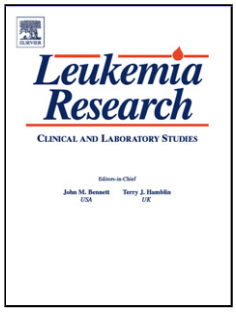

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Subcellular localization of the FLT3-ITD oncogene plays a significant role in the production of NOX- and $p22^{phox}$-derived reactive oxygen species in acute myeloid leukemia.

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Running title: Subcellular localization of FLT3-ITD and ROS generation.

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Highlights

- Tunicamycin and Brefeldin A, induce ER retention of FLT3-ITD.
- ER retention of FLT3-ITD results in post-translational modification of $p22^{phox}$ and NOX4.
- NOX-generated ROS contribute to total pro-survival ROS in AML.
- AKT pathway is vital for FLT3-ITD at the plasma membrane oncogenic effects.

Abstract

Internal tandem duplication of the juxtamembrane domain of FMS-like tyrosine kinase 3 (FLT3-ITD) receptor is the most prevalent FLT3 mutation accounting for 20% of acute myeloid leukemia (AML) patients. FLT3-ITD mutation results in ligand-independent constitutive activation of the receptor at the plasma membrane and 'impaired trafficking' of the receptor in compartments of the endomembrane system, such as the endoplasmic reticulum (ER). FLT3-ITD expressing cells have been shown to generate increased levels of reactive oxygen species (ROS), in particular NADPH oxidase (NOX)-generated ROS which act as pro-survival signals. The purpose of this study is to investigate FLT3-ITD production of ROS at the plasma membrane and ER in the FLT3-ITD expressing AML cell line MV4-11. Receptor trafficking inhibitors; Tunicamycin and Brefeldin A induce ER retention of FLT3-ITD, resulting in a decrease in protein expression of NOX4 and its partner protein $p22^{phox}$, thus demonstrating the critical importance of FLT3-ITD localization for the generation of pro-survival ROS. NOX-generated ROS contribute to total endogenous hydrogen peroxide (H_2O_2) in AML as quantified by flow cytometry using the cell-permeable H_2O_2-probe Peroxy Orange 1 (PO1). We found that PI3K/AKT signaling only occurs when FLT3-ITD is expressed at the plasma membrane and is required for the production of NOX-generated ROS. ER retention of FLT3-ITD resulted in NOX4 deglycosylation and $p22^{phox}$ protein degradation.

The abbreviations used are : FLT3, FMS-like tyrosine kinase 3 receptor; FLT3-ITD, FLT3-internal tandem duplication; FLT3-WT, FLT3- wild type; NOX, NADPH oxidase; $p22^{phox}$, p22 phagocyte

oxidase; ROS, reactive oxygen species; AML, acute myeloid leukemia; ER, endoplasmic reticulum; H_2O_2, hydrogen peroxide; PO1, peroxy orange 1; RTK, receptor tyrosine kinase; COX, cyclooxygenase; DUOX, dual oxidase; DMSO, dimethylsulfoxide.

Keywords:

Acute myeloid leukemia, FLT3-ITD, oncogene, NADPH oxidase, $p22^{phox}$, pro-survival reactive oxygen species.

1. Introduction

Constitutively active mutations in receptor tyrosine kinases (RTKs) are frequently observed in human cancers (1, 2). FMS-like tyrosine kinase 3 (FLT3) is a type III RTK expressed in approximately 90% of acute myeloid leukemia (AML) cases and regulates early steps of hematopoiesis (3, 4). Internal tandem duplication (ITD) of the juxtamembrane domain is the most prevalent mutation of FLT3 present in 20% of AML patients, resulting in ligand-independent constitutive activation of the receptor at the plasma membrane and impaired trafficking of the receptor in compartments of the biosynthetic route, such as the endoplasmic reticulum (ER) (4-8). AML patients with FLT3-ITD mutation have a poor prognosis (9, 10). Ligand-independent constitutive activation of FLT3 stimulates autophosphorylation of the receptor and downstream signaling pathways including PI3K/AKT, ERK and STAT5 resulting in abnormal cell growth, resistance to apoptosis and differentiation block (11-14). Our group demonstrated that cells expressing FLT3-ITD produce higher levels of pro-survival reactive oxygen species (ROS) in comparison to FLT3-wild type (15-18).

Increased production of ROS has been linked to various pathophysiological states including leukemia (19). Little is known about how FLT3-ITD generates such a stress. NADPH oxidases (NOXs) are one of the known sources of ROS in FLT3-ITD expressing cells (18). There are seven NOX isoforms NOX1-5 and dual oxidase 1-2 (DUOX1-2), varying in structure, subcellular localization, biochemical characteristics and regulatory subunit requirements ($p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and Rac1/2). NOX1-4 require $p22^{phox}$ to produce functionally active NOX (20, 21). NOX2 and NOX4 have been shown

previously to be expressed in leukemia (22, 23). Other sources of ROS include mitochondrial ROS, cyclooxygenase (COX), xanthine oxidase, cytochrome p450 enzymes and lipooxygenases (24-26).

Previous studies have looked at the molecular mechanisms through which FLT3-ITD initiates aberrant signaling of pro-survival pathways (PI3K, ERK, STAT5) at the plasma membrane and ER FLT3 using receptor trafficking inhibitors, Tunicamycin and Brefeldin A (27). However, the molecular mechanism describing how mislocalized activation of FLT3-ITD and the aberrant signaling of these downstream pathways leads to the production of ROS and sources of ROS remains unknown. To analyze the role of the cellular localization of FLT3-ITD in the generation of ROS and their signaling outcomes we utilized a panel of inhibitors of ROS production, pro-survival pathways, FLT3-ITD and the 20S proteasome alongside ROS specific antibodies and probes. Experiments were carried out in the FLT3-ITD AML expressing MV4-11 cell line.

