Anti-apoptotic HAX-1 suppresses cell apoptosis by promoting c-Abl kinase-involved ROS clearance

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The anti-apoptotic protein HAX-1 has been proposed to modulate mitochondrial membrane potential, calcium signaling and actin remodeling. HAX-1 mutation or deficiency results in severe congenital neutropenia (SCN), loss of lymphocytes and neurological impairments by largely unknown mechanisms. Here, we demonstrate that the activation of c-Abl kinase in response to oxidative or genotoxic stress is dependent on HAX-1 association. Cellular reactive oxygen species (ROS) accumulation is inhibited by HAX-1-dependent c-Abl activation, which greatly contributes to the antiapoptotic role of HAX-1 in stress. HAX-1 (Q190X), a loss-of-function mutant responsible for SCN, fails to bind with and activate c-Abl, leading to dysregulated cellular ROS levels, damaged mitochondrial membrane potential and eventually apoptosis. The extensive apoptosis of lymphocytes and neurons in Hax-1-deficient mice could also be remarkably suppressed by c-Abl activation. These findings underline the important roles of ROS clearance in HAX-1-mediated anti-apoptosis by c-Abl kinase activation, providing new insight into the pathology and treatment of HAX-1-related hereditary disease or tumorigenesis.

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INTRODUCTION

The anti-apoptotic protein HAX-1, which was initially identified as HS-1 (hematopoietic lineage cell-specific protein)-associated protein [1], is ubiquitously expressed in various tissues and tumors [2, 3]. Similar to BCL-2 family members, it has BH1- and BH2-like domains and a C-terminal transmembrane domain. HAX-1 is critical for maintaining the inner mitochondrial membrane potential and protecting cells against apoptosis [4-8]. Extensive apoptosis in lymphocytes and neurons [9], even in cardiac myocytes [10] and melanoma cells [11], was observed in HAX-1-deficient mice, demonstrating the antiapoptotic role of HAX-1 [1, 12, 13]. Biallelic mutations in the human HAX-1 gene lead to autosomal recessive severe congenital neutropenia (SCN or Kostmann syndrome) and neurological abnormalities, mainly resulting from the loss of mitochondrial control of apoptosis [14-16]. The mitochondrial proteases Parl and HtrA2 have been reported to participate in HAX-1-induced anti-apoptosis by preventing the accumulation of activated Bax [9, 17]. However, the specific mechanism by which HAX-1 mutation leads to a variety of physiological aberrations is still unknown.

The nonreceptor tyrosine kinases c-Abl and Arg (abl-related gene, Abl2) are ubiquitously expressed in mammalian tissues with overlapping functions in cell proliferation, apoptosis, adhesion and cell migration [18-20]. The activities of Abl tyrosine kinases were autoinhibited under normal physiological conditions and activated by oxidative or genotoxic stress to facilitate ROS scavenging and DNA repair. c-Abl plays both proapoptotic and antiapoptotic functions depending on the cellular context [21]. While nuclear c-Abl is required for ion irradiation-induced apoptosis by interactions with p53 and p73 [22-24], the cytoplasmic Bcr-Abl kinase and C-terminal truncated forms of c-Abl (loss of nuclear localization signal) are strong inhibitors of apoptosis [25]. c-Abl−/− progenitor B cells are more sensitive than wild-type cells to apoptosis induced by growth factor deprivation and glucocorticoid treatment [26]. Importantly, embryos deficient in both c-Abl and Arg exhibit defects in neurulation and die by 11 days postcoitus with massive apoptosis in all tissues [27]. Concordantly, fibroblast cells from c-Abl−/− Arg−/− mice are much more sensitive to ROS stimulus than wild-type cells [28]. ROS activate c-Abl [29-31], either by activating ataxia-telangiectasia mutated (ATM) kinase [32, 33], by facilitating PKCδ mediated c-Abl phosphorylation, or activating c-Abl directly. Following the activation by ROS, c-Abl regulates ROS clearance by phosphorylating the key regulators of cellular ROS level such as catalase, glutathione peroxidase, and Prx1 [34].

Our previous work showed that HAX-1 was a candidate association protein of c-Abl by yeast two-hybrid assays. In this study, HAX-1 was determined to be a novel binding partner of c-Abl kinase, and its association with HAX-1 was indispensable for Abl kinase activation induced by oxidative or genotoxic stress.
HAX-1-mediated c-Abl activation also partially contributed to HAX-1-mediated anti-apoptosis and provided a clue for understanding HAX-1-related physiological aberrations.

RESULTS

HAX-1 interacts with c-Abl kinase in vivo and in vitro

To substantiate the potential association of HAX-1 and c-Abl, anti-c-Abl (or IgG as control) immunoprecipitates prepared from MCF-7 cell extracts were subjected to anti-HAX-1 immunoblotting. HAX-1 was detected in anti-c-Abl (but not IgG) immunoprecipitates, indicating the in vivo association of endogenous c-Abl and HAX-1 (Fig. 1A). Furthermore, Flag- or Myc-tagged c-Abl and HAX-1 were exogenously expressed in the cells, and their interactions were also observed by reciprocal immunoprecipitation (Fig. 1B, C). We also noticed that the exogenous Myc-c-Abl level was significantly downregulated by Flag-HAX-1 co-expression (Fig. S1A). In order to obtain a comparable Myc-c-Abl expression level, four fold amount of Myc-c-Abl plasmid was used in Flag-HAX-1 co-transfection than that in Flag-vector co-transfection (Fig. 1C). To define the
interaction domain of c-Abl kinase, exogenously expressed Flag-HAX-1 was incubated with GST-c-Abl-SH2, GST-c-Abl-SH3, or GST-conjugated Sepharose beads in vitro, and the adsorbates were analyzed by immunoblotting. The results showed that HAX-1 associated to either the SH3 or SH2 domain of c-Abl but not to the GST-only protein (Fig. 1D). No detectable tyrosine phosphorylation was observed in HAX-1 co-expressed with c-Abl, which suggests that c-Abl SH2 domain associates with HAX-1 indirectly (e.g. through c-Abl).

