16E-04	4.76E-02
catechol-containing compound biosynthetic process (GO:0009713)	19	7	1.26	+	5.56	8.16E-04	4.74E-02
catecholamine biosynthetic process (GO:0042423)	19	7	1.26	+	5.56	8.16E-04	4.72E-02
clathrin-dependent endocytosis (GO:0072583)	28	10	1.86	+	5.39	7.94E-05	6.86E-03
glutamate secretion (GO:0014047)	30	10	1.99	+	5.03	1.27E-04	1.02E-02
protein localization to Golgi apparatus (GO:0034067)	28	9	1.86	+	4.85	3.44E-04	2.35E-02
vesicle docking involved in exocytosis (GO:0006904)	44	13	2.92	+	4.46	3.67E-05	3.45E-03
mRNA splice site selection (GO:0006376)	31	9	2.06	+	4.38	6.39E-04	3.87E-02
neuron recognition (GO:0008038)	49	14	3.25	+	4.31	2.54E-05	2.48E-03
Golgi to plasma membrane protein transport (GO:0043001)	32	9	2.12	+	4.24	7.74E-04	4.60E-02
Golgi to plasma membrane transport (GO:0006893)	54	15	3.58	+	4.19	1.75E-05	1.84E-03
semaphorin-plexin signaling pathway (GO:0071526)	37	10	2.45	+	4.08	5.22E-04	3.28E-02
synaptic vesicle exocytosis (GO:0016079)	62	16	4.11	+	3.89	2.01E-05	2.03E-03
vesicle docking (GO:0048278)	66	17	4.38	+	3.89	1.14E-05	1.26E-03

organelle transport along microtubule (GO:0072384)	78	20	5.17	+	3.87	2.10E-06	2.92E-04
calcium-ion regulated exocytosis (GO:0017156)	43	11	2.85	+	3.86	4.14E-04	2.77E-02
vesicle cytoskeletal trafficking (GO:0099518)	51	13	3.38	+	3.84	1.31E-04	1.04E-02
exocytic process (GO:0140029)	71	18	4.71	+	3.82	7.65E-06	9.08E-04
dendrite morphogenesis (GO:0048813)	60	15	3.98	+	3.77	4.94E-05	4.54E-03
synaptic transmission, glutamatergic (GO:0035249)	40	10	2.65	+	3.77	8.74E-04	5.00E-02
endosome to lysosome transport (GO:0008333)	52	13	3.45	+	3.77	1.55E-04	1.19E-02
acidic amino acid transport (GO:0015800)	49	12	3.25	+	3.69	3.23E-04	2.24E-02
autonomic nervous system development (GO:0048483)	45	11	2.98	+	3.69	5.75E-04	3.56E-02
vesicle transport along microtubule (GO:0047496)	45	11	2.98	+	3.69	5.75E-04	3.54E-02
synaptic vesicle cycle (GO:0099504)	124	30	8.22	+	3.65	2.01E-08	5.42E-06
transmission of nerve impulse (GO:0019226)	54	13	3.58	+	3.63	2.13E-04	1.58E-02
positive regulation of macroautophagy (GO:0016239)	67	16	4.44	+	3.6	4.50E-05	4.21E-03
autophagosome assembly (GO:0000045)	63	15	4.18	+	3.59	7.94E-05	6.83E-03
presynaptic endocytosis (GO:0140238)	51	12	3.38	+	3.55	4.41E-04	2.87E-02
synaptic vesicle endocytosis (GO:0048488)	51	12	3.38	+	3.55	4.41E-04	2.86E-02
regulation of filopodium assembly (GO:0051489)	47	11	3.12	+	3.53	7.85E-04	4.62E-02
regulation of synaptic vesicle exocytosis (GO:2000300)	82	19	5.44	+	3.49	1.30E-05	1.42E-03
vesicle-mediated transport in synapse (GO:0099003)	134	31	8.88	+	3.49	2.85E-08	7.20E-06
autophagosome organization (GO:1905037)	66	15	4.38	+	3.43	1.24E-04	1.00E-02
axonal transport (GO:0098930)	53	12	3.51	+	3.42	5.93E-04	3.63E-02
regulation of potassium ion transmembrane transporter activity (GO:1901016)	59	13	3.91	+	3.32	4.46E-04	2.87E-02
retrograde transport, endosome to Golgi (GO:0042147)	91	20	6.03	+	3.32	1.52E-05	1.62E-03
regulation of neurotransmitter secretion (GO:0046928)	110	24	7.29	+	3.29	2.47E-06	3.30E-04
cytoskeleton-dependent intracellular transport (GO:0030705)	175	38	11.6	+	3.28	3.66E-09	1.24E-06
regulation of synaptic vesicle cycle (GO:0098693)	117	25	7.76	+	3.22	2.13E-06	2.94E-04
neuron migration (GO:0001764)	123	26	8.15	+	3.19	1.60E-06	2.27E-04
homophilic cell adhesion via plasma membrane adhesion molecules (GO:0007156)	166	35	11.01	+	3.18	2.84E-08	7.28E-06

