Human Molecular Genetics, 2008, Vol. 17, No. 17 **2610–2621**
doi:10.1093/hmg/ddn161
Advance Access published on May 28, 2008

Multistep, sequential control of the trafficking and function of the multiple sulfatase deficiency gene product, SUMF1 by PDI, ERGIC-53 and ERp44

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Received February 21, 2008; Revised April 16, 2008; Accepted May 23, 2008

Sulfatase modifying factor 1 (SUMF1) encodes for the formylglicine generating enzyme, which activates sulfatases by modifying a key cysteine residue within their catalytic domains. SUMF1 is mutated in patients affected by multiple sulfatase deficiency, a rare recessive disorder in which all sulfatase activities are impaired. Despite the absence of canonical retention/retrieval signals, SUMF1 is largely retained in the endoplasmic reticulum (ER), where it exerts its enzymatic activity on nascent sulfatases. Part of SUMF1 is secreted and paracrinally taken up by distant cells. Here we show that SUMF1 interacts with protein disulfide isomerase (PDI) and ERp44, two thioredoxin family members residing in the early secretory pathway, and with ERGIC-53, a lectin that shuttles between the ER and the Golgi. Functional assays reveal that these interactions are crucial for controlling SUMF1 traffic and function. PDI couples SUMF1 retention and activation in the ER. ERGIC-53 and ERp44 act downstream, favoring SUMF1 export from and retrieval to the ER, respectively. Silencing ERGIC-53 causes proteasomal degradation of SUMF1, while down-regulating ERp44 promotes its secretion. When over-expressed, each of three interactors favors intracellular accumulation. Our results reveal a multistep control of SUMF1 trafficking, with sequential interactions dynamically determining ER localization, activity and secretion.

INTRODUCTION

Sulfatases are a large family of prokaryotic and eukaryotic enzymes that catalyze the hydrolysis of ester sulfates, their natural substrates. The consensus sequence of the sulfatase catalytic domain contains a cysteine that is modified to formylglycine (FGly) within the endoplasmic reticulum (ER) by SUMF1/FGE (sulfatase modifying factor/formylglicine generating enzyme), herein called SUMF1 for brevity (1). This unique post-translation modification is essential for sulfatase activity: mutations in the *SUMF1* gene result in the inactivity of all the sulfatases and cause multiple sulfatase deficiency in humans (2,3).

SUMF1/FGE was crystallized and recognized to utilize oxygen to generate FGly in the sulfatases via a cysteine sulfenic acid intermediate (4). Of the eight cysteines present in SUMF1, cysteines 50 and 52 can form intermolecular disulfide bonds yielding SUMF1 homodimers (5,6). Cysteines 336 and 341 are part of the active site of SUMF1, are essential for its function and can exist in different oxidation states (4,7).

Recently, we have demonstrated that SUMF1 is also secreted: when taken up by other cells it is re-localized to

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the ER, where it activates endogenous sulfatases (8). SUMF1 does not contain an ER retention signal, and how its ER localization, secretion and uptake are controlled is still puzzling. Another interesting question is whether and how the activity of SUMF1 is coupled to its trafficking.

To dissect out the mechanisms controlling its folding, trafficking and function, we searched for proteins interacting with SUMF1 and focused on molecules potentially involved in its retention/escape from the ER. Using flagged SUMF1 as a bait, several proteins were co-immunoprecipitated and identified by mass spectrometry (MS). Among others, we further analyzed three specific interactors, PDI (protein disulfide isomerase), ERp44 and ERGIC-53 as strong candidates to control SUMF1 function and trafficking. PDI is an ER resident, multifunctional protein that catalyzes the oxidation, reduction and isomerization of disulfide bonds and acts as a redox sensitive chaperone (9). It consists of four thioredoxin-like domains, abb'a'. The a and a' domains contain CxxC redox-active motifs and are required for oxidase activity. The b and b' domains serve structural functions, participate in substrate binding and are essential for disulfide isomerization (10–12).

ERp44 is a soluble protein of the thioredoxin family (TRX) endowed with an ER-localization signal, RDEL (13), that also accumulates in the ERGIC (ER–Golgi intermediate compartment) and *cis*-Golgi (14–16). It is responsible for thiol-mediated retention of many substrate proteins, including Ero1α, adiponectin and IgM (15–17). ERp44 has been implicated also in ER calcium homeostasis, binding and regulating IP3R1 (18).

ERGIC-53 is a membrane-bound lectin protein that cycles between the ER and Golgi, accumulating in the ERGIC. ERGIC-53 operates as a cargo receptor (19), capturing certain glycoproteins (e.g. Factors V and VIII, pro-catZ) in the ER and releasing them in the Golgi in a pH-dependent manner (20). ERGIC-53 is also the cargo receptor for the LDL receptor-related protein (LRP). It dissociates it from the RAP protein, which blocks LRP-ligand interactions in the early secretory pathway (21). Subsequently, RAP is recycled back to the ER, and LRP proceeds through the Golgi and to secretion. In association with ERp44, ERGIC-53 can assist IgM polymerization (15).

Biochemical and functional assays reveal that PDI retains and activates SUMF1 into the ER, whereas ERGIC-53 and ERp44 exert their activity downstream by exporting the enzyme from, and retrieving it back into the ER, respectively. The interactions with PDI, ERGIC-53 and ERp44 provide a novel, dynamic control of SUMF1 trafficking and enzymatic activity.

RESULTS

Identification of proteins controlling SUMF1 function and trafficking

SUMF1 modifies sulfatases as they enter into the ER (22). However, it does not contain known ER localization signal(s). Furthermore, a fraction of SUMF1 can be secreted and taken up by vicinal cells, where it re-localizes to the ER and activates sulfatases (8). In an attempt to identify SUMF1 interactors controlling its subcellular localization and secretion, extracts from stable HeLa cells expressing *SUMF1-3xFlag* (HL3xFS1 clone) (8) were immunoprecipitated with immobilized anti-Flag antibodies. Upon competition with a Flag peptide, the eluate was analyzed by nanoLC MS/MS and compared with immunoprecipitates from mock-transfected HeLa cells (unpublished data). Among the bands yielding a much higher intensity in the HL3xFS1 lysates, we identified SUMF2, previously shown to form heterodimers with SUMF1 (23) and several novel proteins including PDI, ERp44, ERGIC-53 and ERp57, another thiol-disulfide oxidoreductase (24). We further analyzed these novel interactors as strong candidates for the control of SUMF1 trafficking and localization (Supplementary Material, Table S1).

PDI binding to SUMF1 is mediated by both covalent and non-covalent interactions

To confirm and characterize the association of SUMF1 with PDI, HeLa cells were transfected with *SUMF1-Flag*, *PDI*, or the mutant PDI_{ASAS} that lacks the four active cysteines in a and a' domains (25). In cells over-expressing only *SUMF1-Flag*, two main bands of ∼40 and 80 kDa, likely corresponding to monomers and covalent homodimers (23), were decorated by anti-Flag under non-reducing conditions (Fig. 1A, lane 1). Co-expression of wild-type (wt) PDI drastically decreased the intensity of the SUMF1 homodimers band (lane 2). Two slower migrating bands become detectable (lane 2, see asterisk) likely corresponding to covalent PDI–SUMF1 heterodimers and higher order complexes. These bands disappeared in reducing conditions (unpublished data), were not detectable in cells co-transfected with *SUMF1-Flag* and the redox-inactive PDI_{ASAS} mutant (Fig. 1A, lane 3) and were stained by anti-PDI antibodies (Fig. 1B, lane 2). These experiments demonstrated that PDI binds covalently with SUMF1, and strongly decreases the formation of SUMF1 homodimers. To test if PDI and SUMF1 can also associate via non-covalent interactions the same extracts were immunoprecipitated with anti-Flag. The immunocomplexes were resolved under non-reducing conditions and blots decorated with anti-PDI. Specific bands corresponding to PDI–SUMF1 heterodimers and PDI monomers demonstrated covalent and non-covalent interactions between SUMF1 and PDI, respectively (Fig. 1B, lane 2). In contrast, SUMF1 and PDI_{ASAS} formed mainly non-covalent associations since only the band correspondent to PDI monomers was detectable (Fig. 1B, lane 3, lower band). Of note, a low level of non-covalent association between endogenous PDI and SUMF1 was also seen (Fig. 2B, lane 1). To confirm these results, HL3xFS1 cells transfected with either PDI or PDI_{ASAS} were immunoprecipitated with anti-Flag. Equal amounts of the immunocomplexes were loaded in reducing gels and the amount of PDI co-immunoprecipitated with SUMF1-Flag evaluated by anti-PDI blotting. Clearly, some endogenous PDI co-immunoprecipitated with SUMF1-Flag (Fig. 1C, lane 1). The PDI band was more intense in cells over-expressing PDI with respect to cells over-expressing PDI_{ASAS} which cannot interact covalently with its substrates (Fig. 1C, lanes 2 and 3). These results demonstrate that PDI associates with SUMF1 covalently, via the cysteines in a