The N-terminal of HAX-1 (1–128 a.a.) contains putative but poorly similar Bcl-2 homology domains (BH1 and BH2) and a PEST sequence, and the C-terminus of HAX-1 (129–279 a.a.) contains a conserved C-terminal α-helix domain mainly responsible for interaction with other proteins. Our data showed that only HAX-1 (129–279), but not HAX-1 (1–128), could interact with c-Abl in vivo and in vitro (Fig. 1E, F). In accordance with these findings, HAX-1 (Q190X), a truncated mutant at position 190 leading to neutropenia and neuronal diseases, failed to bind with c-Abl (Fig. 1G). Consistent with Fig. S1A, Flag-c-Abl co-expressed with wild-type HAX-1 demonstrated a significantly decreased protein level (Fig. S1B). To achieve a balanced Flag-c-Abl expression level, 4 fold amount of Flag-c-Abl plasmid was used in wild-type HAX-1 co-transfection than that of HAX-1(Q190X) co-transfection (Fig. 1G). These results implied that the association of c-Abl with the C-terminus of HAX-1 might be involved in the physiological role of HAX-1. Using an in situ proximity ligation assay (in situ PLA), the interaction of endogenous c-Abl and HAX-1 was further confirmed by the observation of fluorescence spots in the cytoplasm (Fig. 2A, upper left panel).

The association of HAX-1 and c-Abl is strengthened by genotoxic or oxidative stimuli

HAX-1 was reported to be involved in the antagonism of apoptotic processes induced by starvation, cytokine withdrawal, irradiation, and genotoxic or oxidative stresses. We then examined whether the association of HAX-1 and c-Abl was regulated under stress conditions, since c-Abl kinase could also be activated by similar stimuli such as genotoxic drugs, ROS, or ionizing radiation [35, 36]. The intensity of the fluorescence signal in the in situ proximity ligation assay (in situ PLA), was observed to be enhanced (~3-fold) by CDDP treatment (Fig. 2A, B), and more strikingly upregulated (~7-fold) by H2O2 treatment (Fig. 2A, B) in a dose-dependent manner (Fig. 2C, D). No fluorescence signal of association was observed in HAX-1 knockout MCF-7 (MCF-7/HAX-1 RNAi) cells, excluding the existence of false-positive signals in wild-type cells (Fig. 2C, D). In concert with these findings by in situ PLA, it was also noted that a substantial amount of HAX-1 (~50%) colocalized with c-Abl in the cytoplasm after CDDP, compared with less than 30% of HAX-1 occupied by c-Abl under normal physiological conditions (Fig. 2E). Upon H2O2 stimulation, a significantly reinforced association of HAX-1 with c-Abl was also detected by immunoprecipitation as expected (Fig. 2F and S2A).

c-Abl kinase is activated by HAX-1 interaction

Previously studies have demonstrated that c-Abl is activated by several c-Abl binding partners by binding the SH3 and SH2 domains to relieve autoinhibition [37–39]. We then investigated whether c-Abl was activated by HAX-1 association. Normalized by immunoprecipitated c-Abl level, the autophosphorylation of c-Abl was significantly enhanced with the co-expression of HAX-1, including the phosphorylation of Y412, a representative phosphorylation site required for c-Abl kinase activation (Fig. 3A and S3A). The increased catalytic activity of c-Abl kinase was also detected in the presence of HAX-1 by an in vitro kinase assay using GST-Crk (120–225) as a substrate (Fig. 3B and S3B). Accordingly, in MCF-7/HAX-1 RNAi cells, the phosphorylation of exogenous c-Abl was significantly lower than that in wild-type cells (Fig. 3C and S3C). Moreover, since c-Abl activity is correlated with its Y412 phosphorylation status, the comparison of Y412 should be made based on the equal c-Abl background level. When normalized by the immunoprecipitated c-Abl protein level, c-Abl phosphorylation was hardly detected by HAX-1 knockdown even with H2O2 treatment, which indicated that c-Abl kinase could not be effectively activated by H2O2 without HAX-1 involvement (Fig. 3D, 6th lane, and Fig. S3D). Similarly, an IR-induced increase in Y412 phosphorylation was observed in wild-type MCF-7 cells but not in MCF-7/HAX-1 RNAi cells (Fig. 3E and S3E). We then rescued HAX-1 expression by concurrent expression of RNase-resistant HAX-1 in MCF-7/HAX-1 RNAi cells. Compared with the control, HAX-1 rescue resulted in enhanced phosphorylation and significant activation of c-Abl kinase in response to H2O2 stimulation (Fig. 3D, 6th lane, and Fig. S3D). Similarly, an IR-induced increase in Y412 phosphorylation was observed in wild-type MCF-7 cells but not in MCF-7/HAX-1 RNAi cells (Fig. 3E and S3E). Concordantly, the HAX-1(Q190X) mutant that was not associated with c-Abl failed to activate ectopically expressed or endogenous c-Abl regardless of the presence of stress stimuli (Fig. 3F, G and S3F–S3G). In these experiments, the cellular c-Abl level normalized by beta-Actin in lysates were also detected and shown in the right panel of Figure S3D–S3G. Additionally, in situ PLA showed that the association of HAX-1 with phosphorylated c-Abl was significantly enhanced in HAX-1-overexpressing cells (Fig. 3H). These results collectively demonstrated a new mechanism of c-Abl kinase activation by HAX-1 association, which is also indispensable for oxidative or genotoxic stress-induced c-Abl activation.

