Peripheral endoplasmic reticulum localization of the Gp78 ubiquitin ligase activity

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Accepted 18 November 2011
Journal of Cell Science 125, 1727–1737

doi: 10.1242/jcs.096396

Summary

Gp78 (also known as AMFR and RNF45) is an E3 ubiquitin ligase that targets proteins for proteasomal degradation through endoplasmic reticulum (ER)-associated degradation (ERAD). In this study, we showed that gp78-mediated ubiquitylation is initiated in the peripheral ER. Substrate monoubiquitylation and gp78 CUE domain integrity restricted substrate to the peripheral ER, where CUE domain interactions and polyubiquitylation reduced gp78 mobility. Derlin-1 and derlin-2, which are involved in the retrotranslocation of ERAD substrates, localized to a central, juxtanuclear ER domain, where polyubiquitylated proteins accumulated upon proteasome inhibition. Transfer of polyubiquitylated substrate to the central ER was dependent on ubiquitin chain elongation and recruitment of the AAA ATPase p97 (also known as VCP). HT-1080 fibrosarcoma cells expressed elevated levels of endogenous gp78, which was associated with segregation of ubiquitylated substrate to the peripheral ER and its polyubiquitin-dependent redistribution to the central ER upon proteasome inhibition. Therefore, the peripheral ER is the site of gp78 ubiquitin ligase activity. Delivery of ubiquitylated substrate to the central ER was regulated by ubiquitin chain elongation and opposing actions of gp78 CUE domain interactions and p97 recruitment.

Key words: Endoplasmic reticulum, gp78 (AMFR), Ubiquitin ligase, ERAD, Proteasome

Introduction

The endoplasmic reticulum (ER) comprises multiple domains implicated in various cellular functions, including lipid synthesis and metabolism, calcium homeostasis, and protein synthesis and degradation (Voeltz et al., 2002). Structurally, the ER comprises central, juxtanuclear rough ER sheets, which are the site of protein biosynthesis, as well as peripheral, smooth membrane tubules, whose expression is defined by reticulons and DP1, which are proteins that form homo-oligomers that drive membrane curvature (Shibata et al., 2010). ER-associated degradation (ERAD) is a quality-control pathway in which misfolded and misassembled proteins are ubiquitylated and targeted for proteasomal degradation (Meusser et al., 2005; Vembar and Brodsky, 2008). A juxtanuclear, ER quality-control (ERQC) compartment where ERAD substrates accumulate upon proteasomal inhibition has been described (Kamhi-Nesher et al., 2001; Spiliotis et al., 2002); however, a role for other ER domains in ERAD remains poorly characterized.

Gp78 (also known as autocrine motility factor receptor, AMFR and RNF45) is an E3 ubiquitin ligase involved in ERAD. The gp78 C-terminal tail contains a RING finger motif, responsible for its ubiquitin ligase catalytic activity, as well as an ubiquitin-binding CUE motif (Fang et al., 2001; Shimizu et al., 1999). The CUE motif is essential for gp78 E4 ubiquitin ligase activity targeting mutant CFTR (ΔF508) for degradation (Morito et al., 2008). The C-terminal extremity of gp78 is the site of interaction with the AAA ATPase p97 (also known as VCP), which provides the driving force to dislocate polyubiquitylated substrates from the membrane into the cytoplasm (Ballar et al., 2006; Lilley and Ploegh, 2005; Ye et al., 2001; Ye et al., 2003; Zhong et al., 2004). p97 also serves as an adaptor protein that links gp78 to components of the retrotranslocation complex, including Derlin-1, VIMP and PNGase (Ballar et al., 2007; Li et al., 2006; Ye et al., 2005). gp78 substrates include unassembled subunits of the T-cell receptors CD3δ and TCRα (Chen et al., 2006; Fang et al., 2001). gp78 substrate regulation has been linked to pathological processes in the case of α1-antitrypsin, CFTRΔF508 and KAI-1, a tumor metastasis suppressor protein (Joshi et al., 2010; Morito et al., 2008; Shen et al., 2006; Tsai et al., 2007). gp78-mediated ERAD is also associated with physiological processes, such as the sterol-dependent ubiquitylation and degradation of HMG-CoA reductase and Insig-1 (Cao et al., 2007; Lee et al., 2006; Song et al., 2005).

Immunoelectron microscopy using the gp78-specific 3F3A monoclonal antibody (Nabi et al., 1990) localized gp78 to a smooth ER domain (Benlimame et al., 1998; Benlimame et al., 1995; Goetz and Nabi, 2006; Wang et al., 1997). Overexpression of FLAG-tagged gp78 results in the distribution of 3F3A labeling to the tubular, peripheral ER, but not to the juxtanuclear, central ER, suggesting that this antibody recognizes a subpopulation of gp78 selectively localized to the smooth, peripheral ER (Goetz et al., 2007). We show here that the peripheral ER is the site of ubiquitin ligase activity of gp78, where CUE domain interaction with ubiquitylated substrate restricts gp78 mobility. Polyubiquitylation and gp78 recruitment of p97 are required for translocation of ubiquitylated substrate to the central ER for proteasomal degradation.

Results

ER domain distribution of ERAD components

To study gp78 function in ERAD, we generated the following N-terminal FLAG-tagged constructs (Fig. 1A): (i) RING finger mutant (FLAG–RINGmut) containing an inhibitory point

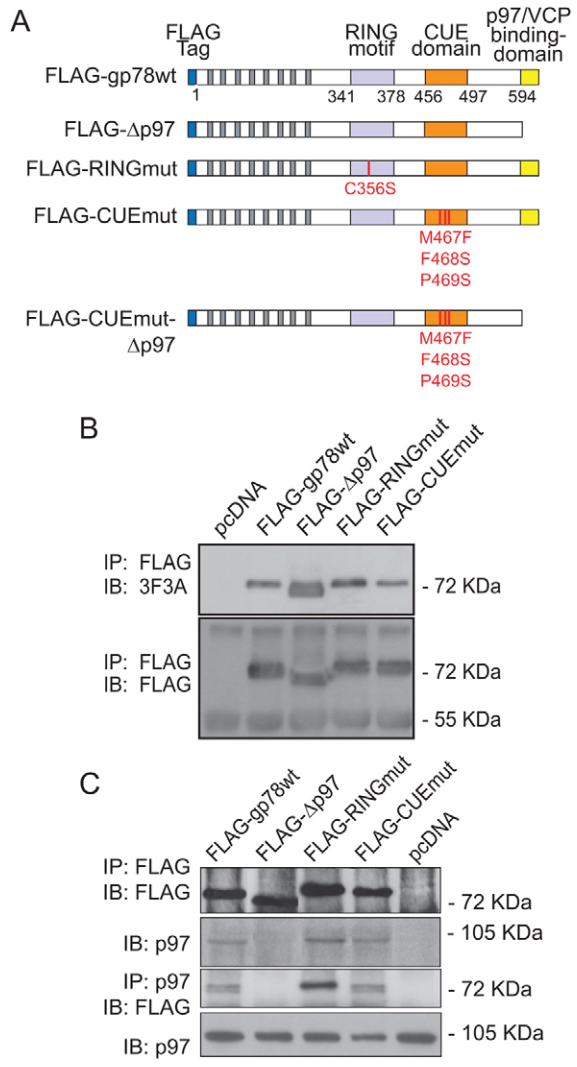

Fig. 1. gp78 construct validation. (**A**) N-terminal FLAG-tagged gp78 constructs: wild type (FLAG–gp78wt); p97-binding domain deletion (FLAG–Δp97); RING finger domain mutation (C356S; FLAG–RINGmut); CUE domain mutation (M467F, F468S, P469S; FLAG–CUEmut); and combined CUE domain mutant and p97-binding domain deletion (FLAG–CUEmut–Δp97). N-terminal grey boxes reflect putative transmembrane domains. (**B**) Lysates of Cos7 cells transfected with empty vector (pcDNA) or the indicated FLAG–gp78 constructs were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted (IB) with 3F3A or anti-FLAG antibodies. (**C**) Lysates of Cos7 cells transiently transfected as in A were immunoprecipitated with anti-FLAG or anti-p97 antibodies and immunoblotted with either anti-FLAG or anti-p97 antibodies, as indicated. Deletion of the p97-binding domain prevented p97 co-immunoprecipitation with FLAG-tagged constructs.

