Severe congenital neutropenia-associated *JAGN1* mutations unleash a calpain-dependent cell death programme in myeloid cells

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Summary

Severe congenital neutropenia (SCN) of autosomal recessive inheritance, also known as Kostmann disease, is characterised by a lack of neutrophils and a propensity for life-threatening infections. Using whole-exome sequencing, we identified homozygous *JAGN1* mutations (p.Gly14Ser and p.Glu21Asp) in three patients with Kostmann-like SCN, thus confirming the recent attribution of *JAGN1* mutations to SCN. Using the human promyelocytic cell line HL-60 as a model, we found that overexpression of patient-derived *JAGN1* mutants, but not silencing of *JAGN1*, augmented cell death in response to the pro-apoptotic stimuli, etoposide, staurosporine, and thapsigargin. Furthermore, cells expressing mutant JAGN1 were remarkably susceptible to agonists that normally trigger degranulation and succumbed to a calcium-dependent cell death programme. This mode of cell death was completely prevented by pharmacological inhibition of calpain but unaffected by caspase inhibition. In conclusion, our results confirmed the association between *JAGN1* mutations and SCN and showed that SCN-associated *JAGN1* mutations unleash a calcium- and calpain-dependent cell death in myeloid cells.

Keywords: apoptosis, calcium, calpain, necroptosis, neutropenia.

Severe congenital neutropenia (SCN) is characterised by early onset, life-threatening infections accompanied by a lack of mature neutrophils, with absolute neutrophil counts (ANCs) of $<0.5 \times 10^9$/l.[1] Furthermore, these patients are at risk of developing myelodysplastic syndrome/leukaemia.[2,3] Previous studies have identified mutations in several genes including neutrophil elastase (*ELANE*), HCLS1 associated protein X-1 (*HAX1*), glucose-6-phosphatase catalytic subunit 3 (*G6PC3*), growth factor independent protein 1 (*GFI1*), and vacuolar protein sorting-associated protein 45 (*VPS45*) in patients with congenital neutropenia, and these gene defects were all linked to the induction of programmed cell death (apoptosis).[4–10] Boztug *et al.*[11] provided the first description of jagunal homologue 1 (*JAGN1*) mutations in SCN, and the authors noted aberrant protein glycosylation and an increased sensitivity to apoptotic stimuli in *JAGN1*-deficient neutrophils. Jagunal was originally identified in the fruit fly and is required for re-organising the endoplasmic reticulum (ER) during oogenesis.[12]

More recent studies have shown that JAGN1 and its murine homologue are required for the anti-fungal actions of neutrophils.[13,14] Nevertheless, the precise role of JAGN1 in the regulation of cell death remains poorly understood.

Myeloid progenitor cells from patients with Kostmann disease, an autosomal recessive form of SCN, display excessive, mitochondria-dependent apoptosis when cultured *ex vivo*.[15] The increased propensity for apoptosis is recapitulated in neutropenia patient-derived induced pluripotent stem cells carrying homozygous *HAX1* mutations.[16] The expression of mutant neutrophil elastase (NE) also drives cells into apoptosis. Mutations in the NE-encoding gene, *ELANE*, result in the production of misfolded NE protein, activation of the unfolded protein response (UPR), and, ultimately, apoptosis of neutrophil precursors.[17–19] The ER is also involved in regulation of calcium homeostasis. Recently, mutations in SEC61 translocon subunit alpha 1 (*SEC61A1*) were identified as a novel cause of autosomal dominant SCN, and this defect was linked to calcium leakage from the ER leading to increased apoptosis.[20] Interestingly, the authors also noted a reduced expression of the B-cell leukaemia/lymphoma 2 apoptosis regulator (*BCL2*) gene in cells with *SEC61A1* mutations. This accords with our observation of defective Bcl-2 expression in the bone marrow of patients with Kostmann disease harbouring *HAX1* mutations.[15] Overall, it thus appears that apoptosis plays a significant role in SCN. It is important to note that cell death (of neutrophils) may involve several different proteases including caspases,[21] as well as calcium-activated calpains.[22–24] However, calpain-dependent cell death has not been studied in the context of SCN. Furthermore, other forms of cell death also need to be considered, as apoptosis is only one of several possible ways in which cells die; indeed, bone marrow cells were also shown to die by regulated necrosis (necroptosis).[25] To further elucidate the aetiology of autosomal recessive SCN, we performed whole exome sequencing in a cohort of patients with SCN and identified homozygous mutations in *JAGN1* in three cases, thus confirming the original discovery of *JAGN1* mutations by Boztug et al.[11] We proceeded to explore the impact of mutant *versus* wild-type *JAGN1* using HL-60 cells, a commonly used model of myeloid cells. Our present results imply that when myeloid cells expressing mutant *JAGN1* are confronted with a stimulus that normally triggers degranulation they undergo a calcium-dependent, calpain-mediated form of cell death.

Patients and methods

Patient cohort

Samples from 21 patients with congenital neutropenia from two paediatric haematology centres in Warsaw and Wroclaw in Poland, and six patients from the Karolinska University Hospital, Stockholm, Sweden, were submitted for whole exome sequencing as described below. The samples were collected in connection with the annual clinical follow-up of these patients. The exome sequencing study was approved by the Regional Ethical Review Board, Stockholm (Dnr 2012/2106-31/4), and informed consent was obtained from the patients and/or their parent(s). Detailed clinical histories of the patients with *JAGN1* mutations can be found in the Extended Methods.

Exome sequencing

To obtain a molecular diagnosis, genomic DNA was isolated from peripheral blood of the patients. Libraries for sequencing on Illumina HiSeq2000 (Illumina, San Diego, CA, USA) were prepared from DNA samples and exome sequences enriched with Agilent SureSelect Human All Exon 50M (Agilent, Santa Clara, CA, USA). Reads were mapped to the human reference genome (hg19) using Mosaik (version 1.0.1388; http://bioinformatics.bc.edu/marthlab/Mosaik). For details on data processing and bioinformatics analysis, refer to the Extended Methods.

Yeast two-hybrid screening

The bait construct for yeast two-hybrid screening was made by subcloning the *JAGN1* complementary DNA (cDNA) into the vector pGBKT7 (Clontech, Mountain View, CA, USA), and baits were mated with a human bone marrow library. The identity of the positive interactors was determined by sequencing. For immunoprecipitation, cell lysates of HL-60 cells transfected with FLAG-tagged *JAGN1* were used.