post-Golgi vesicle-mediated transport (GO:0006892)	133	28	8.82	+	3.18	6.90E-07	1.12E-04
protein transport along microtubule (GO:0098840)	67	14	4.44	+	3.15	4.35E-04	2.86E-02
microtubule-based protein transport (GO:0099118)	67	14	4.44	+	3.15	4.35E-04	2.85E-02
neurotransmitter secretion (GO:0007269)	101	21	6.7	+	3.14	1.94E-05	2.00E-03
signal release from synapse (GO:0099643)	101	21	6.7	+	3.14	1.94E-05	1.99E-03
transport along microtubule (GO:0010970)	154	32	10.21	+	3.13	1.47E-07	3.07E-05
regulation of potassium ion transmembrane transport (GO:1901379)	82	17	5.44	+	3.13	1.20E-04	9.76E-03
cytosolic transport (GO:0016482)	145	30	9.61	+	3.12	3.86E-07	7.05E-05
vesicle-mediated transport to the plasma membrane (GO:0098876)	87	18	5.77	+	3.12	7.86E-05	6.83E-03
lysosomal transport (GO:0007041)	102	21	6.76	+	3.11	2.20E-05	2.21E-03
regulation of postsynapse organization (GO:0099175)	102	20	6.76	+	2.96	6.22E-05	5.52E-03
Rab protein signal transduction (GO:0032482)	72	14	4.77	+	2.93	8.11E-04	4.74E-02
organelle localization by membrane tethering (GO:0140056)	171	33	11.34	+	2.91	4.14E-07	7.40E-05
regulation of macroautophagy (GO:0016241)	173	33	11.47	+	2.88	5.21E-07	9.00E-05
retrograde vesicle-mediated transport, Golgi to endoplasmic reticulum (GO:0006890)	84	16	5.57	+	2.87	4.33E-04	2.86E-02
positive regulation of exocytosis (GO:0045921)	90	17	5.97	+	2.85	3.16E-04	2.22E-02
regulation of neurotransmitter transport (GO:0051588)	147	27	9.75	+	2.77	1.00E-05	1.12E-03
membrane docking (GO:0022406)	180	33	11.93	+	2.77	1.13E-06	1.68E-04
regulation of dendrite morphogenesis (GO:0048814)	93	17	6.17	+	2.76	4.41E-04	2.85E-02
dendrite development (GO:0016358)	115	21	7.62	+	2.75	1.01E-04	8.29E-03
vacuole organization (GO:0007033)	132	24	8.75	+	2.74	3.53E-05	3.34E-03
positive regulation of autophagy (GO:0010508)	122	22	8.09	+	2.72	8.18E-05	6.99E-03
regulation of exocytosis (GO:0017157)	222	40	14.72	+	2.72	1.73E-07	3.45E-05
cell-cell adhesion via plasma-membrane adhesion molecules (GO:0098742)	257	46	17.04	+	2.7	2.10E-08	5.57E-06
multicellular organismal signaling (GO:0035637)	135	24	8.95	+	2.68	4.84E-05	4.47E-03
vesicle localization (GO:0051648)	192	34	12.73	+	2.67	2.02E-06	2.84E-04
vacuolar transport (GO:0007034)	142	25	9.41	+	2.66	6.03E-05	5.39E-03
microtubule-based transport (GO:0099111)	182	32	12.07	+	2.65	4.56E-06	5.81E-04