mutation of the third cysteine (C356S) in the RING–H2 domain (Freemont, 2000); (ii) CUE domain mutant (FLAG–CUEmut) containing inhibitory point mutations of the MFP motif (M467F, F468S and P469S), which are responsible for CUE domain affinity for ubiquitin (Shih et al., 2003); and (iii) a deletion mutant (FLAG–Δp97) lacking the C-terminal p97-binding domain (Ballar et al., 2006; Zhong et al., 2004). Anti-FLAG immunoprecipitates of the gp78 constructs expressed in Cos7 cells were recognized by the gp78-specific 3F3A monoclonal antibody (mAB) (Fig. 1B) (Goetz et al., 2007; Nabi et al., 1990; Registre et al., 2004) and, except for FLAG–Δp97, co-immunoprecipitated with p97 (Fig. 1C).

In Cos7 cells, the 3F3A-labeled smooth ER domain and the extended 3F3A-labeled peripheral ER network formed upon transient transfection of FLAG–gp78wt (Goetz et al., 2007) showed limited overlap with central ER-localized retrotranslocation proteins Derlin-1 and Derlin-2. Transfected Sec61β–GFP co-localized extensively with the Derlins and so serves as an excellent central ER marker (Fig. 2A,B). p97 showed limited association with the 3F3A-labeled ER in untransfected cells, but co-localized extensively with FLAG–gp78wt labeling and was recruited to the peripheral 3F3A-labeled ER in FLAG–gp78wt but not FLAG–Δp97 transfected cells (Fig. 2C). Therefore, gp78 overexpression resulted in an expanded 3F3A-labeled peripheral ER that contained p97 and was spatially distinct from the central ER, which includes Derlin-1 and Derlin-2; these proteins are involved in the retrotranslocation of ubiquitylated substrates and are found in complex with gp78 (Ballar et al., 2007; Ye et al., 2005). This suggests ER domain-specific functions for this ERAD-associated E3 ubiquitin ligase.

Gp78 ubiquitylation is initiated in the peripheral ER

To assess whether exogenous gp78 can ubiquitylate substrate, we co-transfected gp78 with HA-tagged ubiquitin (HA–Ubwt). In the presence of FLAG–gp78wt, the ubiquitylated protein smear was elevated relative to cells transfected with HA–Ubwt alone (Fig. 3A). gp78 is targeted for degradation via the proteasome (Chen et al., 2006) and ubiquitylated FLAG–gp78 is likely to be present in the ubiquitylated smear. However, the ubiquitylated smear detected extends below 78 kDa, suggesting that it includes not only ubiquitylated gp78, but also ubiquitylated gp78 substrates. The ubiquitylated smear showed increased intensity upon transfection with FLAG–Δp97, suggesting that an inability to recruit p97 does not limit gp78 ubiquitin ligase activity, but instead results in accumulation of ubiquitylated substrate by reducing translocation across the ER membrane (Jarosch et al., 2002; Ye et al., 2001). The minimal levels of HA–Ubwt-labeled protein detected in pcDNA-transfected control cells indicated that the ubiquitylated proteins detected upon transfection of FLAG–gp78wt were predominantly substrates of gp78 ubiquitin ligase activity. Therefore, overexpressed gp78 appears to dominate the ERAD machinery in transfected Cos7 cells, such that co-transfected HA–Ubwt represents a faithful reporter for gp78 ubiquitin ligase substrate activity.

In the presence of HA-tagged ubiquitin mutated at three crucial lysine residues (K29, K48 and K63) that are involved in polyubiquitin chain elongation (HA-Ubmono), FLAG–gp78wt or FLAG–Δp97 ubiquitylated protein smears were significantly decreased. Similarly, HA–Ubmono reduced protein polyubiquitylation in pcDNA-transfected cells treated with the proteasome inhibitor MG132 (Fig. 3A). Although K11 linkages have also been reported in ERAD (Xu et al., 2009), the ability of the triple lysine (K29R, K48R and K63R) ubiquitin mutant to reduce gp78 ubiquitylated smears substantially argues that it is not a major linkage in these experiments. The ubiquitylated protein smear was reduced by RING finger and CUE domain mutation, consistent with a role for these domains in gp78 ubiquitin ligase activity (Fig. 3B).

In contrast to the predominantly nuclear labeling of HA–Ubwt in empty vector transfected cells, ubiquitylated substrate co-localized extensively with, and was restricted to, the 3F3A-labeled