HL-60 cell experiments

The human acute promyelocytic leukaemia cell line HL-60 (American Type Culture Collection, Manassas, VA, USA), a commonly used model of myeloid cells, was maintained in phenol red-free RPMI-1640 medium supplemented with 2 mmol/l L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) in 5% CO_2 at 37°C. Cells were transiently transfected with *N*-terminal FLAG-tagged *JAGN1* (myc-DDK-tagged-JAGN1) (OriGene, Rockville, MD, USA). Three FLAG-tagged constructs including two patient-derived point mutation-expressing (G14S and E21D) and one wild type (WT) expressing *JAGN1* constructs were used. To silence endogenous *JAGN1*, HL-60 cells were transfected with either negative control small interfering RNA (siRNA) or siRNAs 1 and 2 (Life Technologies, Gaithersburg, MD, USA) against *JAGN1* using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). For a description of calcium, mitochondrial membrane potential, caspase activation, apoptosis/cell cycle, and cell death measurements, along with reverse transcriptase-polymerase chain reaction (RT-PCR), immunoblotting, transmission electron microscopy (TEM), confocal microscopy, and statistical data analysis, refer to the Extended Methods.

Results

Identification of JAGN1 mutations in patients with Kostmann disease

We performed whole exome sequencing to assign a molecular diagnosis to patients with Kostmann disease in whom mutations in known, disease-associated genes including *HAX1* and *ELANE* had not been identified. Prioritisation for a rare, recessive mutation with high predicted pathogenicity returned *JAGN1*, NM_032492, exon 1, c.40G>A; p.Gly14Ser, as the primary candidate in patient 1. Notably, our survey of public microarray data showed that *JAGN1* mRNA expression is high in normal haematopoietic stem cells (Fig 1A). The mutation was verified by Sanger sequencing (Fig 1B) and further analysis of unaffected family members disclosed that the parents and two of the siblings were heterozygous for the mutation (Fig 1C). We identified a second mutation, p.Glu21Asp, in patient 2 (Fig 1B). Importantly, the affected amino acid positions are conserved in metazoans (Fig 1D). We performed Sanger sequencing and found *JAGN1* mutations in patient 3. Patient 2 and patient 3 harboured the same mutation.

Cell models to study the impact of patient derived JAGN1 mutations

To study the impact of *JAGN1* mutations on myeloid cells, HL-60 cells were transiently transfected with plasmid DNA containing FLAG-tagged *JAGN1* or patient-specific point mutants of *JAGN1* (c.40G>A; p.Gly14Ser and c.63G>T; p.Glu21Asp). The expression of FLAG-tagged constructs was confirmed by Western blot analysis (Fig 2A). Next, we determined the subcellular localisation of JAGN1. Using a fluorescein isothiocyanate (FITC)-labelled FLAG antibody, we found that JAGN1 was predominantly localised to the ER as evidenced by its co-localisation with calnexin (Fig 2B). Jagunal in *Drosophila* is required for reorganising the ER, and a previous study has suggested, based on proteomics-based analysis using HEK293 cells, that JAGN1 may interact with coat protein I (COPI) proteins that coat vesicles transporting proteins from the Golgi complex back to the ER, and between Golgi compartments.[11] To identify potential JAGN1-interacting proteins, we conducted yeast two-hybrid screening using *JAGN1* as a bait. We identified 26 bait-dependent interactors in a human bone marrow library, of which 16 were considered as potential candidates on the basis of their expression in human haematopoietic cells (Table SI). To validate potential binding partners, we performed co-immuno-precipitation experiments in HL-60 cells overexpressing WT and mutant *JAGN1*, and we found that endogenous glucose-regulated protein 78 (Grp78) interacted with both WT and mutant JAGN1 in the HL-60 cell line (Fig 2C). To further assess the role of JAGN1, HL-60 cells were transiently transfected with *JAGN1* specific siRNA and analyzed by RT-PCR;

JAGN1 expression was reduced by >50% (Fig 2D). In subsequent experiments, we compared cells expressing WT and mutant *JAGN1*, and cells in which endogenous JAGN1 expression had been silenced.

JAGN1 modulates cell death by classical apoptosis-inducing stimuli

Previous studies have shown that myeloid progenitor cells from patients with SCN undergo accelerated, mitochondria-dependent apoptosis[15] and subsequent studies revealed that HAX-1 is a major regulator of myeloid homeostasis, acting primarily at the level of mitochondria.[7] HAX-1 also reduces the rate of thapsigargin-induced apoptosis.[26] To test if JAGN1 regulates cellular susceptibility to apoptosis, HL-60 cells overexpressing WT or mutant *JAGN1* were exposed to staurosporine (STS), thapsigargin or etoposide, and apoptosis was evaluated using the propidium iodide (PI)-staining assay for cellular DNA content along with the aspartate-glutamate-valine-aspartate-7-amino-4-methyl-coumarin (DEVD-AMC) assay to monitor for caspase-3-like enzyme activity.[27] As shown in Figure 3A, overexpression of *JAGN1* did not protect against STS-induced apoptosis. However, overexpression of mutant *JAGN1* (p.Gly14Ser and p.Glu21Asp) drastically increased the degree of apoptosis in response to STS, as well as thapsigargin and etoposide (Fig 3B). Similarly, DEVD-AMC cleavage was significantly increased (Fig 3C). Furthermore, dissipation of the mitochondrial membrane potential ($\Delta\psi m$) increased (Figure S1). In fact, we observed that cells overexpressing mutant *JAGN1* displayed a pronounced drop in $\Delta\psi m$ even in the absence of a pro-apoptotic stimulus (Figure S1). In addition, we evaluated cellular sensitivity to apoptosis following transient transfection of HL-60 cells with *JAGN1*-specific siRNA *versus* control siRNA (Fig 2D), but could not detect significant differences in apoptosis, using the assays specified above (data not shown).