HAX-1 facilitated C-Cbl-mediated ubiquitin-proteasomal degradation of c-Abl kinase

Activation of c-Abl leads to its notable degradation through the ubiquitin-proteasomal pathway[40]. Accordingly, endogenous or ectopically expressed c-Abl levels were dramatically downregulated by full-length HAX-1 in a dose-dependent manner but not...
Fig. 2 Interaction of c-Abl with HAX-1 in different physiological contexts. A An in situ proximity ligation assay (in situ PLA) was used for the detection of HAX-1 and c-Abl binding complexes. MCF-7 cells treated with or without CDDP (25 mM, 8 h) or H2O2 (1 mM, 3 h) were incubated with target primary antibodies from two different species or with anti-c-Abl antibody as a control. The red spots reveal c-Abl/HAX-1 interaction. Nuclei are stained with Hoechst 33342. Slides were evaluated using an LSM 510 META confocal microscope (Carl Zeiss). Cell images obtained were exported using the Zeiss LSM Image Browser (Carl Zeiss) in TIF format for further analysis. B Quantification of HAX-1-c-Abl interaction complexes. The number of complexes per cell was counted in at least three fields. Quanti fications were given as the mean±S.D. Representative results are shown from experiments repeated three times. ***p < 0.001, Student’s t test. C MCF-7 or HAX-1 siRNA cells treated with the indicated dosage of H2O2 were subjected to in situ PLA analysis. D Quantification of HAX-1-c-Abl interaction complexes. The number of complexes per cell was counted in at least three fields. Quanti fications were given as the mean ± S.D. Representative results are shown from experiments repeated three times. n.s., not significant; **p < 0.01, ***p < 0.001, Student’s t test. E MCF-7 cells treated with or without CDDP (25 mM, 8 h) were incubated with target primary anti-c-Abl and anti-HAX-1 antibodies and then incubated with FITC- or TRITC-linked secondary antibodies. Nuclei are stained with Hoechst 33342 (left). The relative colocalization ratio of HAX-1 with c-Abl was analyzed by ImageJ software (right). At least 15 cells were analyzed, and the data were shown as mean±S.D. *p < 0.05, Student’s t test. F Lysates prepared from MCF-7 cells treated with or without H2O2 (1 mM, 3 h) were analyzed by immunoprecipitation and immunoblotting.
by the truncated mutant HAX-1 (Q190X) (Fig. 4A, B and S4A–S4C) and were upregulated by HAX-1 knockdown (Fig. 4C and S4D). c-Abl mRNA levels were nearly unchanged by the overexpression or RNAi knockdown of HAX-1 (Fig. S4E). The stimuli dose-dependent increase in HAX-1 levels induced by H2O2 evidently contributed to the c-Abl kinase decrease (Fig. 4D and S4F). Furthermore, it was found that the half-life of endogenous c-Abl was ~6.17 h, which was coincident with a previous report[41] but appreciably reduced to ~3.31 h in the presence of ectopically expressed HAX-1, as determined by a [35S-Met]-labeled pulse-chase assay (Fig. 4E). Significantly short-lived exogenous c-Abl was also observed with the co-expression of HAX-1 in 293 cells in the presence of the protein biosynthesis inhibitor cycloheximide (CHX) (Fig. S4G). These results indicated that the degradation of c-Abl kinase was notably regulated by HAX-1, accompanied by kinase activation.
In line with the finding that the E3 ubiquitin ligase c-Cbl mediated the degradation of activated c-Abl kinase through the ubiquitin-proteasome pathway [41, 42], the c-Abl kinase abundance in wild-type cells was upregulated to a level similar to that in HAX-1 RNAi cells after proteasome inhibitor MG132 treatment (Fig. S4H). Furthermore, the binding of c-Abl with its E3 ubiquitin ligase c-Cbl was remarkably strengthened in the presence of HAX-1 (Fig. 4F and S4I). Consequently, the polyubiquitination of c-Abl in HAX-1 RNAi cells was quite lower than that in wild-type cells and was substantially potentiated by RNAi-resistant HAX-1 rescue (Fig. 4G and S4J), which was coincident with HAX-1-mediated c-Abl level regulation. Importantly, similar to HAX-1 overexpression, IR stimuli not only activated c-Abl kinase but also greatly enhanced the interaction of c-Abl with c-Cbl and c-Abl polyubiquitination (Fig. 4H and S4K) but failed to induce the ubiquitination of c-Abl in HAX-1-deficient cells compared with wild-type or scrambled RNAi cells (Fig. 4I and S4L). These findings collectively indicated that HAX-1 contributes to c-Abl activation and degradation and that the stimulatory factors that activate c-Abl kinase activity are essentially dependent on the presence of HAX-1.

