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Inhibition of USP9X induces apoptosis in FLT3-ITD-positive AML cells cooperatively by inhibiting the mutant kinase through aggresomal translocation and inducing oxidative stress

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ABSTRACT

FLT3-ITD and FLT3-TKD are the most frequent mutations in acute myeloid leukemia (AML) with the former associated with a poor prognosis. Here we show that inhibition of the deubiquitinase USP9X by its inhibitor WP1130 or EOAI3402143 (G9) induces apoptosis preferentially in cells transformed by these mutant kinases, including FLT3-ITD-positive AML cell line MV4-11 and primary AML cells. Mechanistically, WP1130 induced aggresomal translocation of the mutant kinases, particularly FLT3-ITD in its activated and autophosphorylated conformation, to block the downstream signaling events, which was aggravated by knock down of USP9X. Moreover, USP9X physically associated with FLT3-ITD to inhibit its K63-linked polyubiquitination, while FLT3-ITD induced tyrosine phosphorylation and degradation of USP9X through the ubiquitin/proteasome pathway. WP1130 or G9 also induced oxidative stress to stimulate stress-related MAP kinase pathways and DNA damage responses to activate in cooperation with inhibition of FLT3-ITD signaling the intrinsic mitochondria-mediated apoptotic pathway, which was synergistically enhanced by BH3 mimetics and prevented by overexpression of Bcl-xL or Mcl-1. Thus, USP9X represents a promising target for novel therapies against therapy-resistant FLT3-ITD-positive AML.

1. Introduction

FLT3 is a receptor tyrosine kinase expressed on hematopoietic progenitor cells and regulates their proliferation, survival, and differentiation [1,2]. Internal tandem duplication (ITD) mutations in the juxtamembrane domain of FLT3 are the most frequent kinase mutations in AML, occurring in 25–30% of cases, and associated with a poor prognosis. Point mutations within the tyrosine kinase domain (TKD), such as the most frequent D835Y mutation, are also found in 5–10% of AML patients with an undetermined prognostic impact. FLT3-ITD as well as FLT3-TKD results in ligand-independent autophosphorylation and activation of the FLT3 receptor, leading to constitutive activation of various downstream signaling events involving the PI3K/Akt/mTOR and MEK/Erk pathways as well as STAT5 to promote cytokine-independent cell survival and proliferation [1,2]. Although several tyrosine kinase inhibitors specific for FLT3 are currently under development, clinical trials with these used as a single agent have so far shown only transient responses because of emergences of resistance mutations and through other various mechanisms in the case of FLT3-specific inhibitor quizartinib (AC-220) [3,4]. Therefore, novel strategies to target mutant FLT3 are warranted to improve the clinical outcome of AML with FLT3 mutations.

The ubiquitin system is an essential posttranslational mechanism that regulates the function, localization, and turnover of most proteins in normal as well as malignant cells [5]. The polyubiquitin chain attached to target proteins is formed via various linkage(s), which mainly determines the consequences of ubiquitinated proteins. For example, the most prevalent K48 linkage predominantly mediates proteasomal degradation, while the K63 linkage regulates protein-protein interaction involved in signal transduction, protein localization, or DNA damage repair. Protein ubiquitination is a dynamic process mediated by various E3 ubiquitin ligases and reversed by deubiquitinating enzymes (DUBs). While we and others have revealed that the c-Cbl and Cbl-b E3 ligases play important roles in ubiquitination of autophosphorylated FLT3-ITD leading to its proteasomal degradation [6,7], little is known about the DUBs regulating FLT3-ITD, except for a recent report by

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Weisberg et al. [8], in which USP10 was demonstrated to associate with FLT3-ITD to stabilize it.

Among around 100 human DUBs, USP9X plays fundamental roles in development and various cellular functions including protein trafficking and apoptosis [9]. USP9X can cleave various ubiquitin chains including K48 and K63 linkages and is known to associate with and deubiquitinate many proteins such as Mcl-1. Intriguingly, WP1130, a partially selective USP9X inhibitor, was reported to block the aberrant BCR/ABL kinase found in chronic myelogenous leukemia (CML) or cytokine-activated JAK2 by increasing K63-linked polyubiquitination to induce translocation of these tyrosine kinases to the aggresome [10–13], which is a perinuclear inclusion body containing aggregates of misfolded, ubiquitinated proteins and chaperones [14]. Thus, WP1130 induced apoptosis in CML cells. Furthermore, WP1130 or EOAI3402143 (G9), a more potent USP9X inhibitor than WP1130, has been reported to show cytotoxic effects on various solid tumors as well as hematologic malignancies including B-cell lymphoma and multiple myeloma [15–18]. However, neither the effects of these DUB inhibitors on AML cells nor the interaction between USP9X and FLT3-ITD has been reported so far.

In the present study, we find that inhibition of the deubiquitinase USP9X by its inhibitor WP1130 or G9 induces apoptosis efficiently and preferentially in cells transformed by FLT3-ITD, including FLT3-ITD-positive AML cell line MV4-11 and primary AML cells. We further demonstrate that inhibition of USP9X downregulates FLT3-ITD through aggresomal translocation and causes oxidative stress to induce apoptosis cooperatively in AML cells, which is accompanied by caspase-mediated cleavage of Mcl-1 and is synergistically enhanced by BH3 mimetics. Moreover, we reveal intricate regulation mechanisms in which FLT3-ITD and USP9X physically interact with each other to control their expression levels through ubiquitination and deubiquitination. Thus, the present study sheds a new light on regulation mechanisms of FLT3-ITD involving the ubiquitin system and proposes USP9X as a promising molecular target to be employed in novel therapeutic strategies against therapy-resistant FLT3-ITD-positive AML.

2. Materials and methods

Most of cells and reagents used as well as methods for Western blot analysis, immunoprecipitation, analyses of cell proliferation and viability, apoptosis, activation of Bak and Bax, intracellular ROS levels, transfection, lentivirus-mediated knock down, confocal immunofluorescent microscopy, and primary AML cells have been described previously [7,19–22], and the information is provided in detail in Supplementary Materials and Methods. FLT3-ITD-positive AML cases #1, #2, and #3 correspond to those described in our previous report [21], while case #4 was newly diagnosed AML with 96% blasts in the peripheral blood identified to have FLT3-ITD inserted within the juxtamembrane domain (Supplementary Fig. S1) with the allelic ratio estimated as 9.08 by the PCR and sequencing analyses (data not shown). The results shown are representative of experiments repeated at least three times, except for those obtained using primary AML cell samples.

3. Results

3.1. Deubiquitinase inhibitors WP1130 and G9 show potent anti-leukemic effects on FLT3-ITD-driven cells

First, we examined the anti-proliferative effect of WP1130 on various leukemic cell lines including K562, a BCR/ABL-driven CML cell line reported to be susceptible to WP1130 [10,13]. WP1130 remarkably inhibited proliferation of MV4-11, a human leukemic cell line harboring FLT3-ITD, but not other leukemic cell lines with wild-type FLT3 (FLT3-WT) including K562 under the experimental conditions employed (Fig. 1A). Cell cycle analyses further revealed that WP1130 efficiently induced apoptosis in MV4-11 in contrast to other cell lines examined, as judged by increases in population of cells with sub-G1 cellular DNA content (Fig. 1B). Consistent with this, Western blot analysis revealed that WP1130 induced the cleavage of poly ADP ribose polymerase (PARP), a marker of caspase activation in cells undergoing apoptosis, specifically in MV4-11 cells (Fig. 1C).

We also examined the effects of the partially selective but potent USP9X inhibitor G9 [17], and P220477, an USP10 inhibitor that was previously reported to be effective against FLT3-ITD AML [8]. These DUB inhibitors also showed potent anti-leukemic effects on MV4-11 cells as compared with other leukemic cell lines examined (Fig. 1A, B, and C). Further analyses for dose-response effects of WP1130 and G9 on the AML cell lines with or without FLT3-ITD revealed that they inhibited proliferation of HL60 and, to lesser extents, U937 at higher concentrations than that utilized in the experiment shown in Fig. 1A (Supplementary Fig. S2A). Nevertheless, effects of WP1130 at these higher concentrations were observed also more prominently in FLT3-ITD-positive MV4-11 cells than in HL60 or U937, while they did not show any inhibitory effect on K562. Consistent with this, WP1130 induced apoptosis in HL60 and, to a much lesser extent, in U937, but not in K562, at higher concentrations than that used in Fig. 1B, which, however, was much less prominent than apoptosis induced in MV4-11 cells (Supplementary Fig. S2B). Because WP1130 and G9 inhibit USP9X in common, these results suggest the possibility that USP9X, in addition to USP10, could be a therapeutic target against AML with FLT3-ITD.

We then examined the significance of FLT3-ITD as well as FLT3-TKD in induction of apoptosis by WP1130 and G9 by using IL-3-dependent murine hematopoietic 32Dcl3 cells inducibly expressing FLT3-ITD or FLT3-TKD [7,20]. These cells survive and proliferate dependent on FLT3-ITD or FLT3-TKD instead of IL-3 when cultured with doxycycline to induce expression of these mutants. Both WP1130 and G9 induced apoptosis more potently in these cells when cultured dependent on FLT3-ITD or FLT3-TKD than on IL-3 (Fig. 1D and E). These results indicate that WP1130 as well as G9 induced apoptosis remarkably in these cells at least partly by affecting FLT3-ITD or FLT3-TKD.

