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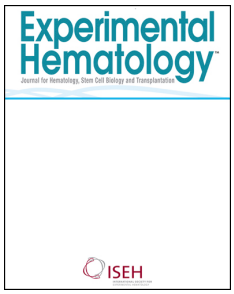

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NOX-driven ROS formation in cell transformation of FLT3-ITD positive AML

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Keywords:

Reactive oxygen species, myeloid leukemia, AML, FLT3-ITD, DNA damage, protein-tyrosine phosphatase (PTP), PTP oxidation

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Abstract

In different types of myeloid leukemia, increased formation of reactive oxygen species (ROS) has been noted and associated with aspects of cell transformation including the promotion of leukemic cell proliferation and migration, as well as DNA-damage and accumulation of mutations. Work reviewed in this article has shown the involvement of NADPH oxidase (NOX)-derived ROS downstream of oncogenic protein-tyrosine kinases in both processes, and the related pathways have been partially identified. FLT3-ITD, an important oncoprotein in a subset of AML, causes activation of AKT and subsequently stabilization of $p22^{phox}$, a regulatory subunit for NOX1-4. This process is linked to ROS formation and DNA damage. Moreover, FLT3-ITD signaling through STAT5 enhances expression of NOX4, ROS formation and inactivation of the protein-tyrosine phosphatase DEP-1/PTPRJ, a negative regulator of FLT3 signaling, by reversible oxidation of its catalytic cysteine residue. Genetic inactivation of NOX4 restored DEP-1 activity and attenuated cell transformation by FLT3-ITD *in vitro* and *in vivo*. Future work is required to further explore these mechanisms and their causal involvement in leukemic cell transformation, which may result in the identification of novel candidate targets for therapy.

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Introduction

The formation of reactive oxygen species (ROS) is essential for normal cell physiology, and has been covered in detail in many excellent recent reviews [1-4]. Only a few aspects, which lay the groundwork for discussing specific ROS functions in leukemic cells, shall be briefly reiterated here.

One important type of ROS are superoxide anions ($O_2^{\cdot-}$) as they are produced as side-products along the respiratory chain in mitochondria [5], by activity of NADPH oxidases 1-3, and 5 [4], or by different other cellular oxidases. Hydrogen peroxide (H_2O_2), considered a particular important signaling molecule [6], can be produced by NADPH oxidase 4 (NOX4) and the Dual oxidases 1 and 2 (DUOX1, 2), or by conversion of superoxide anions through superoxide dismutases (SOD). Further reactive species such as the short-lived hydroxyl radical ($OH^{\cdot}$), lipid hydroperoxides, peroxynitrite (NO_3^-) or hypochloric acid (HOCl) arise by metabolic reactions engaging superoxide or H_2O_2 (Fig.1). Among the specific physiologic functions of ROS is the reversible modification of proteins in the course of signal transduction in many cell types. Some of these modifications may not occur by direct interaction with any of the listed ROS species, but instead be relayed through intermediate oxidation products [3]. The formation of ROS and their reaction products is limited in time and extent by efficient cellular “anti-oxidant” systems. For example, peroxiredoxins (PRDXs) and catalase rapidly decompose H_2O_2. Thioredoxins and glutathione peroxidases can revert oxidative modifications, e.g. of oxidized protein-thiols or of lipid hydroperoxides, respectively. These and further antioxidant systems require NADPH and GSH for their restoration. The expression of several antioxidant proteins/enzymes is under positive control of the transcription factor NRF2, whose protein level and thereby activity is promoted by modification of an upstream “oxidant sensor” designated KEAP1 (Fig. 1). If ROS formation exceeds the capacity of the antioxidant mechanisms, cells are exposed to “oxidative stress”, leading to damage of cellular macromolecules, and potentially cell death.

Different types of cancer cells appear to produce higher levels of ROS than their normal counterparts [7, 8]. For example, early reports described enhanced ROS production in RAS-transformed fibroblasts [9], a finding which has later been corroborated in human CD34-positive hematopoietic progenitor cells [10]. More recently, also enhanced antioxidant activities were noted in cancer cells, for example as consequence of RAS-mediated transformation [11]. Consistent with a contributing role of this mechanism to tumor formation or maintenance, mutations in components of the KEAP1-NRF2 axis were found in different tumor types, leading to constitutive NRF2 activation [12-14]. It is likely that enhanced antioxidant capacity in some tumor entities is required for enabling cell survival under conditions of enhanced ROS production. ROS formation plays also a role in the interaction of cancer cells with their microenvironment, such as the cancer stem cell niche, with inflammatory cells or in the context of tumor angiogenesis. Clearly, alterations in ROS metabolism in cancer are complex,

presumably specific for certain cancer entities, and relate to both alterations in ROS formation and antioxidant defense.