Receptor trafficking inhibitors, Tunicamycin and Brefeldin A, induce ER retention of FLT3-ITD, resulting in a decrease in protein expression of NOX4 and its partner protein $p22^{phox}$ as a result of NOX4 deglycosylation and $p22^{phox}$ protein degradation. This demonstrates the critical importance of FLT3-ITD localization for the generation of pro-survival ROS. Finally, we found that PI3K/AKT signaling only occurs when FLT3-ITD is expressed at the plasma membrane.

2. Materials and Methods

2.1. Cell culture and treatments- The human leukemic cell line MV4-11 (homozygous for the FLT3-ITD mutation) was purchased from DSMZ (Braunschweig, Germany) (DSMZ no.: ACC 102). The cell line was maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO_2.

Tunicamycin (5 µg/ml; Sigma) and Brefeldin A (10 µg/ml; Sigma) induced ER retention of FLT3-ITD overnight. FLT3-ITD was inhibited using PKC412 (50 nM, 200 nM and 250 nM; Tocris) for up to 24 hours. Cyclooxygenase inhibition was achieved using Diclofenac (Sigma) for 2 hours at indicated concentrations. Mitochondrial ROS inhibition was achieved using Rotenone (Tocris) for 1 hour at indicated concentrations. Inhibition of AKT was via LY294002 (50 µM; Cell Signaling) overnight.

GSK3β inhibition was via SB216763 (5 μM; Tocris) overnight. Inhibition of the 20S proteasome was via Lactacystin (5 μM; Merck Millipore) overnight. Dimethylsulfoxide (DMSO) and Ethanol (Brefeldin A) were used as vehicle controls.

2.2. Antibodies- Primary antibodies used for immunoblotting and immunofluorescence included AKT (#9272), phospho-AKT (Ser 473; #9271), GSK3β (#9315), phospho-GSK3β (Ser9; #9336, all from Cell Signaling Technology), Flt-3 (#SC480), $p22^{phox}$ (#SC20781; both from Santa Cruz Biotechnology), NOX4 (#NB110-58849; Novus Biologicals), KDEL (ab12223; Abcam) and β-Actin (#A5441; Sigma).

Microscopy- Mounted slides were viewed on a Leica DM LB2 microscope with Nikon Digital Sight DS-U2 camera, using 40x and 100x objectives. Images were taken using the software NIS-Elements version 3.0, Nikon, Japan.

2.3. Immunofluorescence- MV4-11 cells were cytospun onto glass slides at 500 rpm for 2 mins. Cells were fixed for 10mins using 4% PFA/PBS and permeabilized with 0.2% Triton-X-100 for 5 mins at room temperature. Following washes, the cells were incubated with 50 μl of appropriate primary antibody diluted in 5% FBS/PBS for 1 hour at room temperature in a humidity chamber. Following washes, secondary antibodies conjugated to Alexafluor488 or 594 diluted in 5% FBS/PBS were added for 1 hour in the dark. Slides were washed and mounted using Mowiol. Secondary antibody only controls were used.

2.4. Labelling of the plasma membrane FLT3-ITD– Live cell immunofluorescence of the plasma membrane FLT3-ITD of MV4-11 cells was performed on ice. Following centrifugation at 1,000 rpm for 5 mins, cells were incubated with anti-FLT3 primary antibody diluted in 5% FBS/PBS containing ~0.01% Sodium Azide for 1 hour. Following washes, the cells were incubated with anti-rabbit Alexafluor488 diluted in 5% FBS/PBS for 1 hour. Secondary antibody only controls were used. Cells were washed and viewed under the microscope in ice cold PBS and quantified by flow cytometry using FACSCalibur (BD Biosciences, Europe) and Cellquest Pro software (Becton Dickinson). The mean fluorescent intensity of 10,000 events was determined.

2.5.Measurement of intracellular H_2O_2- The measurement of intracellular H_2O_2 procedure was performed as previously described in (18). Briefly, following treatments, total intracellular H_2O_2 was measured by incubating cells with 5 μM of cell-permeable H_2O_2-probe PO1 (Tocris) for 1 hour at 37°C in the dark. Cells were viewed under the microscope and quantified by flow cytometry using FACSCalibur (BD Biosciences, Europe) and Cellquest Pro software (Becton Dickinson). The mean fluorescent intensity of 10,000 events was determined.

2.6. Western Blotting- The immunoblotting procedure was carried out as previously described (17). Briefly, the cells were lysed in RIPA buffer [Tris–HCl (50mM; pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, NaCl (150mM), EGTA (1mM), sodium orthovanadate (1mM), sodium fluoride (1mM), cocktail protease inhibitors (Roche, Welwyn, Hertforshire, UK) and phenylmethanesulfonyl fluoride (1mM)] for 35-45 minutes on ice, followed by centrifugation at 14,000 rpm for 15 mins. The protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Hemel Hempstead, UK). Equivalent amounts of protein were resolved using SDS-PAGE followed by transfer to nitrocellulose membrane and incubated overnight with primary antibodies. The membrane was incubated in secondary antibody coupled with Alexa Fluor 680 or 800. The signal was detected with an Odyssey infrared imaging system (LI-COR Biosciences).

Statistical Analysis- The results are expressed as a percentage of control, set to 100%. Values are representative of mean ± SD and are representative of three independent experiments. Statistical significance was analyzed by Student's t test with $p<0.05$ representing a significant result.