Mutant JAGN1 drives calpain-dependent cell death in myeloid cells

Neutrophils are densely packed with secretory granules and they release an array of proteases and other soluble mediators upon exocytosis of such granules.[28] Electron microscopy of peripheral blood neutrophils obtained from patient 1 disclosed that numerous granules were present in the periphery of the cell, while granules were dispersed throughout the cytoplasm in neutrophils from a healthy donor (Fig 4C). Furthermore, the granules displayed a striking morphology with condensed, crystalline granular contents, and many granules in the patient cells showed a disrupted membrane. These ultrastructural findings are suggestive of aberrant granule exocytosis. We, therefore, decided to further explore the impact of degranulation-inducing stimuli in our HL-60 model. To this end, HL-60 cells overexpressing WT or mutant *JAGN1* were subjected to treatment with the
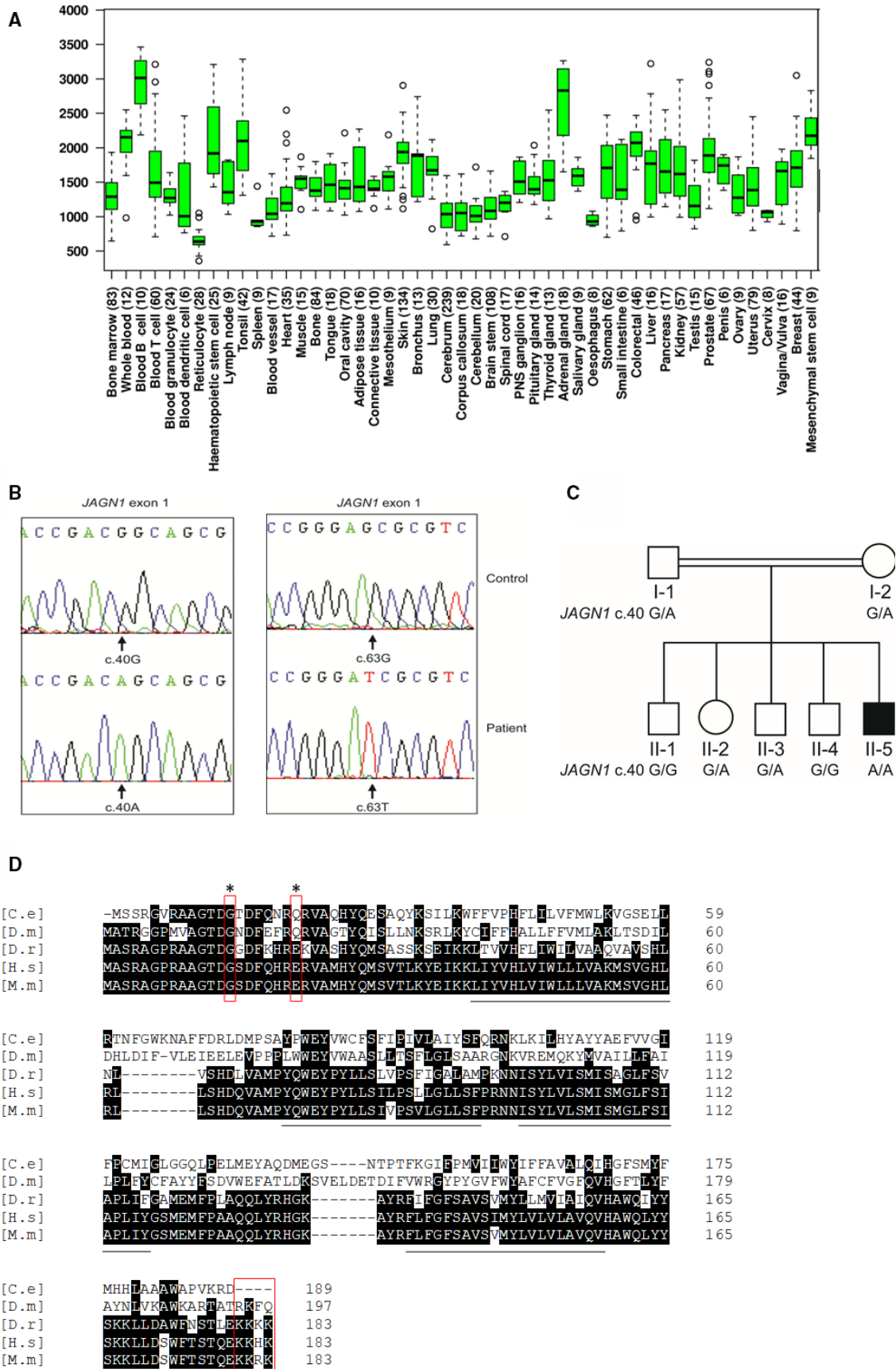
Fig 1. *JAGN1* mutations in patients with severe congenital neutropenia. (A) Box plot depicting *JAGN1* mRNA expression in normal human tissues. The data were derived from the *in silico* transcriptomics database, IST.[43] Each box represents the quartile distribution (25–75%) range with median indicated as a black horizontal line. The 95% range including individual outlier samples is also displayed. The *y*-axis indicates the relative gene expression level. Note high expression of *JAGN1* in haematopoietic stem cells, as well as in mesenchymal stem cells. (B) Sequence chromatograms illustrating the mutation c.40G>A, p.Gly14Ser (left panel) and c.63G>T; p.Glu21Asp (right panel) of *JAGN1*, NM_032492, in patients with SCN compared to the normal sequence in a healthy control. (C) Pedigree of the family of patient 1 illustrating the inheritance of the c.40G>A; p.Gly14Ser mutations in *JAGN1*. The filled symbol denotes the affected patient. (D) Sequence alignment of Jagunal homologues of *C. elegans* (NP_493559.1), *D. melanogaster* (NP_649585.1), *D. rerio* (NP_001005774.1), *H. sapiens* (NP_115881.3), and *M. musculus* (NP_080641.1). The human *JAGN1* mutations identified in the present study are indicated by asterisks. The predicted transmembrane domains[12] are indicated by horizontal lines and the putative ER retention motif is boxed.

chemoattractant, *N*-formylmethionyl-leucyl-phenylalanine (fMLP) in combination with the priming agent, cytochalasin D (CytD). The mobilisation and exocytosis of granules requires an increase in intracellular calcium.[28] As seen in Fig 4A, cells expressing mutant JAGN1 showed a massive increase in cytosolic calcium in response to fMLP+CytD, as determined using the Fluo-4 acetoxymethyl ester (Fluo-4 AM) probe, while this was less pronounced in mock-transfected cells or in cells overexpressing WT JAGN1. Furthermore, cells expressing mutant JAGN1 displayed a significant drop in $\Delta\psi$m after exposure to fMLP+CytD (Fig 4B), and mitochondrial calcium based on Rhod2-AM staining was increased in cells expressing mutant JAGN1 following a brief exposure to degranulation-inducing agonists (Fig 4D). In contrast, no differences in cytosolic calcium levels in response to fMLP+CytD were noted in cells transfected with *JAGN1* siRNA *versus* control siRNA (Figure S2).