Table 1

ROS formation in myeloid neoplasms, underlying mechanisms and functional consequences

Leukemia entity	Observed alterations in ROS pathways	Functional consequences	References
CML	BCR-ABL drives ROS formation through PI3K/AKT and glucose metabolism	General inhibition of phosphatase activity	[15, 16]
CML	ROS formation through PI3K, mitochondria, and AKT	Genomic instability, development of TKI resistant cell clones	[17, 18]
CML	NOX inhibitors synergize with TKI in inhibiting leukemic cells	NOX inhibition as potential therapeutic strategy	[19]
CML	BCR-ABL driven ROS formation involves STAT5	Acquisition of BCR-ABL mutations	[20]
Myeloid neoplasms	ROS overproduction downstream of oncogenic tyrosine kinases	Cell growth and migration	[21]
Myeloid neoplasm	RAS transformation of normal CD34+ cells drives ROS formation through NOX2	Promotion of survival and proliferation	[10]
AML	FLT3-ITD mutation drives ROS formation	DNA damage and misrepair	[22, 23]
AML	ROS production downstream of FLT3-ITD is mediated by $p22^{phox}$	STAT5 activation	[24]
AML	Overproduction of NOX-derived ROS	ROS promoted proliferation in vitro	[25]
AML	FLT3-ITD drives ROS through NOX activation	DNA damage	[26]
AML	FLT3-ITD drives ROS formation and PTP oxidation	Cell proliferation, myeloproliferative disease	[27, 28]
AML	Reduced expression of peroxiredoxin 2 (PRDX2)	Increased ROS levels upon cytokine stimulation; low PRDX2 levels correlated with poor prognosis	[29]

A plethora of data has indicated alterations in ROS metabolism in leukemia, either linked to etiology, prognosis or therapy responses [30, 31]. Many studies have supported the idea that ROS formation

may indeed causally contribute to leukemia growth and malignancy. For example, primary AML patient cells have been shown to frequently produce high levels of superoxide, a phenomenon which could be causally related to AML cell proliferation [25]. Only relatively recently, however, insights into the molecular basis of ROS formation in the leukemic cells were obtained. Importantly, the activity of key oncoproteins in myeloid leukemia, comprising BCR-ABL, FMS-like tyrosine kinase 3 with internal tandem duplications (FLT3-ITD), and Janus kinase 2 (JAKL2) V617F could be mechanistically connected to metabolic alterations leading to enhanced ROS formation (Table 1, Chapter 2). We will summarize here recent findings on these mechanisms, focusing on FLT3-ITD-driven ROS production in AML and its consequences.

Oncogenic kinases as drivers of ROS formation in myeloid leukemia

More than 90% of chronic myelogenous leukemia (CML) cases develop from a chromosomal abnormality known as the Philadelphia chromosome, which result from a reciprocal translocation between chromosomes 9 and 22, generating the chimeric kinase BCR-ABL (reviewed in [32]). BCR-ABL is known to activate down-stream pro-survival pathways, for example, PI3K/AKT, JAK/STAT and RAF/MEK/ERK, resulting in resistance to apoptosis and proliferation [33]. BCR-ABL expressing cells have been shown to generate increased levels of ROS compared to untransformed cells [16]. Various sources of ROS have been examined in CML including leakage from the mitochondrial electron transport chain and NADPH oxidase generated ROS, particularly NOX4. Naughton et al., demonstrated NOX4-generated ROS contributing significantly to total endogenous ROS upon BCR-ABL induction [34]. Treatment of CML cells with the BCR-ABL inhibitors, Imatinib and Nilotinib showed a significant decrease in ROS, coinciding with a post-translational down-regulation of the small membrane-bound protein $p22^{phox}$, a key component of the NOX complex [35]. Treatment of BCR-ABL expressing cells with panNOX inhibitors, DPI or VAS2870 resulted in a reduction in ROS levels. Inhibition of both the PI3K/AKT and RAF/MEK/ERK pathways in combination resulted in p22phox down-regulation. BCR-ABL induced NOX4-generated ROS are dependent on PI3K/AKT and RAF/MEK/ERK activation and GSK3β inhibition [36]. Mitochondrial ROS also appear to contribute to total ROS in CML cells.

The JAK2 V617F mutation is a substitution of a valine for a phenylalanine at amino acid 617 of JAK2 destabilizing the JH2 “pseudokinase” domain of JAK2. It results in loss of the auto-inhibitory function of this regulatory domain, and in turn constitutive tyrosine kinase activity [37]. The mutation is present in approximately 6% of myelodysplastic syndromes (MDS) and 50% of myeloproliferative neoplasms (MPNs) [38]. Signaling of the JAK2 V617F oncoprotein results in constitutive activation of downstream pro-survival signaling, including activation of STAT5, PI3K/AKT and RAF/MEK/ERK, and in turn the formation of ROS [39, 40]. The increase in ROS is concurrent with

elevated levels of DNA damage [40]. In neutrophils from JAK2 V617F positive patients, an increased phosphorylation of the NOX2 subunit $p47^{phox}$ on Ser345 has been observed, suggesting a contribution of NOX2 activation to elevated levels of ROS in MDS [41].