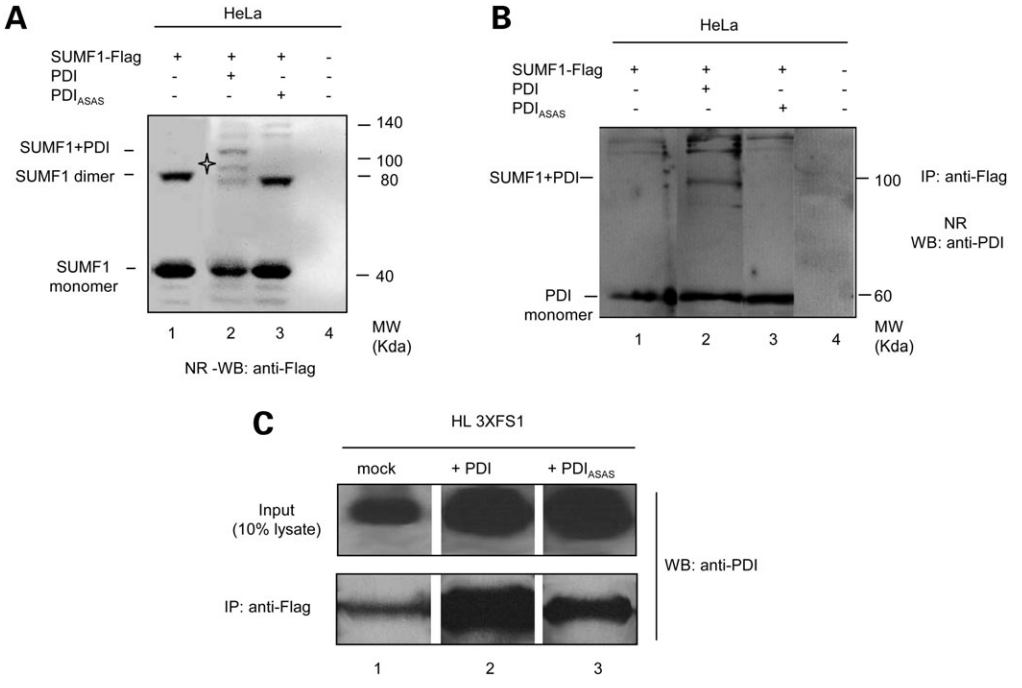

Figure 1. PDI binding to SUMF1. (**A**) Formation of stable PDI–SUMF1 mixed disulfides. HeLa cells were co-transfected with SUMF1-Flag and PDI or PDI_{ASAS}. The cells were treated with N-ethylmaleimide to quench disulfide interchange. Aliquots of the lysates were resolved by non-reducing gradient SDS–PAGE gels. The filters were revealed with anti-Flag antibody. Asterisks indicate bands corresponding to SUMF1-PDI mixed disulfides. (**B**–**C**) PDI association with SUMF1 is mediated also by non-covalent interactions. Transient (B) or stable (C, HL3xFS1 cells), HeLa transfectants expressing SUMF1-Flag were transfected with PDI or PDI_{ASAS}. Protein extracts were immunoprecipitated with anti-Flag antibody and the immunocomplexes were resolved under non-reducing (B) or reducing (C) conditions and blots probed with anti-PDI antibodies.

and a' active sites. Non-covalent interactions also take place, as confirmed by the observation that also PDI_{ASAS} binds SUMF1.

PDI–SUMF1 interactions were also confirmed by their co-distribution upon cell fractionation experiments in Optiprep density gradients (8–24%). SUMF1 monomers and dimers were abundant in the densest ER fractions containing PDI (Supplemantary material, Fig. S1).

N-glycan-dependent interactions between SUMF1 and ERGIC-53

Also ERGIC-53 was identified as a molecule capable of binding SUMF1. To validate the SUMF1-ERGIC-53 interaction, we over-expressed *SUMF1-Flag* in a stable HeLa transfectant that can be induced to express a tagged version of wt ERGIC-53 [KKFF; (26)]. Lysates of cells cultured without tetracycline and expressing both transgenes were immunoprecipitated with anti-ERGIC-53 antibodies. A band corresponding to SUMF1-Flag was clearly detected both in the absence and presence of dithio*bis*(succinimidylpropionate) (Fig. 2A, lane 2). In agreement with low affinity interactions, more SUMF1 was co-immunoprecipitated after cross-linking (Fig. 2A, compare lanes 2), although similar amounts of ERGIC-53 were precipitated by anti-ERGIC-53 antibodies (unpublished data). Mock-IP, PoIP and 20% of the lysates were also analyzed to control the specificity and efficiency of immunoprecipitation (lanes 1, 3, 4). To exclude that the interactions observed were due to over-expression, we analyzed non-transfected HeLa cells (panel B). Specific interactions between the endogenous proteins were detected: bands with the mobility and immunoreactivity of over-expressed SUMF1 (run in lane 3 to facilitate identification of the band) were precipitated by anti-ERGIC-53 also without cross-linking (Fig. 2B, lanes 1 and 2), but not by control antibodies (lane 4).

ERGIC-53 is a lectin that binds substrate glycoproteins via its carbohydrate recognition domain (CRD) in a Ca^{2+}-dependent way (19). To investigate whether also the association between SUMF1 and ERGIC-53 was mediated via carbohydrate–lectin interactions, we transfected HeLa cells expressing tetracycline-inducible ERGIC-53N156A, a CRD inactive mutant (19) with *SUMF1-Flag*. ERGIC-53N156A does not bind substrates and acts as a dominant-negative mutant, recruiting wt ERGIC-53 into hetero-hexameric complexes (27). Consistent with a role of the CRD in binding SUMF1, ERGIC-53N156A did not associate with SUMF1 and inhibited its co-immunoprecipitation with endogenous ERGIC-53 (Fig. 2C, upper panel; compare lane 3 that shows the association of SUMF1-Flag with endogenous ERGIC-53 and lane 1 that shows association of SUMF1-Flag with the left-over endogenous ERGIC-53 not recruited into the hetero-hexameric complex). Next, we transfected KKFF cells with *SUMF1N141A-Flag*, a non-glycosylated SUMF1 mutant (8). Consistent with carbohydrate–lectin-dependent interactions, non-glycosylated SUMF1 mutant did not associate with

