A disease-causing mutation K240E disrupts ferroportin trafficking by SUMO (ferroportin SUMOylation)

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A B S T R A C T

Ferroportin (Fpn/IREG1/MTP1) is the only known transporter mediating iron efflux from epithelial cells and macrophages, and thus regulates how much iron is released into the circulation. Consequently, Fpn mutations are associated with haemochromatosis. Fpn itself is post-translationally regulated by hepcidin (Hepc) which induces its redistribution and degradation in a ubiquitin-dependent process. Together, the two proteins appear to be the nexus for iron homeostasis. Here we show that a rare gain-of-function mutation (K240E) that is associated with iron overload, impedes Fpn binding and subcellular trafficking by the small ubiquitin-like modifier (SUMO). Whereas wild-type Fpn is ensconced within vesicular bodies, the FpnK240E mutant appeared diffused within the cell when co-expressed with SUMO. Furthermore, compared with wild type Fpn, the sumoylation-defective mutant was constitutively-active, resulting in a lower intracellular labile iron pool than the former. These findings suggest that SUMO may regulate iron homeostasis by controlling Fpn trafficking.

1. Introduction

SUMO (small ubiquitin-like modifier) conjugation to a wide array of proteins accounts for its role in processes as diverse as gene regulation, synaptic transmission, cytokinesis, membrane transport, and cellular signaling and cycling [1–7]. There are 5 highly homologous SUMO paralogues in the human genome: SUMO1, SUMO2, SUMO3, SUMO4 and SUMO5 with 101, 95, 103, 95 and 101 amino acids in their primary sequences respectively. The initial "sumoylation" step involves conjugation to ψ-K-X-E/D sites on protein substrates (where K is a lysine, X is any other amino acid and D or E is aspartic acid or glutamic acid residues respectively). Conjugation is optimal where the lysine residue is preceded by a hydrophobic residue, ψ, (I, L, V, F) but sumoylation at non-consensus lysines has also been reported [3–5,7,8]. This process can be reversed by SUMO/sentrin proteases as well as by some bacterial and viral effectors such as listeriolysin O and Gam1 [9–11]. The role of SUMO in regulating the functions of multitudinous substrates and biological processes links it to diseases including cancer, inflammatory and neurodegenerative diseases [12; see references therein]. With very few exceptions, sumoylation of transcription factors invariably leads to gene repression [2,5,13]. Furthermore, although it is almost exclusively linked to nuclear processes such as gene regulation, SUMO has been shown to directly regulate extranuclear functions including the trafficking of transmembrane proteins such as the glucose transporter and kainate receptor involved in glucose uptake and synaptic transmission respectively [3,14–21].

Ferroportin (Fpn)/Slc40A1 is an iron-efflux pump and the only known transporter that regulates systemic iron levels. Increasingly, a spectrum of iron-loading phenotypes (ferroportin disease) has been associated with mutations in Fpn [22]. The only known mechanism by which iron levels are controlled is by hepcidin-dependent internalization, ubiquitination and proteasomal degradation of Fpn [23]. Although this mechanism has been debated [24], an Fpn mutation at K240 (K240E) renders the protein resistant to hepcidin-induced ubiquitin-dependent degradation and in at least one case causes pathologic iron-overload [25–27]. All reports to date also show only partial reduction in iron efflux indicating that Fpn may not be completely degraded. It appears that some of the protein may remain functional but ensconced within intracellular vesicles, suggesting the existence of a non-destructive mechanism of Fpn regulation. We reasoned that SUMO may be an alternative regulator of Fpn trafficking because unlike ubiquitin, it tends to stabilize its substrates. Here we show that SUMO interacts with Fpn through K240 and mutation of this residue to the disease-causing variant K240E leads to altered Fpn trafficking and increased iron efflux from intracellular stores.