FLT3, encoding the class III receptor tyrosine kinase FLT3 is the most frequently mutated gene in AML (up to 35%) [42, 43]. The prevalent mutations (found in 20-25% of AML patients) encode internal tandem duplications of sequence in the FLT3 juxtamembrane or the first part of the kinase domain, giving rise to FLT3-ITD oncoproteins. Since the affected kinase regions have a negative regulatory function for kinase activity, FLT3-ITD mutations result in ligand independent constitutive activation of the FLT3 receptor both at the plasma membrane but also of the newly synthesized kinase at the endoplasmic reticulum [44, 45]. Occurrence of FLT3-ITD has been associated with a worse patient prognosis [46-48]. Constitutive activity of FLT3-ITD activates down-stream pro-survival signaling pathways including PI3K/AKT, STAT5 (whereby STAT5 activation is independent of JAK kinase activation [49]) and RAF/MEK/ERK, which are known to promote survival, proliferation and transformation [50-52]. Recent findings have identified that in order for PI3K/AKT and RAF/MEK/ERK pro-survival pathways to be activated they must be located down-stream of FLT3-ITD at the plasma membrane and STAT5 is located down-stream of FLT3-ITD at the ER [45, 53]. FLT3-ITD expressing cell lines have been shown to produce increased levels of ROS, DNA oxidation and double strand breaks (dsbs) when compared to FLT3-WT expressing cell lines [22, 27]. NOX-generated ROS appear to be a primary source of ROS in FLT3-ITD expressing AML cells. Cells harboring the FLT3-ITD mutant have been shown to produce increased levels of NOX2 and NOX4 and their partner protein $p22^{phox}$ compared to wild type FLT3 cells [26, 28]. Also, stimulation of FLT3-WT expressing cells with FLT3 ligand resulted in an increase in $p22^{phox}$ expression and of endogenous H_2O_2 levels [26]. There was no significant difference in mitochondrial ROS observed in FLT3-ITD or FLT3-WT cells, and cyclooxygenase-driven ROS formation did not contribute to total endogenous H_2O_2 in FLT3-ITD expressing cells ([26] and J. Moloney, T. Cotter, unpublished data). The enhanced ROS formation in FLT3-ITD expressing AML cells serves a signaling function in that it promotes cell proliferation and migration, and thereby contributes to leukemic cell transformation [21, 27]. In addition, FLT3-ITD driven ROS formation has been associated with DNA damage and accumulation of mutations [22, 23, 26]. Both aspects will be discussed in the following parts.

ROS mediated alteration of transforming signal transduction: role of PTP oxidation.

Protein phosphorylation of tyrosine residues plays a fundamental role in diverse cellular functions such as proliferation, growth, metabolism and differentiation. Protein-tyrosine kinases (PTKs) mediated signal transduction is regulated by protein-tyrosine phosphatases (PTPs) and failure of regulation of either protein family can contribute to unfavorable diseases like cancer. The human PTP

superfamily consists of more than 100 members. Many of these enzymes are identified by the unique consensus signature motif HCX_5R involved in the catalytic function. Despite of their sequence and structural similarity, PTPs show a wide range of substrate specificity [54, 55].

In AML, several members of the PTP superfamily were found to be altered by genetic aberration, promoter methylation or gene overexpression. PTPN11 (also known as SHP-2) positively regulates FL ligand mediated FLT3 receptor signaling [56] and not surprisingly, activating mutations (commonly found SHP-2 E76K mutant) were identified in AML [57, 58]. Phosphatase PTEN negatively regulates PI3K signaling downstream of FLT3 receptor and is also mutated, though rarely in AML [59]. Several recent findings claim a role for deregulated gene expression of dual specificity phosphatases such as PRL2, PRL3 and DUSP6 in AML cases with and without FLT3 mutation [60-62]. Recently STS1 and STS2 (also known as UBASH3B and UBASH3A), which belong to a PTP subfamily with histidine-based catalysis [54], were identified to be directly regulating the FLT3 receptor tyrosine phosphorylation in hematopoietic stem cells [63]. However, their potential role in regulating constitutively active FLT3-ITD phosphorylation or FLT3 signaling in AML is yet unknown. Other examples include the transmembrane PTP PTPRD (PTPδ), which is downregulated by promoter methylation and may be a tumor suppressor in pediatric AML [64], and CDC25, which is mutated in familial platelet disorder with predisposition to AML [65].