signal release (GO:0023061)	171	30	11.34	+	2.65	9.70E-06	1.09E-03
membrane fusion (GO:0061025)	127	22	8.42	+	2.61	2.05E-04	1.53E-02
regulation of regulated secretory pathway (GO:1903305)	163	28	10.81	+	2.59	2.50E-05	2.46E-03
establishment of vesicle localization (GO:0051650)	181	31	12	+	2.58	9.14E-06	1.06E-03
axon guidance (GO:0007411)	267	44	17.7	+	2.49	3.27E-07	6.04E-05
positive regulation of synaptic transmission (GO:0050806)	146	24	9.68	+	2.48	1.59E-04	1.22E-02
regulation of neuron projection development (GO:0010975)	506	83	33.55	+	2.47	2.27E-12	2.25E-09
neuron projection guidance (GO:0097485)	270	44	17.9	+	2.46	4.09E-07	7.40E-05
cell morphogenesis involved in neuron differentiation (GO:0048667)	443	72	29.37	+	2.45	1.10E-10	6.71E-08
macroautophagy (GO:0016236)	160	26	10.61	+	2.45	9.64E-05	7.98E-03
protein localization to cell periphery (GO:1990778)	234	38	15.51	+	2.45	2.92E-06	3.87E-04
regulation of dendrite development (GO:0050773)	154	25	10.21	+	2.45	1.34E-04	1.05E-02
synapse organization (GO:0050808)	280	45	18.56	+	2.42	5.86E-07	9.61E-05
neurotransmitter transport (GO:0006836)	178	28	11.8	+	2.37	1.06E-04	8.68E-03
protein localization to plasma membrane (GO:0072659)	191	30	12.66	+	2.37	5.74E-05	5.18E-03
microtubule-based movement (GO:0007018)	268	42	17.77	+	2.36	2.15E-06	2.93E-04
positive regulation of cell projection organization (GO:0031346)	390	61	25.86	+	2.36	9.68E-09	2.90E-06
regulation of synapse organization (GO:0050807)	224	35	14.85	+	2.36	1.37E-05	1.48E-03
Golgi vesicle transport (GO:0048193)	399	62	26.45	+	2.34	1.27E-08	3.60E-06
positive regulation of neuron projection development (GO:0010976)	290	45	19.23	+	2.34	1.03E-06	1.56E-04
axon development (GO:0061564)	415	64	27.51	+	2.33	7.68E-09	2.49E-06
modulation of chemical synaptic transmission (GO:0050804)	450	69	29.83	+	2.31	3.07E-09	1.09E-06
regulation of synapse structure or activity (GO:0050803)	235	36	15.58	+	2.31	1.87E-05	1.95E-03
regulation of trans-synaptic signaling (GO:0099177)	451	69	29.9	+	2.31	3.17E-09	1.10E-06
endosomal transport (GO:0016197)	229	35	15.18	+	2.31	2.61E-05	2.53E-03
axonogenesis (GO:0007409)	381	58	25.26	+	2.3	6.23E-08	1.44E-05
regulation of neurotransmitter levels (GO:0001505)	357	54	23.67	+	2.28	2.38E-07	4.61E-05
regulation of plasma membrane bounded cell projection organization (GO:0120035)	678	101	44.95	+	2.25	2.27E-12	2.13E-09

regulation of cell projection organization (GO:0031344)	687	102	45.55	+	2.24	1.80E-12	2.20E-09
regulation of microtubule cytoskeleton organization (GO:0070507)	196	29	12.99	+	2.23	2.22E-04	1.64E-02
regulation of neuron differentiation (GO:0045664)	663	98	43.96	+	2.23	6.90E-12	5.23E-09
peptide secretion (GO:0002790)	183	27	12.13	+	2.23	4.22E-04	2.80E-02
regulation of axonogenesis (GO:0050770)	190	28	12.6	+	2.22	3.11E-04	2.19E-02
neuron projection development (GO:0031175)	680	100	45.08	+	2.22	4.41E-12	3.69E-09
protein secretion (GO:0009306)	164	24	10.87	+	2.21	7.18E-04	4.31E-02
regulation of cell morphogenesis involved in differentiation (GO:0010769)	308	45	20.42	+	2.2	5.72E-06	7.05E-04
neuron projection morphogenesis (GO:0048812)	494	72	32.75	+	2.2	8.70E-09	2.66E-06
establishment of protein localization to extracellular region (GO:0035592)	165	24	10.94	+	2.19	7.65E-04	4.56E-02
positive regulation of neuron differentiation (GO:0045666)	379	55	25.13	+	2.19	5.34E-07	9.13E-05
plasma membrane bounded cell projection morphogenesis (GO:0120039)	498	72	33.02	+	2.18	1.01E-08	2.98E-06
cell morphogenesis involved in differentiation (GO:0000904)	562	81	37.26	+	2.17	1.25E-09	5.12E-07
regulation of microtubule-based process (GO:0032886)	230	33	15.25	+	2.16	1.43E-04	1.12E-02
cell projection morphogenesis (GO:0048858)	502	72	33.28	+	2.16	1.23E-08	3.55E-06
regulation of autophagy (GO:0010506)	330	47	21.88	+	2.15	7.02E-06	8.45E-04
regulation of Ras protein signal transduction (GO:0046578)	246	35	16.31	+	2.15	8.81E-05	7.49E-03
central nervous system neuron differentiation (GO:0021953)	184	26	12.2	+	2.13	7.77E-04	4.60E-02
cell part morphogenesis (GO:0032990)	524	74	34.74	+	2.13	1.43E-08	3.98E-06
chemical synaptic transmission (GO:0007268)	420	59	27.85	+	2.12	6.95E-07	1.12E-04
anterograde trans-synaptic signaling (GO:0098916)	420	59	27.85	+	2.12	6.95E-07	1.11E-04
organelle localization (GO:0051640)	584	82	38.72	+	2.12	2.71E-09	9.79E-07
neuron development (GO:0048666)	821	115	54.43	+	2.11	2.01E-12	2.28E-09
trans-synaptic signaling (GO:0099537)	439	61	29.1	+	2.1	4.62E-07	8.17E-05
neuron differentiation (GO:0030182)	1012	139	67.09	+	2.07	2.37E-14	6.29E-11
positive regulation of GTPase activity (GO:0043547)	408	56	27.05	+	2.07	2.15E-06	2.94E-04
autophagy (GO:0006914)	278	38	18.43	+	2.06	8.99E-05	7.60E-03
process utilizing autophagic mechanism (GO:0061919)	278	38	18.43	+	2.06	8.99E-05	7.56E-03
microtubule-based process (GO:0007017)	654	89	43.36	+	2.05	2.52E-09	9.32E-07