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2. Materials and methods

2.1. Reagents

Recombinant SUMO1- and SUMO2-conjugated agarose was purchased from Enzo Lifesciences (Exeter, UK). Cell culture media and supplements (FBS, antibiotics) were obtained from ThermoFisher Scientific (Paisley, UK). All analytical grade reagents were purchased from Sigma-Aldrich (Poole, UK).

2.2. Plasmid constructs

Full-length mouse Fpn (FpnFL) cDNA was mutagenized at the canonical sumoylation site LKVE to LEVE with the primer GTA AAA GCT GCT CTC gAG GTA GAG GAG TCA GAA CTG AAG CAG (lower case denotes mutation) using the QuikChange site-directed mutagenesis kit (Agilent, Belgium). The putative Fpn sumoylation domain, FpnSumoD, was also amplified by PCR with the primers: CAT GGA TCC CGC CAG TCA TTG GCT GTG GTT and CAT GAA TTC TTG TTC ACA TTC AAG TTC ACG), and subcloned into the *Bam*HI-*Eco*RI sites of pGEX-5x-1 (GE Healthcare, UK) to generate pGex-5x-FpnSumoD. Fluorescent SUMO constructs were generated by replacing DsRed in pDsRedN1 (Clontech) with monomeric red fluorescent protein RFP (mRFP) cDNA. Both SUMO1 wild-type and mutant were ligated in-frame into the *Bam*HI/*Xba*I sites of the vector backbone to generate mRFP-SUMO1Wt and mRFP-SUMO1Mt respectively. The mammalian two-hybrid (M2H) vectors pM-SUMOWt and pM-SUMOMt were constructed by subcloning the SUMO cDNAs in-frame with the GAL4 DNA-binding domain (DBD) of pM (Clontech) using the primers CAT GAA TTC ATG CCA GTC ATT GGC TGT GGT and CAT GGA TCC TTG TTC ACA TTC AAG TTC ACG; *Eco*RI/*Bam*HI cloning sites are underlined. pCMVAD-FpnFL and pCMVAD-FpnSumoD are M2H vectors in which FpnFL and FpnSumoD (above) were subcloned in-frame with the activation domain of NFκB. All oligonucleotides were synthesized by Eurofins Genomics (Ebersberg, Germany).

2.3. Mammalian 2-hybrid interaction assay

Baby hamster kidney (BHK) cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium with GlutaMAX (DMEM/GlutaMAX) supplemented with antibiotics/antimycotics and 10% foetal calf serum. They were transfected seeded at ~80% confluence in 24-well multi-dishes (Nunc, Denmark) using Lipofectamine 2000 (Invitrogen, Paisley, UK). Each plasmid (pCMVAD-Fpnsumo, pCMVAD-FpnFL, pM-SUMO Wt, and pM-SUMO Mt) was co-transfected with 50 ng pSVβgal (Promega, Southampton, UK) as internal control, and 50 ng of the GAL4 reporter gene pFR-Luc (Agilent). After 48 h, luciferase activity was determined using the luciferase assay reagent (Promega), and β-galactosidase (βgal) activity was determined with the Beta-Glo reagent (Promega). Luminescence measurements were taken in a microplate luminometer (Tropix TR717, Applied Biosystems, MA), and levels of luciferase activity were normalized with respect to βgal activity.

2.4. Fpn sumoylation domain expression and SUMO binding assay

To express the Fpn sumoylation domain, pGex-5x-FpnSumoD was transformed into BL21-CodonPlus (DE3)-RIPL competent cells (Agilent). GST-fusion protein was induced with 0.1 mM IPTG for 3 h; as control GST was similarly expressed from the vector backbone. Cells were lysed in PBS/0.5% Triton-X100/100 μg per ml lysozyme and total lysates of GST or GST-FpnSumoD were used for pull-down assays using 500 μg/ml recombinant SUMO1 or SUMO2 immobilized on agarose. The agarose beads were washed 3x with GST Bind/Wash buffer (Novagen, UK) by spinning at 13,000 rpm for 1 min each, and bound protein complexes were eluted by resuspending the matrix in 2x NUPAGE loading buffer (Invitrogen) and heated for 10 min at 70 °C. Proteins were resolved on 4–12% NuPAGE Tris-MES gels (Invitrogen) and analyzed by staining with Coomassie Brilliant Blue R-250 (National Diagnostics, Nottingham, UK).