3. Results

3.1. Receptor trafficking inhibitors, Tunicamycin and Brefeldin A, induce ER retention of FLT3-ITD.

MV4-11 cell line is a well-established model of AML that expresses homozygous FLT3-ITD. FLT3-ITD is the most prevalent FLT3 mutation resulting in ligand-independent constitutive activation of the receptor at the plasma membrane and impaired trafficking of the receptor in intracellular compartments, such as the ER. Cells were treated with Tunicamycin and Brefeldin A to inhibit glycosylation of FLT3-

ITD resulting in impaired trafficking of FLT3-ITD receptor to the plasma membrane. Tunicamycin prevents glycosylation of plasma membrane receptor, retaining receptors in intracellular compartments by blocking the formation of protein N-glycosidic linkages and consequently blocking the first step of glycoprotein synthesis (28, 29). Brefeldin A inhibits mature and complex glycosylation of plasma membrane receptors through inhibition of guanine-nucleotide exchange factors that are required for ADP-ribosylation factor (ARF) GTPases, which results in disruption of the structure and function of the Golgi apparatus (30). We show that treatment of MV-411 cells with Tunicamycin and Brefeldin A result in retention of FLT3-ITD in a compartment of the biosynthetic pathway such as the ER (KDEL; Figure1A-C). We demonstrated that both of these treatments resulted in a decrease of 50-60% of FLT3-ITD expression at the plasma membrane when compared to vehicle control (Tunicamycin; DMSO and Brefeldin A; Ethanol) as quantified by flow cytometry (Figure 1D-H).

3.2. Impaired trafficking of FLT3-ITD receptor to the plasma membrane results in a decrease in protein expression of NOX4 and its partner protein p22^{phox}.

FLT3-ITD expressing cell lines have been shown to express higher levels of endogenous ROS in comparison to FLT3-WT receptor cell lines (15, 18). The mechanism in which FLT3-ITD driven pro-survival ROS leads to the aggressive form of AML remains unknown. We examined the effect of ER retention of FLT3-ITD on protein expression of p22^{phox} and NOX4. p22^{phox} and NOX4 protein expression decreased significantly following treatment with both receptor trafficking inhibitors, Tunicamycin and Brefeldin A (Figure2A and B). This suggests that for FLT3-ITD to produce NOX-generated pro-survival ROS it has to be located at the plasma membrane. This is supported by previous work from our laboratory showing that ligand-stimulated FLT3-WT results in an increase in p22^{phox} expression (18).

3.3. NOX-generated ROS contribute to total pro-survival ROS in AML.

Our group has shown previously in that knocking down $p22^{phox}$ resulted in almost 20% decrease in total and nuclear H_2O_2 and knocking down NOX4 resulted in approximately 30% decrease of total cellular H_2O_2 and 20% decrease of nuclear H_2O_2 (18). Given that ER retention of FLT3-ITD resulted in a substantial loss of $p22^{phox}$ and NOX4 protein expression we decided to investigate the effect of ER retention of FLT3-ITD on total endogenous H_2O_2 using the probe Peroxy Orange 1 (PO1). This revealed that Tunicamycin treated cells resulted in approximately 35% decrease and Brefeldin A treated cells resulted in approximately 25% decrease of total endogenous H_2O_2 as quantified by flow cytometry. (Figure 3A-D)

3.4. Mitochondrial-generated ROS contribute to total endogenous H_2O_2 in AML. Cyclooxygenase-generated ROS do not contribute to endogenous H_2O_2 in AML.

$p22^{phox}$ and NOX4 contribute to approximately 25-35% total endogenous H_2O_2 (Figure 3C and D). We investigated other potential sources of ROS that may contribute to total endogenous H_2O_2 in FLT3-ITD expressing AML. We used cyclooxygenase inhibitor, Diclofenac and mitochondrial ROS inhibitor, Rotenone and measured their effect on total endogenous H_2O_2 using PO1. Quantification by flow cytometry revealed that cyclooxygenase-generated ROS do not contribute significantly to total endogenous H_2O_2. However, inhibition of mitochondrial-generated ROS using Rotenone at high concentrations resulted in approximately 30% decrease of total endogenous H_2O_2. (Figure 3E and F)

3.5. FLT3-ITD at the plasma membrane is responsible for the activation of AKT and GSK3β pro-survival pathways.

Constitutive activation of FLT3 switches on downstream signaling pathways such as PI3K, MAPK, ERK and STAT5. We investigated the outcome of treating MV4-11 cells with Tunicamycin and Brefeldin A on AKT and GSK3β pro-survival pathways. Impaired trafficking of FLT3-ITD receptor at the plasma resulting in ER retention of FLT3-ITD revealed a decrease in pAKT and pGSK3β (Figure 4A and B). Thus, AKT and GSK3β cell signaling pathways are activated downstream of FLT3-ITD at

the plasma membrane. A decrease in total AKT was observed following treatment with the receptor trafficking inhibitors.

3.6. AKT pathway needs to be activated in order for FLT3-ITD at the plasma membrane to produce its oncogenic effects.

Given that both AKT and GSK3β are switched on downstream of ligand-independent constitutively activated FLT3-ITD receptor we investigated which signaling pathways are responsible for the aberrant production of NOX4-generated pro-survival ROS. Inhibition of AKT pathway using the inhibitor LY294002 (LY) resulted in a decrease in $p22^{phox}$ and NOX4 protein expression, whereas inhibition of GSK3β using the inhibitor SB216763 (SB) showed no effect on the protein expression of NOX4 and $p22^{phox}$ (Figure 4C). Thus, the AKT pathway needs to be phosphorylated and activated in order for FLT3-ITD at the plasma membrane to produce is oncogenic effects. The decrease in total AKT expression is result of AKT being hyper-phosphorylated leading to the aberrant signaling of FLT3-ITD. Inhibition of FLT3-ITD using PKC412, a drug in clinical trial to treat AML (31), resulted in a decrease in protein expression of $p22^{phox}$, NOX4, pAKT and pGSK3β as expected (Figure 4D).