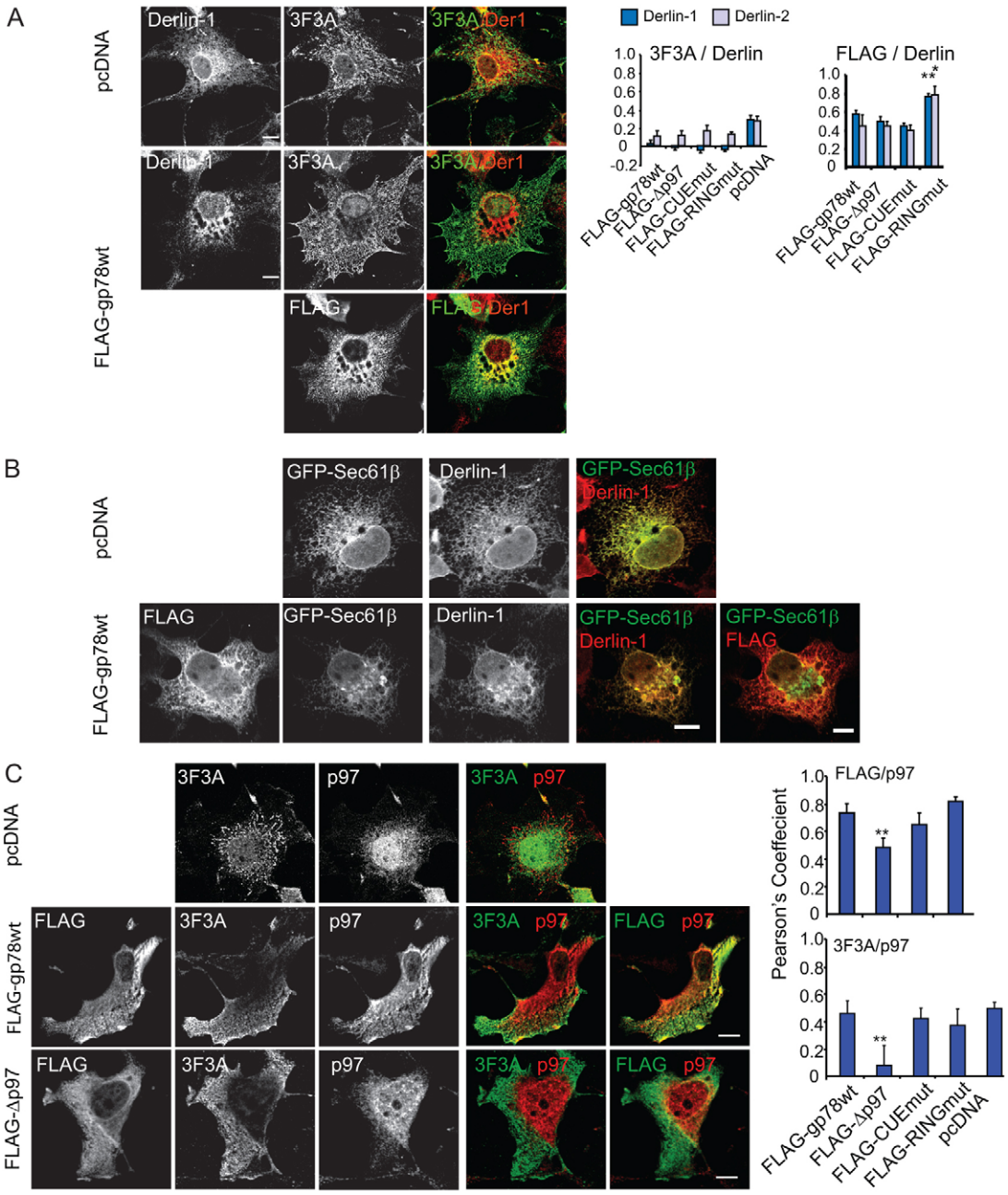

Fig. 2. ER domain distribution of gp78 and ERAD proteins. (**A**) Cos7 cells transfected with the indicated FLAG–gp78 constructs or empty vector (pcDNA) were immunofluorescently labeled with anti-FLAG, 3F3A antibody and either anti-Derlin-1 or anti-Derlin-2. Representative images of pcDNA and FLAG–gp78wt transfected cells labeled for Derlin-1 and 3F3A are shown. Bar graphs present Pearson's co-localization coefficients between 3F3A (top) or FLAG (bottom) and Derlin-1 or Derlin-2 labeling [24 cells per condition from three distinct experiments, data are mean ± s.e.m., $*P<0.05$, $**P<0.001$; scale bar: 10 μm]. (**B**) Cos7 cells were transfected with GFP–Sec61β alone or with FLAG–gp78wt, as indicated, then fixed and labeled with anti-FLAG and anti-Derlin-1 antibodies and appropriate species-specific secondary antibodies. GFP was visualized as is. FLAG–gp78wt labeling extended to the peripheral ER, but its overexpression induced concentration of GFP–Sec61β and Derlin-1 to the central ER. Scale bar: 10 μm. (**C**) Cells were transfected as in A and then fixed and immunofluorescently labeled with anti-FLAG, 3F3A mAB and anti-p97, as indicated, followed by the appropriate species-specific secondary antibodies. Representative images of FLAG–gp78wt and FLAG–Δp97 transfected cells are shown. Bar graphs present Pearson's coefficients of p97 and FLAG or 3F3A co-localization, as indicated. (24 cells per condition from three distinct experiments; data are mean±s.e.m., $**P<0.001$; scale bar: 10 μm.)

peripheral ER in FLAG-gp78wt and HA-Ubwt co-transfected cells (Fig. 3C; supplementary material Fig. S1). The dramatic redistribution of HA–Ubwt labeling from a predominant nuclear localization in pcDNA-transfected control cells to the ER upon transfection of FLAG–gp78wt suggests that the ER-associated ubiquitylated proteins detected are substrates of gp78 ERAD activity. Upon co-transfection of HA–Ubwt with FLAG–CUEmut, polyubiquitylated proteins were present not only in the peripheral, but also the central ER, as evidenced by HA–Ubwt labeling of the nuclear envelope. In FLAG–gp78wt- or FLAG–CUEmut-transfected