The anti-apoptosis mediated by HAX-1 was partially dependent on c-Abl activation

We then deeply investigated the biological effects of the HAX-1 c-Abl association. As reported previously, c-Abl kinase activated by ROS regulates the activity of catalase and glutathione peroxidase 1 [28, 43]. Considering that HAX-1 plays important role in neurons apoptosis [9, 44], HAX-1-mediated anti-apoptosis was evaluated not only in MCF-7 cells, but also in neuroblastoma SH-SY5Y cells. HAX-1 c-Abl association and HAX-1-regulated c-Abl expression were observed in SH-SY5Y cells similarly, indicating that ROS scavenging function of HAX-1 c-Abl axis did not limit to a certain cell line (Fig. S5A–S5D). As expected, HAX-1 but not HAX-1 (Q190X) expression dramatically reduced ~40% of cellular ROS in neuron-like SH-SY5Y cells (Fig. 5A). Moreover, HAX-1 knockdown resulted in increased intracellular ROS levels, similar to the knockdown of c-Abl/Arg (Fig. 5B). Notably, compared with the respective knockdown, simultaneous knockdown of both HAX-1 and c-Abl/Arg in the same cell did not lead to a more serious increase in ROS, suggesting that HAX-1 and c-Abl/Arg might regulate cellular ROS levels by the same pathway, in which HAX-1-mediated c-Abl activation functions as ROS scavenger (Fig. 5B). Accordingly, HAX-1 knockdown-induced ROS increases could be partly rescued by ectopic expression of c-Abl with constitutive activity (Fig. 5C). In contrast, ectopic expression of HAX-1 in c-Abl/Arg knockdown cells had only a minor effect (Fig. 5C).

Furthermore, the mitochondrial membrane potential was dramatically reduced by HAX-1 knockdown (Fig. 5D). Moreover, treatment with the Abl kinase-specific inhibitor STI571 also resulted in a greatly deleterious effect on mitochondrial membrane integrity in wild-type cells but not in HAX-1-deficient cells (Fig. 5D). Accordingly, ~40% of HAX-1 knockdown cells treated with H2O2 were subjected to apoptosis, which was much higher than the ~20% apoptosis ratio of wild-type cells with the same treatment. However, the difference in ROS-induced apoptosis between wild-type and HAX-1 knockdown cells was not observed after STI571 treatment (Fig. 5E), indicating that HAX-1-mediated anti-apoptosis was c-Abl kinase dependent.

Next, we extensively compared H2O2-induced apoptosis in HAX-1−/−, c-Abl/Arg−/−, or both-knockdown cells. Similar to STI571 treatment, knockdown also led to substantially increased apoptosis after H2O2 stimulation compared with wild-type cells (Fig. 5F, G). Notably, knockdown of c-Abl/Arg in HAX-1 RNAi cells did not result in more serious apoptosis, suggesting that the individual rescue of either HAX-1 or c-Abl/Arg expression could not relieve ROS-induced apoptosis in HAX-1 c-Abl/Arg triple-knockdown cells (Fig. 5F, G). These findings provide a new mechanism of HAX-1-mediated anti-apoptosis, by which HAX-1 may antagonize ROS-induced cell apoptosis and protect cells from oxidative damage primarily dependent on c-Abl kinase activity.

**HAX-1 insufficiency-induced ROS accumulation and cell death could be rescued by c-Abl activation**

We next investigate whether HAX-1 insufficiency-induced ROS accumulation and cell death could be rescued by c-Abl activators. c-Abl showed a compromised activity by HAX-1 knocking-down, as shown by decreased Y412 autophosphorylation, or Y207 phosphorylation of CrkL, and was activated by DPH (5-(1,3-diaryl-1H-pyrazol-4-yl) hydantoin), a small-molecule reagent that binds to the myristoyl binding site to activate cellular c-Abl, at a concentration of 10 μM (Fig. 6A and S6A-S6B) in scramble as well as HAX-1 knockdown cells. The cellular c-Abl level normalized by beta-Actin in lysates were also detected and shown in the right panel of Fig. 6A. In accordance, loss of HAX-1 resulted in increased levels of ROS and increased apoptosis in MCF-7 cells, whereas the increase was markedly prevented by treatment with DPH (Fig. 6B). Similarly, mice PMN cells (Fig. 6C) and neuron-like SH-SY5Y cells (Fig. 6D) also exhibited an increased ROS level and increased apoptosis by HAX-1 knockdown (Fig. 6B, C and S6C). Consistently, treating HAX-1 RNAi cells with DPH led to a reduction
in cellular ROS level, and decreased apoptotic cells, which was not observed by nilotinib treatment, an inhibitor of c-Abl kinase (Fig. 6C, D). This finding indicated that apoptosis caused by HAX-1 insufficiency could be partially rescued by c-Abl activation. Further, in concert with the previous studies showed that Hax1-null mice exhibited extensive apoptosis of neurons in the striatum and cerebellum [9], administration of DPH via the tail vein in Hax-1-null mice exhibited severely decreased apoptosis to ~20% in the striatum and ~1% in the cerebellum, in comparison to Hax-1-null mice injected with vehicle (Fig. 6E and S6D). These results indicate that activation of c-Abl by DPH treatment protected nerve and PMN cells from HAX-1 deficiency-induced apoptosis. And, importantly, glutathione, a ROS scavenger, showed significant protective effect on HAX-1 insufficiency-induced cell apoptosis (Fig. 6C).