3.2. WP1130 rapidly downregulates the autophosphorylated and activated form of FLT3-ITD to block downstream signaling pathways

Next, we examined the effect of WP1130 on FLT3-ITD protein. WP1130 time dependently downregulated FLT3-ITD in MV4-11 cells (Fig. 2A). Importantly, the downregulation of FLT3-ITD was more rapidly and prominently observed for FLT3-ITD in the activated conformation with autophosphorylation on Y591 as compared with total FLT3-ITD (Fig. 2B). Accordingly, inhibition of the kinase activity with quizartinib attenuated WP1130-induced downregulation of FLT3-ITD (Fig. 2A). WP1130 also rapidly and prominently inhibited STAT5 and the MEK/Erk pathway activated downstream of FLT3-ITD in parallel with downregulation of the activated form of FLT3-ITD (Fig. 2B and C). WP1130 also downregulated FLT3-ITD and induced apoptosis, as judged by the cleavage of PARP by caspases, in primary leukemic cells from all the three cases with FLT3-ITD-positive AML we reported in our previous report (Fig. 2D) [21] as well as from an additional AML case (Case #4) with FLT3-ITD (Supplementary Figs. S1 and S3A). WP1130 also inhibited STAT5 in Case #2 and #4, in which we could evaluate the phosphorylation status of STAT5. Furthermore, it was confirmed in primary AML cells that FLT3-ITD autophosphorylated on Y591 was more prominently downregulated by WP1130 (Supplementary Fig. S3B). Under the similar conditions, WP1130 induced apoptosis only modesty in primary AML cells from 2 patients without FLT3-ITD we could examine, although it was demonstrated that FLT3 was downregulated by WP1130 (Supplementary Fig. S3C). Consistent with these Western blotting data, cell cycle analyses confirmed that WP1130 increased the apoptotic sub-G1 fraction prominently in primary AML cells with FLT3-ITD (Case #4) and only modestly in those without FLT3-ITD (Supplementary Figs. S3D and E).

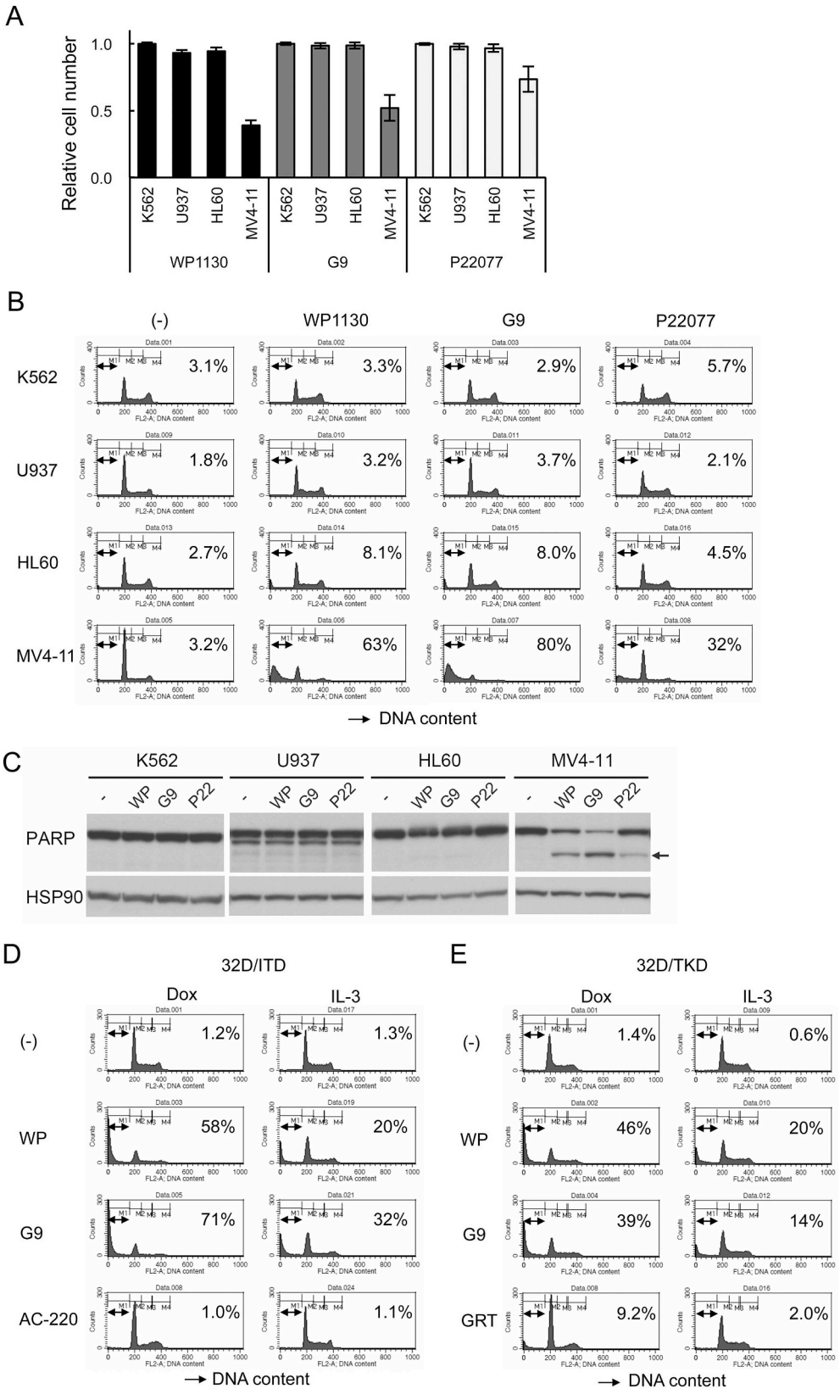

Fig. 1. Deubiquitinase inhibitors WP1130 and EOAI3402143 (G9) show potent anti-leukemic effects on FLT3-ITD-driven cells. (A) K562, U937, HL60, and MV4-11 cells were left untreated as control or treated for 24 h with 1.25 μM WP1130, 0.75 μM G9, or 7.5 μM P22077, as indicated. Viable cell numbers were measured by the colorimetric assay. Each column represents the mean of triplicate cultures, with error bars indicating standard deviations, and is expressed as a ratio to the control cell numbers. (B) Cells were left untreated as control (−) or treated for 6 h with 1.25 μM WP1130, 1 μM G9, or 10 μM P22077, as indicated, and analyzed for DNA content by flow cytometry. Percentages of apoptotic cells with the sub-G1 DNA content are indicated. (C) Cells were left untreated as control or treated for 5 h with 1.25 μM WP1130 (WP), 1 μM G9, or 15 μM P22077 (P22), as indicated. Cells were lysed and subjected to immunoblot analysis with antibodies against indicated proteins. HSP90 was used for a loading control. The arrow indicates the position of cleaved PARP. D, E. 32D/ITD (D) or 32D/TKD (E) cells cultured either with doxycycline (Dox) or IL-3 were left untreated as control (−) or treated for 24 h with 2.5 μM WP1130, 1.5 μM G9, 20 nM quizartinib (AC-220), or 50 nM gilteritinib (GRT), as indicated, and analyzed.

WP1130 downregulated FLT3-ITD and FLT3-TKD expressed in 32D cells similarly (Fig. 2E). Furthermore, WP1130 downregulated wild-type FLT3 similarly with FLT3-ITD in human leukemic UT7 cells exogenously expressing these kinases (Fig. 2F). Thus, the downregulation of FLT3 itself was dependent on neither the mutational status of FLT3 nor cell types.

Consistent with the data that MV4-11, but not K562, was sensitive to up to 2 μM WP1130 (Fig. 1A–C, Supplementary Figs. S2A and B), BCR/ABL in K562 was not affected by 2 μM WP1130 in contrast to FLT3-ITD in MV4-11, although higher concentrations of WP1130

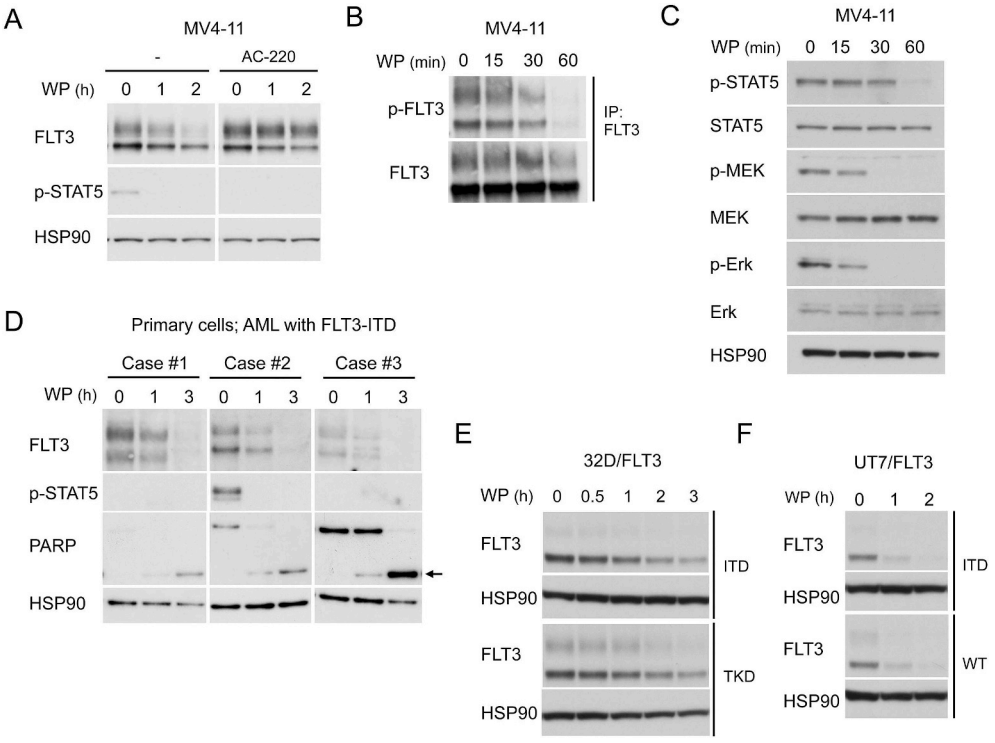

Fig. 2. WP1130 rapidly downregulates the autophosphorylated and activated form of FLT3-ITD to block downstream signaling pathways. (A) MV4-11 cells were left untreated or pre-treated for 1 h with 50 nM quizartinib (AC-220) and subsequently treated with 2 μM WP1130 (WP) for indicated times. Cells were lysed and subjected to immunoblot analysis with antibodies against indicated proteins. HSP90 was used for a loading control. Abbreviation: p-STAT5, phospho-Y694-STAT5. (B) MV4-11 cells were treated with 5 μM WP1130 for indicated times and subjected to immunoprecipitation (IP) with anti-FLT3 antibody followed by immunoblot analysis. Abbreviation: p-FLT3, phospho-Y591-FLT3. (C) MV4-11 cells were treated with 5 μM WP1130 for indicated times and analyzed. Abbreviations: p-MEK, phospho-S217/S221-MEK; p-Erk, phoshpo-T202/Y204-Erk1/2. (D) Primary cells from three FLT3-ITD-positive AML patients were treated with 5 μM WP1130 for indicated times and analyzed. The arrow indicates the position of cleaved PARP. (E) 32D/ITD (ITD) or 32D/TKD (TKD) cells cultured with doxycycline were treated with 3 μM WP1130 for indicated times and analyzed. (F) UT7/FLT3-ITD (ITD) or UT7/FLT3-WT (WT) cells were treated with 5 μM WP1130 for indicated times and analyzed.

downregulated BCR/ABL and induced apoptosis in accordance with previous reports (Supplementary Fig. S4) [10,13].