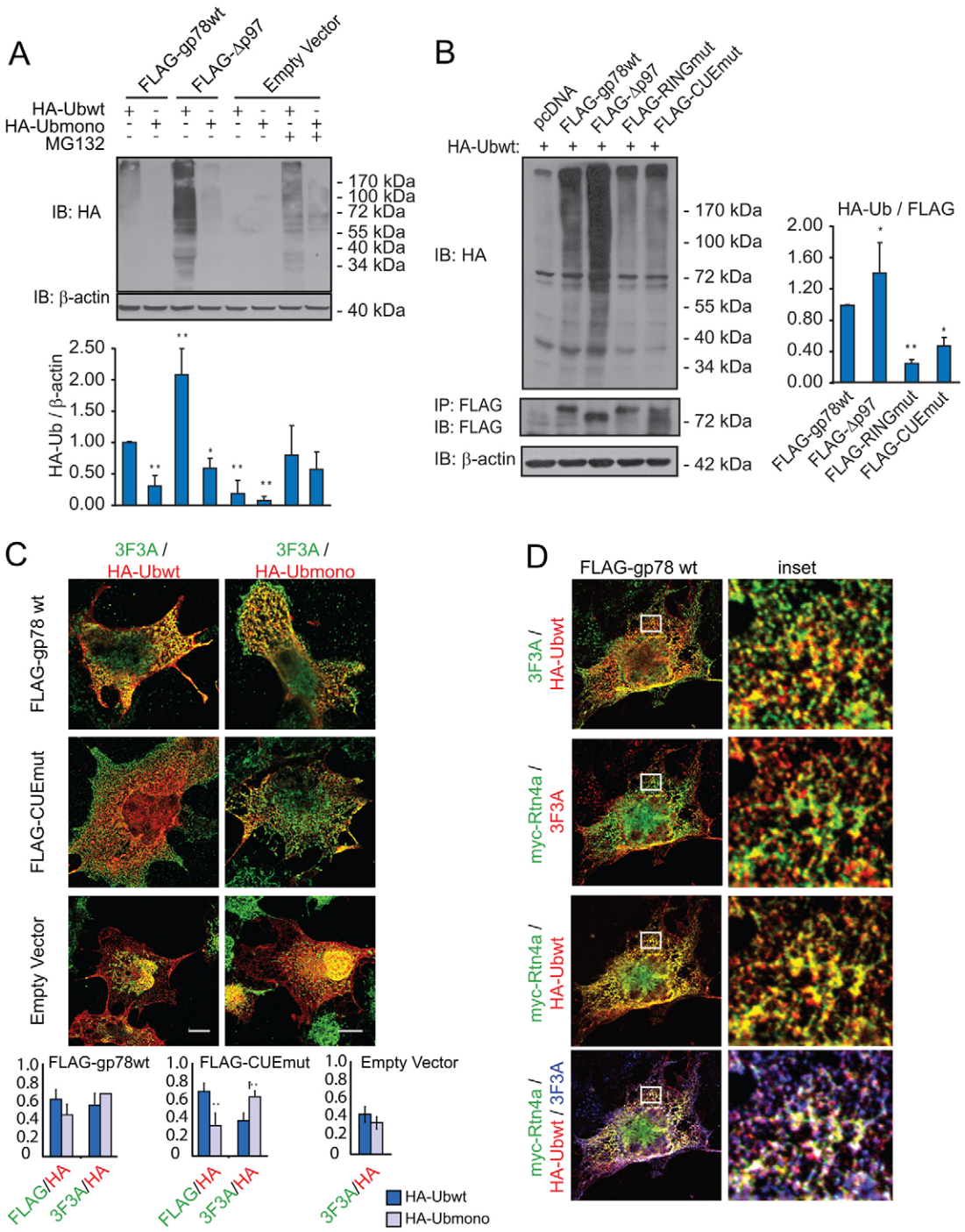

Fig. 3. Ubiquitin ligase activity of gp78 is initiated in the peripheral ER. (**A**) Cos7 cells were co-transfected with FLAG–gp78wt, FLAG–Δp97 or empty vector (pcDNA) and either HA–Ubwt or HA–Ubmono. pcDNA-transfected cells were also treated with MG132 (30 μg/ml) for 8 hours (*P<0.05, **P<0.001 relative to FLAG–gp78wt and/or HA–Ubwt). (**B**) TX-100 soluble cell lysates of Cos7 cells co-transfected with pcDNA or the indicated FLAG–gp78 constructs and HA-tagged ubiquitin (HA–Ubwt) were immunoblotted with anti-HA antibodies (top) or immunoprecipitated and immunoblotted with anti-FLAG antibody. The bar graph represents the density of the ubiquitin smears relative to anti-FLAG signal (*P<0.05, **P<0.001 relative to FLAG–gp78wt). (**C**) Cos7 cells were co-transfected with FLAG–gp78wt, FLAG–CUEmut or empty vector and either HA–Ubwt or HA–Ubmono and immunofluorescently labeled with anti-HA, 3F3A and anti-FLAG antibodies. Bar graphs present Pearson's co-localization coefficients between HA and either FLAG or 3F3A labeling (n=24, data are mean ± s.e.m., **P<0.001). (**D**) Peripheral regions of Cos7 cells transfected with FLAG–gp78wt, myc–Rtn4a and HA–Ubwt were labeled with anti-HA, 3F3A and anti-myc antibodies, as indicated. Scale bar: 2 μm.

cells, prevention of polyubiquitylation by co-transfection with HA–Ubmono restricted ubiquitylated substrates to the peripheral ER, increasing co-localization with the 3F3A-labeled peripheral ER (Fig. 3C; supplementary material Fig. S1). Tubules of the peripheral ER of FLAG–gp78wt-transfected cells, defined by reticulon (myc-Rtn4a) positivity were also labeled for 3F3A, as previously reported

(Goetz et al., 2007). HA–Ubwt showed extensive co-localization with both reticulon and 3F3A-labeled elements of the peripheral ER (Fig. 3D), suggesting that initiation of gp78-mediated ubiquitylation occurs in the peripheral ER.

CUE domain-polyubiquitylated substrate interaction reduces gp78 mobility in the peripheral ER

The central ER accumulation of polyubiquitylated substrate upon mutation of the gp78 CUE domain led us to determine the role of CUE domain integrity and ubiquitylated substrate interaction on gp78 mobility in the ER. Mobility of gp78 and mutants in the peripheral and central ER was measured using fluorescent recovery after photobleaching (FRAP). gp78 tagged with GFP, either N- or C-terminally, accumulates in the central ER, exhibits reduced ubiquitin ligase activity and, when tagged C-terminally, interferes with p97 recruitment (St. Pierre and Nabi, 2012). To visualize gp78 by live cell imaging, we added an eight amino acid tetracysteine (4c) tag between the N-terminal FLAG tag and gp78 cDNA. The 4c tag binds selectively to FlAsH and ReAsH, which are arsenic-based compounds that fluoresce when bound to the 4c tag (Adams et al., 2002; Martin et al., 2005). Transfected FLAG–4c–gp78wt labeled with FlAsH distributed to peripheral and central ER elements that were best visualized in live cells by maximum projections over time (Fig. 4A). Compared with GFP-Sec61β, which diffuses freely throughout the ER (Shibata et al., 2008), and shows 75–80% recovery after bleaching, FLAG–4c–gp78wt showed a highly restricted recovery of <20% in both the peripheral and central ER (Fig. 4B; supplementary material Table S1). This suggests that the bulk of gp78 exists within stable complexes that do not freely diffuse within the ER. FLAG–4c–CUEmut exhibited a significantly increased mobile fraction (~40%) in both the peripheral and juxtanuclear ER, suggesting that CUE domain-dependent interaction with ubiquitylated substrate stabilizes the gp78 complex motility. Loss of the p97-binding domain did not affect the gp78 mobile fraction. However, FLAG–4c–RINGmut, which was assessed only in the central ER where it concentrates (Fig. 2A), exhibited a highly restricted diffusion rate that was significantly reduced relative to