PTPs can modulate signal transduction in many ways, both negatively and positively. For example, they prevent the non-specific activation of PTKs, e.g. by averting the ligand-independent activation of RTKs. In other contexts, PTPs can promote signaling by activation of SRC family kinases or of the RAS pathway [55]. PTP activity is regulated by several mechanisms [66] and one such regulatory process is the reversible oxidation of the catalytic cysteine by ROS. H_2O_2 is considered an important ROS species in the PTP oxidation process. Upon oxidation, the active-site thiol moiety (-SH) is converted to a sulfenyl moiety (-SOH), which further reacts to more stable reaction products in intramolecular reactions, like sulfenylamides or disulfides. The widely presumed role of H_2O_2 in PTP oxidation may in fact be indirect [3] and other oxidants, such as lipid peroxides, can also effectively oxidize PTPs [67, 68]. PTP oxidation is typically transient, and reduction back to the active state is accomplished by interaction with cellular antioxidants like GSH or thioredoxin [67]. Reversible PTP inactivation facilitates the efficient RTK signal transduction in the cells upon ligand/growth factor stimulation [1]. Emerging reports claim, however, that PTPs are also important targets of pathologically generated ROS and that in such circumstances ROS mediated PTP inactivation could contribute to diseases like cancer. In support that such processes play a role in leukemia, an early study showed that high ROS levels in BCR-ABL transformed cells were associated with low levels of overall PTP activity, and treatment with antioxidants reverted these effects [15]. As outlined above, apart from BCR-ABL, also other myeloid leukemia-specific PTK oncoproteins, JAK2 V617F and FLT3-ITD, cause constitutive formation of elevated levels of ROS and their possible consequences

for PTP deregulation deserve attention. The transmembrane PTP PTPRJ (also known as DEP-1, and CD148) was previously identified as *bona fide* PTP negatively regulating FLT3 receptor signaling in myeloid cells [69]. DEP-1 regulates FL ligand-induced FLT3 receptor signaling by associating with [70] and dephosphorylating FLT3 directly, thereby attenuating the activation of FLT3. When the role of DEP-1 for regulation of the FLT3-ITD oncoprotein was analyzed, DEP-1 was discovered to be oxidized and partially inactivated due to high levels of sustained ROS generation leading to elevated FLT3 activity and promotion of downstream signaling pathways, including STAT5 and RAS/ ERK1/2 activation, causally contributing to cellular transformation [27]. Investigating the relevant ROS sources it could recently be convincingly shown that NOX4 mRNA and protein expression are elevated in FLT3-ITD positive AML cells and that NOX4 expression is directly transcriptionally regulated by STAT5 [28]. The *NOX4* promoter possesses STAT binding elements, and STAT5 was demonstrated by ChIP assays to bind to these elements in a FLT3-ITD dependent manner. General interference with ROS formation by different means, downregulation of NOX4 with RNAi, or treatments with potential small molecule NOX4 inhibitors caused a pronounced decrease in ROS levels, rescued DEP-1 PTP activity, and attenuated transforming FLT3-ITD-driven signaling and cell transformation *in vitro* and *in vivo*. Double depletion of DEP-1 and NOX4 partially rescued the effect of NOX4 depletion on transformation *in vitro*, suggesting that DEP-1 reactivation is essential for the inhibitory effect of NOX4 depletion. Interestingly, murine hematopoietic stem cells transduced with a combination of FLT3-ITD with other potent oncogenic drivers (Hoxa9/Meis1, or MLL-AF9) and with genetic inactivation or downregulation of *Nox4*, did not grow in absence of cytokines *in vitro*, and were impaired in their capacity to elicit a myeloproliferative disease in sublethally irradiated recipient mice *in vivo*, respectively [28]. These findings revealed an important role played by NOX4 dependent ROS formation for oxidation of DEP-1, a *bonafide* PTP of FLT3 as a transforming event in FLT3-ITD harboring aggressive AMLs (Figure 2). It will be interesting to know whether NOX4 dependent oxidative inactivation of DEP-1 is a selective mechanism or reflects a more general attenuation of PTPs in FLT3-ITD cells. While NOX4 may indeed be of interest as a therapeutic target in FLT3-ITD subtype AMLs, there are still several other potential sources of ROS formation, whose investigation is warranted.

ROS-mediated DNA damage and potential implications for leukemia biology

Genomic instability has been suggested to be the main cause of genetic diversity in cancer [71, 72]. Also disease progression in leukemia is associated with the accumulation of multiple mutated genes, resulting in resistance to apoptosis, abnormal cell growth and a block in differentiation [43, 73]. To explain the multiple mutations, the leukemic cells must likely acquire some form of genetic instability. Increasing evidence has shown that an increase in ROS formation, which is

associated with an increased DNA damage, may be important in this context. Oxidative DNA damage can cause a wide range of DNA alterations such as base pair mutations, insertions and deletions [74]. DSBs are one of the most dangerous lesions resulting in translocations and deletions. Alternative mechanisms involved in the generation of genomic instability include unfaithful or insufficient repair of DNA damage [75]. There are two DNA repair systems responsible for DNA dsb repair: a precise homologous recombination and a less precise non-homologous end-joining (NHEJ). Increased activity of the unfaithful AEJ repair pathway and down-regulation of the faithful NHEJ pathway were found associated with FLT3-ITD and BCR-ABL oncogenic signaling [23, 76, 77]. For example, an earlier study revealed that inhibition of FLT3-ITD using PKC412 resulted in significant decrease in dsbs and non- homologous repair of DNA damage. On the other hand, PKC412 had no effect on dsbs or the DNA repair pathways in FLT3-WT expressing cells [78], showing the importance and involvement of FLT3-ITD oncogene in genomic instability. It was also shown that increased repair of FLT3-ITD stimulated DNA damage contributes to drug resistance, which coincides with the high relapse rate associated with FLT3-ITD expressing AML cases [78]. The BCR/ABL mutation in CML is involved in a cycle of genomic instability similar to the FLT3-ITD mutation. The oncogenic effects of BCR/ABL cause increased levels of ROS production leading to enhanced DNA damage and compromised DNA repair [79]. Not only levels of DNA damage are much higher in BCR/ABL transformed cells compared to non-transformed cells, also the rate of DNA repair by unfaithful end joining systems is much higher. Importantly, the resulting accumulation of DNA damage and genetic abnormalities contributes to resistance against drugs that are commonly used in the treatment of CML including Imatinib [80, 81].