3.3. WP1130 and G9 downregulate FLT3 through aggresomal translocation

It has been reported that WP1130 blocks BCR/ABL and cytokine-stimulated JAK2 signaling through translocation of these proteins into perinuclear aggresomes, which could be detectable within the detergent-insoluble fraction of cell lysates [11–13]. Thus, we next examined if FLT3-ITD was also translocated to the aggresomes by WP1130. As shown in Fig. 3A and Supplementary Fig. S3F, WP1130 induced a time-dependent decrease or increase in FLT3-ITD in the detergent-soluble or -insoluble fraction, respectively, in MV4-11 cells as well as primary AML cells with FLT3-ITD. Confocal microscopy of MV4-11 cells confirmed that FLT3-ITD (green) accumulated within the perinuclear aggresomes (red) after treatment with WP1130 (Fig. 3B). In 293T cells transfected with FLT3, the aggresomal translocation was observed for both FLT3-ITD and FLT3-WT (Fig. 3C).

Next, we compared the effects of various DUB inhibitors on FLT3-ITD to elucidate the DUB(s) involved in WP1130-mediated effect. WP1130 inhibits USP9X, USP5, USP14, and UCHL5 [11], while G9 inhibits USP9X, USP24, and USP5 [17]. On the other hand, P22077 inhibits USP10 and USP7 [8], and b-AP15 inhibits USP14 and UCHL5, two proteasome-associated DUBs that are also inhibited by WP1130 [23]. PR-619 is a non-selective, pan-deubiquitinase inhibitor. All these DUB inhibitors increased ubiquitination of cellular proteins in MV4-11 cells, as expected, and downregulated FLT3-ITD in the detergent-soluble fraction to similar extents expect for PR-619, which showed a distinctively lesser effect (Fig. 3D). On the other hand, the aggresomal translocation of FLT3-ITD, as judged by its appearance in the detergent-insoluble fraction, was induced distinctively only by PR-619, G9, or WP1130. These results suggest that inhibition of USP9X and/or USP5 by WP1130 or G9, but not the proteasomal DUBs USP14 and UCHL5, may play a role in aggresomal translocation of FLT3-ITD. In this regard, it is notable that WP1130 or G9 also downregulated the USP9X expression in these cells (Fig. 3D).

3.4. FLT3-ITD physically interacts with USP9X to induce its phosphorylation, ubiquitination, and proteasomal degradation

To further explore the association of DUBs and FLT3-ITD, we examined the interaction between FLT3-ITD and USP9X by co-expressing FLT3-ITD and Flag-tagged USP9X along with ubiquitin in 293T cells. We could detect a physical association of FLT3-ITD with USP9X by co-immunoprecipitation assays (Fig. 4A). USP9X bearing the inactivating C1566S (CS) mutation similarly associated with FLT3-ITD (Fig. 4A). Interestingly, USP9X was tyrosine phosphorylated and ubiquitinated when co-expressed with FLT3-ITD (Fig. 4B). Meanwhile, the expression level of USP9X was decreased by co-expression of FLT3-ITD, which was partially rescued by the proteasome inhibitor MG-132 (Fig. 4B). These results indicate that FLT3-ITD induces tyrosine phosphorylation and ubiquitination of USP9X to promote its proteasomal degradation. On the other hand, the inactive CS mutant of USP9X was robustly ubiquitinated without co-expression of FLT3-ITD (Fig. 4B), suggesting that catalytically competent USP9X may deubiquitinate itself to escape from downregulation through proteasomal degradation, which may explain the decrease in expression level of USP9X induced by its inhibitors (Fig. 3D).

In MV4-11 cells, USP9X physically associated with FLT3-ITD irrespective of its activity inhibited by quizartinib, which decreased tyrosine phosphorylation of USP9X (Fig. 4C). The physical association with USP9X was observed for FLT3-ITD and to a slightly lesser extent for FLT3-TKD also in 32D/ITD and 32D/TKD cells, respectively (Fig. 4D). Inhibition of FLT3-ITD and FLT3-TKD by gilteritinib did not affect the physical association but decreased tyrosine phosphorylation of USP9X in these cells as well. Furthermore, a long-term inhibition of FLT3-ITD in MV4-11 cells increased the expression level of USP9X, which was accompanied by a modest increase in FLT3-ITD expression (Fig. 4E). Together, these results indicate that FLT3-ITD as well as FLT3-TKD physically interacts with USP9X independent of its kinase

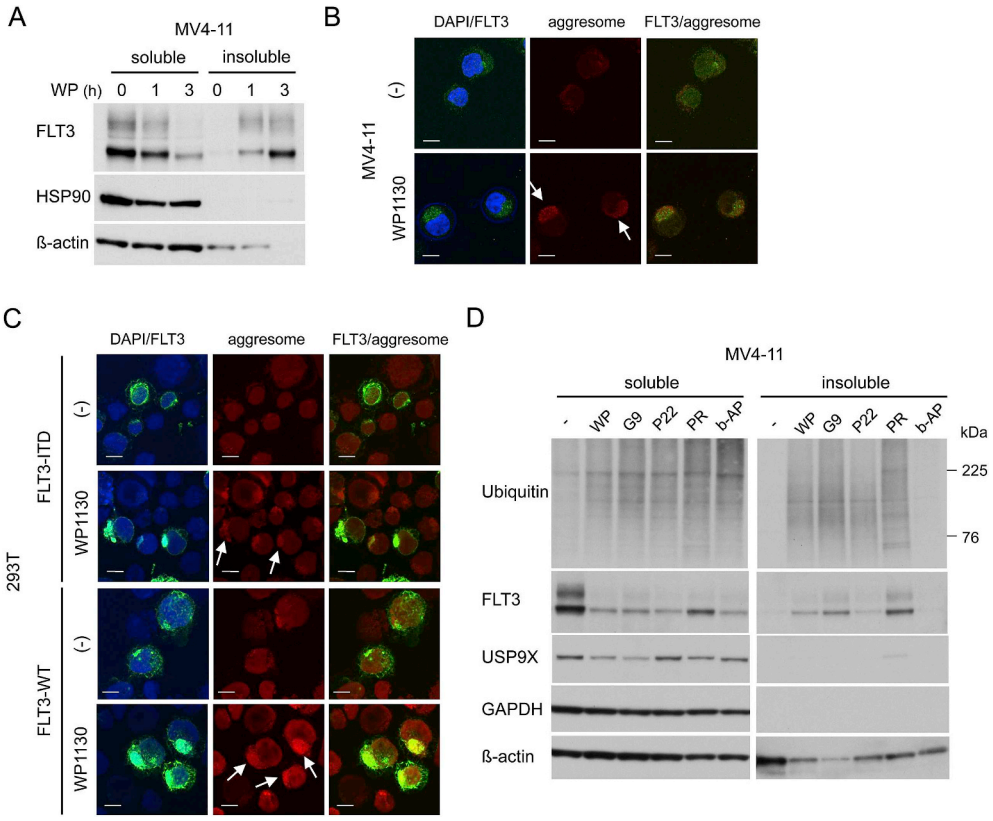

Fig. 3. WP1130 and G9 downregulate FLT3-ITD through aggresomal translocation of the FLT3. (A) MV4-11 cells were treated with 5 μM WP1130 (WP) for indicated times. Cells were lysed and detergent-soluble and -insoluble proteins were extracted as described in Materials and Methods and subjected to immunoblot analysis with antibodies against indicated proteins. HSP90 and ß-actin were used for loading controls. (B) MV4-11 cells were left untreated as control (−) or treated for 2 h with 5 μM WP1130 and stained with anti-FLT3 antibody followed by secondary antibody conjugated with Alexa Fluor 647 (green). Cells were also stained with the PROTEOSTAT aggresome detection reagent (red) as well as with DAPI (blue) for nuclear staining and analyzed by confocal immunofluorescence microscopy. Representative images of cells stained for aggresome and merged images are shown. Scale bars correspond to 10 μm. The arrows indicate perinuclear aggresomes. (C) 293T cells were transfected with plasmids coding for ubiquitin along with either FLT3-ITD or FLT3-WT, as indicated. Cells were left untreated as control (−) or treated for 4 h with 2.5 μM WP1130 and analyzed by confocal immunofluorescence microscopy. (D) MV4-11 cells were left untreated as control or treated for 3 h with 3 μM WP1130, 3 μM G9, 20 μM P22077 (P22), 25 μM PR-619 (PR), or 1 μM b-AP15 (b-AP) as indicated, and analyzed. Positions of size markers are indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

activity and may induce its tyrosine phosphorylation and destabilization in cells including AML cells.

3.5. WP1130 enhances K63-linked polyubiquitination of FLT3-ITD through inhibition of USP9X

We next examined if WP1130 enhances K63-linked polyubiquitination of FLT3-ITD, in a similar manner with BCR/ABL or JAK2 demonstrated in previous reports [12,13], using FLAG-K63-TUBE that specifically binds to K63-linked polyubiquitin chains for purification. As shown in Fig. 5A, autophosphorylated FLT3-ITD was polyubiquitinated in the K63-linked manner in MV4-11 cells, which was inhibited by quizartinib. As expected, WP1130 enhanced the K63-linked polyubiquitination of FLT3-ITD, although that of autophosphorylated FLT3-ITD was diminished, most likely due to its rapid downregulation by translocation to the aggresome (Fig. 5B).