3.7. Inhibition of FLT3-ITD cell surface expression results in proteasomal degradation of $p22^{phox}$ and deglycosylation of NOX4.

Our group has shown that inhibition of FLT3-ITD using PKC412 results in proteasomal degradation of $p22^{phox}$ by the ubiquitin proteasome pathway (17). Given that $p22^{phox}$ and NOX4 protein expression decreases significantly following treatment with Tunicamycin and Brefeldin A, we decided to investigate whether impaired trafficking of FLT3-ITD resulted in post-transcriptional regulation of $p22^{phox}$ and NOX4. To this end, we treated cells with Tunicamycin and Brefeldin A in the presence of 20S proteasome inhibitor, Lactacystin. ER retention of FLT3-ITD resulted in NOX4 deglycosylation and $p22^{phox}$ degradation by the proteasome. Inhibition of the 20S proteasome resulted in recovery of

$p22^{phox}$ protein expression (Figure 5A and B). Interestingly, this increase in $p22^{phox}$ protein expression to basal level did not coincide with an increase in endogenous H_2O_2 (Figure 5C and D).

4. Discussion

Oncogenic kinases act as drivers of ROS production in myeloid leukemia (32). Mutations in tyrosine kinases are commonly found in cancer and act as primary or secondary mediator of oncogenic signalling (1). FLT3-ITD is the most prevalent FLT3 mutation expressed in 20% of AML cases. Constitutive activation of FLT3-ITD at the plasma membrane and ER is associated with poor prognosis (33). A number of studies have investigated the function of ROS, specifically NOX4-generated ROS in AML. Our group has shown that NADPH oxidases (NOX), in particular NOX4 and $p22^{phox}$, a small membrane subunit of the NOX complex, are major sources of ROS in AML (17, 18). However, the molecular mechanism describing how mislocalized activation of FLT3-ITD and the aberrant signaling of downstream pathways (PI3K/AKT, ERK/MAPK and STAT5) leads to the production of ROS remains unknown.

NOX-derived ROS have been shown to have numerous effects in leukemia including differentiation block, cell proliferation and resistance to apoptosis (34, 35). Leukemic oncogenes have been widely documented in the regulation of the expression of the NOX family and their partner protein, $p22^{phox}$ (22, 34, 36, 37). $p22^{phox}$ is a membrane-bound protein and is an essential component required for fully functioning NOX1-4 (21). Our group demonstrated that cells expressing FLT3-ITD produce higher levels of pro-survival reactive oxygen species (ROS) in comparison to FLT3-wild type (18).

In this study, we investigated the role of trafficking of the oncogenic FLT3-ITD receptor and its effect on the production of ROS utilizing receptor trafficking inhibitors, Tunicamycin and Brefeldin A. We found that Tunicamycin and Brefeldin A cause ER retention of FLT3-ITD (Figure 1) by inhibiting the glycosylation of FLT3-ITD receptor (27). $p22^{phox}$ and NOX4 protein expression decrease significantly following ER retention of FLT3-ITD (Figure 2) suggesting that $p22^{phox}$ and NOX4 protein expression and activation is dependent on FLT3-ITD being present at the plasma membrane. Importantly, to our

knowledge, this is the first study that finds FLT3-ITD cellular organization to play an essential role in the production of NOX4-generated pro-survival ROS and $p22^{phox}$ stability.

Our group has shown that inhibition of FLT3-ITD results in post-translational regulation of $p22^{phox}$ (17). Here, we show that a decrease in $p22^{phox}$ and NOX4 protein expression following ER retention of FLT3-ITD correlates with a decrease in endogenous H_2O_2 (Figure 3). Thisdata suggests that $p22^{phox}$- and NOX4-generated H_2O_2 contribute to total endogenous H_2O_2 in FLT3-ITD AML. Also we found that mitochondrial-generated ROS contribute to endogenous H_2O_2 in FLT3-ITD expressing AML (Figure 3). Interestingly, we have shown that cyclooxygenase-generated ROS do not contribute to total pro-survival ROS in FLT3-ITD expressing AML (Figure 3).

Three major survival signaling pathways activated downstream of FLT3-ITD are PI3K/AKT, Raf/MEK/ERK1/2 and STAT5 pathways. In this study, we looked at the effect of ER retention of FLT3-ITD on PI3K/AKT and ERK pathways. As shown, both pathways are activated downstream of FLT3-ITD at the plasma membrane (Figure 4). Inhibition of both of these signaling pathways revealed that the PI3K/AKT pathway is responsible for the activation and generation of NOX4-generated ROS (Figure 4). Although ERK pathway is located downstream of FLT3-ITD, it has minimal effect on $p22^{phox}$ and NOX4 expression (Figure 4). This result demonstrated that AKT regulates $p22^{phox}$ and NOX4 expression and is responsible for the production of pro-survival ROS in FLT3-ITD expressing AML.

Tunicamycin and Brefeldin A inhibit glycosylation of many proteins. For this reason they are not suitable for the treatment of FLT3-ITD expressing AML cases. They have however previously been used to examine the effects of cellular localization of oncogenic FLT3-ITD and its effect on pro-survival signaling pathways (27). In this study, a mutant of FLT3-ITD was created that contained a deletion of the extracellular ligand-binding domain of FLT3-ITD (FLT3-ITD ΔECD). This mutation eliminated many potential sites of glycosylation, resulting in glycosylation-independent trafficking of the FLT3-ITD receptor. Inhibition of FLT3-ITD ΔECD with PKC412 resulted in a loss of pro-survival signaling, as indicated by a decrease in pERK1/2 and pAKT. Due to the mutation in glycosylation sites recognized by Tunicamycin, treatment with this inhibitor had no effect on trafficking of the receptor and pERK1/2

and pAKT levels remained high. Brefeldin A inhibits glycosylation of receptors indirectly by disruption of the structure and function of the Golgi apparatus. Treatment with Brefeldin A therefore successfully inhibited trafficking of the receptor to the plasma membrane and as a result, pERK1/2 and pAKT levels decreased. As further support, they showed that wild-type FLT3, found only at the plasma membrane, in the presence of FLT3 ligand leads to the activation of PI3K and ERK signaling (27). These findings not only support the current study, highlighting a crucial role for FLT3-ITD at the plasma membrane in stimulating pro-survival signaling, but it also endorses the use of inhibitors, such as Tunicamycin and Brefeldin A, as a method to investigate the effect of subcellular localization of FLT3-ITD on the production of pro-survival ROS.