**DISCUSSION**

HAX-1 was first noted to be a Bcl-2 family member based on its homology with the anti-apoptotic protein [1] and was then found to be a regulator of calcium signaling [45, 46] participating in mitochondria [4] and postmitochondrial apoptosis [47]. HAX-1 was reported to contribute to the processing and activation of the antiapoptotic factor HtrA2 by the mitochondrial protease PARL, thus preventing the accumulation of proapoptotic Bax in the outer mitochondrial membrane [9]. However, the conclusion was later questioned by the observation that HAX-1 lacks BH modules and is peripherally associated with heavy membranes and cannot be mechanistically coupled to PARL because the two proteins are confined in distinct cellular compartments in vivo [48]. These observations suggested that HAX-1 may function in cell apoptosis by as yet unrevealed mechanisms.
C-Abl plays a vital role in the complex regulation of apoptosis, cell proliferation, survival, and cell spreading, including the responses to oxidative stress and DNA damage [18]. It maintained relatively low activity in a normal state and was activated following exposure to many genotoxic agents (e.g., IR, cisplatin, methyl methane sulfonate, mitomycin) and ROS. Protein crystallographic structures of the c-Abl autoinhibited fragment show that the SH3 and SH2 domains are docked onto the surface of the kinase domain distal to the active site and that the kinase is activated through a conformational change in the SH2/SH3 domain [49]. c-Abl interacting proteins may either inhibit (Pag/MSP23 and Aap1) [50, 51] or activate (Crk26 and the DNA-binding protein RXF113) c-Abl [37, 38]. Additionally, oligomerized Abi-1 interacts with c-Abl and contributes to the modulation of autophosphorylation and kinase activity in both normal and oncogenic processes [52]. c-Abl activation also requires and results in autophosphorylation on several tyrosine residues, including Y412 in its activation loop [39]. The crystal structure of the nonphosphorylated Abl kinase domain shows the activation loop tyrosine pointing into the interior of the kinase, making it inaccessible to phosphorylation. The binding of Hck to c-Abl may increase the structural plasticity of the activation loop or induce a conformational change that exposes Y412 for phosphorylation [39], thus resulting in the activation of the kinases. In this study, it activated c-Abl significantly upon ROS stimulation, thereby promoted c-Abl-mediated activation of catalase and glutathione peroxidase to carry out ROS clearance. As reported previously, a decreased stability of activated c-Abl kinase was also observed not only by stress stimuli but also by HAX-1 over-expression. During this process, the activation of c-Abl and c-Abl-dependent stress-responsive signaling pathways is a more major and rapid event than the subsequent degradation of c-Abl. Although c-Abl activation is transient, its downstream signal transduction lasts until the phosphorylated substrates effector was exhausted. Essentially, it is Abl activation but not degradation that contributes to HAX-involved anti-apoptosis.

Our results also support a central connection between HAX-1 and c-Abl in ROS level modulation. HAX-1/c-Abl interactions were dramatically potentiated by H2O2, and the expression of HAX-1 strikingly potentiated c-Abl kinase activation (Figs. 2, 3). Similar to c-Abl/Arg knockout, downregulation of HAX-1 expression conferred significant increases in intracellular ROS levels, which were partially rescued by c-Abl overexpression. Simultaneous knockdown of HAX-1 and c-Abl/Arg kinases failed to show any synergizing effect, supporting HAX-1 and c-Abl/Arg regulation of cellular ROS by the same pathway (Fig. 5). Similarly, HAX-1 controlled cytoplasmic oxidative stress-induced apoptosis by activating c-Abl and Arg, which were shown to activate antioxidative enzymes, with the subsequent elimination of H2O2 or other reactive oxidative species (ROS), generating a protective effect against oxidative stress [28, 43]. It has been reported that HAX-1 blocks cell apoptosis by inhibiting the activation of the initiator caspase-9 and death caspase-3 [10, 54]. According to our study, these contributions may be mediated through the regulation of cellular ROS levels by HAX-1 because increasing ROS levels could also activate caspase-9/3 [55, 56]. Loss-of-function mutations of HAX-1 resulted in Kostmann disease, an inherited severe congenital neutropenia syndrome (SCN)[14]. HAX-1-deficient neutrophils in SCN patients, but not SCN neutrophils expressing functional HAX-1, showed evidence of enhanced production of ROS. Neutrophils isolated from the patient exhibited spontaneous apoptosis and loss of inner

**Fig. 4** HAX-1 contributes to a reduction in c-Abl expression levels. A Western blot analysis with specific antibodies for determination of the levels of total Flag-c-Abl status in HEK 293 cells expressing different dosages of Myc-HAX-1 protein. All cells were transfected with 1 μg Flag-c-Abl, and lanes 2, 3 and 4 were transfected with 0.5, 1.0 and 2.0 μg Myc-HAX-1, respectively. Mock (Lane 1) served as a control in which empty vector was used to transfect the cells. Cellular tubulin was used as an internal control for comparison of protein load in each lane. B Western blot analysis of HEK 293 cells transfected with the indicated plasmids. The expression of β-actin served as a loading control. C Western blot using anti-HAX-1 antibody identified the MCF-7 clones that were transfected with HAX-1-over-expressed HEK 293 cells. The expression of c-Abl was also determined for comparison of protein expression levels, and the expression of HAX-1 was also determined. The expression of c-Abl in the transfection was determined by autoradiography. The expression level of c-Abl and c-Abl was normalized to the tubulin loading control. D HEK 293 cells were treated with increased concentrations of H2O2 for 3 h and subjected to Western blot analysis. E Flag-c-Abl was co-expressed with the Myc-vector or with Myc-HAX-1 in HEK 293 cells. Cells were pulsed with [35S] methionine for 45 min, washed and then incubated in [35S] methionine-free DMEM for the indicated time. Lysates were immunoprecipitated with anti-Flag antibody and analyzed by SDS-PAGE and autoradiography. The half-life of c-Abl was calculated according to the intensity of the signals of three independent experiments. F Flag-c-Abl was transfected into HEK 293 cells with or without Myc-HAX-1-over-expressed HEK 293 cells. Cell lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-c-Cbl or anti-Flag antibody. G MCF-7 scramble c-Abl in transfected with Myc-HAX-1, and HAX-1 knockout cells were transfected with a Myc-HAX-1 rescue construct (Mut). For the ubiquitination assay, cells were treated with MG132 (10 μM) 12 h before harvesting. Cell lysates were immunoprecipitated with anti-c-Abl antibody or IgG as a control. The precipitates were analyzed by Western blot using indicated antibodies. H MCF-7 cells transfected with or without HAX-1 were IR treated (10 Gy) as indicated. Cell lysates were immunoprecipitated with anti-c-Abl antibody and immunoblotted with indicated antibodies. I MCF-7 scramble and two HAX-1 knockdown clones overexpressing Myc-Ub were IR (10 Gy) treated. After 2 h, cells were harvested, and cell lysates were immunoprecipitated with anti-Myc antibody. The ubiquitination levels of the precipitates were assessed by Western blot.
mitochondrial membrane potential, which were further enhanced upon treatment with hydrogen peroxide [57, 58]. HAX-1 was also shown to be protective in MCF-7 cells and neuron-like SHY5Y cells from H₂O₂-induced cell death. Recently, it was observed that HAX-1 negatively regulates integrin-mediated adhesion that affects uropod detachment and neutrophil chemotaxis, a process that may be key to the pathogenesis of congenital neutropenia syndromes, such as Kostmann disease [15]. Similarly, c-Abl/Arg knockdown resulted in elevated ROS levels. c-Abl also regulates human neutrophil chemotactic activity [59, 60], and the Abl kinase selective inhibitor STI571 can induce neutropenia [61]. Since HAX-1 activates c-Abl to downmodulate cellular ROS levels, SCN neutrophils, where HAX-1 is often functionally mutated or not expressed [14, 62], will be compromised for c-Abl activation, resulting in cellular ROS accumulation and neutrophil death. HAX-1-modulated c-Abl activation may be responsible for the regulation of myeloid cell migration and has a likely role in the pathogenesis of SCN. Our findings underscore the important coordinated role of HAX-1 and c-Abl and form a foundation for further study of critical pathways in cellular stress responses, and patients suffered from Kostman disease may be benefited from c-Abl activators such as DPH, or ROS scavengers.
**MATERIALS AND METHODS**