We then examined the effects of knock down of USP9X. Although the phosphorylation and expression levels of FLT3-ITD were not affected, 293T cells knocked down of USP9X showed enhanced susceptibility to WP1130-induced downregulation of phospho-FLT3-ITD (Fig. 5C). We next isolated four clones of MV4-11 cells expressing USP9X shRNA and two clones of control cells expressing GFP shRNA. Knock down of USP9X was confirmed by Western blot analysis (Supplementary Fig. S5A), and representative clones for each group were used in the following experiments. While the expression level of FLT3-ITD remained unchanged, K63-linked polyubiquitination of FLT3-ITD was increased in USP9X knock down cells as compared with control cells (Fig. 5D). Furthermore, MV4-11 cells with USP9X knock down were much more susceptible than control cells to WP1130-induced FLT3-ITD downregulation and apoptosis (Fig. 5E and F and Supplementary Fig. S5B). Taken together, these results indicate that activated and autophosphorylated FLT3-ITD is constitutively polyubiquitinated in the K63-linked manner and is undergoing deubiquitination by USP9X. Thus, its inhibition by WP1130 induced FLT3-ITD downregulation and apoptosis in MV4-11 cells.

3.6. WP1130 activates the mitochondria-mediated intrinsic apoptotic pathway, which is synergistically enhanced by BH3 mimetics and is blocked by overexpression of Bcl-xL or Mcl-1

We next examined the mechanisms involved in induction of apoptosis by WP1130 in MV4-11 cells. WP1130 rapidly activated the Bcl-2 family pro-apoptotic effector proteins Bak and Bax before apoptosis was distinctively observed by the cell cycle analysis (Fig. 6A and B). Furthermore, the activation of Bak and Bax was not inhibited by the caspase inhibitor Boc-d-FMK, which almost completely blocked WP1130-induced apoptosis as well as PARP cleavage (Fig. 6A–C). Thus, WP1130 initially activated Bak/Bax to induce caspase-dependent apoptosis subsequently.

Next, we examined the roles of anti-apoptotic Bcl-2 family proteins, such as Bcl-xL, Bcl-2, and Mcl-1, which reside on the mitochondrial outer membrane and directly and/or indirectly inhibit Bak/Bax to activate the intrinsic mitochondrial apoptotic pathways [24]. As shown in Fig. 6D, WP1130-induced apoptosis in MV4-11 cells was synergistically enhanced by the BH3 mimetic navitoclax, A-1210477, A-1331852, or venetoclax, which respectively inhibits Bcl-2/Bcl-w/Bcl-xL, Mcl-1, Bcl-xL, or Bcl-2. However, these BH3 mimetics failed to show any synergistic effect with WP1130 on induction of apoptosis in the FLT3-ITD-negative AML cell lines HL60, U937, and K562 (Supplementary Fig. S6). On the other hand, overexpression of Mcl-1 or Bcl-xL in MV4-11 cells very efficiently inhibited WP1130-induced activation of Bak and Bax as well as apoptosis (Fig. 6E and Supplementary Fig. S5C).

In accordance with previous studies using various cell lines [11,13,15–17], WP1130 downregulated Mcl-1 in MV4-11 cells as well

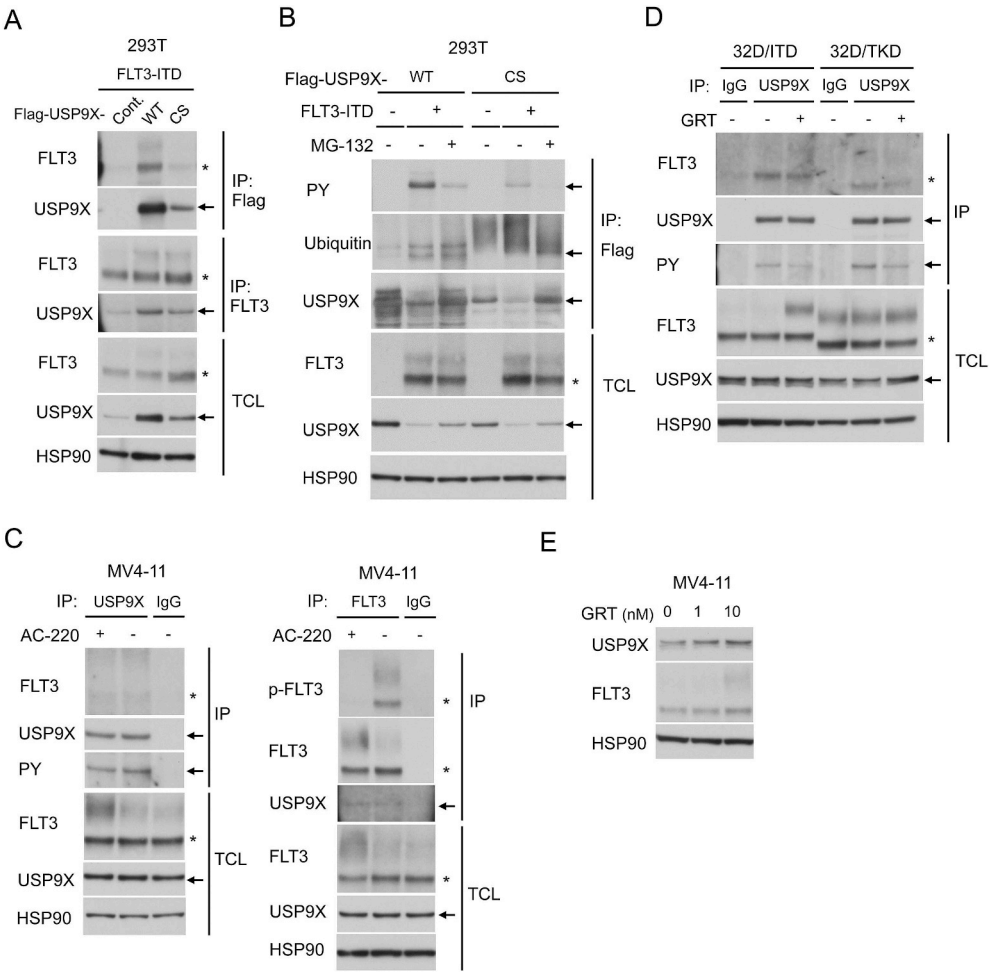

Fig. 4. FLT3-ITD physically interacts with USP9X to induce its phosphorylation, ubiquitination, and proteasomal degradation. (A) 293T cells were transfected with plasmids coding for FLT3-ITD and ubiquitin along with Flag-tagged USP9X-WT or the CS mutant or an empty vector (Cont.), as indicated. Immunoprecipitates (IP) with anti-Flag affinity gel or anti-FLT3 antibody and total cell lysates (TCL) were subjected to immunoblot analysis with antibodies against indicated proteins. The asterisks and arrows indicate the positions of FLT3 (lower band) and USP9X, respectively. HSP90 was used for a loading control. (B) 293T cells were transfected with plasmids coding for indicated proteins and ubiquitin. Cells were left untreated as control or treated for 5 h with 10 μM MG-132 before harvest for analysis. Abbreviation: PY, phosphotyrosine. (C) MV4-11 cells were treated for 3 h with or without 10 nM quizartinib (AC-220), as indicated, and lysed. Immunoprecipitates (IP) with antibodies against USP9X or FLT3 as well as normal rabbit IgG as a control were analyzed. Abbreviation: p-FLT3, phospho-Y591-FLT3. (D) 32D/ITD or 32D/TKD cells were treated for 3 h with or without 100 nM gilteritinib (GRT), as indicated and analyzed. (E) MV4-11 cells were treated for 24 h with indicated concentrations of gilteritinib and analyzed.

(Fig. 6C). It was notable, however, that the Mcl-1 downregulation in MV4-11 cells was associated with an appearance of a faster-migrating extra band and was prevented by Boc-d-FMK. Thus, the downregulation of Mcl-1 should be mostly due to its cleavage by caspases under these conditions. WP1130 also downregulated USP9X in the same manner with Mcl-1 (Fig. 6C). Similarly, WP1130 downregulated Mcl-1, though only modestly, in primary AML cells with FLT3-ITD with the appearance of faster-migrating extra band (Supplementary Fig. S3A). However, Boc-d-FMK did not effectively prevent the cleavage of Mcl-1 as well as PARP in these cells. USP9X was also downregulated by WP1130 in these primary AML cells, although the cleaved fragment could not be identified by Western blot analysis (Supplementary Fig. S3A). Nevertheless, Boc-d-FMK partly prevented the downregulation of USP9X in these cells. Although the discordant effects of the caspase inhibitor in MV4-11 and primary AML cells remain to be explained, these results implicate activation of caspases in downregulation of USP9X as well as Mcl-1 in these cells. On the other hand, downregulation of FLT3-ITD was only slightly rescued by Boc-d-FMK, thus indicating that cleavage by caspases may not play a considerable role in downregulation of FLT3-ITD in contrast to Mcl-1 and USP9X (Fig. 6C and Supplementary Fig. S3A).

3.7. Inhibition of USP9X induces apoptosis at least partly through oxidative stress activating stress-related MAP kinase pathways and DNA damage responses

Although WP1130 downregulated FLT3-ITD as well as FLT3-TKD and efficiently induced apoptosis in 32Dcl3 cells transformed by these FLT3 mutants, inhibition of the kinase activity by quizartinib or gilteritinib hardly induced apoptosis during the same time period in these cells (Fig. 1D and E). Consistent with this, WP1130, but not quizartinib, rapidly activated Bak and Bax to induce apoptosis in MV4-11 cells, although signaling downstream of FLT3-ITD was more distinctively inhibited by quizartinib (Fig. 7A–C). Intriguingly, WP1130, but not quizartinib, induced activation of stress-related JNK and p38 MAP kinases as well as phosphorylation of histone H2A.X involved in DNA damage responses (Fig. 7C). Consistent with this, WP1130, but not quizartinib, induced activation of p38 and phosphorylation of H2A.X in primary AML cells with FLT3-ITD, while quizartinib abrogated STAT5 activation without inducing apoptosis in these cells (Fig. 7D and Supplementary Fig. S3A). Similar results were observed also with G9 in MV4-11 (Supplementary Figs. S7A and B), which implies that inhibition of USP9X may induce cellular stresses and DNA damages in addition to inhibition of FLT3-ITD to induce apoptosis efficiently in these cells.