As outlined above, FLT3-ITD expressing cells have been shown to generate increased levels of ROS. $p22^{phox}$ and $p22^{phox}$ dependent NOX isoforms, particularly NOX4, have been shown to be the primary source of ROS in FLT3-ITD expressing cells [24, 28]. Recently, further research has therefore been carried out to investigate the specific pathways leading to ROS formation and ROS-mediated DNA damage and genomic instability in FLT3-ITD positive AML cells [26]. MV4-11 cells, a human AML-derived cell line with endogenous FLT3-ITD, and 32D cells, a murine immortalized myelobast-like cell line stably transfected with FLT3-ITD or FLT3-WT expressing plasmids, were employed in these studies. Inhibition of FLT3-ITD, NOX and $p22^{phox}$ (by siRNA) in MV4-11 cells resulted in a significant decrease in nuclear H_2O_2 measured with Nuclear Peroxy Emerald 1 (NucPE1). NOX4 and $p22^{phox}$ were shown to co-localize in the nucleus, thus reinforcing that nuclear NOX activity may contribute to genomic instability in AML [26]. FLT3-ITD expressing 32D cells exhibited a 100% increase in endogenous H_2O_2 compared to FLT-WT expressing cells as quantified by flow cytometry using the H_2O_2 specific probe Peroxy Orange 1 (PO1), and a 25% increase in nuclear H_2O_2. There was, however, no significant difference in mitochondrial generated ROS between FLT3-ITD- and FLT3-WT-expressing cells. FLT3-ITD mediated DNA damage was

characterized using two different readouts: Phosphorylated histone H2AX (γH2AX), one of the most widely used marker for detecting DNA dsbs [82], and 8-hydroxy-2' deoxyguanosine (8-OHdG), the predominant form of ROS-induced DNA lesion, also widely used as a marker of oxidative stress [83]. MV4-11 cells showed a 50% increase in dsbs compared to the FLT3-WT expressing AML cell line HL-60. In 32D cells, similar results were obtained. There was a 75% increase in levels of dsbs in 32D cells with FLT3-ITD compared to 32D cells with FLT3-WT. Moreover, in FLT3-ITD expressing 32D cells 100% higher levels of the oxidative stress marker 8-OHdG were detectable as compared to FLT3-WT expressing cells [26]. Knockdown of $p22^{phox}$ and thereby inhibition of $p22^{phox}$ dependent NOXs in MV4-11 cells resulted in a 30% decrease in the number of dsbs and DNA oxidation. Thus, in FLT3-ITD expressing cells $p22^{phox}$ is necessary for NOX-generated ROS to oxidatively damage DNA. Conversely, $p22^{phox}$ knockdown in FLT3-WT expressing cells showed no effect on endogenous H_2O_2 and no alterations in dsbs. However, when 32D FLT3-WT expressing cells were stimulated with the FLT3 ligand FL, an increase in $p22^{phox}$ protein expression was observed, and concurrently a 40% increase in endogenous H_2O_2 and a 20% increase in nuclear H_2O_2. The increase in $p22^{phox}$ protein expression coincided with a 50% increase in the number of dsbs, demonstrating the DNA damaging capacity of also H_2O_2 produced downstream of ligand-stimulated FLT3-WT. The possible contribution of individual NOXs in FLT3-ITD expressing 32D cells to ROS formation and dsbs was investigated by siRNA experiments. NOX4 knockdown had the largest effect resulting in a 30% decrease in endogenous H_2O_2 levels and dsbs. NOX2 knockdown resulted in a 20% decrease in endogenous H_2O_2 and a 30% decrease in dsbs. In contrast, NOX1 knockdown resulted only in a marginal decrease in both H_2O_2 and DNA damage. Taken together, these data identified a $p22^{phox}$ and NOX2/4 axis for ROS formation in FLT3-ITD expressing cells causing DNA damage and genetic instability [26] (Figure 3).

Unfortunately, ROS-mediated damage in AML and CML has major implications in the treatment of leukemia. It is increasingly more difficult to treat leukemia due to the accumulation in genetic abnormalities leading to resistance to protein tyrosine kinases inhibitors, for example, PKC412 and imatinib and further progression of the malignancy (Fig. 3).