2.5. Fluorescence confocal microscopy

To detect in-cell Fpn-SUMO interaction, Chinese hamster ovary (CHO) cells stably expressing wild-type Fpn or FpnK240E were passaged onto chamber slides (Nunc) and transfected with mRFP-SUMO1Wt. After 48 h cells, were washed with PBS and fixed with 4% paraformaldehyde for 15 min, washed 3x with PBS and mounted in Vectashield (Vector Laboratories, UK). Confocal images were acquired with a Zeiss LSM 510 confocal microscope and processed with Volocity Software (PerkinElmer).

2.6. Determination of intracellular chelatable iron pool by flow cytometry

Stable CHO cell lines expressing Egfp-Fpn Wt and Egfp-FpnK240E were grown to confluence on 6-cm dishes (PAA) using DMEM/GlutaMAX (Invitrogen) containing 800 mg/ml G418 (Invitrogen). Intracellular iron content was quantified by flow cytometry as previously reported [28]. Briefly, the cells were washed 3x with PBS and then incubated with 5 μM or 10 μM Phen Green SK (dipotassium salt; Invitrogen) diluted in PBS. After 20 min incubation, the cells were washed 3x with PBS and detached with PBS/0.5 mM EDTA. They were then centrifuged for 5 min at 2000 rpm and resuspended in 1 ml PBS for FACs analysis using a CyAn ADP flow cytometer (Beckman Coulter).

2.7. Intrinsic disorder analyses

Since protein interaction domains or interfaces are usually intrinsically disordered, we used algorithms in PONDR (Predictors of Natural Disordered Regions) to determine whether the sumoylation domain was unstructured. We analyzed Fpn (UniProtKB/Swiss-Prot #Q9JHI9.1; NCBI Reference Sequence NP_058613.2) for disorder based on criteria including compositional bias (towards Gly, Gln, Ala, Arg, Glu, Lys, Pro and Ser), net charge, hydropathy/solvent accessibility, low representation of hydrophobic residues (Val, Leu, Ile, Met, Phe, Trp and Tyr), energy content, flexibility, coordination number, propensity to form β-sheets, bulkiness and sequence length [29–34]. We also used IUPred [35], DISOPRED2 [36], and PrDOS [37], to assign regions of disorder. In all cases, the threshold for disorder probability was set at a false positive rate of 5%.

2.8. Statistical analysis

Data were analyzed and plotted using GraphPad Prism version 5.04 (GraphPad Software, Inc., San Diego, USA). All data points were presented as means ± S.E.M.