To pursuit this possibility further, we examined the possible involvement of reactive oxygen species (ROS) in the WP-1130-induced cellular responses. In contrast to quizartinib, WP1130 or G9 rapidly increased intracellular ROS levels of MV4-11 cells, which was augmented by cotreatment with hydrogen peroxide (H_2O_2) and prevented by the ROS scavenger N-acetyl-L-cysteine (NAC), as expected (Fig. 7E and Supplementary Fig. S7C). Furthermore, H_2O_2 or NAC respectively enhanced or inhibited activation of p38, phosphorylation of H2A.X, and apoptosis induced by inhibition of USP9X (Fig. 7A, F, Supplementary Figs. S7B and D). Consistent with this, NAC also prevented apoptosis induced by WP1130 in primary AML cells with FLT3-ITD (Supplementary Fig. S3D). Finally, it was revealed that quizartinib and H_2O_2 cooperatively induced apoptosis in MV4-11 cells (Fig. 7A). Together, these results suggest that WP1130 efficiently induces apoptosis

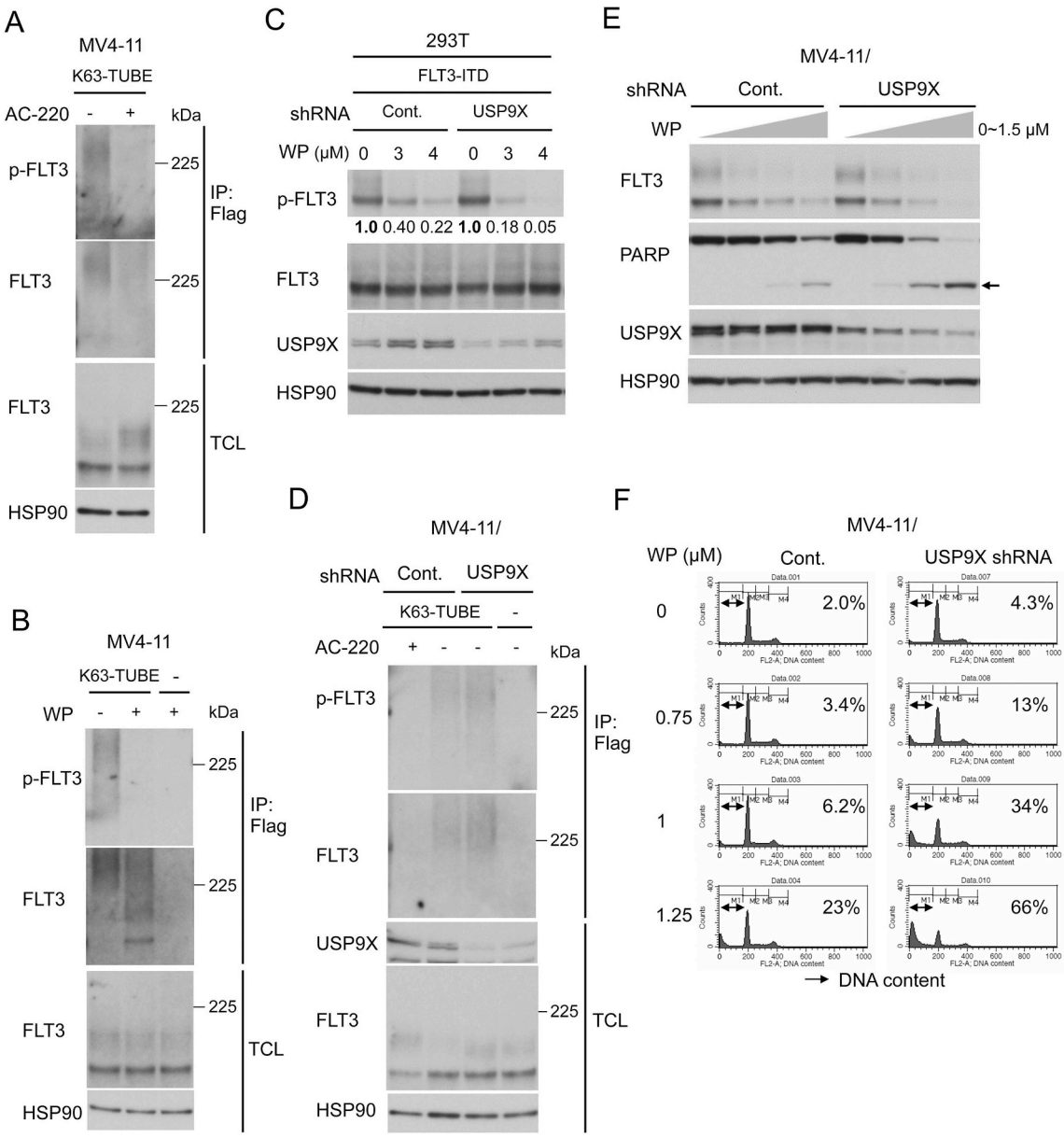

Fig. 5. WP1130 enhances K63-linked polyubiquitination of FLT3-ITD through inhibition of USP9X. (A) MV4-11 cells were treated for 4 h with or without 10 nM quizartinib (AC-220), as indicated. K63-polyubiquitinated proteins were isolated by immunoprecipitation with anti-Flag after incubation of cell lysates with FLAG-K63-TUBE (K63-TUBE). Immunoprecipitates (IP) as well as total cell lysates (TCL) were subjected to immunoblot analysis with antibodies against indicated proteins. HSP90 was used for a loading control. Positions of size marker are indicated. Abbreviation: p-FLT3, phospho-Y591-FLT3. (B) MV4-11 cells were treated for 20 min with or without 5 μM WP1130 (WP), as indicated. Cell lysates were incubated with or without K63-TUBE, as indicated, and analyzed. (C) 293T cells were transfected with plasmids coding for FLT3-ITD and ubiquitin along with that expressing USP9X shRNA or GFP shRNA (Cont.). Cells were treated for 1 h with indicated concentration of WP1130 and analyzed. Relative levels of FLT3-ITD phosphorylated on Y591 were determined by densitometric analysis and are shown below the panel. (D) MV4-11/USP9X-shRNA or MV4-11/GFP-shRNA (Cont.) cells were treated for 3 h with or without 10 nM quizartinib, as indicated. Cell lysates were incubated with or without FLAG-K63-TUBE, as indicated, and analyzed. (E) MV4-11/USP9X-shRNA or MV4-11/GFP-shRNA (Cont.) cells were treated for 3 h with increasing concentration of WP1130 (0, 1, 1.25 and 1.5 μM), as indicated, and analyzed. The arrow indicates the position of cleaved PARP. (F) MV4-11/USP9X-shRNA or MV4-11/GFP-shRNA (Cont.) cells were treated for 5 h with indicated concentration of WP1130. Cells were analyzed for DNA content by flow cytometry. Percentages of apoptotic cells with the sub-G1 DNA content are indicated.

through the intrinsic mitochondrial pathway by activating the cellular stress and DNA-damage responses through increased ROS levels in co-operation with inhibition of FLT3-ITD-mediated survival signaling in AML cells with FLT3-ITD.

4. Discussion

In the present study, we have shown that the USP9X inhibitor WP1130 or G9 induced aggresomal translocation of FLT3-ITD to block its downstream signaling to induce apoptosis cooperatively by causing oxidative stress in cells transformed by this mutant kinase. Although these two inhibitors are not specific for USP9X, comparison of specificities of various DUB inhibitors utilized and their effects on FLT3-ITD strongly suggests that inhibition of USP9X or USP5 should be mainly responsible for the effects observed with WP1130 and G9. The possible involvement of USP5 has remained to be studied in future studies in view of a recent report that its inhibition by WP1130 caused destabilization of c-Maf to induce apoptosis in multiple myeloma cells [25].