regulation of GTPase activity (GO:0043087)	481	65	31.89	+	2.04	5.42E-07	9.16E-05
regulation of small GTPase mediated signal transduction (GO:0051056)	348	47	23.07	+	2.04	1.98E-05	2.02E-03
vesicle organization (GO:0016050)	304	41	20.15	+	2.03	6.70E-05	5.92E-03
small GTPase mediated signal transduction (GO:0007264)	319	43	21.15	+	2.03	6.01E-05	5.40E-03
synaptic signaling (GO:0099536)	465	62	30.83	+	2.01	1.46E-06	2.11E-04
Ras protein signal transduction (GO:0007265)	248	33	16.44	+	2.01	4.24E-04	2.81E-02
plasma membrane bounded cell projection organization (GO:0120036)	1114	148	73.86	+	2	4.38E-14	9.95E-11
response to insulin (GO:0032868)	234	31	15.51	+	2	7.31E-04	4.37E-02
cell projection organization (GO:0030030)	1159	153	76.84	+	1.99	2.13E-14	6.77E-11
generation of neurons (GO:0048699)	1550	203	102.76	+	1.98	1.11E-18	8.81E-15
cell morphogenesis (GO:0000902)	724	94	48	+	1.96	8.66E-09	2.70E-06
cellular component morphogenesis (GO:0032989)	826	107	54.76	+	1.95	6.86E-10	3.03E-07
regulation of nervous system development (GO:0051960)	935	121	61.99	+	1.95	5.83E-11	3.87E-08
regulation of neurogenesis (GO:0050767)	827	107	54.83	+	1.95	7.11E-10	3.05E-07
microtubule cytoskeleton organization (GO:0000226)	467	60	30.96	+	1.94	5.97E-06	7.30E-04
regulation of cell morphogenesis (GO:0022604)	499	64	33.08	+	1.93	3.24E-06	4.26E-04
cell-cell adhesion (GO:0098609)	502	64	33.28	+	1.92	3.55E-06	4.59E-04
neurogenesis (GO:0022008)	1649	210	109.33	+	1.92	5.70E-18	3.02E-14
regulation of membrane potential (GO:0042391)	433	55	28.71	+	1.92	2.41E-05	2.38E-03
regulation of transporter activity (GO:0032409)	293	37	19.43	+	1.9	4.99E-04	3.16E-02
establishment of organelle localization (GO:0051656)	368	46	24.4	+	1.89	1.26E-04	1.01E-02
positive regulation of neurogenesis (GO:0050769)	483	60	32.02	+	1.87	1.90E-05	1.97E-03
nervous system development (GO:0007399)	2357	289	156.27	+	1.85	6.85E-23	1.09E-18
positive regulation of nervous system development (GO:0051962)	549	67	36.4	+	1.84	8.75E-06	1.02E-03
establishment of localization in cell (GO:0051649)	394	48	26.12	+	1.84	1.97E-04	1.49E-02
regulation of vesicle-mediated transport (GO:0060627)	552	67	36.6	+	1.83	9.44E-06	1.07E-03
endomembrane system organization (GO:0010256)	423	51	28.04	+	1.82	1.51E-04	1.17E-02
regulation of cell development (GO:0060284)	950	113	62.98	+	1.79	1.70E-08	4.66E-06
regulation of ion transmembrane transport (GO:0034765)	491	58	32.55	+	1.78	7.36E-05	6.43E-03