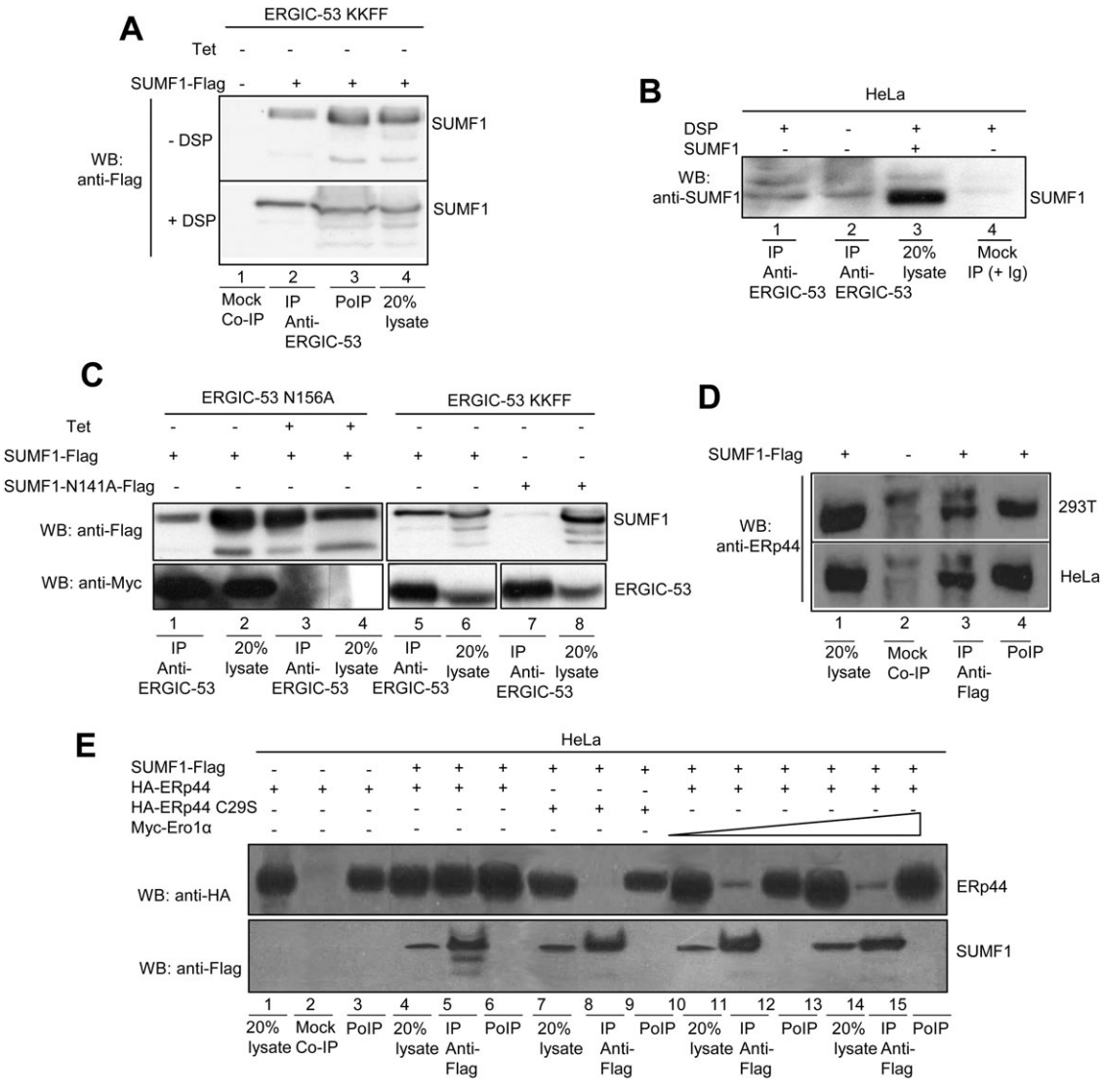

Figure 2. SUMF1 binds ERGIC-53 and ERp44 via glycan- and thiol-dependent interactions. (**A**) Co-precipitation between SUMF1 and ERGIC-53. KKFF cells incubated for 48 h without tetracycline to induce expression of myc-ERGIC53 were transfected with or without SUMF1-Flag and then immunoprecipitated with anti-ERGIC-53 antibodies before or after cross-linking with dithio*bis*(succinimidylpropionate) (DSP). Immunoprecipitates were resolved under reducing conditions and blots decorated with anti-Flag to reveal exogenous SUMF1 molecules associating with ERGIC-53. (**B**) Interactions between endogenous SUMF1 and ERGIC-53. Extracts from untransfected HeLa cells were immunoprecipitated as in A and blots decorated with anti-SUMF1 to detect endogenous molecules. Over-expressed SUMF1 was run in lane 3 to facilitate identification of the relevant band. (**C**) Binding between SUMF1 and ERGIC-53 is N-glycan dependent. ERGIC-53N156A or ERGIC-53KKFF cells were transfected with SUMF1-Flag or non-glycosylated SUMF1N141A-Flag as indicated, incubated for 48 h ± tetracycline to modulate ERGIC-53 expression and cross-linked with DSP. IP and Western blotting of the immunocomplexes were carried out with the indicated antibodies. (**D**) Endogenous ERp44 associates with over-expressed SUMF1. HeLa and 293T cells were transfected with the SUMF1-Flag-expressing plasmid. The extracts were immunoprecipitated with anti-Flag antibodies and blots probed with anti-ERp44. (**E**) Over-expression of Ero1α inhibits the association between SUMF1 and ERp44. HeLa cells were transfected with SUMF1-Flag, HA-ERp44, HA-ERp44C29S and Myc-ERO1α, as indicated. Cell extracts were immunoprecipitated with anti-Flag antibodies and blots probed with anti-HA or anti-Flag.

ERGIC-53 (Fig. 2C, lanes 5 and 7, lower panels). These data demonstrate that binding of SUMF1 and ERGIC-53 is mediated by *N*-glycan–lectin interactions.

Thiol-dependent interactions between SUMF1 and ERp44

The interactions between SUMF1-Flag and endogenous ERp44 were confirmed in 293T and HeLa cells (Fig. 2D). Non-reducing gels revealed the presence of a dithiothreitol-sensitive band with motility (86 kDa) and immunoreactivity consistent with a SUMF1–ERp44 disulfide complex (Supplementary Material, Fig. S2). Interestingly, ERp44 homodimers (17) became barely detectable in cells over-expressing *SUMF1-Flag* (Supplementary Material, Fig. S2), suggesting that SUMF1–ERp44 intermolecular interactions competed with ERp44 homodimerization and might involve ERp44 Cys29, as for other substrates of thiol-mediated quality control (13,16,17). Accordingly, unlike wt ERp44, the ERp44C29S mutant did not co-precipitate SUMF1 (Fig. 2E, compare lanes 5, 8, upper panel), confirming that

efficient binding largely depends on Cys29 of ERp44. Similar data were independently obtained by Mariappan *et al*. (6). From the latter study, it appears that a mutant ERp44 in which Cys 29 was replaced by Alanine, bound non-covalently to SUMF1. The discrepant behavior of the two mutants could reflect the different hydrophilicity of the two residues used to replace the cysteine in the active site.

The specificity and functional relevance of the ERp44–SUMF1 interaction was further underscored by experiments in which ERp44 was co-expressed with Ero1α, an oxidase that establishes strong interactions with ERp44. When over-expressed, Ero1α inhibits the interactions between ERp44 and its endogenous partners (16,17). In a similar way, Ero1α competed with SUMF1 for binding to ERp44 (Fig. 2E, lanes 10–15). These data demonstrate that ERp44 interacts covalently with SUMF1 via Cys 29 and this interaction can be inhibited by active Ero1α.

Subcellular distribution of SUMF1

Next, we determined the co-distribution of SUMF1 with PDI, ERp44 and ERGIC-53 by triple and double immunofluorescence (IF) staining (Fig. 3A). As expected, we found that most SUMF1 co-localizes with PDI in the ER, as also previously shown (3) as well as with ERp44. Co-localization of SUMF1 with ERGIC-53 was detectable in ER, while it was poorly if at all evident in the ERGIC perhaps because interactions are very transient in this compartment. This suggests that endogenous SUMF1 is efficiently and rapidly retrieved to the ER. The distribution of SUMF1 in different compartments of the early secretory apparatus was confirmed by cell fractionation experiments in Optiprep density gradients. SUMF1 immunoreactivity was abundant in the densest fractions containing most PDI and part of ERp44. However, a minor amount of SUMF1 co-distributed with ERp44 and ERGIC-53 within the less dense fractions (Fig. 3B).