3. Results and discussion

A single nucleotide substitution (c.718A > G) in Fpn has previously been identified in a patient [25] with high serum iron (200 μg/dL; normal range, 59–158 μg/dL), high transferrin saturation index (71%; normal range, 20–45%) and liver iron content that was over twice the normal level. The causative mutation changed K240 to glutamic acid, E240 (hereafter referred to as FpnK240E). Scanning the sequence with SUMOplot (http://www.abgent.com/sumoplot), we identified one high-scoring canonical SUMO ligation site at K240 (Fig. 1A and B) in all Fpn orthologues including humans, and suggests functional conservation. Although putative auxiliary sumoylation sites (e.g. K117) were also identified, we focused on K240 for the above reasons. Secondary structure prediction [38] showed that this residue lies within the large intracellular/cytosolic loop of Fpn (Fig. 1C), indicating that K240 may be readily accessible for SUMO conjugation. Next, we used site-directed
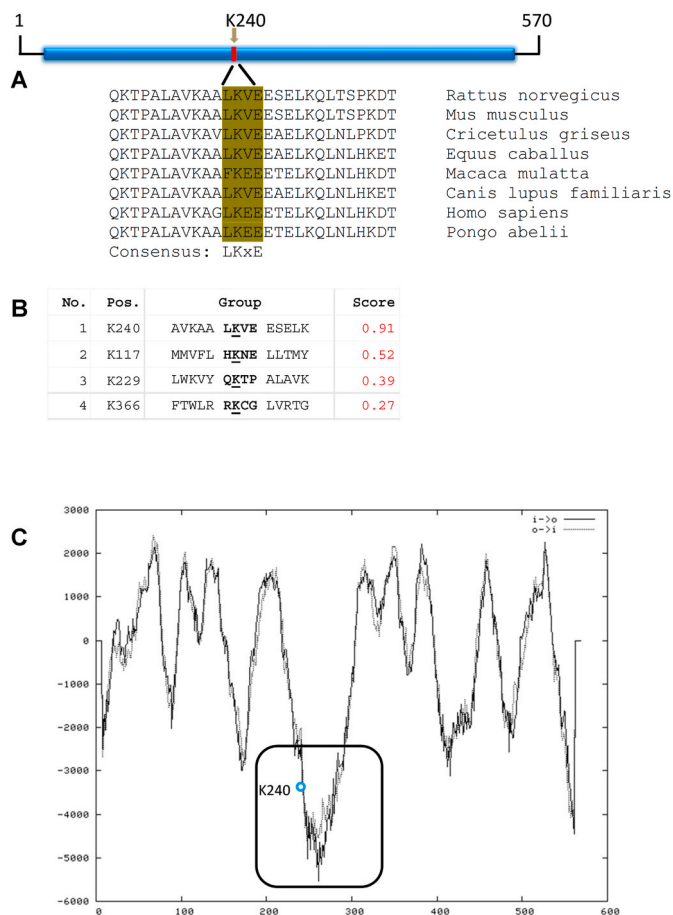
Fig. 1. Simplified Fpn structure. (**A**) A putative sumoylation motif with the consensus sequence LKxE is highly-conserved in Fpn orthologues (where L is leucine, E is glutamic acid, and x is any other amino acid). (**B**) Fpn sequence (NP_058613.2) analysis with SUMOplot identified four putative SUMO conjugation sites (underlined lysine, K, residues). K240 (site # 1) has the highest probability of being a sumoylation site, closely matching the SUMO signature motif ΨKxD/E (where Ψ is a large hydrophobic residue such as leucine, valine and isoleucine; D and E are aspartic and glutamic acid residues respectively). (**C**) Fpn secondary structure prediction suggests that K240 is located (blue circle) within the intracellular/cytosolic loop (boxed). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mutagenesis to recreate the pathogenic mutation K240E to assess how it might regulate iron efflux. First we assessed interaction between FpnFL or the Fpn sumoylation domain (FpnSumoD) and SUMO using mammalian two-hybrid assays. Here, wild-type and mutant SUMO were fused to yeast GAL4 DBD while FpnFL and FpnSumoD were tethered to NFκB activation domain. Cotransfections with a GAL4 reporter gene into BHK cells showed that while the vector backbone (pM) showed only background reporter activity, interaction between AD-FpnSumoD and wild-type but not mutant SUMO increased reporter expression, and that this interaction was strongest with FpnFL (Fig. 2A).