Future directions

Despite the significant advances in understanding mechanisms of ROS formation in myeloid leukemia, notably downstream of the AML oncoprotein FLT3-ITD as outlined above, there are obviously many open questions. For example, more work appears warranted with respect to the topology of NOX-mediated ROS formation in the leukemic cells. H_2O_2, which may directly oxidize targets such as PTP molecules, is a stable molecule but still short-lived in cells due to very efficient cellular antioxidants. Therefore close proximity of target and H_2O_2 source may be essential for

interaction [2]. While nuclear p22^{phox} and NOX4 may be important for eliciting DNA damage [26], the topological relationships of NOX4 and DEP-1/PTPRJ remain to be assessed. It appears also likely that in FLT3-ITD transformed cells, in addition to DEP-1/PTPRJ, other signaling molecules may be modified by oxidation such as other PTPs, protein kinases, or transcriptional regulators thereby contributing to cell transformation. Generic methods to detect oxidative protein modifications [84] may be helpful in identifying these targets. More work will also be needed to further establish the putative causal role of ROS formation for the biology of myeloid leukemia. For example, mouse strains with constitutive or conditional deficiency of NOX enzymes and several regulators are available [85] and may be investigated in transplantation-based or transgenic leukemia models. These studies may help in establishing components in the pathways for ROS formation in cells of myeloid leukemia as candidate drug targets. Inhibition of AML-cell proliferation and attenuation of the development of a FLT3-ITD driven myeloproliferative disease in mice by compounds reported to inhibit NOX4 have been recent promising findings [28]. However, many currently available compounds for interference with ROS formation are not entirely specific or controversial with respect to their mechanism of action [86]. Given the importance of NOX mediated ROS formation not only in leukemia but many other pathological contexts, the improvement of compounds can be expected and their testing in leukemia models will be an exciting perspective.

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Figure legends

Figure 1. **Reactive oxygen (ROS) species, their origins, and cellular systems involved in ROS metabolism.** Major ROS sources are the mitochondria and NADPH oxidases, and several oxidases/peroxidases also contribute to ROS formation. Superoxide anions, hydrogen peroxide, lipid peroxides, hypochloride, and the hydroxyl radical can oxidize and thereby modify cellular macromolecules. This can serve essential signaling functions ("green" range), but also cause deleterious effects ("red" range) designated oxidative stress, potentially leading to cell death. Several enzyme systems can modify the formed ROS, have antioxidant activity for preventing damage and reverting macromolecule oxidations (right part). NADPH and GSH are required for efficient antioxidant responses. The KEAP-NRF2 system is a master regulator of genes for antioxidant response.

Figure 2. **Role of ROS formation in leukemic cell transformation by the oncoprotein FLT3-ITD.** FMS-like tyrosine kinase 3 (FLT3) with internal tandem duplications (FLT3-ITD) causes elevated ROS levels in cells of Acute Myeloid Leukemia (AML). This involves activation of signal transducer and activator of transcription 5 (STAT5), which can directly bind to the promoter of NADPH oxidase 4 (NOX4), leading to elevated transcription. Increased NOX4 levels cause elevated formation of ROS, which oxidize the catalytic cysteine of density enhanced phosphatase-1 (DEP-1; a transmembrane protein-tyrosine phosphatase, also designated PTPRJ or CD148). In contrast to its activity in normal cells, the oxidized and thereby (reversibly) inactivated DEP-1 can no longer dephosphorylate FLT3-ITD, enabling elevated signal transduction and promoting cell transformation.

Figure 3. **Oncoprotein-driven ROS formation in myeloid cells causes DNA damage.** FLT3-ITD but also ligand-activated FLT3 or the BCR-ABL oncoprotein can drive oxidative DNA damage through a signaling chain involving AKT activation, elevated expression of $p22^{phox}$, and activation of $p22^{phox}$-interacting NADPH oxidases. DNA damage, involving DNA oxidation and generation of double-strand breaks, contributes to genetic instability and the accumulation of mutations associated with aggressive phenotypes, drug resistance and relapse.