regulation of transmembrane transport (GO:0034762)	573	67	37.99	+	1.76	2.85E-05	2.75E-03
brain development (GO:0007420)	739	86	48.99	+	1.76	2.22E-06	2.99E-04
cell projection assembly (GO:0030031)	447	52	29.64	+	1.75	3.24E-04	2.24E-02
plasma membrane bounded cell projection assembly (GO:0120031)	432	50	28.64	+	1.75	3.79E-04	2.57E-02
behavior (GO:0007610)	581	67	38.52	+	1.74	4.53E-05	4.21E-03
positive regulation of cell development (GO:0010720)	557	63	36.93	+	1.71	1.31E-04	1.04E-02
cell adhesion (GO:0007155)	920	104	60.99	+	1.71	7.80E-07	1.23E-04
intracellular transport (GO:0046907)	1529	172	101.37	+	1.7	1.34E-10	7.63E-08
biological adhesion (GO:0022610)	926	104	61.39	+	1.69	8.68E-07	1.34E-04
head development (GO:0060322)	784	88	51.98	+	1.69	7.86E-06	9.26E-04
protein localization to membrane (GO:0072657)	518	58	34.34	+	1.69	3.06E-04	2.16E-02
protein transport (GO:0015031)	1528	171	101.3	+	1.69	1.92E-10	1.02E-07
peptide transport (GO:0015833)	1558	174	103.29	+	1.68	1.43E-10	7.82E-08
cytoskeleton organization (GO:0007010)	1095	121	72.6	+	1.67	2.42E-07	4.63E-05
establishment of protein localization (GO:0045184)	1621	179	107.47	+	1.67	2.06E-10	1.06E-07
membrane organization (GO:0061024)	855	94	56.69	+	1.66	7.06E-06	8.44E-04
amide transport (GO:0042886)	1592	175	105.55	+	1.66	4.08E-10	1.96E-07
cellular localization (GO:0051641)	2396	262	158.85	+	1.65	8.62E-15	3.43E-11
nitrogen compound transport (GO:0071705)	1861	203	123.38	+	1.65	2.20E-11	1.59E-08
organelle assembly (GO:0070925)	725	79	48.07	+	1.64	5.35E-05	4.89E-03
positive regulation of cellular component organization (GO:0051130)	1226	133	81.28	+	1.64	1.52E-07	3.11E-05
protein localization (GO:0008104)	2178	236	144.4	+	1.63	6.57E-13	9.50E-10
regulation of cytoskeleton organization (GO:0051493)	547	59	36.27	+	1.63	6.10E-04	3.72E-02
cell development (GO:0048468)	1633	175	108.27	+	1.62	2.31E-09	8.75E-07
central nervous system development (GO:0007417)	975	103	64.64	+	1.59	1.33E-05	1.44E-03
regulation of ion transport (GO:0043269)	704	74	46.67	+	1.59	2.67E-04	1.92E-02
cellular macromolecule localization (GO:0070727)	1626	170	107.8	+	1.58	2.35E-08	6.12E-06
metal ion transport (GO:0030001)	641	67	42.5	+	1.58	6.29E-04	3.82E-02
cellular protein localization (GO:0034613)	1618	169	107.27	+	1.58	2.92E-08	7.26E-06