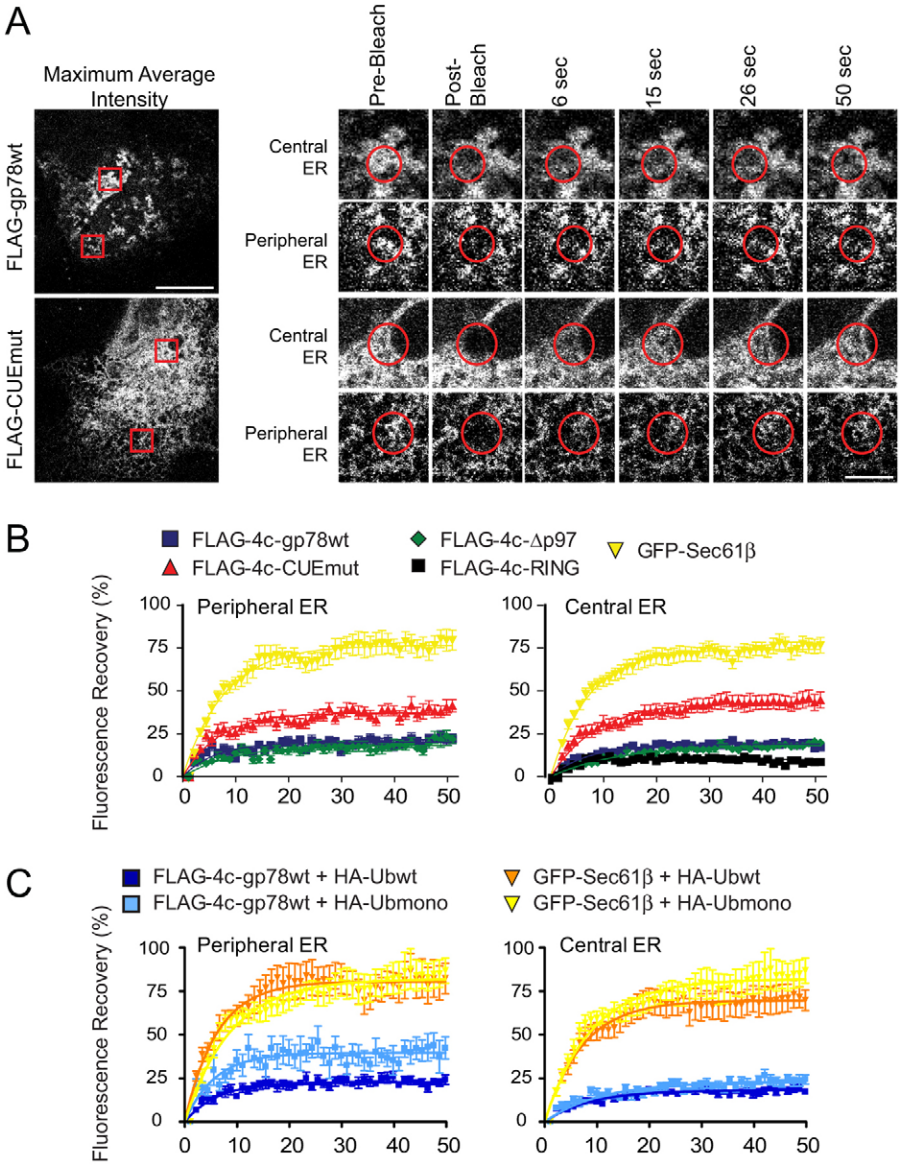

Fig. 4. Polyubiquitylated substrate–CUE motif interaction stabilizes gp78 in the peripheral ER. (**A**) Cos7 cells were transfected with FLAG–4c–gp78wt, FLAG–4c–Δp97, FLAG–4c–RINGmut or FLAG–4c–CUEmut constructs and visualized in live cells with FlAsH reagent. Representative maximum projections (50 images over 52 sec) of FLAG–4c–gp78wt and FLAG–4c–CUEmut are shown. Peripheral and central ER regions (boxes) are shown pre-bleach, immediately after bleach and 6, 15, 26 and 50 sec after bleach (circles). (n=12, scale bar: 10 µm; inset: 2 µm). (**B**) Graphs show normalized recovery after photobleaching of indicated FLAG–4c–gp78 constructs as well as GFP–Sec61β (yellow), as a control, in the peripheral (left) and central (right) ER. (**C**) Graphs show recovery after photobleaching of FLAG–4c–gp78wt or GFP–Sec61β co-transfected with HA–Ubwt or HA–Ubmono, as indicated.

FLAG–4c–gp78wt (Fig. 4B; supplementary material Table S1). Whereas co-transfection with HA–Ubwt did not affect FLAG–4c–gp78wt mobility, co-transfection with HA–Ubmono increased FLAG–4c–gp78wt motility to levels similar to FLAG–4c–CUEmut, but only in the peripheral ER (Fig. 4C; supplementary material Table S1). These data indicate that mobility of gp78 requires active ubiquitin ligase activity. The ability of both CUE domain mutation and coexpression of HA–Ubmono to enhance gp78 mobility in the peripheral ER argues that gp78 mobility is regulated, at least in part, by interaction with polyubiquitylated substrate.

Transfer of gp78-polyubiquitylated substrate to the central ER

Based on the discussion above, initiation of ubiquitylation and interaction with polyubiquitylated substrate appear to be peripheral ER events. However, Derlins are concentrated in the central ER, where they co-localize with GFP–Sec61β and are depleted from the 3F3A-labeled peripheral ER in gp78-overexpressing cells (Fig. 2A,B). To identify the site of proteasomal targeting, we treated cells with the proteasome inhibitor MG132, resulting in accumulation of ubiquitylated protein. As observed previously (Fig. 3C), HA–Ubwt-labeled protein was excluded from the GFP–Sec61β-labeled central ER when co-transfected with FLAG–gp78wt. However, upon treatment with MG132, ubiquitylated proteins accumulated in the central ER (Fig. 5A,B), as previously reported for ERQC (Kamhi-Nesher et al., 2001; Spiliotis et al., 2002). This suggests that proteasomal targeting occurs in the central ER but, in the absence of proteasome inhibition, ubiquitylated substrates do not accumulate and are rapidly degraded.

In cells co-transfected with HA–Ubwt and FLAG–Δp97, ubiquitylated substrates were restricted to the peripheral ER, even in cells treated with MG132 (Fig. 5A,B). Therefore, p97 recruitment by gp78 to the peripheral ER where polyubiquitylation is initiated appears to be required for subsequent transfer of the complex to the central ER. To verify this, we deleted the p97-binding site from FLAG–CUEmut (FLAG–CUEmut–Δp97) (Fig. 1A). In the absence of p97 binding, expression of the CUE mutant no longer resulted in central ER accumulation of polyubiquitylated substrate (Fig. 5A,B). Furthermore, central ER accumulation of ubiquitylated substrate in FLAG-CUEmut transfected cells and in MG132-treated FLAG–gp78wt-transfected cells was prevented by expression of mutant HA–Ubmono (Fig. 5A,B). This suggests that CUE domain interaction with polyubiquitin chains promotes stabilization of a gp78 complex that restricts, and potentially regulates, its exit from the peripheral ER. Substrate polyubiquitylation and p97 recruitment to gp78 appear to be required for central ER distribution of ubiquitylated substrates, irrespective of CUE domain integrity.

To demonstrate directly the transfer of ERAD substrates from their site of ubiquitylation in the peripheral ER to their site of proteasome targeting in the central ER, we performed a MG132 washout experiment. We measured the intensity of the HA–Ubwt signal in the GFP–Sec61β-defined central ER after MG132 treatment and over time following transfer to inhibitor-free media. In MG132-treated FLAG–gp78wt-transfected cells, up to ~50% of the polyubiquitylated protein was localized in the central ER. After subsequent washout of the proteasome inhibitor, the amount of ubiquitylated protein in the central ER decreased rapidly, reaching the level of untreated cells (~10%) after 90 minutes (Fig. 5C). In FLAG–CUEmut-transfected cells, MG132 only slightly increased ubiquitylated protein accumulation in the juxtanuclear ER. MG132 treatment did not induce the accumulation of ubiquitylated protein in the central ER in cells co-transfected with either wild-type or CUE mutant gp78 and HA–Ubmono, indicating that central ER ubiquitylated substrates are polyubiquitylated. Although the precise hierarchy of events remains to be more clearly defined, the fact that levels of polyubiquitylated proteins in the central ER remained essentially unchanged after MG132 washout (Fig. 5C) argues that CUE domain integrity is important for the efficient proteasomal degradation of substrates.

Gp78 ubiquitin ligase activity localizes to the peripheral ER of HT-1080 fibrosarcoma cells

To determine whether gp78 ubiquitin ligase activity is localized to the peripheral ER in the absence of exogenous gp78 overexpression, we used HT-1080 cells that express elevated gp78 levels and concomitant reduced expression of the gp78 substrate, KAI1 (Fig. 6A). Consistent with elevated functional gp78 levels in HT-1080 cells, transfected HA–Ubwt showed a more peripheral distribution of ubiquitylated substrate in HT-1080 relative to HEK293t cells (Fig. 6B). Although HA–Ubwt was closely associated with the peripheral, tubular ER network defined by myc-Rtn4a (Voeltz et al., 2006) in both cell lines, increased co-localization of 3F3A with both reticulon and HA–Ubwt was observed in HT-1080 cells compared with HEK293t cells (Fig. 6C).