We have demonstrated that ER retention of FLT3-ITD results in proteasomal degradation of $p22^{phox}$ (Figure 5). Receptor trafficking inhibitors, Tunicamycin and Brefeldin A inhibit glycosylation. NOX4 is glycosylated at two positions, amino acid position 133 and 230 (38). Treatment of FLT3-ITD AML expressing cells with these inhibitors results in deglycosylation of NOX4. $p22^{phox}$ protein expression was recovered following treatment with the 20S proteasome inhibitor Lactacystin (Figure 5). Interestingly, $p22^{phox}$ function was not recovered, as observed by no significant increase in endogenous H_2O_2.

In conclusion, we propose that FLT3-ITD at the plasma membrane is responsible for the activation and expression of $p22^{phox}$- and NOX4-generated pro-survival ROS in FLT3-ITD expressing AML cells in (Figure 6). $p22^{phox}$ is essential for the maintenance of pro-survival signaling in AML. For FLT3-ITD to generate its oncogenic effects it has to be located at the plasma membrane. ER retention of FLT3-ITD results in NOX4 deglycosylation and $p22^{phox}$ proteasomal degradation. This study presents FLT3-ITD at the plasma membrane as a potential therapeutic target, in preventing downstream ROS-driven oncogenic effects.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

Conceived and designed the experiments: JNM TGC JS. Performed the experiments: JNM. Analyzed the data: JNM JS TGC. Wrote the paper: JNM TGC.

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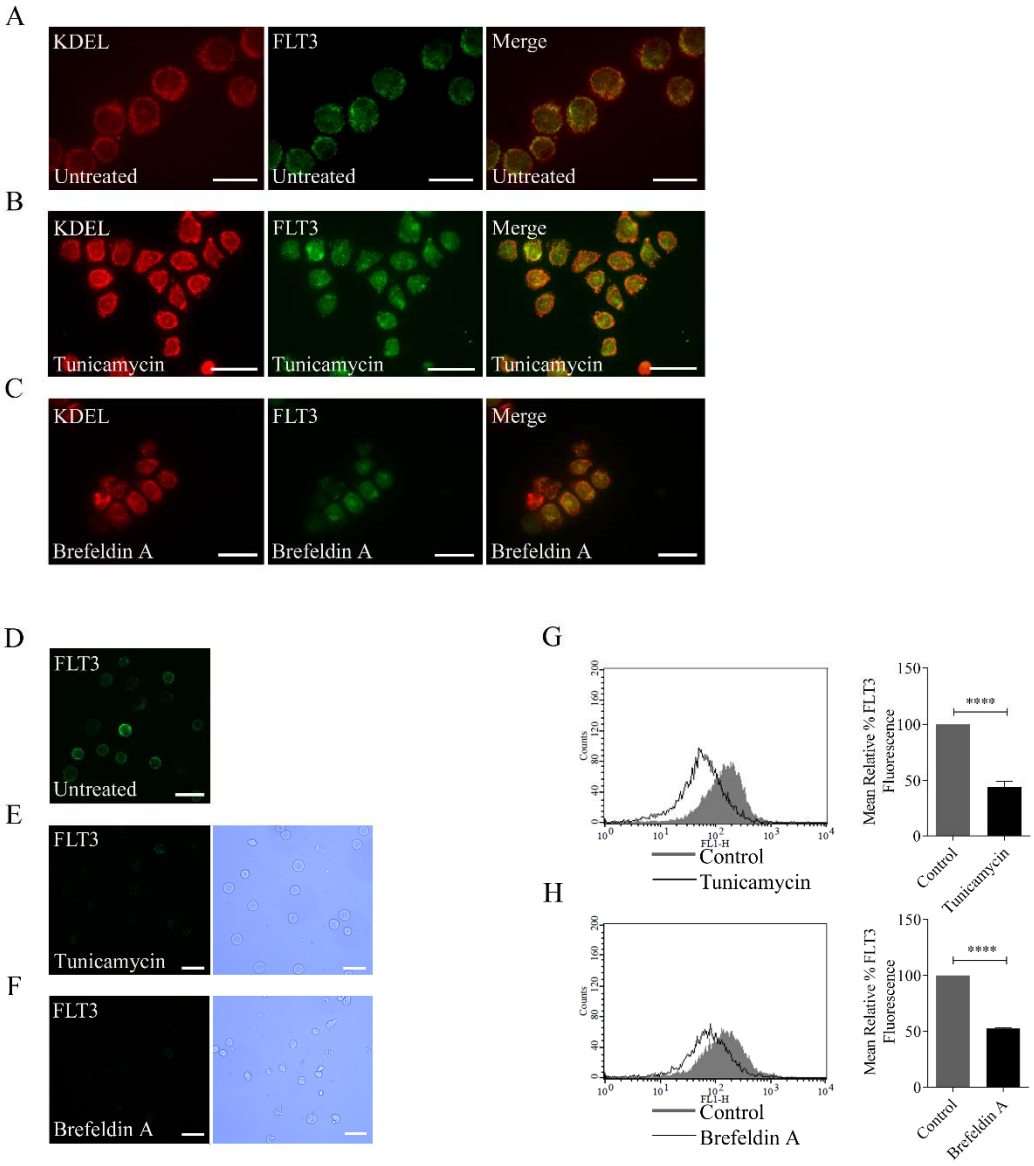