PDI, ERGIC-53 and ERp44 co-operatively modulate SUMF1 retention/secretion and its enzymatic activity

Having confirmed that SUMF1 establishes specific interactions with PDI, ERp44 and ERGIC-53, we tested their role in the subcellular localization of SUMF1. First, we determined whether the over-expression of *PDI*, PDI_{ASAS} or *PDIΔKDEL*, a mutant lacking the C-terminal KDEL sequence and hence secreted (15,28) impacted SUMF1 traffic. As shown in Fig. 4A, PDI prevented SUMF1 secretion and increased its intracellular accumulation (Fig. 4A, lanes 1 and 2), indicating that PDI can retain SUMF1. PDI_{ASAS} had a smaller effect on SUMF1 retention/secretion, reflecting its capability of establishing non-covalent interactions (Fig. 4A, lane 3). As expected, the secretable *PDIΔKDEL* mutant impacted marginally SUMF1 localization. Interestingly, ERp57, another thiol-disulfide oxidoreductase identified in the MS analysis as a SUMF1 interactor, also inhibited SUMF1 secretion (Fig. 4A, lane 5). These results were confirmed by quantitative pulse and chase assays (Fig. 4B). Clearly, wt PDI was more efficient than PDI_{ASAS}, *PDIΔKDEL* and *ERp57* in favoring the intracellular retention of SUMF1.

Next, we analyzed their effects on the activity of IDS, a sulfatase that depends on SUMF1 to exert its enzymatic function. When IDS activity was measured in the extracts of the *PDI* over-expressing cells, a significant increase in IDS activity (compared with mock not-transfected cells) was observed. In contrast, PDI_{ASAS}, *PDIΔKDEL* had a slight inhibitory effect on IDS activity, whereas *ERp57* did not affect IDS activity (Fig. 4C). The stimulatory effects of PDI were specific since ERp57, although retaining SUMF1, did not impact SUMF1-dependent IDS activity. These results were confirmed by the findings that PDI wt, but not its mutants or ERp57, enhanced the activity of over-expressed IDS in the presence or absence of over-expressed SUMF1 (Supplementary Material, Fig. S3A). Furthermore, to confirm that PDI controls SUMF1 retention and activity, we silenced *PDI* in HeLa cells. A significant decrease of SUMF1 in the cellular pellet with an increase of secretion was observed in the interfered cells (Fig. 4D). Accordingly, IDS and SGSH activities were significantly decreased upon *PDI* silencing (Fig. 4D). Importantly, the inhibitory effect was not due to lower sulfatases protein levels, as the amount of SGSH remained constant upon PDI silencing (Fig. 4D). The inhibitory effects of down-regulating PDI were even more evident in cells over-expressing *IDS* and *SUMF1* (Supplementary Material, Fig. S3B). These findings indicate that PDI controls retention of SUMF1 and modulates the activity of SUMF1 and of sulfatases in a redox-dependent way.

Next, we examined the effect of modulating the ERGIC-53 or ERp44 levels on retention/secretion of SUMF1. When ERGIC-53 was over-expressed, little if any effect was observed compared with mock not-transfected HeLa cells (unpublished data), suggesting that ERGIC-53 is not rate-limiting for SUMF1 transport. However, expression of KKAA ERGIC-53, a mutant that accumulates in the ER (29), caused intracellular accumulation of SUMF1, at the expense of the secreted fraction (Fig. 5A). Since the KKAA mutant is known to act as a dominant negative for ERGIC-53-dependent transport (26), these findings confirm that functional interactions occur between ERGIC-53 and SUMF1. The effects of ERp44 over-expression on SUMF1 retention/secretion were next evaluated. When increasing amounts of *HA-ERp44* were co-transfected with *SUMF1-Flag* in HeLa cells, secretion of SUMF1 was strongly reduced (Fig. 5B). Interestingly, the co-expression of *HA-ERp44* and two increasing amounts of *Ero1α* resulted in increased secretion of SUMF1-Flag (Supplementary Material, Fig. S4A), likely owing to the preferential association between ERp44 and Ero1α. An increase in endogenous SUMF1 secretion was also observed upon over-expression of *Ero1α* (Supplementary Material, Fig. S4B) or treatment of cells with β-mercaptoethanol (Supplementary Material, Fig. S4C). These results demonstrated that the association of either ERGIC-53 or ERp44 impacts SUMF1 localization/secretion and that formation of intermolecular disulfide bonds is critical for SUMF1 retention.

To further dissect the function of ERGIC-53 and ERp44 in controlling SUMF1 trafficking we depleted HeLa cells of either or both proteins by silencing assays. Intriguingly, upon ERGIC-53 silencing the amount of SUMF1 detectable in cell extracts sensibly decreased with respect to controls, but the

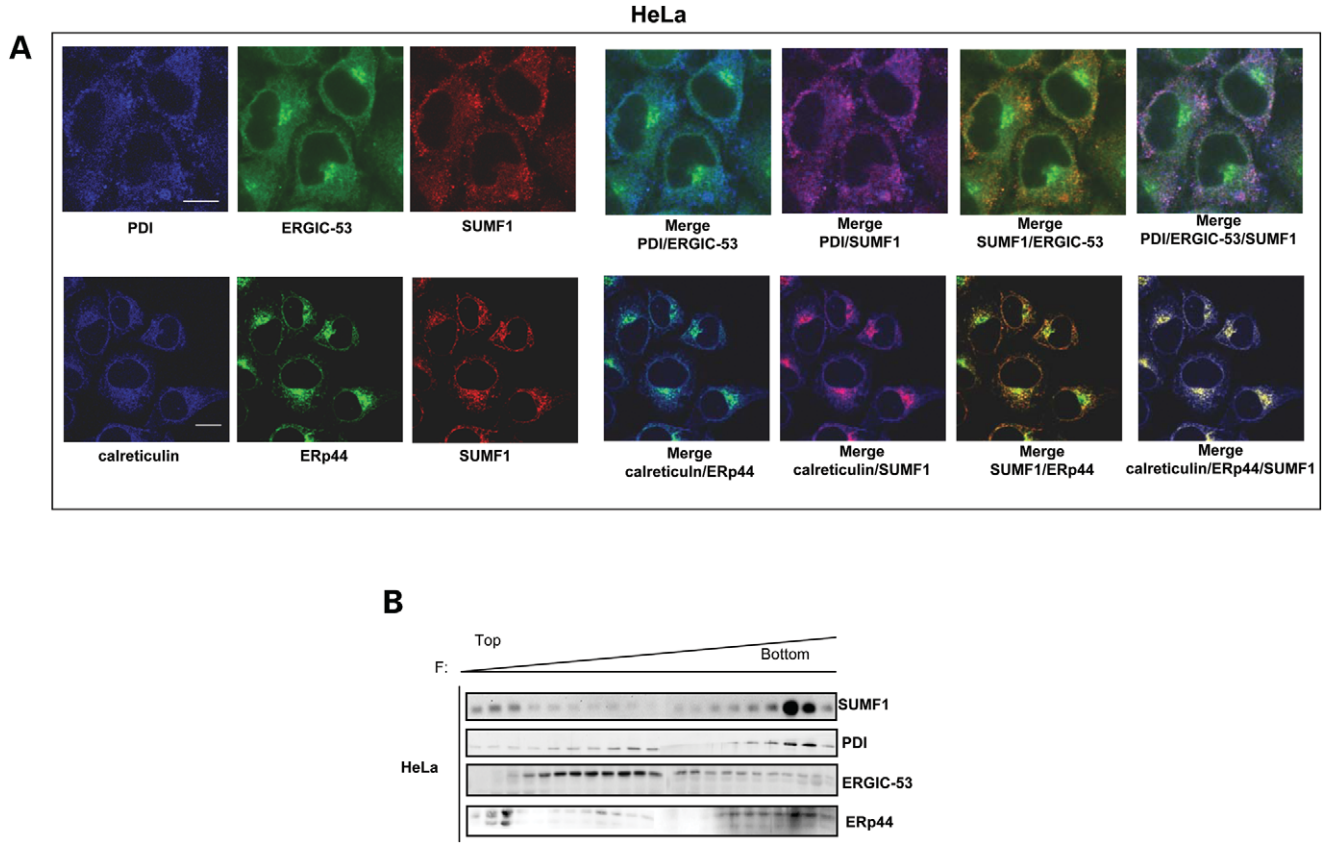

Figure 3. Subcellular distribution of SUMF1. (**A**) Immunofluorescence localization of SUMF1 within PDI, ERp44 and ERGIC-53 enriched compartment. HeLa cells were co-stained with fluorescent antibodies specific for SUMF1, ERp44, PDI, Calreticulin and ERGIC-53, as indicated. Scale bar, 5 μm. (**B**) Co-fractionation of SUMF1, ERp44, ERGIC-53 and PDI. Postnuclear cellular membranes from HeLa cells were fractionated by continuous Optiprep gradients (8–24%). Twenty different fractions were collected and resolved in SDS-gels under reducing conditions. Blots were probed for SUMF1, ERp44, ERGIC-53 or PDI, as indicated.

fraction of SUMF1 secreted into the media was not significantly increased (Fig. 5C). Silencing ERp44 also caused a similar decrease in the intracellular pool of SUMF1. However, differently from what observed with ERGIC-53-specific siRNAs, reducing ERp44 levels increased SUMF1 secretion (Fig. 5D).