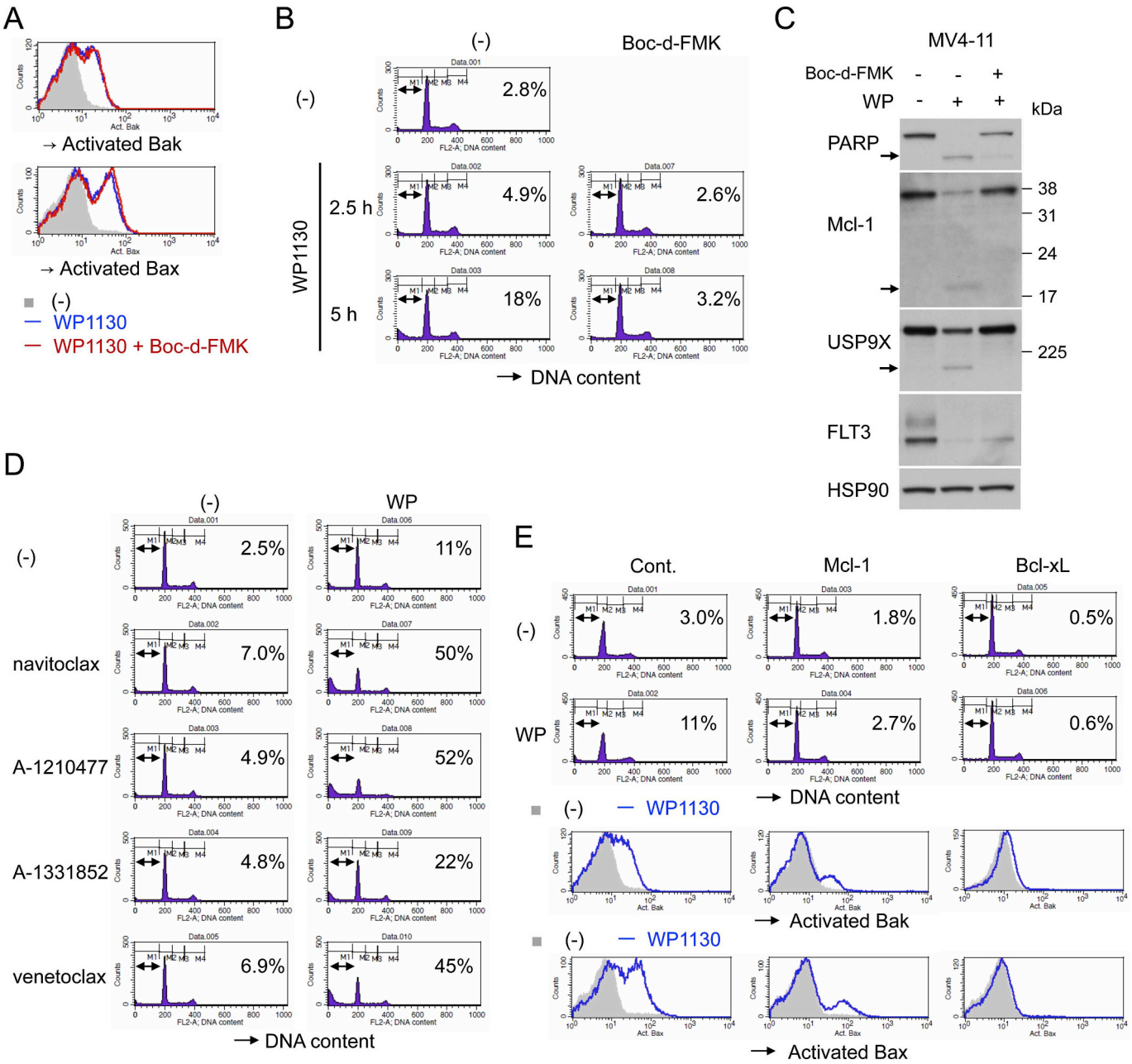

Fig. 6. WP1130 activates the mitochondria-mediated intrinsic apoptotic pathway, which is synergistically enhanced by BH3 mimetic inhibitors and is blocked by overexpression of Bcl-xL or Mcl-1. (A) MV4-11 cells were left untreated as control (−) or treated for 2.5 h with 1.5 μM WP1130 in the presence of absence of 100 μM Boc-d-FMK, as indicated. Cells were analyzed for the activated Bak and Bax by flow cytometry. (B) MV4-11 cells were left untreated as control (−) or treated for 2.5 or 5 h with 1.5 μM WP1130 in the presence or absence of 100 μM Boc-d-FMK, as indicated. Cells were analyzed for DNA content by flow cytometry. Percentages of apoptotic cells with the sub-G1 DNA content are indicated. (C) MV4-11 cells were pretreated in the absence or presence of 50 μM Boc-d-FMK for 30 min and then treated for 3 h with or without 3 μM WP1130 (WP), as indicated. Cells were lysed and subjected to immunoblot analysis with antibodies against indicated proteins. HSP90 was used for a loading control. The arrows indicate cleaved fragments of proteins detected. Positions of size markers are indicated. (D) MV4-11 cells were left untreated as control (−) or treated for 5 h with 0.25 μM navitoclax, 0.5 μM A-1210477, 1 μM A-1331852, or 10 nM venetoclax in the absence or presence of 1 μM WP1130, as indicated, and analyzed. (E) MV4-11/Mcl-1 (Mcl-1), MV4-11/Bcl-xL (Bcl-xL), or vector control cells (Cont.) were left untreated as control (−) or treated for 3 h with 2 μM WP1130, as indicated. Cells were analyzed for DNA content and the activated Bak and Bax by flow cytometry.

Nevertheless, enhancement of the effects of WP1130 in cells knocked down of USP9X in the present study indicates that its inhibition should play a substantial role in downregulation of FLT3-ITD and induction of apoptosis. The physical as well as functional interaction between FLT3-ITD and USP9X observed in cells including AML cells further supports the notion that USP9X is the target molecule for cytotoxic effects of these inhibitors on FLT3-ITD-positive AML cells.

Although USP9X has been reported to associate physically with or deubiquitinate various proteins [9], this is the first report that FLT3 physically and functionally interacts with USP9X. The present study is, however, in line with the previous report that inhibition of USP9X by WP1130 downregulated the cytoplasmic tyrosine kinase BCR/ABL by increasing its K63-linked ubiquitination and inducing its aggresomal translocation in K562 and other cell lines expressing BCR/ABL, although the authors failed to observe the physical interaction of BCR/ABL with USP9X [13]. On the other hand, these authors reported that neither the membrane associated tyrosine kinases JAK2 and Lyn nor the receptor tyrosine kinases including FLT3 and c-Kit were affected by

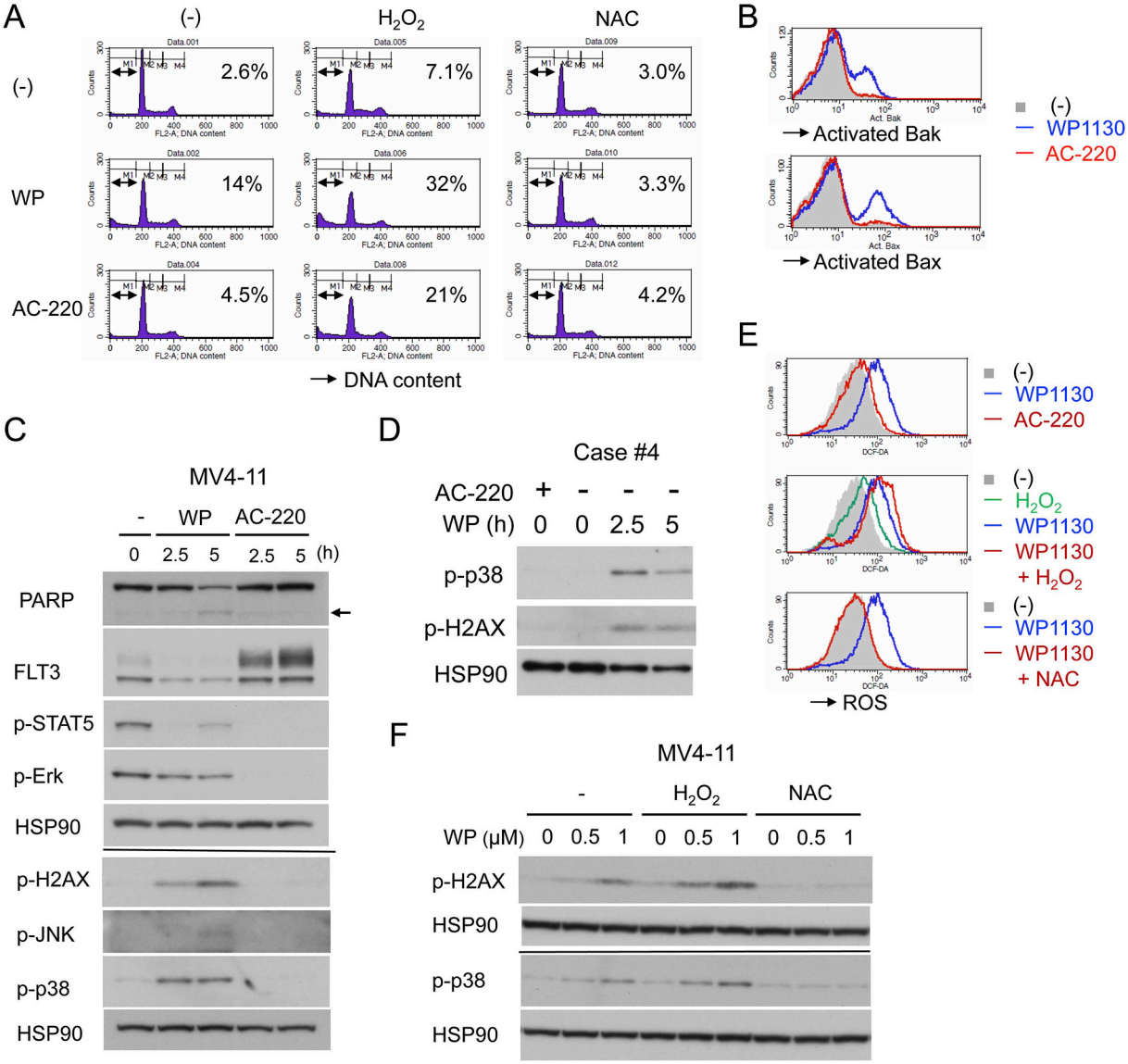

Fig. 7. WP1130 induces apoptosis at least partly through oxidative stress to activate stress-related MAP kinase pathways and DNA damage responses. (A) MV4-11 cells were left untreated as control (−) or treated for 5 h with 1 μM WP1130 or 50 nM quizartinib (AC-220) in the presence of absence of 0.2 mM H_2O_2 or 5 mM NAC, as indicated. Cells were analyzed for DNA content by flow cytometry. Percentages of apoptotic cells with the sub-G1 DNA content are indicated. (B) MV4-11 cells were left untreated as control (−) or treated for 1.5 h with 1.5 μM WP1130 or 50 nM quizartinib, as indicated. Cells were analyzed for the activated Bak and Bax by flow cytometry. (C) MV4-11 cells were left untreated as control (−) or treated either with 1.5 μM WP1130 (WP) or 50 nM quizartinib for indicated times. Cells were lysed and subjected to immunoblot analysis with antibodies against indicated proteins. The results obtained from duplicate gels are shown above or below a horizontal line. HSP90 was used for a loading control. Abbreviations: p-STAT5, phospho-Y694-STAT5; p-Erk, phoshpo-T202/Y204-Erk1/2; p-H2AX, phospho-S139-histone H2AX; p-JNK, phospho-T183/Y185-JNK; p-p38, phospho-T180/Y182-p38. (D) Primary AML cells from FLT3-ITD-positive case (Case #4) were cultured with 2 μM WP1130 (WP) for indicated times or with 10 nM quizartinib (AC-220) for 5 h, as indicated. Cells were lysed and subjected to immunoblot analysis with antibodies against indicated proteins. (E) MV4-11 cells were left untreated as control (−) or treated for 4 h with 1 μM WP1130, 50 nM quizartinib, 0.2 mM H_2O_2, and/or 5 mM NAC, as indicated. Cells were analyzed for reactive oxygen species (ROS) by flow cytometry. (F) MV4-11 cells were left untreated or pre-treated for 2 h with 0.2 mM H_2O_2, as indicated. Cells were then treated for 4 h with indicated concentrations of WP1130 in the absence or presence of 5 mM NAC, as indicated, and analyzed.