To further explore the impact of fMLP+CytD on cells, we asked whether cell death was induced. We detected an increase in apoptosis after exposure for 24 h to fMLP+CytD, but there was no difference between mock or WT transfected cells and mutant *JAGN1* transfected cells (Figure S3A). Interestingly, apoptosis in this model was not inhibited by the pan-caspase inhibitor, zVAD-fmk, while the calpain inhibitor, PD150606 suppressed cell death (Figure S3A). We reasoned that cell death under these conditions might occur through a non-apoptotic programme. To ensure that the overall degree of cell death (not only apoptosis) was captured, we switched from the PI-staining assay to the lactate dehydrogenase (LDH) release assay that monitors loss of integrity of the plasma membrane. We found that fMLP+CytD induced significant, time-dependent cell death in HL-60 cells, and this was greatly aggravated in cells overexpressing the JAGN1 mutants (p.Gly14Ser and p.Glu21Asp) (Fig 5A). In contrast, no differences were seen in HL-60 cells transfected with *JAGN1* siRNA *versus* control siRNA (Fig 5B). As shown in Fig 5C, fMLP+CytD-induced cell death was completely abrogated by the calpain inhibitor, PD150606, while zVAD-fmk had no effect. In light of the finding that fMLP+CytD also triggered a loss of $\Delta\psi$m, we tested whether bongkrekic acid (BA), a specific inhibitor of mitochondrial permeability transition (MPT) pore opening,[29] could rescue the cells. Indeed, BA partially blocked cell death

in fMLP+CytD-treated cells. We also asked whether necrostatin-1 (nec-1), an inhibitor of the necroptosis-associated receptor interacting serine/threonine kinase 1 (RIPK1), could block cell death and noted a modest, yet significant effect on fMLP+CytD-induced cell death (Fig 5C). The results shown in Fig 5C were obtained at 12 h of exposure and similar results were seen at 24 h, hence, PD150606 completely blocked cell death, and the effect of BA was even more robust at 24 h (Figure S3B), implying that MPT plays a significant role in the current model. In sum, exocytosis agonists trigger calpain-mediated cell death in cells expressing mutant JAGN1 protein.

Discussion

In the present study, we confirmed the presence of *JAGN1* mutations in patients with clinical symptoms consistent with autosomal recessive SCN or Kostmann disease. Furthermore, we established a cellular model by silencing endogenous *JAGN1* expression in HL-60 cells, and by overexpressing WT JAGN1 or patient-specific JAGN1 mutant proteins and we could demonstrate that JAGN1 regulates cell death in HL-60 cells. HL-60 cells (derived from a patient with acute promyelocytic leukaemia) and other cell lines, such as U937 (derived from a patient with histiocytic lymphoma), are commonly used models to study myeloid cell biology.[17,30] However, further investigations in primary patient cells or patient-derived induced pluripotent stem cells[16] are required to understand the role of calpain-mediated cell death in the pathogenesis of *JAGN1*-associated SCN.

Boztug *et al.*[11] identified nine distinct homozygous *JAGN1* mutations in 14 individuals with SCN. The two mutations identified in three individuals in the present study (p.Gly14Ser, p.Glu21Asp) correspond to previously identified mutations. The affected amino acids are located in the well-conserved *N*-terminal region of the protein that is predicted to face the cytoplasm, and the mutations could perhaps affect interactions with other cytosolic protein(s). To address the possibility of JAGN1-interacting proteins, we conducted a yeast two-hybrid screen using *JAGN1* as the bait gene mated with a human bone marrow library. We identified 16 candidate interactors and confirmed the binding of FLAG-tagged JAGN1 with endogenous Grp78.
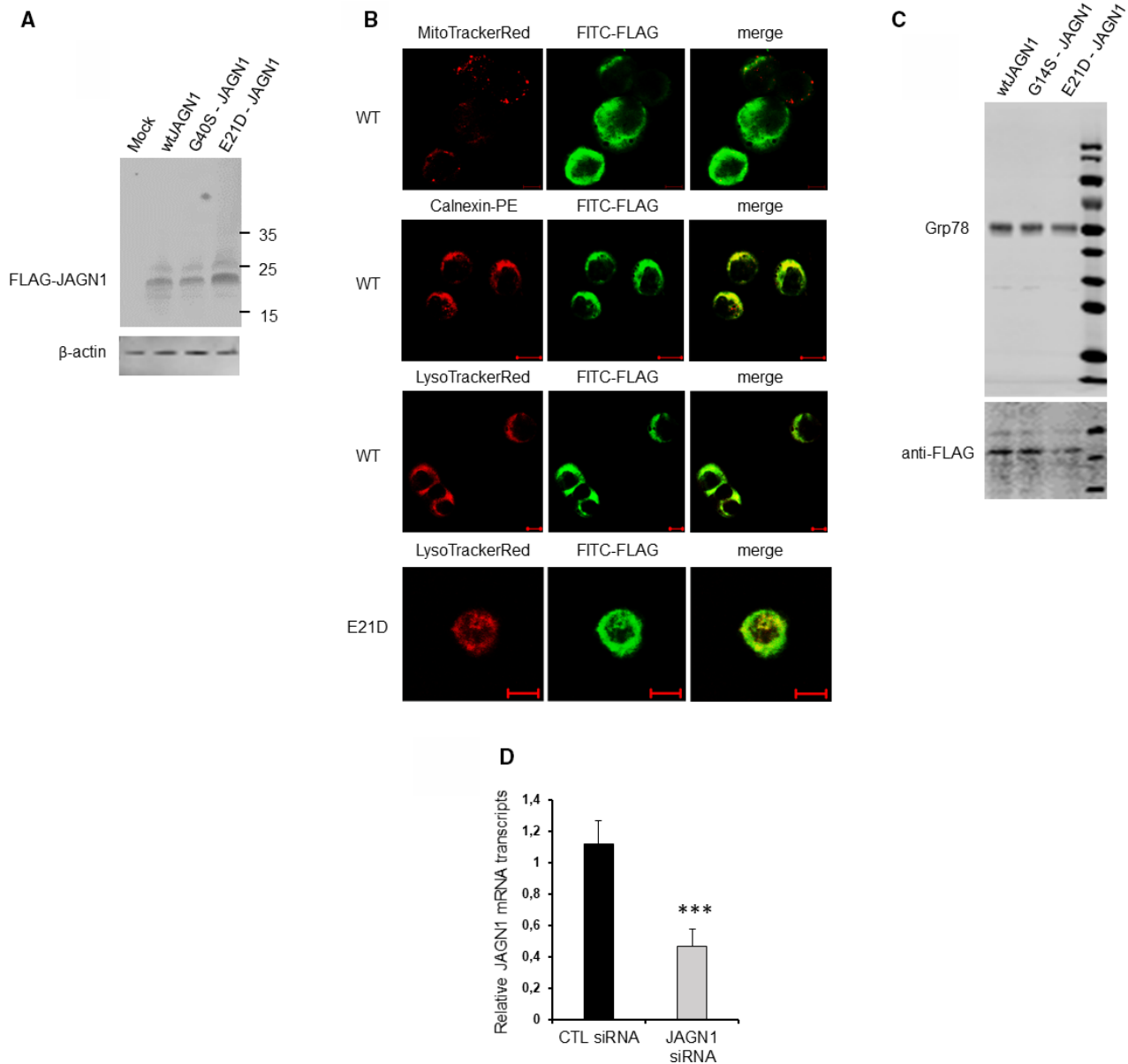
Fig 2. Expression of patient-derived mutant JAGN1 in HL-60 cells. (A) HL-60 cells were transfected with either FLAG-tagged wild-type (wt) *JAGN1* or patient-derived *JAGN1* mutation-expressing plasmids. JAGN1 expression in HL-60 cells was confirmed by Western blot using an anti-FLAG antibody. The membrane was re-probed for β-actin to control for equal loading. (B) FLAG-tagged JAGN1 co-localises with the ER protein, calnexin. HL-60 cells were transfected with FLAG-tagged wt-*JAGN1* or E21D-*JAGN1* and cells were incubated with MitoTracker™ (red) or Lyso-Tracker™ (red) before fixation or phycoerythrin (PE)-labelled anti-calnexin antibody (red) after fixation and subsequently stained with a FITC-labelled anti-FLAG antibody (green). Images were acquired with a confocal laser scanning microscope fitted with a ×63 objective. Scale bars, 20 μm. (C) Immunoprecipitation of FLAG-tagged wt- and mutant-JAGN1 proteins (G14S and E21D) with the endogenous ER protein, Grp78 in HL-60 cells (see Table SI for yeast two-hybrid screen results). (D) HL-60 cells were transfected with either scrambled siRNA or *JAGN1* specific siRNA and *JAGN1* mRNA levels relative to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA were quantified using RT-PCR. Results are mean values ± SD ($n = 3$). ***$P < 0.005$, by Student's *t*-test.