As expected, the loss of intracellular SUMF1 in ERGIC-53- and ERp44-depleted HeLa cells caused a significant decrease in the activity of different sulfatases (Fig. 5C and D). Likewise, cells from a ERp44$^{+/-}$ ES trapped clone had lower ARSC, IDS and SGSH activities, further confirming that the ERp44-mediated retention is important for guaranteeing SUMF1 function (Fig. 5E). Taken together, these data suggest that both ERp44 and ERGIC-53 modulate SUMF1 trafficking and activity; however, they have a different function. ERp44 plays an important role in localizing SUMF1 in the ER, likely through RDEL-dependent retrieval from distal stations of the early secretory apparatus, as also recently suggested (15).

Binding to ERGIC-53 inhibits proteasomal degradation of SUMF1

Why would ERGIC-53 silencing cause loss of intracellular SUMF1 without simultaneous secretion? We hypothesized that the loss of SUMF1 was caused by increased degradation. To test this hypothesis, we cultured HeLa ERGIC-53- and ERp44-interfered cells in the presence or absence of the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal (LLN). Clearly, SUMF1 was stabilized by LLN in ERGIC-53 silenced cells (Fig. 6A, compare lanes 5 and 6), but not in ERp44-silenced or mock-treated cells. As expected, increased secretion of SUMF1 was seen in cells silenced for ERp44. In fact, proteasome inhibition did not affect the amounts of secreted SUMF1.

To further confirm that the absence of ERGIC-53 caused degradation of SUMF1, we performed pulse-chase experiments using HL3xFS1 cells silenced for either ERp44 or ERGIC-53. In untreated cells, SUMF1 was relatively stable. In contrast, silencing of ERp44 or of ERGIC53 caused a marked decrease in the amount of intracellular SUMF1-Flag (Fig. 6B). Consistent with the previous results, a simultaneous increase of SUMF1 secretion was seen upon ERp44 silencing, but not in the medium of ERGIC-53 siRNA cells (Fig. 6B). During the chase, this result was reverted after addition of proteasome inhibitors that stabilized SUMF1 only in ERGIC-53-interfered cells (Fig. 6C). Taken together, these findings indicated that ERGIC-53 depletion increased proteasomal degradation of SUMF1 and that part of it could still be secreted possibly via lectin-independent mechanisms; this is also supported by the observation that non-glycosylated SUMF1 is also secreted (8).

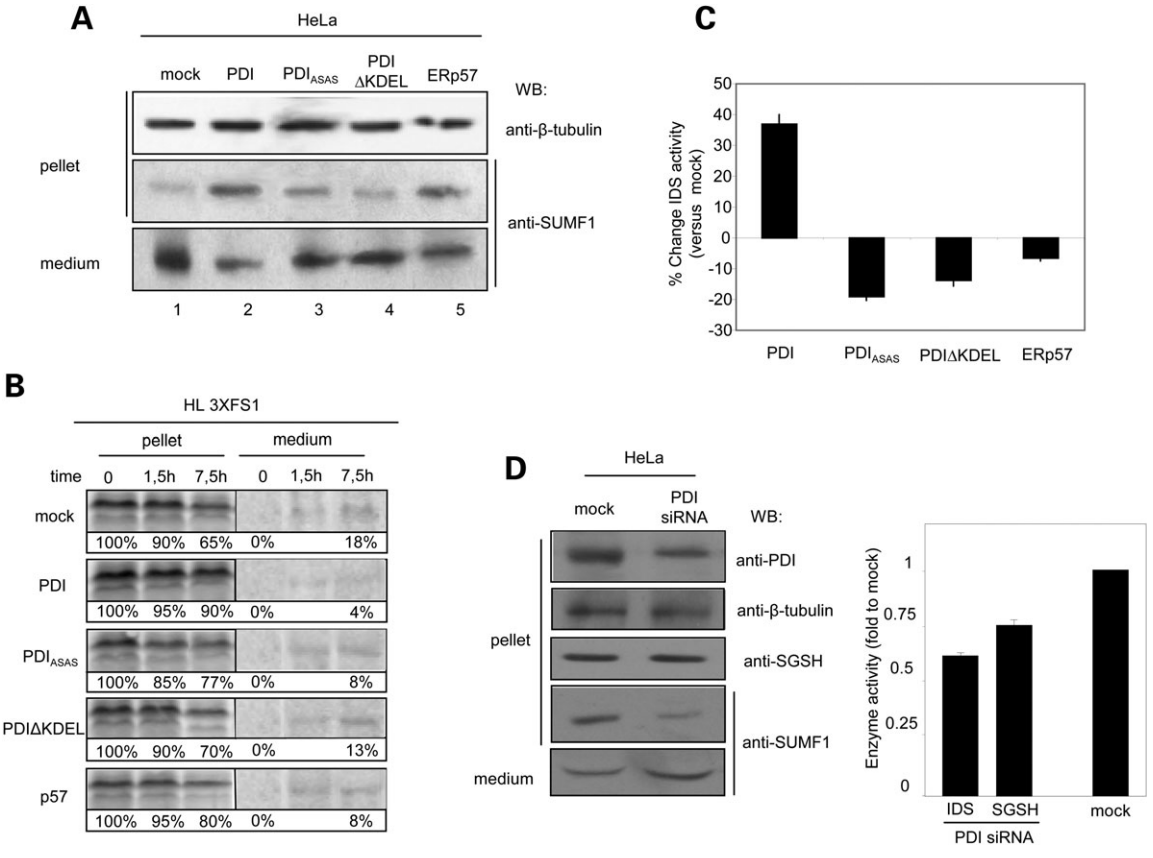

Figure 4. PDI controls ER retention and activity of SUMF1. (**A** and **B**) SUMF1 retention/secretion upon over-expression of PDI wt, PDI mutants and ERp57. (A) HeLa cells were transfected with PDI, PDI_{ASAS}, PDIΔKDEL or ERp57 expression plasmids. Two days after transfection, proteins extracts from cellular pellets deriving from 2×10^6 cells and the correspondent media (conditioned for 16 hours) were probed with anti-SUMF1. Anti-tubulin antibodies were used as loading controls. (B) HL3xFS1 stable cells transfected with PDI, PDI_{ASAS}, PDIΔKDEL or ERp57 expression plasmids were pulsed with [^{35}S] aminoacids for 15 min. Pellets and media were collected at the indicated chase times and immunoprecipitated with anti-Flag antibody to reveal SUMF1-Flag proteins. The amount of SUMF1 recovered in the pellet and in the medium at different chase times is quantified as the percentage of total SUMF1 present in cells at time 0 (100%). (**C**) PDI potentiates intracellular IDS activity. Forty-eight hours after transfection the activity of IDS was quantified in cellular extracts, and expressed as the percentage relative to the activity of mock transfected cells. (**D**) Silencing of PDI affects retention and activity of SUMF1. HeLa cells were mock transfected or transfected with PDI-directed siRNA expressing plasmid. Twenty-four hour after transfection cell pellets and the correspondent conditioned media were analyzed by Western-Blotting with the indicated specific antibodies (left). The IDS and SGSH activities were measured in the same extracts. Data are expressed as fold induction relative to mock transfected cells (right).

In contrast, ERp44 depletion leads to loss of intracellular SUMF1 that is not degraded but efficiently secreted.