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Figure 1. Tunicamycin and Brefeldin A induce ER retention of FLT3-ITD. (A-C) Colocalization of FLT3 with ER marker KDEL in MV4-11 cell line. Untreated (A), Tunicamycin treated (5µg/ml overnight) (B) and Brefeldin A treated (10µg/ml overnight) (C). Scale bar represents 30µm. (D-F) Live cell immunofluorescence of FLT3 at the plasma membrane in MV4-11 cell line, Untreated (D), Tunicamycin treated (E) and Brefeldin A treated (F). Scale bar represents 30µm. (G and H) Flow cytometric analysis of mean relative FLT3 fluorescence at the plasma membrane in MV4-11 cell line

treated with Tunicamycin (G) and Brefeldin A (H). Bar charts show relative mean FLT3 fluorescence of treated cells expressed as % of control. Results are representative of three independent experiments. Asterisks indicate statistically significant difference (****; $p<0.0001$) as analyzed by Student t-test. Error bars represent ± SD.

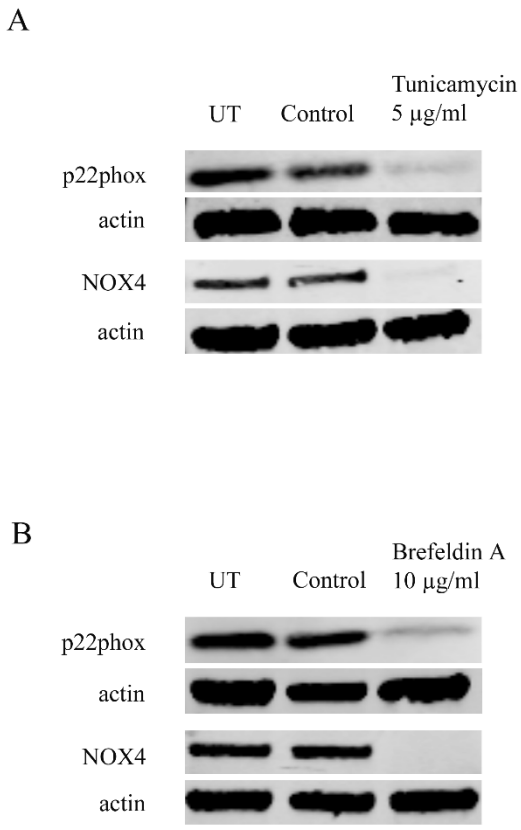

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Figure 2. Plasma membrane FLT3-ITD stabilizes $p22^{phox}$ and NOX4 expression at the protein level. (A and B) Western Blot analysis of $p22^{phox}$ and NOX4 protein expression in untreated (UT), vehicle control (control) and following treatment with Tunicamycin (5 µg/ml) overnight (A) and Brefeldin A (10 µg/ml) overnight (B). β-Actin was used as a loading control.