To further confirm interaction between Fpn and SUMO, we generated a recombinant Fpn fragment (amino acids 201–280) containing the SUMO motif by expression in *E. coli* as a GST fusion protein. This region contains half of transmembrane domain 4, the large cytosolic loop of the protein and the SUMO motif. Pull-down assays with SUMO-conjugated agarose followed by gel electrophoresis and Coomassie staining showed almost complete recovery and strong binding of GST-FpnSumoD to both SUMO1 and SUMO2 compared with GST alone (Fig. 2B). Strikingly, we found that SUMO2 induced more high-molecular weight complexes [(Fpn-SUMO$_n$)] than SUMO1, suggesting that FpnK240 may
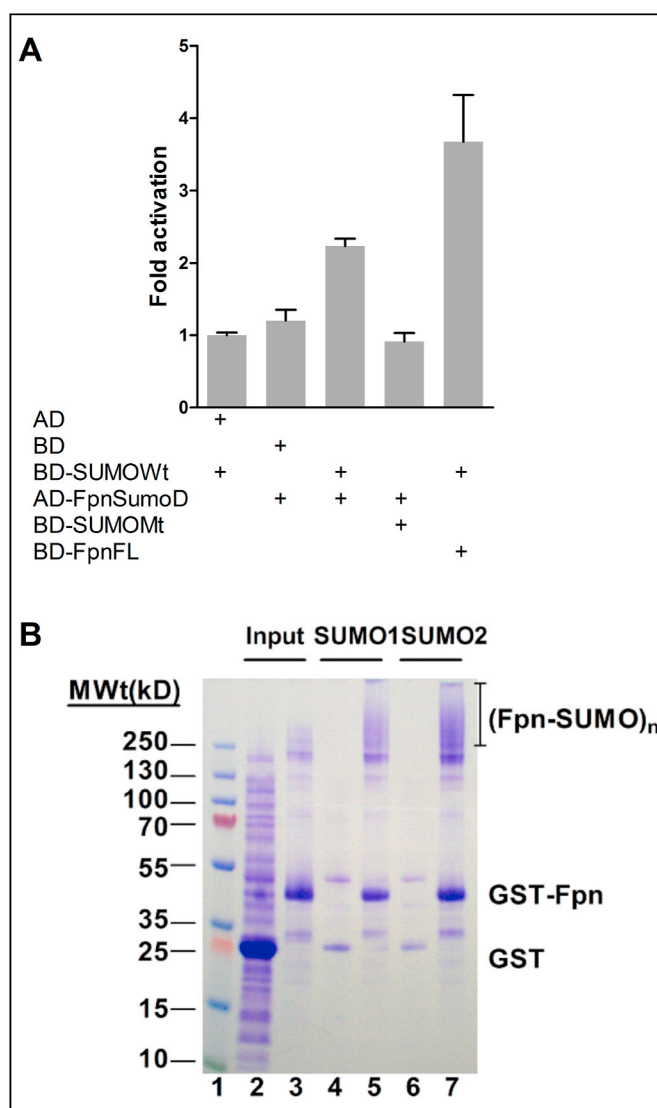

Fig. 2. Ferroportin interacts with SUMO. (**A**) Mammalian two-hybrid assay was performed in BHK cells with GAL4 DNA binding domain (BD)-SUMOWt or BD-SUMOMt, and activation domain (AD) vectors encoding FpnL and FpnSumoD. Fold activation was calculated from GAL4 reporter activity normalized to β-galactosidase internal control; pM (BD) and pCMV-AD vector backbones were used as negative controls. (**B**) GST or GST-FpnSUMO was incubated with agarose conjugated to SUMO1 (lanes 4 and 5) or SUMO2 (lanes 6 and 7). Lanes 2 and 3 show starting amounts (Input) of GST in cell lysate or partially purified GST-FpnSUMO respectively; lanes 4, 6 and 5, 7 contain samples of GST or GST-FpnSUMO pulled down with SUMO respectively. (Fpn-SUMO)$_n$ shows putative poly-SUMO conjugates of Fpn where n indicates multiplicity.

be a nucleation site for SUMO polymers. Proteomic analysis (data not shown) confirmed that 63.78% and 64.57% of these high-molecular weight complexes were SUMO1 and SUMO2 conjugates of GST-Fpn respectively. This preference for SUMO2 was unexpected but is consistent with the fact that it is more efficient in forming polySUMO chains than SUMO1 because it has the requisite lysine residue (K11) that is lacking in the latter [39]. Furthermore, similar polymeric conjugates have been noted with proliferating cell nuclear antigen and topoisomerase 1 [40,41] but in those cases conjugating enzymes were used. These observations were also surprising because polymeric SUMO conjugates formed in the absence of activating enzymes (SAE1/2) or the SUMO ligase Ubc9. One explanation for this could be that the immobilized SUMO1 and SUMO2 used for the pull-down assays were mature, i.e. the requisite diglycine motifs were pre-exposed and primed for conjugation

[42]. It may also be that interaction with the Fpn fragment was a non-covalent one involving SUMO-interacting motifs, SIMs. These motifs are targets for ubiquitination by E3 ligases such as RNF4 [43–46] and it is unclear whether putative SIMs (data not shown) in Fpn could contribute to polysumoylation to presage its degradation [26,27].