Upon proteasome inhibition with MG132, HA–Ubwt redistributed to the central ER of HT-1080 cells, but not in the presence of HA–Ubmono (Fig. 7A). As observed in gp78-overexpressing Cos7 cells (Figs 3, 5), polyubiquitylation appears to be required for central ER delivery of ubiquitylated substrate in HT-1080 cells. To determine the role of gp78, we generated stable gp78-knockdown HT-1080 cells that have reduced gp78 mRNA transcript levels, ~50% reduced gp78 expression and increased KAI1 expression by western blot (Fig. 7B). In these cells, ubiquitylated substrate remained localized to the peripheral ER and MG132 treatment did not induce the central ER redistribution of ubiquitylated substrate (Fig. 7C). Therefore, these data in HT-1080 fibrosarcoma cells support the peripheral ER as the site of gp78-dependent ubiquitylation and suggest that transfer of ubiquitylated substrate to the central ER for proteasomal degradation occurs in response to elevated gp78 ERAD activity.

Discussion

Taken together, these data show that gp78 ERAD activity occurs in the peripheral ER, where the CUE domain mediates the interaction of gp78 with ubiquitylated substrate. Indeed, in mice deficient for TAP, which is required for assembly of the MHC complex, ubiquitin-associated heavy chain (HC) and β_2-microglobulin (β_2m) subunits accumulate in ER tubules that are morphologically similar to 3F3A-labeled smooth ER, suggesting the presence of a subcompartment of the ER where misassembled proteins are recognized and ubiquitylated for degradation (Benlimame et al., 1995; Raposo et al., 1995). Selective 3F3A labeling of FLAG–gp78 in the peripheral but not the central ER is consistent with functional and conformational differences of gp78 in this domain. A role for ubiquitin chain elongation and CUE domain interaction in restricting ubiquitylated substrate to the

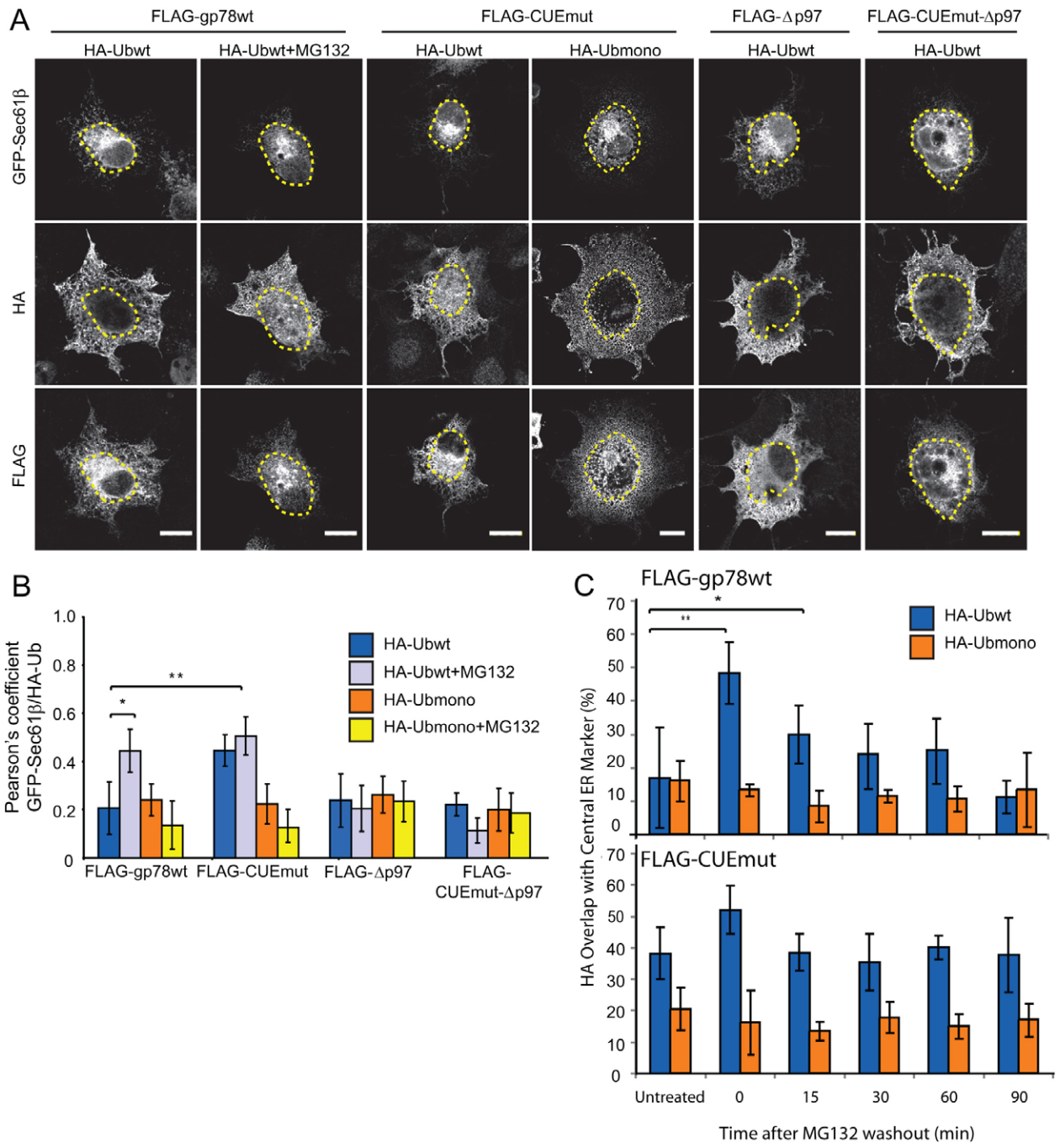

Fig. 5. Proteasomal degradation of gp78 ERAD substrates in the central ER. (**A**) Cos7 cells transfected with the indicated FLAG–gp78 constructs, either HA–Ubwt or HA–Ubmono and the central ER marker GFP–Sec61β (yellow line) were treated with or without MG132 for 8 hours, then labeled for HA and FLAG. Scale bar: 20 µm. (**B**) Co-localization between GFP–Sec61β and HA–Ubwt labeling was determined for the indicated FLAG–gp78 constructs in the presence of HA–Ubwt or HA–Ubmono with or without MG132 treatment. (**C**) Cos7 cells transfected as in A were treated with MG132 for 8 hours then incubated in MG132-free media for the indicated times. Using GFP–Sec61β to define the central ER, the intensity of HA labeling in the peripheral ER was determined relative to total HA intensity. (n=24, data are mean ± standard error of the mean, *P<0.05, **P<0.001.)

peripheral ER suggests that gp78 ubiquitylation of, and interaction with, its substrates in this domain is a highly regulated process. It has been proposed that monoubiquitylation acts as an ER retention signal to allow folding of newly synthesized membrane proteins functioning as a quality-control mechanism in ERAD (Feldman and van der Goot, 2009). Correct protein folding would result in deubiquitylation, whereas protein misfolding would lead to polyubiquitin chain extension and degradation. Our demonstration that monoubiquitylated gp78 substrates are restricted to the peripheral ER supports monoubiquitylation as an ER retention signal and identifies the peripheral ER as the putative site of this ERAD quality-control mechanism.