Grp78, also known as immunoglobulin heavy-chain binding protein or binding-immunoglobulin protein (BiP), is a multifunctional protein belonging to the HSP70 family of molecular chaperones.[31] Grp78 is a key regulator of the unfolded protein response (UPR) which is induced in cells upon ER stress. Grp78 is also a regulator of calcium homeostasis in the ER, and previous work has shown that Grp78/BiP is required to limit calcium leakage mediated by the Sec61 channel.[32] Boztug *et al.*[11] showed that Grp78/BiP expression was elevated in neutrophils of two patients with SCN with *JAGN1* mutations when compared to a healthy control. The authors attempted to identify interaction
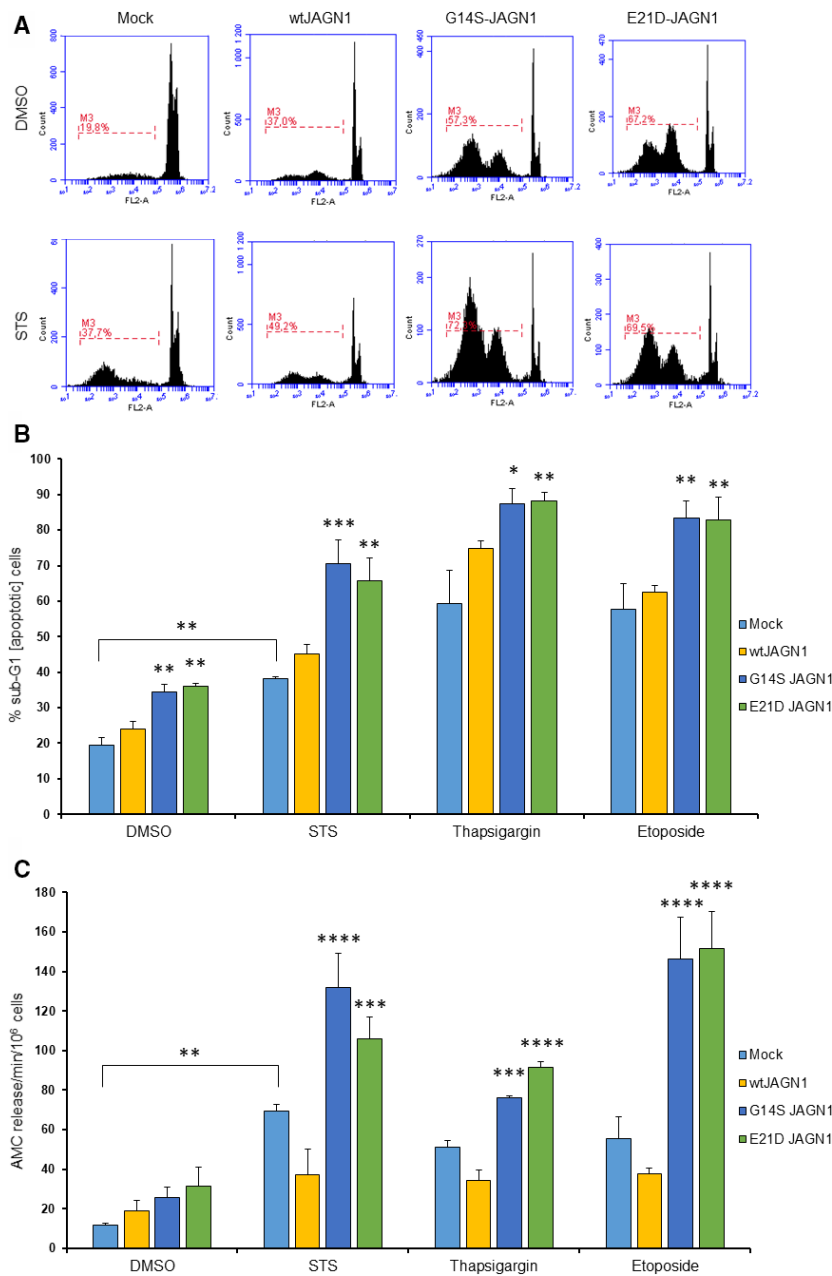
Fig 3. Increased apoptosis in mutant JAGN1-expressing HL-60 cells. HL-60 cells expressing wt-*JAGN1*, G14S-*JAGN1*, or E21D-*JAGN1* were treated with vehicle alone or exposed to the indicated, pro-apoptotic stimuli. (A) Representative flow cytometry results showing apoptosis as evidenced by the emergence of a sub-G1 peak following treatment with staurosporine (STS) (2 μmol/l) for 3 h. (B) Quantification of apoptosis in cells stimulated with STS (2 μmol/l) for 3 h or thapsigargin (1 μmol/l) for 2 h, or stimulated for 6 h with etoposide (10 μmol/l). Results shown are mean values ± SD ($n = 3$). (C) Quantification of caspase-3-like activity as determined by DEVD-AMC cleavage in HL-60 cells expressing wt- or mutant-JAGN1 stimulated as detailed above. Results shown are mean values ± SD ($n = 5$). *$P < 0.05$, **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$.