The results shown so far suggested that soon after translocation in the ER, SUMF1 folds and interacts with PDI. The redox competency of PDI is important for the SUMF1-dependent activation of sulfatases in the ER. Part of SUMF1 binds to ERGIC-53 that likely delivers it to the *cis*-Golgi. Here ERp44 retrieves SUMF1 to the ER in an RDEL-dependent mechanism.

In the above scenario, a fraction of SUMF1 should be present in ERGIC and *cis*-Golgi: this was confirmed by cell fractionation experiments in Optiprep density gradients. However, triple and double IF staining of non-transfected HeLa cells revealed that the vast majority of SUMF1 accumulates in PDI-containing compartments (Fig. 3A). Only traces of it could be detected in ERGIC-53 and/or ERp44-positive downstream compartments. These observations suggest that endogenous SUMF1 is efficiently and rapidly retrieved to the ER, a notion that is consistent to the low level of SUMF1 secretion by non-transfected cells.

However, consistent with the biochemical and functional data shown in Figures 5 and 6, silencing ERp44 or ERGIC-53 had profound effects on the subcellular distribution of SUMF1. In both ERp44- and ERGIC-53-silenced cells, the fraction of SUMF1 co-localizing with the ER markers was drastically reduced (Supplementary Material, Fig. S5). While in ERGIC-53-depleted cells we observed primarily a loss of intracellular staining, consistent with the observed proteasomal degradation of SUMF1 (see Supplementary Material, Fig. S5).

DISCUSSION

SUMF1 activates all sulfatases in the ER operating a post-translational oxidation of a specific cysteine of their catalytic domain (22). However, SUMF1 lacks ER localization signals and part of it is secreted in the medium. How are these features harmonized and regulated? We identified three proteins, PDI, ERGIC-53 and ERp44 that by orchestrating

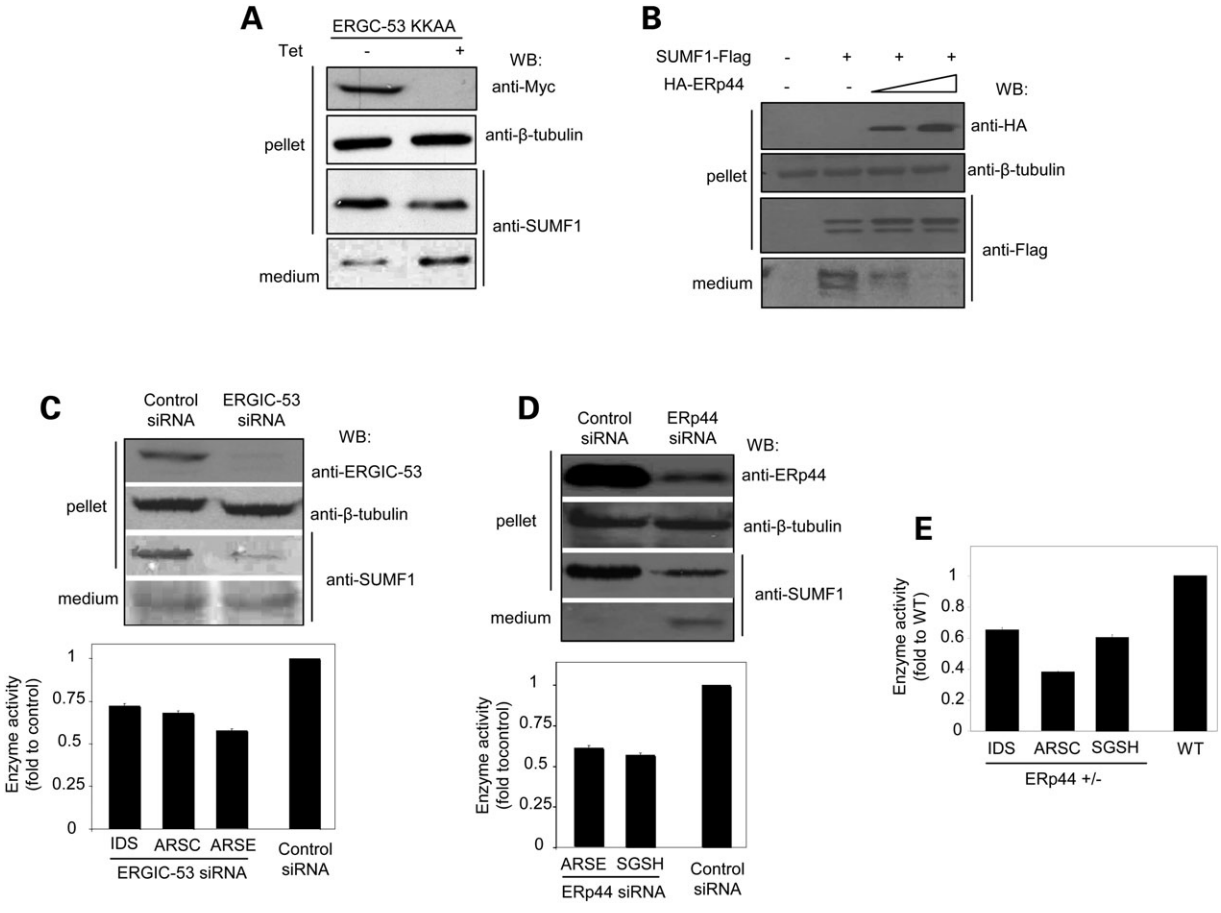

Figure 5. ERGIC-53 and ERp44 control SUMF1 traffic and activity. (**A**) ERGIC-53KKAA retains SUMF1 in the ER. Conditioned media (16 h) and lysates of KKAA cells cultured for 48 h ± tetracycline were analyzed by Western blotting with the indicated antibodies. (**B**) Over-expression of ERp44 inhibits SUMF1 secretion. HeLa cells were transfected with SUMF1-Flag alone or with increasing levels of HA-ERp44. Western blotting of conditioned media (16 h) and cellular extracts was carried out with the indicated antibodies. (**C**) Silencing ERGIC-53 causes loss of intracellular SUMF1 without increasing its secretion. HeLa cells were transfected with fluorescent ERGIC-53-directed siRNA or control duplexes. 3 days after transfection the cellular pellets and media (16 h conditioned) were probed with the indicated antibodies. Different sulfatases activities were measured in protein extracts obtained from FACS-sorted cells to enrich siRNA-treated cells. (**D**) Silencing ERp44 increases SUMF1 secretion. HeLa cells were transfected with fluorescent ERp44 siRNA and control duplexes. 3 days after transfection cell pellets and media (16 h conditioned) were probed with the indicated antibodies. The indicated sulfatases activities were measured in protein extracts obtained from FACS-sorted cells to enrich siRNA-treated cells. (**E**) Increased SUMF1 secretion in ERp44$^{+/-}$ ES cells. Different sulfatases activities were measured in extracts of ES trapped clones containing a single copy of ERp44 gene. The activities were compared to control ES wt cells.

the balance between SUMF1 retention in the ER and its secretion modulate its activity. After its translocation into the ER, SUMF1 must fold. It does so likely under the assistance of resident chaperones, lectins and enzymes, including calnexin and ERp57. Indeed, peptides derived from the latter two molecules were also found in our MS analyses (see Supplementary Material, Table S1).