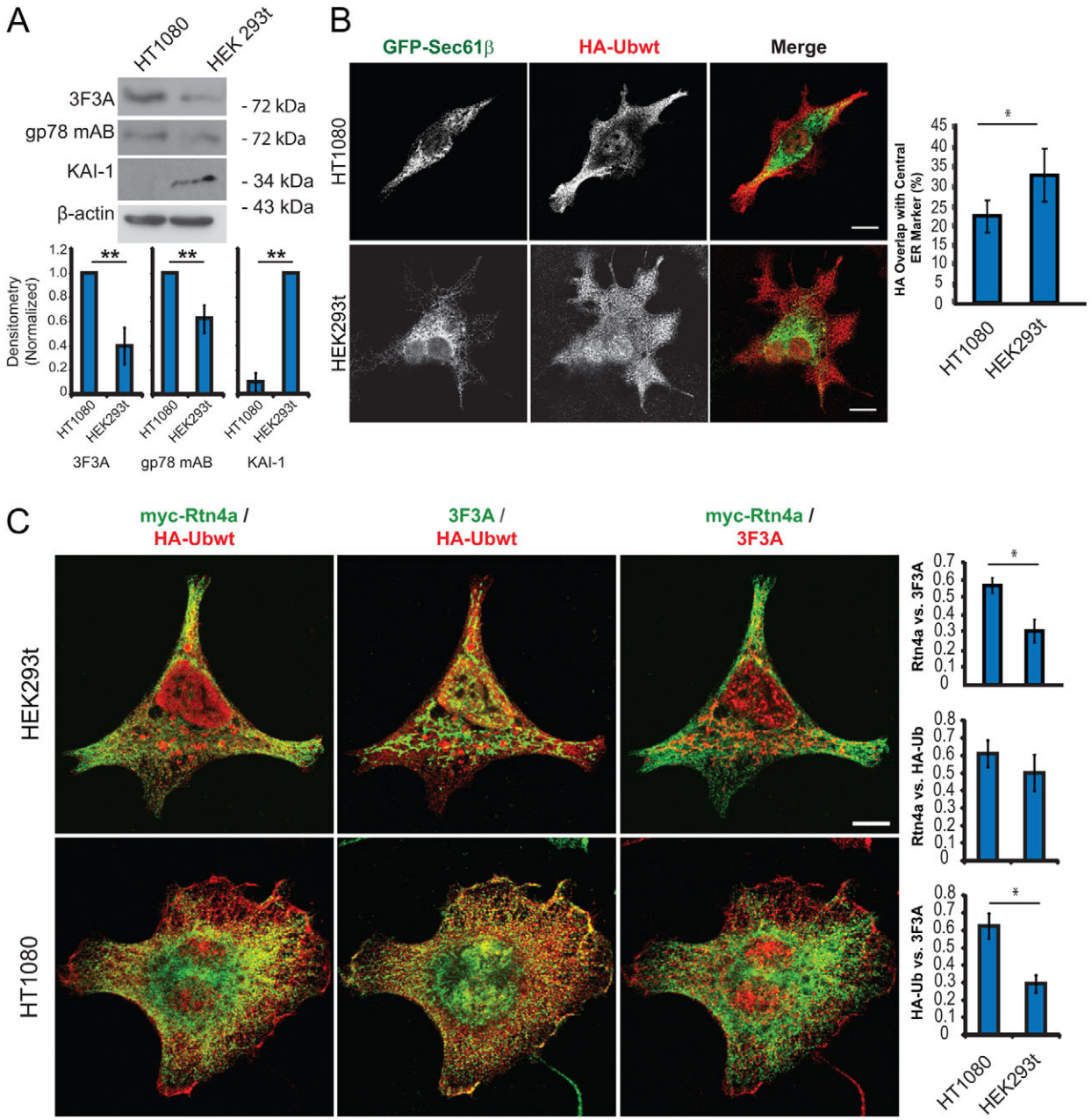

Fig. 6. Elevated gp78-mediated ERAD in HT-1080 fibrosarcoma cells. (**A**) HT-1080 and HEK293t cell lysates were western blotted with 3F3A and mouse monoclonal anti-gp78 (gp78 mAb), KAI1 and β-actin, and the 3F3A and KAI1 band intensity was quantified relative to β-actin. (**B**) HT-1080 and HEK293t cells were transiently transfected with GFP–Sec61β and HA–Ubwt; and HA–Ubwt labeling overlap with the central ER marker GFP-Sec61β was quantified ($n=18$ cells, scale bar: 10 μm). (**C**) HT-1080 and HEK293t cells were co-transfected with myc–Rtn4a and HA–Ubwt and labeled for myc, HA and 3F3A. Representative images of peripheral cellular regions are presented and Pearson's co-localization coefficients determined. ($n=12$, data are mean ± s.e.m., $^{*}P<0.05$, $^{**}P<0.001$, scale bar: 10 μm.)

Prolonged proteasomal inhibition results in retention of unassembled subunits of the class I major histocompatibility complex in the juxtanuclear ERQC (Kamhi-Nesher et al., 2001; Spiliotis et al., 2002). Similarly, we observed the accumulation of ubiquitylated substrate in the central ER of gp78-expressing cells upon proteasome inhibition. The fact that ERAD substrate accumulation in the central ERQC is predominantly observed upon proteasome inhibition argues that central ER proteasomal degradation is a highly efficient process. Gp78-dependent recruitment of ubiquitylated substrate to the central ER requires polyubiquitylation and p97 recruitment. Consistently, juxtanuclear, ubiquitin-associated aggregates that accumulate after MG132 treatment are dispersed throughout the ER upon p97 silencing (Wojcik et al., 2004). Moreover, expression of a dominant negative mutant of p97 ATPase function is sufficient to induce accumulation of p97 itself, Derlin-1 and polyubiquitylated substrates in the ERQC, identifying this site as the potential retrotranslocation site in the ER (Wakana et al., 2008).