partners of JAGN1 by a different approach using HEK293T cells. However, none of our candidates (Table SI) overlapped with the proteins identified in the latter study. The differences may be due to the fact that Boztug *et al.*[11] used a non-haematopoietic cell line, whereas we have used a human bone marrow library. Further studies are warranted to explore the other candidates identified in the present screen, but our present results confirm that JAGN1 resides in the ER. Moreover, our observations are compatible with a role of JAGN1 in the modulation of cytosolic calcium levels at the level of the ER (discussed below).

Neutrophil degranulation is a co-ordinated, stepwise process in which calcium signalling plays a key role.[33] However, it should be noted that while calcium plays important
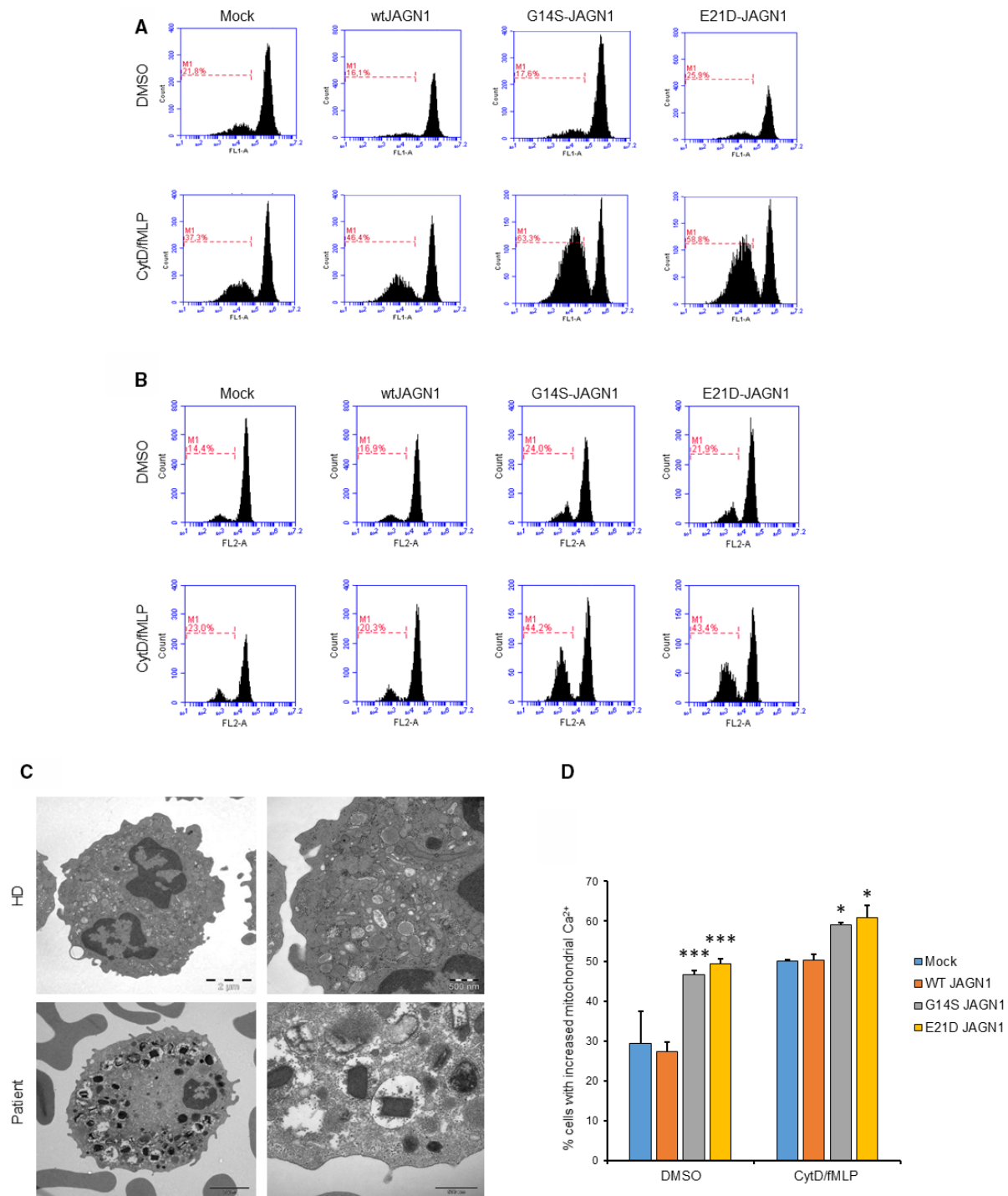
Fig 4. Impact of degranulation agonists in cells expressing mutant JAGN1. HL-60 cells expressing wt-*JAGN1*, G14S-*JAGN1*, or E21D-*JAGN1* were exposed to vehicle alone or stimulated with cytochalasin D (10 ng/ml) and fMLP (10 µmol/l). (A) Cytosolic calcium was determined after 20 min by flow cytometry using the calcium-sensitive probe, Fluo4-AM. (B) Dissipation of the mitochondrial membrane potential ($\Delta\psi$m) was determined at 2 h using the fluorescent probe, tetramethylrhodamine ethyl ester (TMRE). The experiments were performed three times, and representative results are shown. (C) TEM images of peripheral blood neutrophils from a human donor (HD) and from patient 1, respectively. Note polarisation of granules to the periphery of the cell in patient neutrophils giving the impression of granule 'congestion', along with varying contents of dark, dense material, crystal-like in appearance, but without discernible crystal structure, as well as several instances of disrupted granule membranes. Scale bars, 2 µm (left) and 500 nm (right). (D) HL-60 cells were either mock-transfected or transfected with wt- or mutant-*JAGN1* constructs. Exposure to vehicle alone increased mitochondrial calcium levels in cells expressing mutant *JAGN1* and calcium levels were further increased by CytD/fMLP as shown by flow cytometric analysis after rhodamine-2-acetoxymethyl ester (Rhod2-AM) staining. Data are mean values ± SD ($n = 3$). *$P < 0.05$, ***$P < 0.005$. DMSO, dimethyl sulphoxide. [Colour figure can be viewed at wileyonlinelibrary.com]