**Plasmids and Generation of HAX-1 Mutants**
Flag-tagged vectors containing c-Abl, c-Abl (K290R), and HAX-1 were constructed by cloning the full-length human HAX-1 (NM_006118) genes into the pcDNA3 vector (Invitrogen, V7952). Myc-tagged expression plasmids were prepared by cloning into the pcMV-Myc vector (Clontech, 6356589). GST fusion proteins were generated by expression in pGEX4T-2-based vectors (Amersham Biosciences Biotech Inc.) in *Escherichia coli* BL21 (DE3).

Deletion mutants were generated by PCR amplification using HAX-1 plasmid as a template, and the following sets of primers: (i) sense primer-1: 5′-CGGGATCCATGCTTAAGTATCCAGATAGTCACCAG-3′ and antisense primer-1: 5′-GGAGATCTCTACCGGGACCGGAACCAACGT-3′; (ii) sense primer-2: 5′-GAAGATCTCTACCGGGACCGGAACCAACGT-3′ and antisense primer-1: 5′-GGAGATCTCTACCGGGACCGGAACCAACGT-3′; (iii) sense primer-2: 5′-GGAGATCTCTACCGGGACCGGAACCAACGT-3′ and antisense primer-2: 5′-GGAGATCTCTACCGGGACCGGAACCAACGT-3′. These primers were used for generation of a HAX-1 lacking the N-terminal 128 amino acids which contains BH1, BH2 as well as PEST motifs, (ii) sense primer-2: 5′-GAAGATCTCTACCGGGACCGGAACCAACGT-3′ and antisense primer-1: 5′-GGAGATCTCTACCGGGACCGGAACCAACGT-3′; (iii) sense primer-2: 5′-GAAGATCTCTACCGGGACCGGAACCAACGT-3′ and antisense primer-2: 5′-GGAGATCTCTACCGGGACCGGAACCAACGT-3′. All antisense primers contained a BglII site for insertion into the pCMV-myc vector and generation of Myc-tagged fusion proteins. The primer 5′-TGATCCTTTTTCGATCCAAGACCCAG-GAGGG-3′ was used to construct the HAX-1 Q190X mutant. The authenticity of all constructs was verified by DNA sequencing.

**Cells and transfection**
Human embryonic kidney (HEK) 293 cells, the human breast adenocarcinoma cell line MCF-7 and the human neuroblastoma cell line SH-SY5Y were obtained from ATCC (https://www.atcc.org/). The cells were verified and were free of mycoplasma contamination based on the results of the Mycoplasma Stain Assay Kit (Beyotime). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Sigma, RPN3541) supplemented with 10% heat-inactivated fetal bovine serum (Teconco, F801-500), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transient transfections were performed with Lipofectamine 2000 (Invitrogen, 11668019). Cells were treated with STI571 (matinib, Novartis, 22012757-1), cisplatin (Sigma, 1566327-1), H2O2 (Sigma, 7722-84-1), MG132 (Sigma, MB6899) or lactacystin (Biomy, HY-165949) as indicated.