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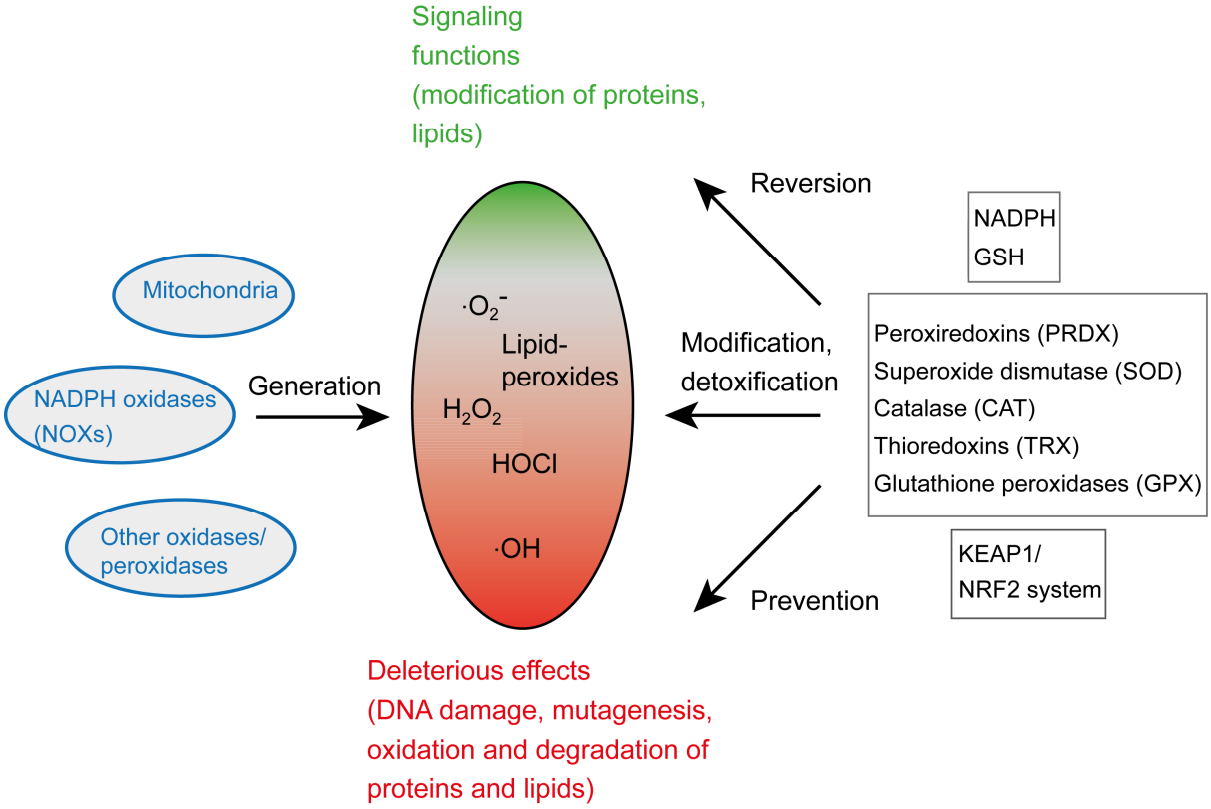

Figure 1

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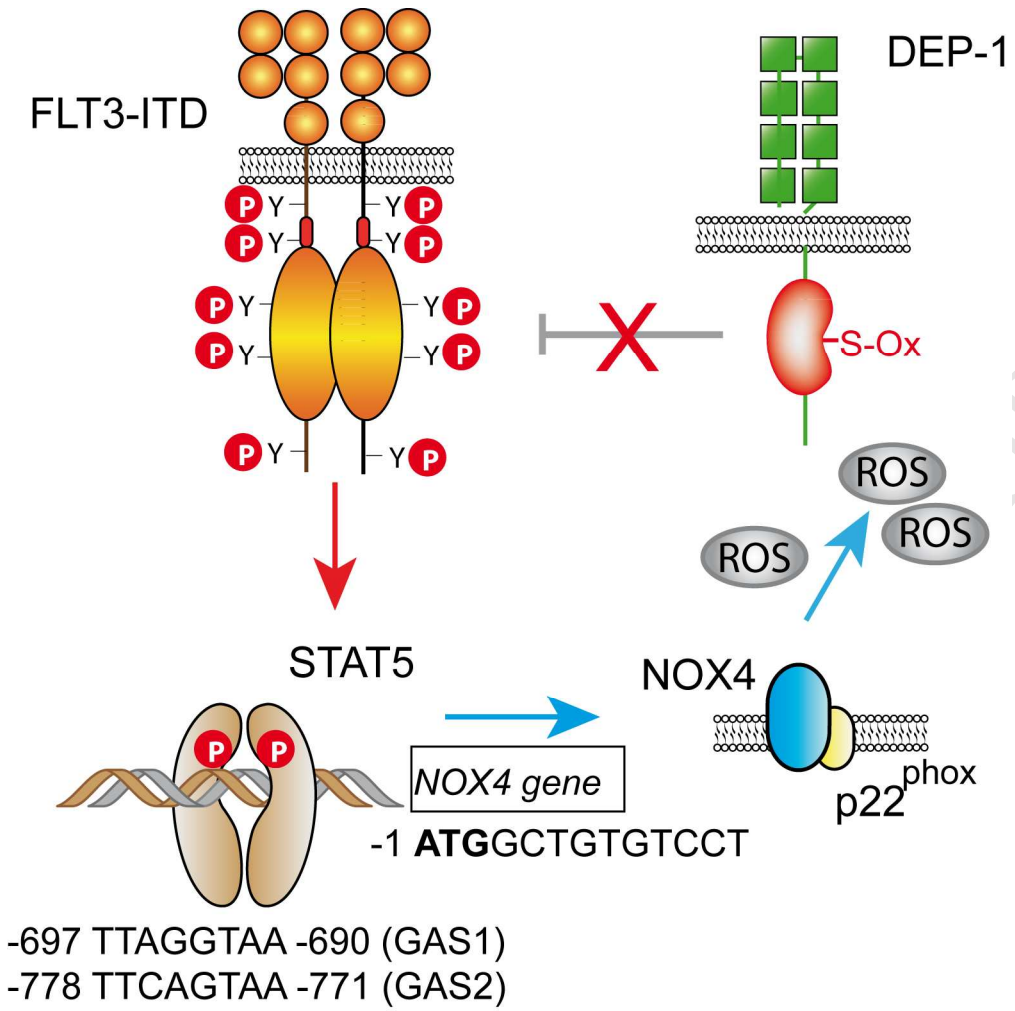
FLT3-ITD
DEP-1
S-Ox
ROS
ROS
ROS
STAT5
NOX4
NOX4 gene
p22phox
-1 ATGGCTGTGTCCT
-697 TTAGGTAA -690 (GAS1)
-778 TTCAGTAA -771 (GAS2)