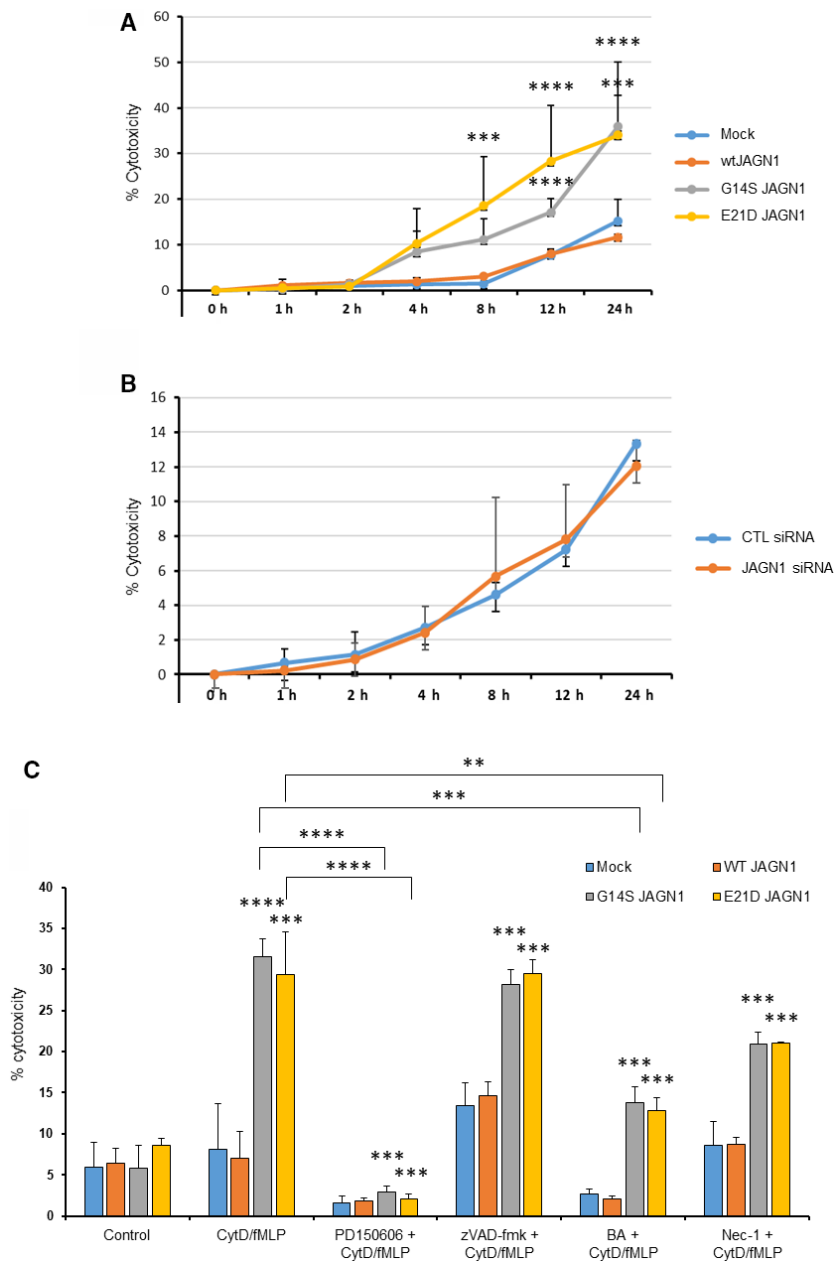
Fig 5. Degranulation agonist-induced cell death is calpain-dependent. (A) HL-60 cells were mock-transfected or transfected with wt- or mutant-*JAGN1* (G14S and E21D). Cells were then stimulated with cytochalasin D (10 ng/ml) + fMLP (10 μmol/l) for the indicated time-points and cell death was determined using the LDH release assay. (B) HL-60 cells were transiently transfected with *JAGN1* specific siRNA *versus* control siRNA and stimulated as indicated in (A). Data shown in panels (A) and (B) are mean values ± SD (n = 3–4). (C) Cells were pretreated for 1 h with various inhibitors [i.e. the calpain inhibitor, PD150606 (60 μmol/l), the pan-caspase inhibitor, zVAD-fmk (10 μmol/l), the MPT inhibitor, bongkrekic acid (BA) (50 μmol/l), or the RIP1 kinase inhibitor, necrostatin-1 (40 μmol/l)] prior to stimulation with cytochalasin D (10 ng/ml) + fMLP (10 μmol/l) for 12 h. Data are mean values ± SD (n = 3). *$P < 0.05$, **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com]

roles in degranulation, cytosolic calcium overload, or perturbation of the intracellular compartmentalisation of calcium, are also linked to the induction of cell death by apoptosis and/or necrosis.[34] Indeed, MPT-driven necrosis is initiated by perturbation of the intracellular microenvironment including calcium overload.[35] The adenine nucleotide translocator (ANT) is implicated in the regulation of MPT,

and BA, a specific inhibitor of ANT, was shown to prevent the release of the so-called apoptosis-inducing factor (AIF) from mitochondria in a classical model of glucocorticoid-induced apoptosis of thymocytes.[29] More recent studies have shown that calpain-mediated cleavage of AIF in mitochondria is required for its subsequent release into the cytosol.[36] We found that BA afforded significant protection