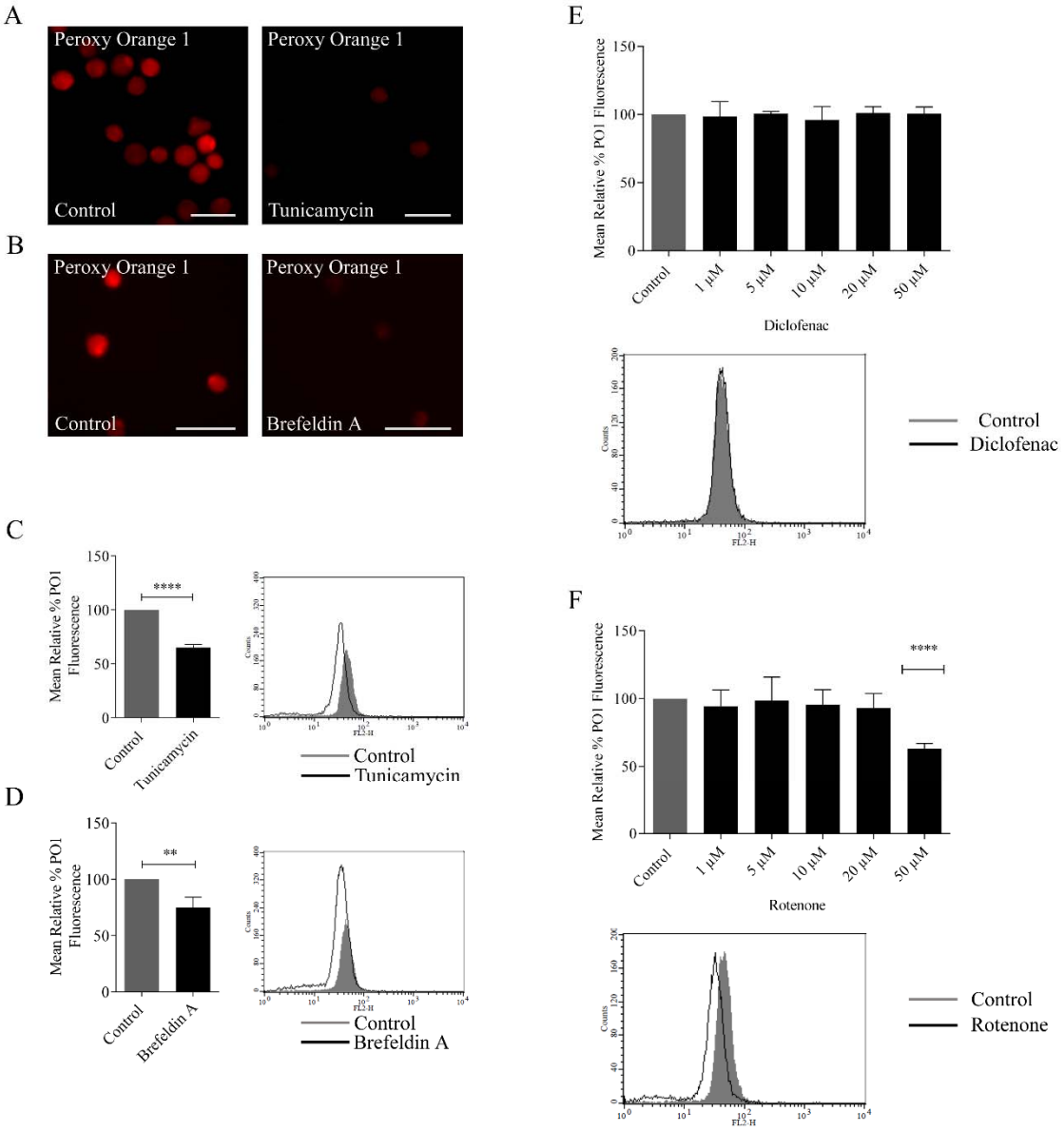

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Figure 3. NOX and mitochondrial-generated ROS contribute to total endogenous H_2O_2 in FLT3-ITD expressing cells. (A and B) Live cell immunofluorescence of total cellular H_2O_2 levels in vehicle control (control) and Tunicamycin treated (5 µg/ml overnight) (A) and Brefeldin A treated (10 µg/ml overnight) (B) treated MV4-11 cells as measured by H_2O_2-probe, Peroxy Orange 1 (PO1). Scale bar represents 30µm. (C-F) Flow cytometric analysis of mean relative PO1 fluorescence in MV4-11 cell line treated with Tunicamycin (C), Brefeldin A (D), Diclofenac for 2 hours at indicated concentrations

(E) and Rotenone for 1 hour at indicated concentrations (F). Bar charts show relative mean PO1 fluorescence of treated cells expressed as % of control. Results are representative of three independent experiments. Asterisks indicate statistically significant difference (**; $p<0.01$) and (****; $p<0.0001$) as analyzed by Student t-test. Error bars represent ± SD.

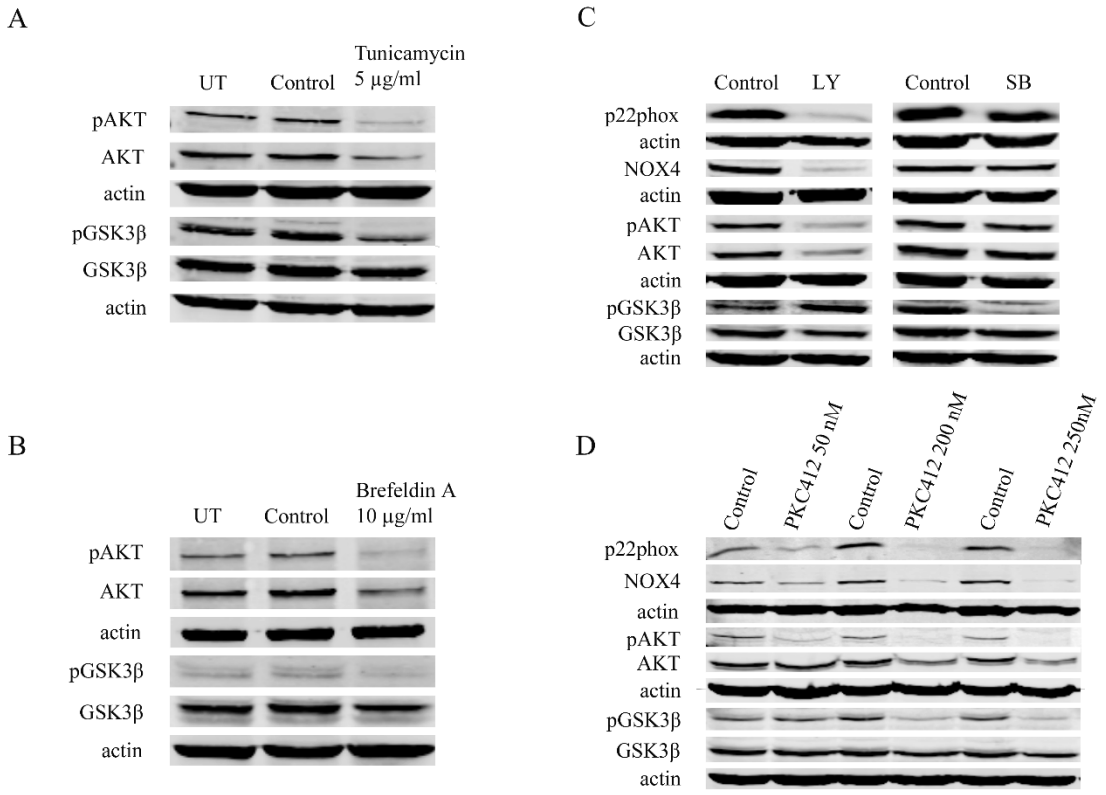

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Figure 4. Tunicamycin and Brefeldin A inhibit FLT3-ITD-induced PI3K signaling. NOX4-generated pro-survival ROS requires AKT activation. (A and B) Western blot analysis of AKT and GSK3β signaling in untreated (UT), vehicle control (control), and following treatment with Tunicamycin (5 µg/ml) overnight (A) and Brefeldin A (10 µg/ml) overnight (B). Western blot analysis of $p22^{phox}$ and NOX4 protein expression in MV4-11 cells following treatment with LY294002 (LY; 50µM) and SB216763 (SB; 5µM) overnight (C). Western blot analysis of $p22^{phox}$, NOX4, pAKT, AKT, pGSK3β and GSK3β protein expression in vehicle control (control) and PKC412 treated MV4-11 cells (D). β-Actin was used as a loading control.