Since protein-protein interaction domains are often disordered, we analyzed Fpn for intrinsic disorder using predictors that cover criteria including compositional bias towards polar and charged residues, low representation of bulky hydrophobic residues, and low sequence complexity [29–37]. We found that all the algorithms assigned intrinsic disorder to Fpn regions that include the sumoylation domain, with K240 at the proximal end of the longest region (43 residues) of intrinsic disorder (Fig. 3A and B). Interestingly, these disordered regions are conserved in Fpn orthologues (data not shown); they also overlap predicted molecular recognition features, i.e. they may participate in protein-protein interactions [47]. Why may disorder in the sumoylation domain be important? Intrinsically disordered protein domains appear to be hubs for multitudinous fuzzy or transient interactions with effector proteins involved in cell signaling, transcriptional regulation, proteostasis, molecular recognition, the inflammatory response, the cell cycle and phase separation [48–54]. They are flexible both in their conformation and choice of interaction partners. By undergoing dynamic conformational changes, they are able to bind different effector proteins, trading low affinity for high-specificity, i.e. although their interactions are transient they have high specificity. Another important feature is that they are tightly regulated and hence are able to avoid spurious interactions. This may be teleologically important given that diverse structurally distinct proteins can bind to the same disordered domain. Of note, loss of intrinsic disorder is associated with several diseases including cancer, neurodegeneration and diabetes [55–59]. It is therefore possible that intrinsic disorder in the sumoylation domain of Fpn may be permissive to binding by globular proteins such as SUMO and ubiquitin [26,27]. We suggest that this domain may also act as a hub for other proteins that might regulate iron metabolism through Fpn.
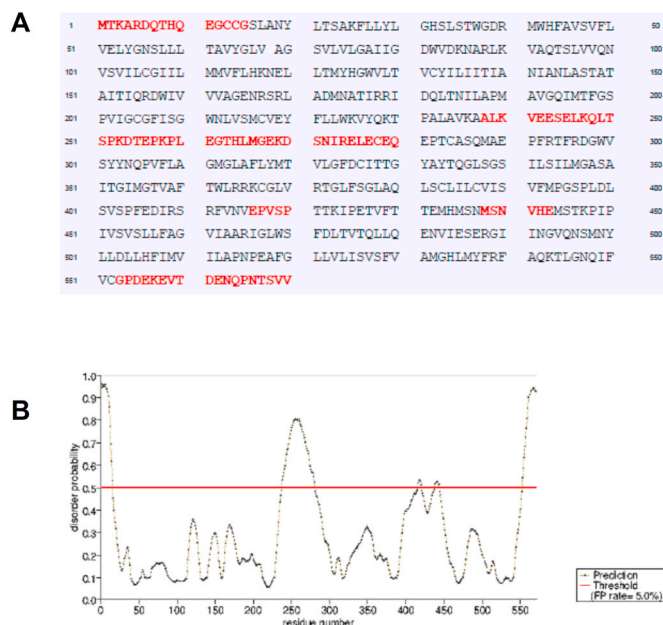

Fig. 3. Prediction of intrinsic disorder within Fpn. (**A**) Disordered residues/regions (in red font) of Fpn were predicted with PrDOS. As shown, the sumoylation domain is the longest disordered region (43 amino acids). (**B**) Disorder probability curve of full-length Fpn; disorder analysis was performed using a 5% false positive rate i.e. a 95% chance of correctly assigning intrinsic disorder). The red line marks disorder threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