against CytD/fMLP-induced cell death. We also noted that cell death in the present model was at least partially mediated by RIPK1, as evidenced by the protective effect of nec-1. AIF is known as a key factor in caspase-independent cell death[37] and has also been implicated in regulated necrosis (necroptosis).[38] Further studies are needed to unravel the potential contribution of AIF and RIPK1 in CytD/fMLP-induced cell death in myeloid cells that express mutant *JAGN1*. Nevertheless, we may conclude that CytD/fMLP-induced cell death in the present model is caspase-independent, as zVAD-fmk had no effect. Instead, the fact that PD150606, a selective calpain inhibitor,[39] rescued cells from CytD/fMLP-induced cell death points toward a key role of the calcium-dependent protease, calpain (Fig 6). The convergence of cell death pathways on mitochondria has been shown in several different forms of SCN, e.g. in *HAX1* and adenylate kinase 2 (*AK2*) deficiency.[7,40] However, it should be noted that the actions of calpain are not restricted to the mitochondrial compartment and it is entirely plausible that calpain activation also transpires in the cytoplasm. Our previous studies showed that myeloid progenitor cells from patients with SCN with *HAX1* mutations undergo classical, caspase-dependent apoptosis.[15] Boztug *et al.*[11] suggested that neutrophils from *JAGN1*-deficient individuals are more prone to apoptosis insofar as they could show, using the annexin V assay, that the degree of STS-induced cell death was increased in two cases when compared to healthy controls. However, neutrophils are programmed to undergo apoptosis from the moment they are released into the blood and the fate of such cells may not be reflective of what goes on in progenitor cells in the bone marrow. Using the same annexin V assay, we found that myeloid stem cells isolated from patient 1 (prior to bone marrow transplantation) were highly prone to spontaneous cell death as evidenced by phosphatidylserine (PS) exposure (unpublished observations). However, while *HAX1*-deficiency was shown to result in mitochondria-dependent apoptosis with caspase activation,[7] there is currently no evidence that myeloid cells harbouring *JAGN1* mutations undergo classical, caspase-dependent apoptosis. In fact, PS exposure, commonly used as a surrogate marker for apoptosis, is not unique to apoptosis and has also been documented in cells undergoing necroptosis.[41,42] Instead, on the basis of our present results, we suggest that neutropenia-associated *JAGN1* mutations promote a non-apoptotic, calpain-dependent form of cell death. It is notable that the silencing of endogenous *JAGN1* in the present study did not affect the susceptibility of HL-60 cells to apoptotic or non-apoptotic cell death. Furthermore, mice with haematopoietic lineage specific deletion of *JAGN1* exhibited normal numbers of neutrophils in the bone marrow, secondary lymphoid organs and blood.[14] One may speculate that the pro-death potential of pathogenic *JAGN1* mutations is unleashed only in cells that have encountered pro-death stimuli and/or stimuli that trigger degranulation.
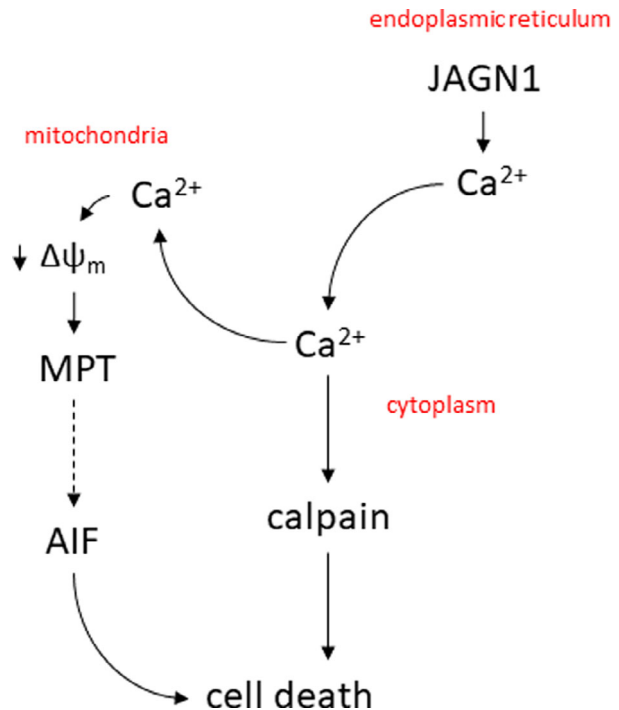

Fig 6. Schematic figure illustrating the putative role of JAGN1 in the calcium- and calpain-dependent cell death pathway described in the present study. JAGN1 was found to interact with Grp78, a key regulator of calcium homeostasis in the ER (not shown). Cell death was completely prevented by PD150606, a selective calpain inhibitor. Bongkrekic acid, a specific inhibitor of ANT, also blocked cell death, pointing to a role for MPT in the present model, while the involvement of AIF remains to be demonstrated. Furthermore, a role for RIPK1, a key player in necroptosis, seems likely, as evidenced by the protective effect of nec-1. However, caspase involvement was excluded in the present model. JAGN1, jagunal homolog 1; MPT, mitochondrial permeability transition; AIF, apoptosis-inducing factor (note that AIF also participates in non-apoptotic cell death); ANT, adenine nucleotide translocator; $\Delta\psi_m$, mitochondrial membrane potential; RIPK1, receptor interacting serine/threonine kinase 1. [Colour figure can be viewed at wileyonlinelibrary.com]

In conclusion, we identified homozygous *JAGN1* mutations in three patients with Kostmann disease, thus confirming the original findings of Boztug *et al.*[11] Furthermore, we showed that mutant JAGN1 regulates the sensitivity of myeloid cells to conventional pro-apoptotic stimuli. We also provided evidence for a role of JAGN1 in the control of calpain-mediated cell death and could show that this transpired with a drastic increase in cytosolic calcium and a subsequent drop of the mitochondrial membrane potential. These results shed light on the regulation of myeloid cell death by the recently identified neutropenia-associated factor, JAGN1.

Acknowledgements

Author Contributions

Avinash Khandagale, Teresa Holmlund, and Miriam Entesarian performed *in vitro* experiments and analysed data, Daniel Nilsson performed the bioinformatics analysis; Göran Carlsson, Maja Klaudel-Dreszler, Krzysztof Kalwak provided patient samples and clinical data; Jan-Inge Henter, Magnus Nordenskjöld, and Bengt Fadeel interpreted the data and supervised the experimental work; Bengt Fadeel secured funding, co-ordinated the study, and wrote the manuscript; all co-authors approved the final version.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig S1. Decay of mitochondrial membrane potential in *JAGN1*-transfected cells.

Fig S2. Degranulation-induced calcium changes are not affected by *JAGN1* silencing.

Fig S3. Degranulation agonist-induced cell death is calpain-dependent.

Table SI. Yeast two-hybrid screening for proteins that interact with JAGN1.

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