vesicle-mediated transport (GO:0016192)	1954	203	129.55	+	1.57	1.17E-09	4.91E-07
positive regulation of hydrolase activity (GO:0051345)	771	80	51.12	+	1.57	2.10E-04	1.57E-02
macromolecule localization (GO:0033036)	2517	260	166.87	+	1.56	4.37E-12	3.86E-09
movement of cell or subcellular component (GO:0006928)	1535	158	101.77	+	1.55	1.92E-07	3.77E-05
cell-cell signaling (GO:0007267)	1111	114	73.66	+	1.55	1.42E-05	1.53E-03
import into cell (GO:0098657)	675	69	44.75	+	1.54	8.61E-04	4.94E-02
organic substance transport (GO:0071702)	2224	227	147.45	+	1.54	4.44E-10	2.08E-07
regulation of transport (GO:0051049)	1877	188	124.44	+	1.51	7.39E-08	1.68E-05
regulation of cellular component organization (GO:0051128)	2491	248	165.15	+	1.5	4.49E-10	2.04E-07
regulation of cellular component biogenesis (GO:0044087)	946	93	62.72	+	1.48	3.67E-04	2.49E-02
anatomical structure morphogenesis (GO:0009653)	2153	205	142.74	+	1.44	5.13E-07	8.97E-05
regulation of cell differentiation (GO:0045595)	1824	172	120.93	+	1.42	9.26E-06	1.07E-03
locomotion (GO:0040011)	1304	122	86.45	+	1.41	2.80E-04	1.99E-02
regulation of multicellular organismal development (GO:2000026)	2047	191	135.71	+	1.41	4.63E-06	5.85E-04
system development (GO:0048731)	4424	412	293.3	+	1.4	5.05E-13	8.03E-10
regulation of localization (GO:0032879)	2790	258	184.97	+	1.39	1.14E-07	2.53E-05
regulation of organelle organization (GO:0033043)	1298	120	86.06	+	1.39	5.13E-04	3.24E-02
positive regulation of catalytic activity (GO:0043085)	1440	133	95.47	+	1.39	2.74E-04	1.96E-02
cellular component assembly (GO:0022607)	2473	227	163.96	+	1.38	1.47E-06	2.10E-04
multicellular organism development (GO:0007275)	5026	460	333.22	+	1.38	1.40E-13	2.78E-10
organelle organization (GO:0006996)	3418	308	226.61	+	1.36	4.36E-08	1.02E-05
intracellular signal transduction (GO:0035556)	1680	151	111.38	+	1.36	3.25E-04	2.24E-02
cellular component organization (GO:0016043)	5642	504	374.06	+	1.35	2.07E-13	3.66E-10
anatomical structure development (GO:0048856)	5422	482	359.47	+	1.34	2.14E-12	2.27E-09
positive regulation of molecular function (GO:0044093)	1797	159	119.14	+	1.33	4.07E-04	2.73E-02
regulation of developmental process (GO:0050793)	2592	229	171.85	+	1.33	1.67E-05	1.77E-03
transport (GO:0006810)	4550	400	301.66	+	1.33	2.20E-09	8.52E-07
establishment of localization (GO:0051234)	4681	410	310.34	+	1.32	2.08E-09	8.27E-07
regulation of cell communication (GO:0010646)	3540	310	234.7	+	1.32	5.76E-07	9.54E-05

cellular component organization or biogenesis (GO:0071840)	5830	509	386.52	+	1.32	5.25E-12	4.18E-09
regulation of signaling (GO:0023051)	3581	312	237.41	+	1.31	7.81E-07	1.22E-04
cellular developmental process (GO:0048869)	3806	331	252.33	+	1.31	3.25E-07	6.07E-05
cell differentiation (GO:0030154)	3710	321	245.97	+	1.31	9.17E-07	1.40E-04
cellular component biogenesis (GO:0044085)	2699	233	178.94	+	1.3	5.65E-05	5.13E-03
developmental process (GO:0032502)	5862	506	388.64	+	1.3	4.63E-11	3.20E-08
localization (GO:0051179)	5765	496	382.21	+	1.3	1.28E-10	7.55E-08
regulation of multicellular organismal process (GO:0051239)	3147	269	208.64	+	1.29	2.24E-05	2.24E-03
cellular protein modification process (GO:0006464)	3084	256	204.46	+	1.25	2.56E-04	1.87E-02
protein modification process (GO:0036211)	3084	256	204.46	+	1.25	2.56E-04	1.86E-02
regulation of biological quality (GO:0065008)	4073	335	270.03	+	1.24	3.38E-05	3.24E-03
regulation of signal transduction (GO:0009966)	3154	259	209.1	+	1.24	4.52E-04	2.90E-02
signaling (GO:0023052)	5393	436	357.55	+	1.22	5.44E-06	6.76E-04
cell communication (GO:0007154)	5500	443	364.64	+	1.21	6.23E-06	7.56E-04
multicellular organismal process (GO:0032501)	6973	560	462.3	+	1.21	1.29E-07	2.77E-05
positive regulation of cellular process (GO:0048522)	5465	424	362.32	+	1.17	3.37E-04	2.31E-02
regulation of cellular process (GO:0050794)	10981	827	728.02	+	1.14	2.46E-07	4.65E-05
regulation of biological process (GO:0050789)	11688	875	774.9	+	1.13	1.52E-07	3.14E-05
biological regulation (GO:0065007)	12387	922	821.24	+	1.12	8.21E-08	1.84E-05
cellular process (GO:0009987)	14575	1047	966.3	+	1.08	3.91E-06	5.02E-04
biological_process (GO:0008150)	17840	1255	1182.76	+	1.06	3.32E-08	7.99E-06
multi-organism process (GO:0051704)	2832	144	187.76	-	0.77	7.04E-04	4.24E-02
immune system process (GO:0002376)	2806	139	186.03	-	0.75	2.24E-04	1.65E-02
Unclassified (UNCLASSIFIED)	3156	137	209.24	-	0.65	3.32E-08	8.11E-06
positive regulation of immune system process (GO:0002684)	1150	46	76.24	-	0.6	2.70E-04	1.93E-02
defense response (GO:0006952)	1342	50	88.97	-	0.56	9.39E-06	1.07E-03
immune response (GO:0006955)	1868	68	123.85	-	0.55	4.01E-08	9.51E-06
regulation of immune response (GO:0050776)	1139	41	75.51	-	0.54	2.31E-05	2.30E-03
G protein-coupled receptor signaling pathway (GO:0007186)	1315	47	87.18	-	0.54	3.31E-06	4.31E-04