To determine intracellular trafficking dynamics and colocalization of Fpn and SUMO1, we performed confocal microscopy with cells expressing mRFP-SUMO1Wt and Fpn-Egfp (wild-type) or the sumoylation-defective mutant FpnK240E-Egfp. As anticipated, Fpn-Egfp was discretely localized to the cell membrane when expressed alone (Fig. 4A). Cotransfection of FpnK240E-Egfp and mRFP-SUMOWt showed extensive cytosolic, perinuclear localization and diffuse distribution of the transporter throughout the cell (Fig. 4B). In contrast, cotransfection of wild-type Fpn-Egfp and mRFP-SUMOWt showed vesiculation and colocalization of the two proteins to discrete multivesicular bodies (Fig. 4C). Thus FpnK240E appears to be defective in its trafficking to endosomes by SUMO, leading to its sequestration in cytosolic compartments. While there was residual expression on the cell surface, most FpnK240E was intracellular.

We hypothesized that the intracellular localization of FpnK240E would render it constitutively active in exporting iron. To test this, we loaded cells expressing mutant or wild-type Fpn with Phen Green (PG) to measure the respective amounts of non-transferrin bound iron (labile iron pool) as previously described [28]. FACs analysis showed that at comparable loading concentrations of PG, FpnK240E-expressing cells contained lower amounts of labile iron compared with cells expressing wild-type Fpn (Fig. 4D). This suggests that FpnK240E may constitutively efflux iron. Mechanistically this would explain the low intracellular iron and the relatively high serum iron and transferrin saturation seen in the patient expressing this mutant. The results therefore suggest that SUMO may restrain Fpn from uncontrolled release of iron into the circulation by binding to Fpn K240. However, we did not test whether FpnK240E was resistant to hepcidin-dependent degradation. Although it is possible that SUMO might compete with ubiquitin [26,27] for K240-binding, the downstream sequelae (Fpn stabilization versus degradation) may be dependent on hepcidin. There is also evidence that polysumoylated targets are susceptible to ubiquitination and degradation by the RING domain-containing SUMO-targeted ubiquitin ligases both in yeast and in mammals [27,45,46]. Hence, since Fpn degradation appears to be preceded by its ubiquitination, it is possible that monosumoylation might stabilize the transporter while polysumoylation could make it a target of ubiquitin E3 ligases and subsequent degradation through SIM-dependent sumoylation [27,44–46]. These suppositions will need confirmation by further study.