WP1130 in BCR/ABL-expressing cells, though we failed to analyze FLT3 in K562 cells by Western blot analysis (Supplementary Fig. S4A). Nevertheless, these authors later found that JAK2 activated by cytokines in other cell types was susceptible to the very similar effects of WP1130 demonstrated for BCR/ABL [12]. Thus, as suggested by these authors, functional roles and inhibitory effects of USP9X may differ depending on cellular contexts and other conditions. In this regard, it should be noted that downregulation of FLT3-ITD in MV4-11 resulting in apoptosis was induced at much lower concentrations of WP1130 than that of BCR/ABL in K562 causing apoptosis (Fig. 1B and C, and Supplementary Figs. S2B and S4C), which may reflect the close physical association of USP9X with FLT3-ITD but not with BCR/ABL that could be observed in these cells. Therefore, FLT3-ITD in AML cells should provide a very promising target as compared with BCR/ABL in Ph-positive leukemia for novel therapies with USP9X inhibitors.

Weisberg et al. [8] have very recently reported that inhibition of USP10 induced degradation of FLT3-ITD but not wild-type FLT3, which was partially rescued by the lysosome inhibitor chloroquine, and induced apoptosis preferentially in AML cells expressing FLT3-ITD. In the present study, we have confirmed that inhibition of USP10 by P22077

downregulated FLT3-ITD and induced apoptosis preferentially in MV4-11 cells as compared with other leukemic cell lines examined. However, inhibition of USP10 did not induce the aggresomal translocation of FLT3-ITD. On the other hand, inhibition of USP9X affected wild-type FLT3 as well, although its effect was more rapidly and prominently observed on the autophosphorylated form of FLT3-ITD. Our previous study showed that the RING family E3 ligases c-Cbl and Cbl-b induced K48-linked polyubiquitination of autophosphorylated FLT3-ITD to facilitate degradation through the lysosome and proteasome systems and implicated the HECT family E3 ligase NEDD4 in K63-linked polyubiquitination of FLT3-ITD [7], which is in accordance with a more recent study revealing that NEDD4 is a K63-specific ligase [26]. These data suggest that inhibition of USP10 or USP9X downregulated FLT3-ITD through different mechanisms by protecting K48- or K63-linked polyubiquitination of FLT3-ITD, respectively, which may be induced by different types of E3 ligases. Future studies are required to address the functional significance of K63-linked polyubiquitination of FLT3-ITD observed preferentially for autophosphorylated FLT3-ITD and to more precisely define E3 ligases involved in this process.

The present study has shown that inhibition of USP9X by WP1130 decreased the expression level of Mcl-1 in MV4-11 as well as in primary AML cells, which is in accordance with previous reports [11,13,15–17]. These previous studies have implicated proteasomal degradation as the molecular mechanism involved based on the apparent recovery by the proteasome inhibitor MG132 [11,17], which is concordant with the previous report that USP9X deubiquitinated Mcl-1 to protect it from proteasomal degradation [27]. However, the present study has revealed that the decrease was mainly caused not by proteasomal degradation but by cleavage by caspases, because it was prevented by inhibition of caspases in MV4-11 and was accompanied by the appearance of a cleaved fragment. This is consistent with a recent report that the stabilization of Mcl-1 by deubiquitination was mediated not by USP9X but by USP13 or other DUBs in some cell types [28]. Furthermore, inactivation of Mcl-1 or its conversion to a pro-apoptotic protein by the cleavage by caspases has also been reported mainly in hematopoietic cells treated with proteasome inhibitors [29,30]. Irrespective of the mechanisms involved, the decrease in Mcl-1 expression should contribute to induction of apoptosis by USP9X inhibition, because the synergistic enhancement by BH3 mimetics was observed in the present as well as previous studies [15,16].

Regulation of the USP9X expression and its post-translational modification largely remain to be known. It has been reported that phosphorylation of USP9X at S1600 induced by T-cell receptor activation enhanced catalytic activity of USP9X against ZAP70 [31]. On the other hand, phosphorylation of other serine, threonine and tyrosine residues of USP9X has been revealed only by using proteomic discovery mass spectrometry according to the PhosphoSitePlus database (www.phosphosite.org). Thus, neither the regulatory mechanisms nor significance of phosphorylation of these residues has been examined. In the present study, we found that FLT3-ITD induced tyrosine phosphorylation of USP9X, which led to its ubiquitination and decreased expression in 293T cells. The decrease was partly prevented by the proteasome inhibitor MG132, while inactivation of the DUB activity by the CS mutation drastically increased its ubiquitination with a concomitant decrease in expression. In accordance with this, inhibition of USP9X by WP1130 as well as G9 or that of FLT3-ITD by gilteritinib decreased or increased the expression level of USP9X, respectively, in MV4-11 cells. These data indicate that FLT3-ITD phosphorylates USP9X on tyrosine to induce its ubiquitination and degradation through the proteasome system, which is partly prevented by its own deubiquitinating activity. Thus, there may exist a negative feed back regulation mechanism in which the stabilizing effect of USP9X on FLT3 is antagonized by the aberrant kinase activity of FLT3-ITD. It is tempting to speculate that interruption of this mechanism by FLT3 kinase inhibitors may contribute at least partly to development of the therapy resistance. We have also found that USP9X is downregulated through the caspase-dependent mechanism similar to Mcl-1 in cells undergoing apoptosis, which may enhance cytotoxic effects of not only USP9X inhibitors but also other cytotoxic agents by affecting FLT3-ITD and various other USP9X substrates. These possibilities as well as the tyrosine residues phosphorylated and E3 ligases involved need to be examined in future studies.

The present study has revealed that both inhibition of FLT3-ITD signaling and induction of oxidative stress signaling were necessary for USP9X inhibitors to induce apoptosis cooperatively in cells transformed with FLT3-ITD. The former was proved to be necessary because WP1130 induced apoptosis in 32D cells transformed by FLT3-ITD but not by those surviving dependent on IL-3. On the other hand, inhibition of FLT3-ITD by its kinase inhibitor alone failed to induce apoptosis in FLT3-ITD-transformed cells as rapidly and efficiently as USP9X inhibitors unless oxidative stress responses were simultaneously elicited by adding H_2O_2, while the ROS scavenger NAC prevented apoptosis induced by inhibition of USP9X. However, it was previously reported that treatment with WP1130 did not increase intracellular ROS level in CML and mantle cell lymphoma cell lines [11,13]. Thus, USP9X inhibition may induce oxidative stress responses in cell type specific manners. In this regard, elevated intracellular ROS levels have been observed in hematological malignancies, including AML, and treatment with pro-oxidants to amplify the pre-existent oxidative stress is expected to cause catastrophic damages in these cells [32]. Furthermore, it has been reported that FLT3-ITD induces increased ROS production leading to DNA damage in AML cells and sensitizes these cells to pro-oxidant chemotherapeutic or molecular targeting agents [33,34]. This may partly explain the very high sensitivity of FLT3-ITD-transformed cells to WP1130 inhibitors. However, the mechanisms involved in increased ROS production induced by USP9X inhibition have remained to be known. In this regard, it is notable that a portion of USP9X is present at the mitochondria, the major source of ROS generation, and deubiquitinates polyubiquitinated Mcl-1 [9,27]. DUBs are involved in quality control of the mitochondria, disturbance of which increases ROS generation leading to oxidative damage [35]. Thus, it is possible that inhibition of USP9X may affect Mcl-1 and other mitochondrial proteins causing mitochondrial dysfunction to increase the ROS levels in AML cells irrespective of the FLT3 status. Future studies are needed to elucidate the mechanisms involved in induction of oxidative stress by inhibition of USP9X and the roles of stress-related MAP kinase activation and DNA damage responses in induction of apoptosis.

Author contributions

HA and OM contributed to project conception and the design of experiments. HA and YU performed most of the experiments and analyzed the results. SI, KO, and AN also performed experiments and analyzed results. HA and OM wrote the paper with contributions from all of the other coauthors. All authors reviewed the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.046.

References

[1] S. Meshinchi, F.R. Appelbaum, Structural and functional alterations of FLT3 in acute myeloid leukemia, Clin. Cancer Res. 15 (2009) 4263–4269.

[2] M.A. Hospital, A.S. Green, T.T. Maciel, I.C. Moura, A.Y. Leung, D. Bouscary, J. Tamburini, FLT3 inhibitors: clinical potential in acute myeloid leukemia, OncoTargets Ther. 10 (2017) 607–615.

[3] C.C. Smith, A. Paguirigan, G.R. Jeschke, K.C. Lin, E. Massi, T. Tarver, C.S. Chin, S. Asthana, A. Olshen, K.J. Travers, S. Wang, M.J. Levis, A.E. Perl, J.P. Radich, N.P. Shah, Heterogeneous resistance to quizartinib in acute myeloid leukemia revealed by single-cell analysis, Blood 130 (2017) 48–58.

[4] C.C. Smith, Q. Wang, C.S. Chin, S. Salerno, L.E. Damon, M.J. Levis, A.E. Perl, K.J. Travers, S. Wang, J.P. Hunt, P.P. Zarrinkar, E.E. Schadt, A. Kasarskis, J. Kuriyan, N.P. Shah, Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia, Nature 485 (2012) 260–263.