cellular amide metabolic process (GO:0043603)	799	26	52.97	-	0.49	6.77E-05	5.95E-03
response to biotic stimulus (GO:0009607)	1353	44	89.7	-	0.49	1.21E-07	2.63E-05
response to other organism (GO:0051707)	1327	43	87.98	-	0.49	1.70E-07	3.42E-05
response to external biotic stimulus (GO:0043207)	1329	43	88.11	-	0.49	1.31E-07	2.78E-05
detection of stimulus (GO:0051606)	693	22	45.94	-	0.48	1.48E-04	1.16E-02
defense response to other organism (GO:0098542)	952	28	63.12	-	0.44	1.33E-06	1.93E-04
adaptive immune response (GO:0002250)	599	17	39.71	-	0.43	9.15E-05	7.65E-03
peptide metabolic process (GO:0006518)	542	15	35.93	-	0.42	1.70E-04	1.30E-02
amide biosynthetic process (GO:0043604)	542	15	35.93	-	0.42	1.70E-04	1.29E-02
innate immune response (GO:0045087)	766	21	50.78	-	0.41	5.43E-06	6.80E-04
response to bacterium (GO:0009617)	701	16	46.48	-	0.34	5.71E-07	9.56E-05
peptide biosynthetic process (GO:0043043)	419	9	27.78	-	0.32	1.02E-04	8.33E-03
translation (GO:0006412)	393	8	26.06	-	0.31	9.43E-05	7.85E-03
detection of stimulus involved in sensory perception (GO:0050906)	543	10	36	-	0.28	1.04E-06	1.57E-04
defense response to bacterium (GO:0042742)	333	5	22.08	-	0.23	3.49E-05	3.32E-03
keratinocyte differentiation (GO:0030216)	268	4	17.77	-	0.23	3.20E-04	2.23E-02
keratinization (GO:0031424)	226	3	14.98	-	0.2	4.93E-04	3.15E-02
cellular response to biotic stimulus (GO:0071216)	227	3	15.05	-	0.2	4.95E-04	3.15E-02
detection of chemical stimulus (GO:0009593)	519	6	34.41	-	0.17	8.14E-09	2.59E-06
sensory perception of chemical stimulus (GO:0007606)	534	6	35.4	-	0.17	4.02E-09	1.33E-06
humoral immune response (GO:0006959)	372	4	24.66	-	0.16	1.29E-06	1.91E-04
cellular response to lipopolysaccharide (GO:0071222)	194	2	12.86	-	0.16	5.37E-04	3.36E-02
cellular response to molecule of bacterial origin (GO:0071219)	203	2	13.46	-	0.15	3.80E-04	2.56E-02
detection of chemical stimulus involved in sensory perception (GO:0050907)	482	3	31.96	-	0.09	2.36E-10	1.17E-07
complement activation, classical pathway (GO:0006958)	161	1	10.67	-	0.09	8.03E-04	4.71E-02
humoral immune response mediated by circulating immunoglobulin (GO:0002455)	167	1	11.07	-	0.09	5.48E-04	3.42E-02
positive regulation of B cell activation (GO:0050871)	167	1	11.07	-	0.09	5.48E-04	3.40E-02
complement activation (GO:0006956)	176	1	11.67	-	0.09	2.46E-04	1.80E-02

sensory perception of smell (GO:0007608)	462	2	30.63	-	0.07	8.02E-11	5.10E-08
detection of chemical stimulus involved in sensory perception of smell (GO:0050911)	433	0	28.71	-	< 0.01	1.13E-12	1.50E-09
regulation of DNA-templated transcription in response to stress (GO:0043620)	126	0	8.35	-	< 0.01	5.89E-04	3.62E-02
antimicrobial humoral response (GO:0019730)	142	0	9.41	-	< 0.01	1.73E-04	1.31E-02