Figure 2

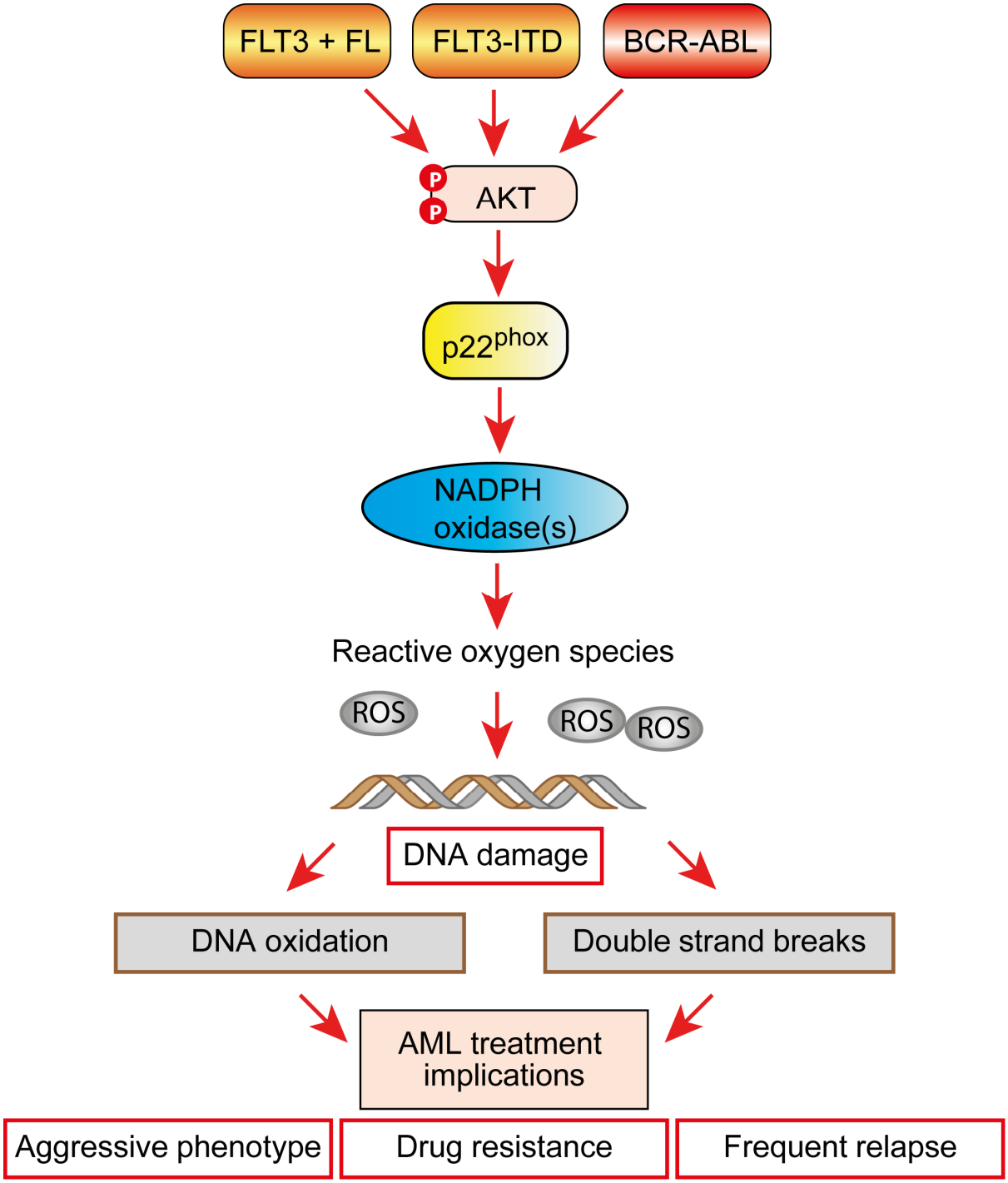
FLT3 + FL
FLT3-ITD
BCR-ABL
P
P
AKT
p22phox
NADPH oxidase(s)
Reactive oxygen species
ROS
ROS
ROS
DNA damage
DNA oxidation
Double strand breaks
AML treatment implications
Aggressive phenotype
Drug resistance
Frequent relapse

Figure 3