Once folded, a large fraction of SUMF1 must remain in the ER, so as to exert its function on incoming sulfatases. Increasing or decreasing PDI levels show that the interaction with PDI is important in maintaining SUMF1 in the ER. PDI is a redox-dependent chaperone that mediates the formation, isomerization and reduction of disulfide bridges of numerous substrates, through the formation of transient mixed disulfides. In general, it is difficult to trap these intermediates in living cells, unless the second cysteine in the PDI CxxC motifs, which resolves the intermediates, is deleted. SUMF1 is clearly a special substrate of PDI, in that abundant heterodimers are easily detected in cells, provided that disulfide interchange reactions are inhibited by alkylants. In this respect, SUMF1 has profound similarities with Ero1α (25). Our data also demonstrate that the redox activity of PDI is essential for SUMF1 function. The ASAS mutant, that lacks all four cysteines in the a and a' domains, fails to potentiate sulfatase activities. In addition, ERp57 over-expressing cells display normal sulfatase activity despite their larger pool of intracellular SUMF1. These experiments suggest that retention can be uncoupled from activation and that PDI might act as a cofactor in the SUMF1-dependent sulfatase activation. PDI increases sulfatase activity, also without the concomitant SUMF1 overexpression. This observation may reflect increased folding of IDS, further activation on endogenous SUMF1, or both. It is also possible that PDI favors the interaction between SUMF1 and IDS, acting as a binding platform or maintaining the key cysteines involved in sulfatase modifications in a reaction-competent state. Whatever the exact mechanism(s)

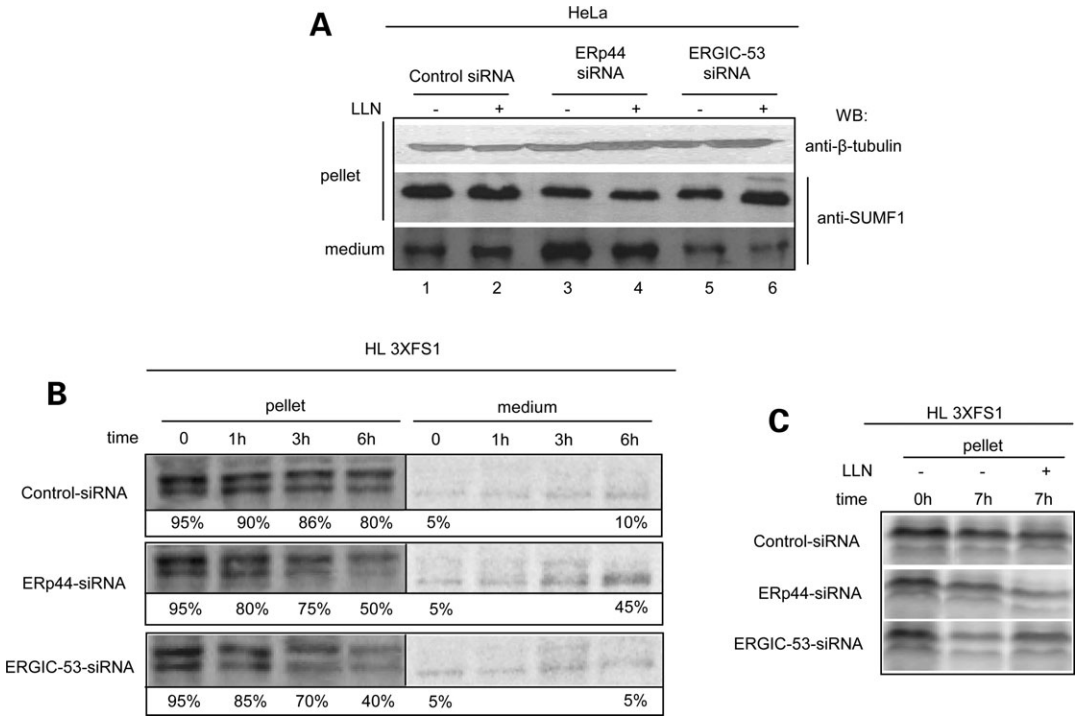

Figure 6. Different fate of SUMF1 in cells lacking ERGIC-53 or ERp44. (**A**) Proteasomal degradation of SUMF1 in ERGIC-53-depleted cells. HeLa cells were transfected with ERp44- or ERGIC53-specific siRNA or control duplexes, as indicated, and cultured with or without the proteasome inhibitor LLN for 12 h. Cellular pellets and media were probed with the indicated specific antibodies. Note the increased secretion in ERp44-depleted cells, and the increased intracellular pool in LLN-treated cells only in cells silenced for ERGIC-53. (**B**) Increased SUMF1 secretion upon ERp44, but not ERGIC-53 silencing. HL3xFS1 stable cells were transfected with ERp44- or ERGIC53-specific siRNA or control duplexes and then pulsed, chased and handled as in Figure 4B. (**C**) ERGIC-53 silencing promotes proteasomal degradation of SUMF1. The lysates of cells treated as above were collected after a 7 h chase carried out in presence or absence of the proteasome inhibitor LLN.

involved, our data imply that PDI folds, retains and activates SUMF1 in the ER.

However, the life cycle of SUMF1 is not limited to the ER: part of it is secreted and taken up in distant cells (Fig. 7). How does SUMF1 embark into secretion? Our results identify SUMF1 as a novel ERGIC-53 substrate. ERGIC-53 mediates the forward transport of selected glycoproteins (19). It interacts with SUMF1 via its CRD domain, and could hence promote its export from the ER via COPII-coated vesicles. Once in the *cis*-Golgi, the lower pH might favor SUMF1dissociation from ERGIC-53 (Fig. 7), as described for other substrates (20). Surprisingly, the destiny of SUMF1 in the absence of ERGIC-53 entails proteasomal degradation, rather than ER accumulation. This observation suggests that degradation and export are in competition for a fraction of SUMF1 molecules (30). Whether and how the molecules that are rapidly degraded in ERGIC-53-depleted cells differ from the stable SUMF1 pool is at presently unknown.

Once in the *cis*-Golgi, SUMF1 can proceed with forward membrane traffic or bind to ERp44 and be retrieved into the ER (Fig. 7). This notion is supported by several lines of evidence: first, over-expression of active ERp44 inhibits secretion. Second, SUMF1-ERp44 mixed disulfides are easily detected in different cell types. Third, impairment of ERp44 function increases significantly the secretion of the enzyme. Similar findings have been recently obtained independently (6). An intriguing difference emerged with our studies is that a Cys29Ala ERp44 mutant retained SUMF1 binding capabilities (6). The different phenotypes observed with the two ERp44 mutants might be explained with the insertion of a more hydrophilic residue in position 29, and hence within the active binding site, that could partially inhibit the non-covalent interactions that ERp44 establishes with its client proteins.

In all cell lines analyzed so far, ERp44 localizes also downstream of the ER, and a considerable fraction accumulates in the ERGIC and *cis*-Golgi. In these compartments, ERp44 is thought to capture oxidative folding intermediates (e.g. IgM, adiponectin) and retrieve them to the ER via RDEL-dependent mechanisms (15,16). Our data suggest that ERp44 rapidly retrieves SUMF1 back into the ER (Fig. 7). Therefore, ERp44 is essential for SUMF1 function, as cells depleted of ERp44 secrete SUMF1 and nascent sulfatases will be partially inactivated thereof.

Confocal IF and subcellular fractionation analyses reveal that in untransfected HeLa cells most SUMF1 is present in compartments that are positive for PDI. Therefore, little SUMF1 is present in compartments downstream of the ER at steady state. Nonetheless, silencing either ERGIC-53 or ERp44 causes a significant decrease in the total intracellular SUMF1 pool, and a redistribution of the remaining molecules that can be documented by microscopy and fractionation assays. Taken together, these findings suggest a highly dynamic localization mechanism.