In summary, although it has been shown that K240 mediates Fpn ubiquitination and turnover by hepcidin [26,27], our data suggest that this residue may also be a target for Fpn sumoylation. It is noteworthy that SUMO can also serve an intermediary role in protein turnover [60–62] and were Fpn to be conjugated to polymeric SUMO, that may signal its ubiquitination and degradation. It is therefore possible that dynamic cross-talk between SUMO and ubiquitin may regulate iron transport by Fpn. Interestingly, evidence shows that sumoylated nuclear proteins accumulate when ubiquitination is inhibited [63]. Hence it is possible that whereas (mono)sumoylation may regulate Fpn trafficking to endosomes, ubiquitination may signal for its degradation. Taken together with previous observations [26,27] we propose that: (1) the balance between K240 sumoylation or ubiquitination may singularly determine susceptibility to ferroportin disease [25]; (2) SUMO may be an important regulator of iron homeostasis independent of hepcidin-mediated Fpn turnover, and (3) the disease-causing mutant K240E may be a gain-of-function variant that renders Fpn constitutively-active, resulting in uncontrolled iron release into the circulation. This may be evinced by the high serum iron levels and transferrin saturation reported [25] and by the low level of chelatable iron in cells expressing FpnK240E (see Fig. 3D). We further posit that K240 may be required for Fpn restitution whereas K240E may impede Fpn trafficking or cycling to and from the plasma membrane by SUMO. This therefore hints at a direct role for SUMO in iron homeostasis through Fpn regulation and suggests how dynamic changes in sumoylation e.g. due to oxidative stress [12,64–68] may impact iron metabolism.
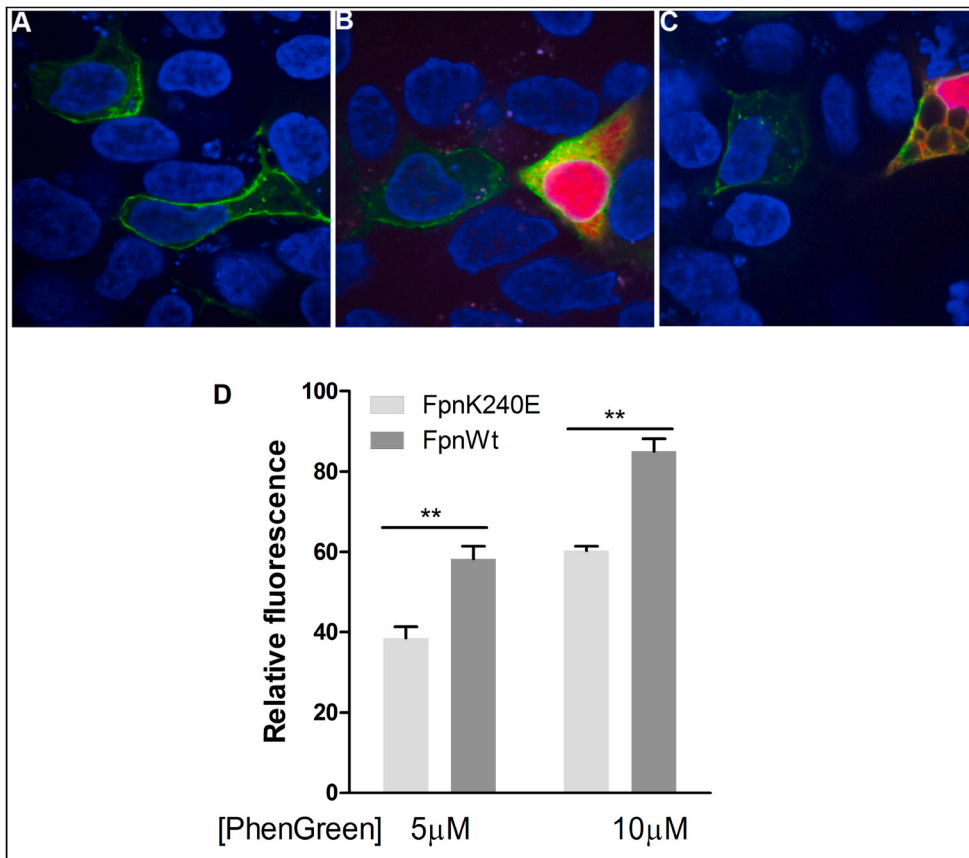
Fig. 4. Confocal microscopy. (**A**) Cells expressing wild-type Fpn-Egfp alone. (**B**) Cells co-expressing FpnK240E-Egfp and mRFP-SUMO1Wt show high constitutive internalization of FpnK240E. (**C**) With wild-type Fpn-Egfp, SUMO trafficked the transporter to, and induced the vesiculation of endosomal membranes. Fpn is coloured green and SUMO red; colocalization of the two proteins is indicated by yellow punctae. Cell nuclei were stained with DAPI. (**D**) Determination of differences in intracellular labile iron pool between cells expressing wild-type (Wt) Fpn and the sumoylation-defective mutant FpnK240E by flow cytometry as described under "Materials and methods". Data are representative of two independent experiments; $**P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Author contribution statement

Henry K. Bayele and Surjit S. K. Srai developed the concept; H.K.B performed experiments and wrote the manuscript. Both authors analyzed the data, critically reviewed the manuscript, approved it for publication and are responsible for the veracity of its contents.

Declaration of competing interest

The authors have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrep.2020.100873.

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