**Immunoprecipitation and immunoblot analysis**
Cell lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5 mM EDTA) supplemented with complete protease inhibitor cocktail (Roche,4069313001). Soluble protein was subjected to immunoprecipitation with anti-Flag antibody (agarose-conjugated, M-2, Sigma-Aldrich, A2220), anti-c-Abl antibody (K-12, Santa Cruz Biotechnology, Cat# SC-131), or anti-rabbit IgG antibody (agarose-conjugated, Sigma-Aldrich). An aliquot of the total lysate (5%, v/v) was included as a control. Immunoblot analysis was performed with anti-HAX-1 antibody (FL-279, Santa Cruz Biotechnology, sc-28268), anti-c-Abl antibody (K12 rabbit polyclonal antibody, Santa Cruz), anti-c-Cbl antibody (Santa Cruz, sc-1651), anti-tubulin antibody (Sigma-Aldrich, Cat# T8282), anti-β-actin(Santa Cruz, Cat# SC-1616), or HRP-conjugated anti-Flag antibody (Sigma-Aldrich, Cat# A8592), anti-Myc antibody (Santa Cruz, Cat# SC-40), anti-β-actin antibody (Bio-Rad, LS-B219), anti-Ub antibody (Bio-Rad, NSJ-R30490) and anti-phosphotyrosine antibody (4G10, Millipore, Cat# 16-105). The antigen-antibody complexes were visualized by chemiluminescence (ECL, Millipore, WBKLS0500). Data shown are representative of three independent experiments. The relative intensity of WB bands was quantified by gray scanning and presented as mean±S.D. The intensity of the following bands was calculated as the mean±S.D. of three independent experiments. n.s., not significant; *p < 0.05; **p < 0.01; ***p < 0.001, Student’s t test.

**Glutathione transferase (GST)-pull down assay**
GST fusion proteins were generated by expression in pGEX4T-2 (Amersham Pharmacia Biotech Inc.) vectors in *E. coli* BL21 (DE3) (Novagen) and purified by affinity chromatography using glutathione Sepharose beads (GE Healthcare). Cells lysates were incubated with 2 μg of GST, GST-HAX-1 and GST-c-Abl/Arg/HAX-1 immobilized on the beads for 2 h at 4°C. The adsorbates were washed with lysis buffer and then subjected to SDS-PAGE and immunoblot analysis. An aliquot of the total lysate (2%, v/v) was included as a loading control. Data shown are representative of three independent experiments.

**Pulse-chase Assays**
HEK 293 cells transfected with Flag-c-Abl/Myc-Vector or Flag-c-Abl/Myc-HAX-1 were washed with Met/Cys-free DMEM (Gibco), and then incubated with Met/Cys-free DMEM containing 10 μM (135S) methionine (Amer sham Biosciences Biotech Inc.) for 45 min. The cells were then washed and cultured in complete DMEM containing 10% heat-inactivated FBS and harvested at the indicated time points. Anti-Flag immunoprecipitates were subjected to SDS-PAGE and autoradiography. The bands were then excised and subjected to liquid scintillation counting for quantification. Data shown are representative of three independent experiments.

**c-Abl tyrosine kinase assay**
 Purified recombinant GST-HAX-1 (0.5 μg) or GST-Crk was incubated with recombinant c-Abl (0.02 μg; Upstate Biotechnology Inc.) in kinase reaction buffer (20 mM HEPES (pH 7.5), 75 mM KCl, 10 mM MgCl2, and 10 mM MnCl2) containing 2 mM ATP for 30 min at 37°C. The reaction products were analyzed by SDS-PAGE and immunoblot. A fusion protein GST-Crk containing the c-Abl phosphorylation site in the adapter protein CRK was used as a specific substrate to assay c-Abl kinase. Data shown are representative of three independent experiments. The relative intensity of WB bands was quantified by gray scanning and presented as mean±S.D. of three independent analysis. ***p < 0.001, Student’s t test.

Silencing HAX-1 with short interfering RNAs (siRNA)
The HAX-1 siRNA sequences were selected by using an siRNA selection program. Synthesized and purified oligonucleotides were annealed and...
Fig. 6  c-Abl activation protected nerve cells from HAX-1 insufficiency-induced ROS accumulation and death. A The MCF-7 scramble or HAX-1 knockdown cell line (MCF-7/siHAX-1) was treated with the indicated concentration of DPH for 12 h, and cell lysates were analyzed by immunoprecipitation and immunoblotted with indicated antibodies. The immunoprecipitates were normalized by c-Abl level. B MCF-7 scramble or HAX-1 knockdown cell lines (MCF-7/siHAX-1) with the indicated H2O2 (3 h) or DPH (4 h) treatment were subjected to flow cytometry analysis to evaluate ROS levels (left panel) and ratio of cell death (right panel). n.s., not significant; *p < 0.05, Student’s t test. C PMN-like cells were transfected with lentivirus-HAX-1-siRNA or lentivirus-Scramble-siRNA (MOI = 5), and HAX-1-siRNA cells were treated with DPH (10 μM, 12 h) or GSH (1 mM, 3 h). ROS levels and ratio of cell death of all the cells were measured after exposure to 1 mM H2O2 for 3 h (left panel) or for 8 h (right panel), respectively. *p < 0.05, **p < 0.01, Student’s t test. D Scramble cells, siRNA-HAX-1 SY5Y cells, and siRNA-HAX-1 SY5Y cells treated with nilotinib (5 μM, 18 h) or DPH (10 μM, 18 h) were subjected to 0.5 mM H2O2 treatment for 2 h, and the death rate was analyzed. n.s., not significant; *p < 0.05, **p < 0.01, Student’s t test. E WT or Hax-1 null mice were tail vein injected with DPH or vehicle daily for 30 days, and the striatum were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. TUNEL-positive cells are marked in green, and nuclei are marked with DAPI. Right panel, quantification and statistical analysis of TUNEL-positive cells. **p < 0.01, Student’s t test.
cloned into the pSUPER-retro-neo plasmid (Oligo Engine, Inc.). The HAX-1 siRNA construct was named pSuper-GFP-HAX-1-siRNA. The scrambled control plasmid (pSuper-GFP-C) encodes an shRNA that did not match any sequence found in the human genome database. The sequence used for constructing pSuper-GFP-HAX-1 siRNA was 5'-GATCCCCAGAGGAGGAACATATGCTTT-CAGAGAGATCATTGCTCTCTGGTTTTTTTA-3'. The scrambled sequence used as a control was 5'-GATCCCCAGAGGAGGAACATATGCTTT-CAGAGAGATCATTGCTCTCTGGTTTTTTTA-3'. The primer 5'-CTTAGAAACCAGGAGAATAGTGCTCTTTTTTA-3' was used to construct a HAX-1 mutant that could not be influenced by RNAi. Stable cell lines were obtained by treating the cells with 800 μg/mL G418 and identified by GFP and HAX-1 protein expression.