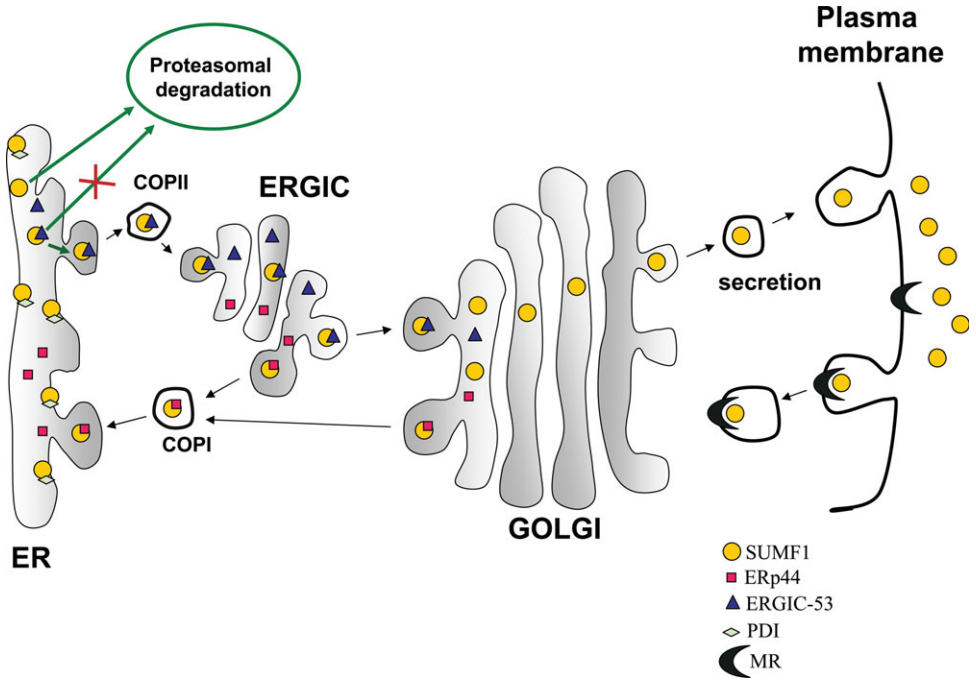

Figure 7. SUMF1 trafficking: a working model. Within the ER, SUMF1 folds and interacts with PDI. The redox activity of PDI is important for the SUMF1-dependent activation of sulfatases. SUMF1 also binds to ERGIC-53 through lectin–glycan interactions. This interaction protects SUMF1 form proteasomal degradation and delivers it to the *cis*-Golgi. Here, part of SUMF1 is retrieved by ERp44 through RDEL-dependent mechanisms and part of it can move forward and reach the medium. Secreted SUMF1 can be taken up as previously shown (8).

Many chaperones and enzymes reside in the ER because they possess C-terminal KDEL-like sequences. Their absence in SUMF1 suggests that an evolutionary pressure exists that favors the multi-level localization control described here, allowing regulated secretion. Particularly relevant in this context is the observation that over-expression of active Ero1α increases SUMF1 secretion, which may reflect competition with ERp44 and/or enforced dimerization of SUMF1 through cysteines 50–52 (5). In analogy with adiponectin, SUMF1 could be rapidly secreted in response to Ero1α-dependent redox changes.

In conclusion, SUMF1 can be retained into the ER or secreted into the extracellular matrix, depending on the concerted action of PDI, ERp44 and ERGIC-53, and this multistep control modulates in turn its activity on sulfatases. Our data reveal a fine tuned localization mechanism involving recycling of enzymatically active, transport-competent molecules. This dual localization provides a pool of SUMF1 molecules easily mobilized into secretion. The identified multistep control of interactions tunes the amounts of circulating SUMF1. Increased secretion of SUMF1 upon certain physiological stimuli, e.g. ER hyperoxidation, might thus allow an additional level of regulation of the sulfatase activities in some cells and tissues.

MATERIALS AND METHODS

Cells and reagents

KKFF, KKAA and N156A cells were gifts from H.-P. Hauri (University of Basel). ERp44$^{+/-}$ ES cells (clone # XR1070) were purchased from Mutant Mouse Regional Resource Center (MMRRC). Antibodies: monoclonal and polyclonal anti-calreticulin from ABCAM, monoclonal anti-ERGIC53 from Alexis, monoclonal anti-SUMF1 from R&D, rabbit anti-Myc from Santa Cruz Biotechnology, polyclonal anti-PDI (kind gift from Ineke Braakman, Utrecht, NL), monoclonal anti-Flag, monoclonal anti-β-tubulin and polyclonal anti-HA from Sigma. The ERp44-specific monoclonal 2D5, 36C9 are available in our laboratory and previously described (15). The *ERp44-HA*, *ERp44C29S-HA*, *Myc-Ero1α*, *IDS*, *SUMF1-Flag*, *SUMF1N141A-Flag*, *PDI*, *PDI*$_{ASAS}$, *PDIΔK-DEL* and *ERp57* expressing plasmids were previously described (8,13,17,23,25). The proteasome inhibitor LLN was purchased from Sigma.

Immunoaffinity purification of SUMF1-associated proteins

SUMF1 complexes were affinity purified from total extracts prepared from 6×10^8 HL3XFS1 cells (stable HeLa cells expressing SUMF1-3XFlag). Cells were washed in PBS and lysed in 50 mM Tris/HCl, pH 7.9, 200 mM NaCl, 0.5% Triton, 1 mM EDTA, 50 mM HEPES and protease inhibitors (Sigma). After clarification, extracts were incubated with M2 anti-FLAG agarose-conjugated antibody (Sigma) overnight, followed by extensive washes with BC100 buffer (100 mM NaCl, 10% glycerol, 20 mM Tris/HCl, pH 7.9, 0.1% NP40) and FLAG peptide elution in BC100. The eluted extracts were TCA precipitated before loading onto 5–20% SDS–PAGE. The gels were stained with colloidal Coomassie blue (Invitrogen). Protein bands were excised and analyzed by MS.

siRNA transfection

Cells were transiently transfected with siRNA duplexes using a 5 nM final oligonucleotide concentration with the Lipofectamine RNAiMax reagent (Invitrogen), according to manufacturer instructions. The siRNA duplexes were purchased from Qiagen; the sequences of the siRNA oligonucleotides against ERGIC-53 were as previously described (31): 5′-GGACAG AAUCGUAUUCAUCdTdT-3′ and 5′-GAUGAAUACGAUU CUGUCCdTdT-3′. The sequences of ERp44 siRNA oligonucleotides were as previously described (15): 5′-CAACUCUG GCAAACACUAC-3′ and 3′-UUGAGACCGUUUGUGAUG -3′. PDI silencing was obtained by transfection with a pSilencer2.1-U6 neo vector expressing a siRNA for human PDI [(a kind gift of Dr K. Ahn (Seoul, Korea) (32)].

Cross-linking and immunoprecipitation

This technique is performed following the protocol of Appenzeller *et al*. (19).

MS, sulfatase enzymatic activity assays, non-reducing and reducing western-blotting

These techniques were carried out as previously described (8).

Immunofluorescence staining

Cells were grown on coverslips and fixed in PBS (pH 7.4) 4% paraformaldehyde for 20 min. The cells were permeabilized for 15 min in 0.1% Tween in PBS and then incubated for 30 min in PBS, 10% FBS (blocking). Cells were then incubated with appropriate primary and secondary antibodies (diluted in PBS + 1% FBS). Coverslips were mounted in Vectashield (Vector Laboratories) and viewed under confocal microscopy (Leica TCS SP2 AOBS confocal microscope with a x63 Neofluor Pan-Apo 1.3-nm oil objective).

Preparation of cellular membranes, Optiprep density sedimentation and immunoblotting

Cellular membranes from human HeLa cells as well as the Optiprep gradients were prepared according to the manufacturer's instruction. All fractions were dissolved in reducing and not-reducing sample buffer, resolved on 10% SDS–PAGE, transferred to PVDF and immunoblotted.

Pulse-chase assays

HL3xFS1 cells were transfected with control or ERGIC-53- or ERp44-specific siRNA oligos. Seventy-two hours after transfection, the cells were starved for 30 min in methionine/cysteine-free DMEM and then labeled for 15 min in the same medium containing [^{35}S]-met and [^{35}S]-cys (Amersham Promix). Cells and media were then collected at different time points after the pulse. Cell pellets were lysed in 50 mM Tris/HCl, pH 7.9, 200 mM NaCl, 0.5% Triton, 1 mM EDTA, 50 mM HEPES and protease inhibitors (Sigma). Cell extracts and media were subjected to immunoprecipitation with anti-Flag antibodies and the immunocomplexes resolved on SDS–gels. Radio-labeled proteins were revealed and densitometrically quantified by phosphoimaging analysis (Typhoon, Amersham).

Data analysis

Data were analyzed by one-way analysis of variance. A P-value <0.05 was considered to be statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We are grateful to Drs K. Ahn, I. Braakman, H.P. Hauri for providing excellent reagents.

Conflict of Interest statement. None declared.

FUNDING

Financial support from the Italian Telethon Foundation, Associazione Italiana per la Ricerca sul Cancro, Fondazione Cariplo and EMBO YIP are gratefully acknowledged.

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