[5] E. Oh, D. Akopian, M. Rape, Principles of Ubiquitin-dependent Signaling, Annual Review of Cell and Developmental Biology, (2018).

[6] B. Sargin, C. Choudhary, N. Crosetto, M.H.H. Schmidt, R. Grundler, M. Rensinghoff, C. Thiessen, L. Tickenbrock, J. Schwable, C. Brandts, B. August, S. Koschmieder, S.R. Bandi, J. Duyster, W.E. Berdel, C. Muller-Tidow, I. Dikic, H. Serve, Flt3-dependent transformation by inactivating c-Cbl mutations in AML, Blood 110 (2007) 1004–1012.

[7] G. Oshikawa, T. Nagao, N. Wu, T. Kurosu, O. Miura, c-Cbl and Cbl-b ligases mediate 17-allylaminodemethoxygeldanamycin-induced degradation of autophosphorylated Flt3 kinase with internal tandem duplication through the ubiquitin proteasome pathway, J. Biol. Chem. 286 (2011) 30263–30273.

[8] E.L. Weisberg, N.J. Schauer, J. Yang, I. Lamberto, L. Doherty, S. Bhatt, A. Nonami, C. Meng, A. Letai, R. Wright, H. Tiv, P.C. Gokhale, M.S. Ritorto, V. De Cesare, M. Trost, A. Christodoulou, A. Christie, D.M. Weinstock, S. Adamia, R. Stone, D. Chauhan, K.C. Anderson, H.S. Seo, S. Dhe-Paganon, M. Sattler, N.S. Gray, J.D. Griffin, S.J. Buhrlage, Inhibition of USP10 induces degradation of oncogenic FLT3, Nat. Chem. Biol. 13 (2017) 1207–1215.

[9] M. Murtaza, L.A. Jolly, J. Gecz, S.A. Wood, La FAM fatale: USP9X in development and disease, Cell. Mol. Life Sci. 72 (2015) 2075–2089.

[10] G.A. Bartholomeusz, M. Talpaz, V. Kapuria, L.Y. Kong, S. Wang, Z. Estrov, W. Priebe, J. Wu, N.J. Donato, Activation of a novel Bcr/Abl destruction pathway by WP1130 induces apoptosis of chronic myelogenous leukemia cells, Blood 109 (2007) 3470–3478.

[11] V. Kapuria, L.F. Peterson, D. Fang, W.G. Bornmann, M. Talpaz, N.J. Donato, Deubiquitinase inhibition by small-molecule WP1130 triggers aggresome formation and tumor cell apoptosis, Cancer Res. 70 (2010) 9265–9276.

[12] V. Kapuria, A. Levitzki, W.G. Bornmann, D. Maxwell, W. Priebe, R.J. Sorenson, H.D. Showalter, M. Talpaz, N.J. Donato, A novel small molecule deubiquitinase inhibitor blocks Jak2 signaling through Jak2 ubiquitination, Cell. Signal. 23 (2011) 2076–2085.

[13] H. Sun, V. Kapuria, L.F. Peterson, D. Fang, W.G. Bornmann, G. Bartholomeusz, M. Talpaz, N.J. Donato, Bcr-Abl ubiquitination and Usp9x inhibition block kinase signaling and promote CML cell apoptosis, Blood 117 (2011) 3151–3162.

[14] R.R. Kopito, Aggresomes, inclusion bodies and protein aggregation, Trends Cell Biol. 10 (2000) 524–530.

[15] G. Karpel-Massler, M.A. Banu, C. Shu, M.E. Halatsch, M.A. Westhoff, J.N. Bruce, P. Canoll, M.D. Siegelin, Inhibition of deubiquitinases primes glioblastoma cells to apoptosis in vitro and in vivo, Oncotarget 7 (2016) 12791–12805.

[16] C. Peddaboina, D. Jupiter, S. Fletcher, J.L. Yap, A. Rai, R.P. Tobin, W. Jiang, P. Rascoe, M.K. Rogers, W.R. Smythe, X. Cao, The downregulation of Mcl-1 via USP9X inhibition sensitizes solid tumors to Bcl-xl inhibition, BMC Canc. 12 (2012) 541.

[17] L.F. Peterson, H. Sun, Y. Liu, H. Potu, M. Kandarpa, M. Ermann, S.M. Courtney, M. Young, H.D. Showalter, D. Sun, A. Jakubowiak, S.N. Malek, M. Talpaz, N.J. Donato, Targeting deubiquitinase activity with a novel small-molecule inhibitor as therapy for B-cell malignancies, Blood 125 (2015) 3588–3597.

[18] L.V. Pham, A.T. Tamayo, C. Li, W. Bornmann, W. Priebe, R.J. Ford, Degrasyn potentiates the antitumor effects of bortezomib in mantle cell lymphoma cells in vitro and in vivo: therapeutic implications, Mol Cancer Ther 9 (2010) 2026–2036.

[19] M. Iiyama, K. Kakihana, T. Kurosu, O. Miura, Reactive oxygen species generated by hematopoietic cytokines play roles in activation of receptor-mediated signaling and in cell cycle progression, Cell. Signal. 18 (2006) 174–182.

[20] A. Nogami, G. Oshikawa, K. Okada, S. Fukutake, Y. Umezawa, T. Nagao, T. Kurosu, O. Miura, FLT3-ITD confers resistance to the PI3K/Akt pathway inhibitors by protecting the mTOR/4EBP1/Mcl-1 pathway through STAT5 activation in acute myeloid leukemia, Oncotarget 6 (2015) 9189–9205.

[21] K. Okada, A. Nogami, S. Ishida, H. Akiyama, C. Chen, Y. Umezawa, O. Miura, FLT3-ITD induces expression of Pim kinases through STAT5 to confer resistance to the PI3K/Akt pathway inhibitors on leukemic cells by enhancing the mTORC1/Mcl-1 pathway, Oncotarget 9 (2018) 8870–8886.

[22] Y. Umezawa, H. Akiyama, K. Okada, S. Ishida, A. Nogami, G. Oshikawa, T. Kurosu, O. Miura, Molecular mechanisms for enhancement of stromal cell-derived factor 1-induced chemotaxis by platelet endothelial cell adhesion molecule 1 (PECAM-1), J. Biol. Chem. 292 (2017) 19639–19655.

[23] P. D'Arcy, S. Brnjic, M.H. Olofsson, M. Fryknäs, K. Lindsten, M. De Cesare, P. Perego, B. Sadeghi, M. Hassan, R. Larsson, S. Linder, Inhibition of proteasome deubiquitinating activity as a new cancer therapy, Nat. Med. 17 (2011) 1636–1640.

[24] F. Llambi, T. Moldoveanu, S.W. Tait, L. Bouchier-Hayes, J. Temirov, L.L. McCormick, C.P. Dillon, D.R. Green, A unified model of mammalian BCL-2 protein family interactions at the mitochondria, Mol Cell 44 (2011) 517–531.

[25] S. Wang, J. Juan, Z. Zhang, Y. Du, Y. Xu, J. Tong, B. Cao, M.F. Moran, Y. Zeng, X. Mao, Inhibition of the deubiquitinase USP5 leads to c-Maf protein degradation and myeloma cell apoptosis, Cell Death Dis. 8 (2017) e3058.

[26] E. Maspero, E. Valentini, S. Mari, V. Cecatiello, P. Soffientini, S. Pasqualato, S. Polo, Structure of a ubiquitin-loaded HECT ligase reveals the molecular basis for catalytic priming, Nat. Struct. Mol. Biol. 20 (2013) 696–701.

[27] M. Schwickart, X. Huang, J.R. Lill, J. Liu, R. Ferrando, D.M. French, H. Maecker, K. O'Rourke, F. Bazan, J. Eastham-Anderson, P. Yue, D. Dornan, D.C. Huang, V.M. Dixit, Deubiquitinase USP9X stabilizes MCL1 and promotes tumour cell survival, Nature 463 (2010) 103–107.

[28] S. Zhang, M. Zhang, Y. Jing, X. Yin, P. Ma, Z. Zhang, X. Wang, W. Di, G. Zhuang, Deubiquitinase USP13 dictates MCL1 stability and sensitivity to BH3 mimetic inhibitors, Nat. Commun. 9 (2018) 215.

[29] M. Herrant, A. Jacquel, S. Marchetti, N. Belhacene, P. Colosetti, F. Luciano, P. Auberger, Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis, Oncogene 23 (2004) 7863–7873.

[30] K. Podar, S.L. Gouill, J. Zhang, J.T. Opferman, E. Zorn, Y.T. Tai, T. Hideshima, M. Amiot, D. Chauhan, J.L. Harousseau, K.C. Anderson, A pivotal role for Mcl-1 in Bortezomib-induced apoptosis, Oncogene 27 (2008) 721–731.

[31] E. Naik, V.M. Dixit, Usp9X is required for lymphocyte activation and homeostasis through its control of ZAP70 ubiquitination and PKCβ kinase activity, J. Immunol. 196 (2016) 3438–3451.

[32] P.S. Hole, R.L. Darley, A. Tonks, Do reactive oxygen species play a role in myeloid leukemias? Blood 117 (2011) 5816–5826.

[33] K.A. Doshi, R. Trotta, K. Natarajan, F.V. Rassool, A.E. Tron, D. Huszar, D. Perrotti, M.R. Baer, Pim kinase inhibition sensitizes FLT3-ITD acute myeloid leukemia cells to topoisomerase 2 inhibitors through increased DNA damage and oxidative stress, Oncotarget 7 (2016) 48280–48295.

[34] A. Sallmyr, J. Fan, K. Datta, K.T. Kim, D. Grosu, P. Shapiro, D. Small, F. Rassool, Internal tandem duplication of FLT3 (FLT3/ITD) induces increased ROS production, DNA damage, and misrepair: implications for poor prognosis in AML, Blood 111 (2008) 3173–3182.

[35] J.A. Harrigan, X. Jacq, N.M. Martin, S.P. Jackson, Deubiquitylating enzymes and drug discovery: emerging opportunities, Nature reviews, Drug discovery 17 (2018) 57–78.