Table A8.4 pathway-BMPR2

BMPR2 co-expressed genes

Analysis Type: PANTHER Overrepresentation Test (Released 20190711)
Annotation Version and Release Date: PANTHER version 14.1 Released 2019-03-12
Analyzed List: upload_1 (Homo sapiens)
Reference List: Homo sapiens (all genes in database)
Test Type: FISHER
Correction: FDR

PANTHER Pathways	#	#	Expected		Fold	Raw P	FDR
Hedgehog signaling pathway (P00025)	21	9	2.34	+	3.84	2.05E-03	2.40E-02
Histamine H1 receptor mediated signaling pathway (P04385)	44	16	4.91	+	3.26	2.04E-04	3.35E-03
Oxytocin receptor mediated signaling pathway (P04391)	58	20	6.47	+	3.09	6.33E-05	1.30E-03
5HT2 type receptor mediated signaling pathway (P04374)	67	22	7.47	+	2.94	5.10E-05	1.19E-03
PI3 kinase pathway (P00048)	53	17	5.91	+	2.88	4.39E-04	6.01E-03
Thyrotropin-releasing hormone receptor signaling pathway (P04394)	60	19	6.69	+	2.84	2.38E-04	3.55E-03
Endothelin signaling pathway (P00019)	86	25	9.59	+	2.61	9.31E-05	1.70E-03
Gonadotropin-releasing hormone receptor pathway (P06664)	230	66	25.64	+	2.57	5.05E-10	8.28E-08
Ras Pathway (P04393)	74	21	8.25	+	2.55	4.41E-04	5.57E-03
FGF signaling pathway (P00021)	120	34	13.38	+	2.54	9.42E-06	3.09E-04
EGF receptor signaling pathway (P00018)	134	37	14.94	+	2.48	7.99E-06	3.28E-04
P	174	44	19.4	+	2.27	6.58E-06	3.60E-04
Wnt signaling pathway (P00057)	312	71	34.79	+	2.04	3.12E-07	2.56E-05
Angiogenesis (P00005)	173	35	19.29	+	1.81	2.33E-03	2.55E-02
Unclassified (UNCLASSIFIED)	18383	1978	2049.66	-	0.97	3.80E-05	1.04E-03

Table A8.5 pathway-BMPR1A

BMPR1A co-expressed genes

Analysis Type:	PANTHER Overrepresentation Test (Released 20190711)
Annotation Version and Release Date:	PANTHER version 14.1 Released 2019-03-12
Analyzed List:	upload_1 (Homo sapiens)
Reference List:	Homo sapiens (all genes in database)
Test Type:	FISHER
Correction:	FDR

PANTHER Pathways	**#**	**#**	**Expected**		**Fold**	**Raw P**	**FDR**
DNA replication (P00017)	28	12	2.46	+	4.88	4.85E-05	7.96E-03
p53 pathway (P00059)	87	20	7.64	+	2.62	4.44E-04	3.64E-02

Table A8.6 Pathway-BMPR1B

BMPR1B co-expressed genes

Analysis Type:	PANTHER Overrepresentation Test (Released 20190711)
Annotation Version and Release Date:	PANTHER version 14.1 Released 2019-03-12
Analyzed List:	upload_1 (Homo sapiens)
Reference List:	Homo sapiens (all genes in database)
Test Type:	FISHER
Correction:	FDR

PANTHER Pathways	**#**	**#**	**Expected**		**Fold**	**Raw P**	**FDR**
Adrenaline and noradrenaline biosynthesis (P00001)	29	9	2.21	+	4.08	1.09E-03	3.56E-02
Dopamine receptor mediated signaling pathway (P05912)	57	13	4.34	+	2.99	1.13E-03	3.09E-02
Oxytocin receptor mediated signaling pathway (P04391)	58	13	4.42	+	2.94	1.30E-03	3.04E-02
5HT2 type receptor mediated signaling pathway (P04374)	67	15	5.1	+	2.94	5.75E-04	2.36E-02
Gonadotropin-releasing hormone receptor pathway (P06664)	230	42	17.52	+	2.4	1.91E-06	3.13E-04
Wnt signaling pathway (P00057)	312	49	23.76	+	2.06	1.07E-05	8.76E-04
Unclassified (UNCLASSIFIED)	18383	1349	1400	-	0.96	3.28E-04	1.79E-02