**Apoptosis analysis by FITC-Annexin V and propidium iodide staining**
To monitor the association of HAX-1 with cell apoptosis, MCF-7 cells were analyzed by flow cytometry using FITC-Annexin V (FITC-ANV) and propidium iodide (PI) staining. Apoptotic cells were identified as ANV+PI+ on a BD Biosciences FACSCalibur. The mean frequencies of apoptotic cells were calculated as the mean±S.D. of three independent experiments. n.s., not significant; *p < 0.05, **p < 0.01, Student’s t test.

**Detection of ROS**
Cells were harvested using trypsin, washed twice with serum-free DMEM, and then incubated with DCFH-DA (Beyotime) at a final concentration of 10 μM for 20 min at 37 °C in the dark. Then, the cells were washed once with serum-free DMEM and analyzed immediately by flow cytometry. Data were shown as mean±S.D. of three independent analysis. n.s., not significant; *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t test.

**Measurement of mitochondrial membrane potential by JC-1**
Cells were harvested using trypsin and stained with JC-1 (Beyotime) according to the manufacturer’s protocol. The ratio of JC-1 aggregate/JC-1 monomer was determined by calculating the mean FL2/FL1 fluorescence detected by flow cytometry. Data were shown as mean±S.D. of three independent analysis. n.s., not significant; **p < 0.01, Student’s t test.

**In situ proximity ligation assay (in situ PLA)**
Duolink in situ PLA (Duolink Detection kit, Olink Bioscience, Uppsala, Sweden) was used to detect interactions between HAX-1 and c-Abl. Briefly, MCF-7 cells plated on glass coverslips were treated with cisplatin for 24 h and fixed as described above. The fixed cells were incubated with rabbit anti-HAX-1 and mouse anti-c-Abl (Sigma-Aldrich) primary antibodies. The Duolink system provides oligonucleotide-labeled secondary antibodies (PLA probes) to each of the primary antibodies that, in combination with a DNA amplification-based reporter system, generate a signal only when the two primary antibodies are in close enough proximity. The signal from each detected pair of primary antibodies was visualized as a spot (see the manufacturer’s instructions). Slides were evaluated using an LSM 510 META confocal microscope (Carl Zeiss). Cell images obtained were exported in TIF format for further analysis. Interactions per cell were determined with the Duolink image tool, which was developed by Olink Bioscience and was counted in at least three fields. Quantifications were given as the mean±S.D. n.s., not significant; **p < 0.01, ***p < 0.001, Student’s t test. Representative results are shown from experiments repeated three times.

**Confocal microscopy**
Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 for 10 min at room temperature, and nonspecifically blocked with PBS buffer containing 1% goat serum for 1 h. The cells were then incubated with primary antibody for 1 h and secondary antibody for another 1 h at room temperature. Nuclei were stained with Hoechst33342. Images were randomly obtained using the Confocal Image Browser (Carl Zeiss) in TIF format. Representative results are shown from experiments repeated three times.

**Mice**
C57 Hax-1+/− mice were purchased from Cyagen Biosciences Inc., and heterozygous mice were crossed to generate homozygosity. All mice were bred and maintained in the animal facility of the Military Medical Research Institute according to the institutional and national guidelines for animal care and use. The animal studies were approved by the Institutional Ethics Committee of Military Medical Science.

**Apoptosis detection of neurons in the striatum and cerebellum**
Twenty-eight-day-old WT or Hax-1 null female mice (eight mice per group) were randomly divided into groups before the experiment, then tail vein injected with DPH (5 μg) or vehicle daily for 30 days. The mice were sacrificed and immediately perfused with 4% paraformaldehyde. The striatum and cerebellum were extracted and fixed in 4% paraformaldehyde and then subjected to terminal deoxynucleotidy transferase-mediated dUTP nick end labeling (TUNEL) staining according to the standard protocol of the Roche In Situ Cell Death Detection Kit. TUNEL-positive cells are marked in green, and nuclei are marked with DAPI. Representative images are shown. The TUNEL assays were performed by Wuhan Servicebio Technology Co., Ltd. Investigators were blinded to the order of samples.

**Statistical analysis**
All experiments were replicated at least three times, and Statistical analysis was carried out using unpaired two-tailed Student’s t test by GraphPad Prism 7. No data were excluded from the analyses unless indicated. Data were considered significant when p < 0.05.

**DATA AVAILABILITY**
All data generated or analyzed during this study are included in the article and its supplementary files, and available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS
CC and XL designed the study. QD, DL, HZ, XZ, YY, GW, YL, HL, YH, LZ, TG, XN, TZ, CS, JJ, DW, and YB performed the experiments. QD, XL, and CC analyzed data. ZL, YJ, and PL developed protocols and provided reagents. QD, CC, and XL wrote the manuscript.

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COMPETING INTERESTS
The authors declare no competing interests.

ETHICS STATEMENT
The animal studies were approved by the Institutional Ethics Committee of Military Medical Science.
ADDITIONAL INFORMATION

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