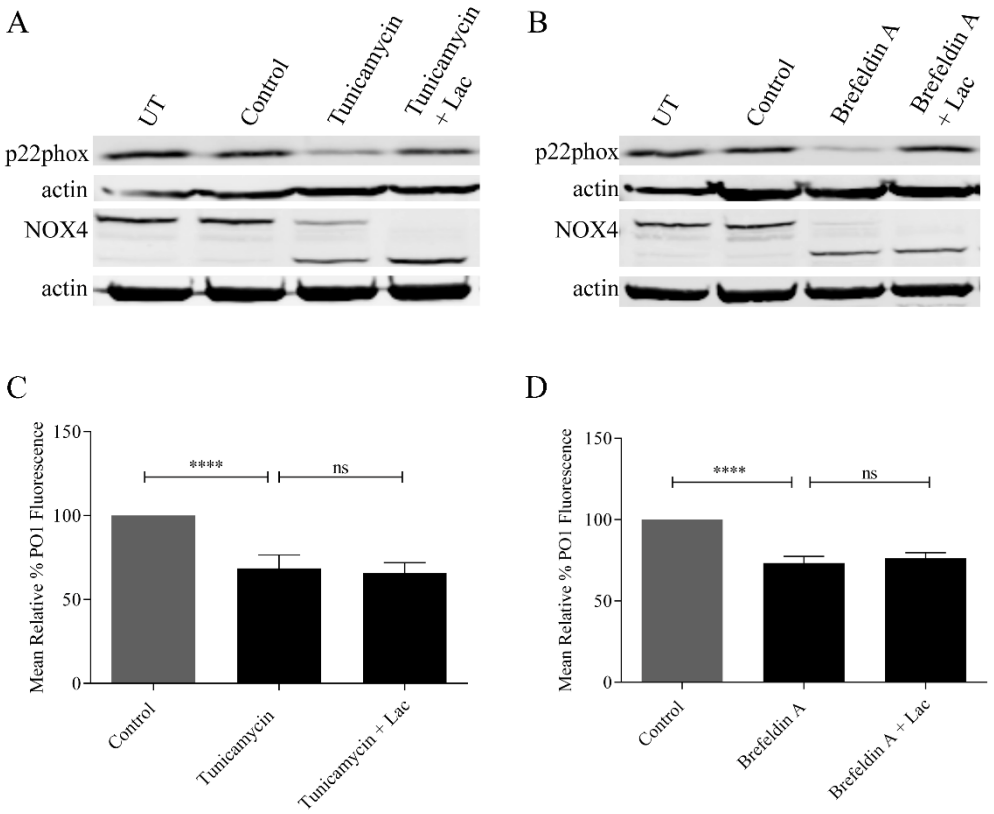

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Figure 5. Tunicamycin and Brefeldin A induce ER retention of FLT3-ITD, resulting in NOX4 deglycosylation and p22^{phox} proteasomal degradation. (A and B) Western blot analysis of p22^{phox} and NOX4 signaling in untreated (UT), vehicle control (control), and cells treated with Tunicamycin (5 μg/ml) and Lactacystin (5 μM) overnight (A) and Brefeldin A (10 μg/ml) and Lactacystin (5 μM) overnight (B). β-Actin was used as a loading control. (C and D) Flow cytometric analysis of mean relative PO1 fluorescence in MV4-11 cell line treated with vehicle control (control), and cells treated with Tunicamycin (5 μg/ml) and Lactacystin (5 μM) overnight (C) and Brefeldin A (10 μg/ml) and Lactacystin (5 μM) overnight (D). Bar charts show relative mean PO1 fluorescence of treated cells expressed as % of control. Results are representative of three independent experiments. Asterisks indicate statistically significant differences (****; $p<0.0001$) as analyzed by Student t-test. Error bars represent ± SD.

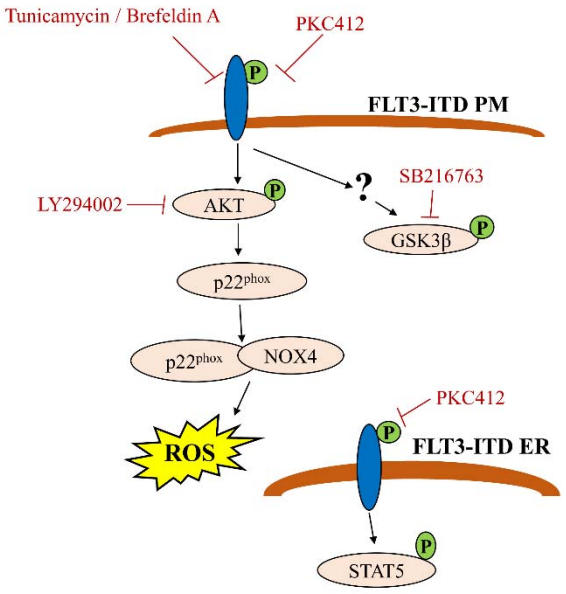

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Figure 6. A schematic of proposed mechanism in which FLT3-ITD at the plasma membrane and its downstream pro-survival pathways leads to the production of NOX4 pro-survival ROS in FLT3-ITD AML cells. FLT3-ITD at the plasma membrane is responsible for activation and phosphorylation of AKT signalling pathway and the production of $p22^{phox}$-generated H_2O_2. Inhibition of FLT3-ITD generated-ROS at the plasma membrane leads to NOX4 deglycosylation and $p22^{phox}$ proteasomal degradation.