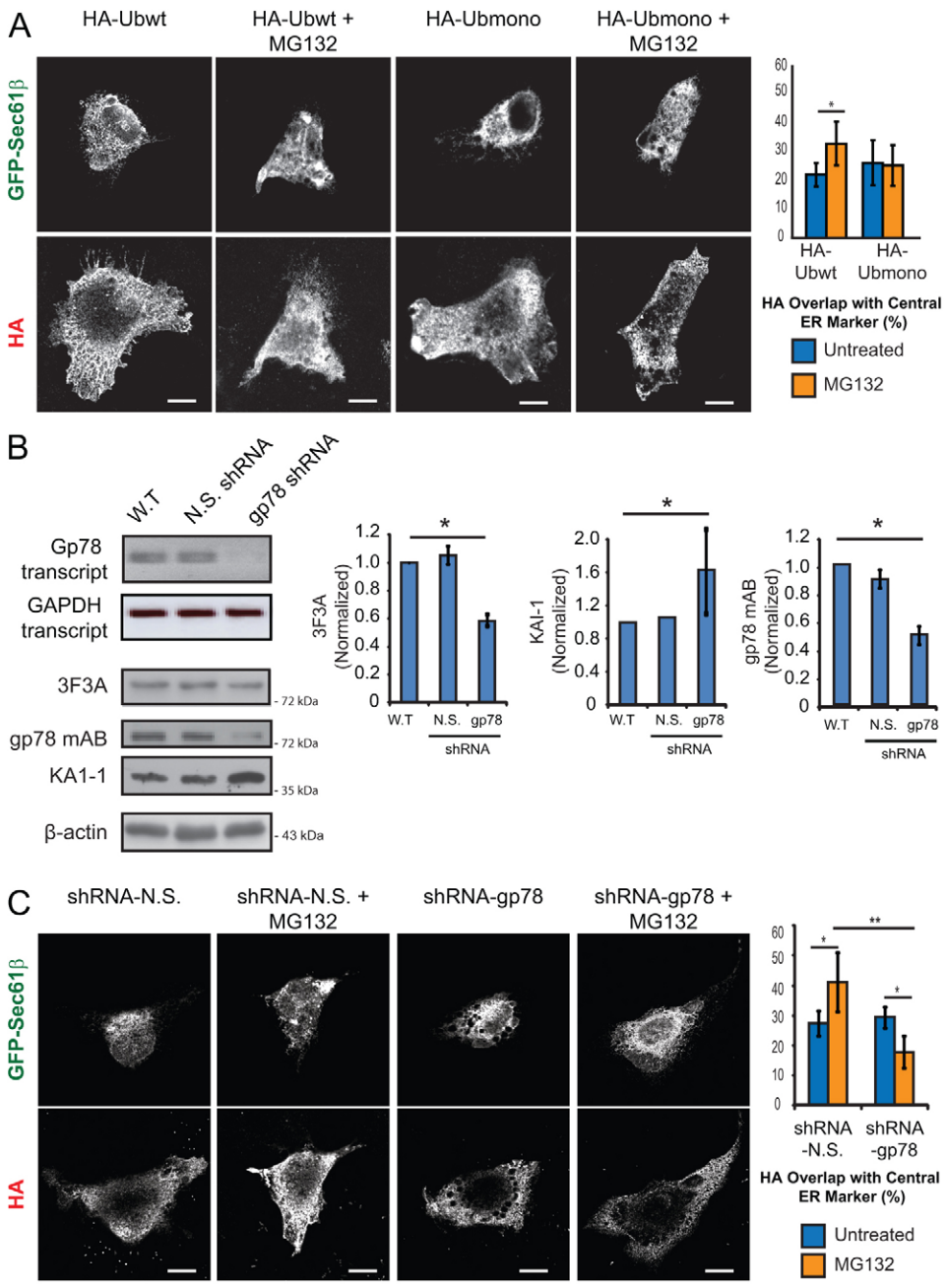

Fig. 7. ERAD domain segregation is gp78 dependent in HT-1080 fibrosarcoma cells. (**A**) HT-1080 cells co-transfected with GFP–Sec61β and either HA–Ubwt or HA–Ubmono were treated with or without MG132 for 8 hours and overlap of HA–Ubwt labeling with the central ER marker GFP–Sec61β was quantified. (**B**) Stable gp78-shRNA-expressing HT-1080 cells show reduced gp78 mRNA by RT-PCR, reduced 3F3A and mouse anti-gp78 mAb and increased KAI1 by immunoblot relative to wild-type or non-silencing (NS) shRNA-transfected cells. Densitometric quantification of 3F3A, gp78 mAb and KAI1 band intensity was determined relative to β-actin. (**C**) Gp78- or NS-shRNA-expressing HT-1080 cells were transiently transfected with GFP–Sec61β and HA–Ubwt and treated with or without MG132 for 8 hours. Overlap of HA–Ubwt labeling with the central ER marker GFP–Sec61β was quantified. (n=24, data are mean ± s.e.m., *P<0.05, **P<0.001; scale bar: 10 μm.)

A role for central ER translocation of polyubiquitylated substrate in proteasomal targeting is consistent with the previously reported interaction of gp78 not only with p97 (Ballar et al., 2007; Li et al., 2005; Zhong et al., 2004), but also with various components of the retrotranslocation machinery, including the Derlins, Sec61 and JAMP1, which recruit proteasomes to the ER (Li et al., 2005; Tcherpakov et al., 2009; Ye et al., 2005). Localization of CLIMP-63, a transmembrane protein involved in maintaining the architecture of ER sheets, is stabilized via interaction with ribosomes (Shibata et al., 2010). The basis for differential protein localization in the ER might be the result of complex domain-specific interactions. For instance, whereas CUE domain mutation enhances gp78 mobility in both the central and peripheral ER, expression of HA–Ubmono selectively enhances gp78 mobility in the peripheral but not the central ER. Therefore, only now are the mechanisms that regulate the mobility and distribution of gp78 beginning to be understood.

The inefficient removal of ubiquitylated substrate from the central ER in FLAG–CUE mutant transfected cells suggests that CUE-dependent retention and processing of substrate in the peripheral ER impacts on efficient proteasomal degradation of gp78 ERAD substrates. CUE domains exhibit preferential binding to monoubiquitin, and structural studies show that the contact surface of the CUE domain with monoubiquitin encompasses K48, potentially inhibiting polyubiquitin chain elongation (Kang et al., 2003; Shih et al., 2003). By contrast, polyubiquitin is a p97 recognition signal and p97 restricts polyubiquitin chain length to that required for efficient proteasomal targeting and degradation (Flierman et al., 2003; Richly et al., 2005; Ye et al., 2003). It is tempting to speculate that the differential specificity of CUE

domain for monoubiquitin and p97 for polyubiquitin underlie their opposing roles as regulators of the peripheral–central distribution of ubiquitylated substrates. By retaining substrates carrying mono- or short ubiquitin chains in the peripheral ER, CUE domain interactions might ensure that only proteins with extended polyubiquitin chains are delivered to the central ER for proteasomal degradation. Such a mechanism would enhance the efficiency of proteasomal degradation and explain the accumulation of ubiquitylated substrate in the central ER upon mutation of the gp78 CUE domain.

Gp78 knockdown in HT-1080 cells increased KAI1 levels and, although ubiquitylated substrate was still distributed to the peripheral ER, it no longer redistributed to the central ER upon proteasome inhibition. This supports the peripheral ER as the site of gp78-mediated ERAD and suggests that central ER accumulation of ubiquitylated substrate is related to gp78 expression levels and overall ERAD activity. gp78 overexpression upon transfection results in the clear separation of peripheral, smooth and central, rough ER. However, previous EM studies have shown that gp78-positive smooth ER structures are interspersed with, and connected to, ribosome-studded rough ER (Benlimame et al., 1998; Benlimame et al., 1995; Wang et al., 1997). Therefore, domain segregation of gp78 ubiquitylation and proteasomal targeting might occur on a more intimate scale within ER tubules and cisternae. Indeed, synthetic and degradatory machineries can function in parallel within the same ER structures, a process named ‘co-translational degradation’, allowing for tight regulation of protein synthesis (Fisher and Ginsberg, 2002; Oyadomari et al., 2006). By regulating delivery of ubiquitylated substrate to the central ER, spatial segregation of ERAD functions might limit access by unwanted ER proteins to common ER translocation machinery, thereby ensuring efficient production of essential, newly synthesized proteins. Sequestration of gp78 ubiquitin ligase activity to an expanded peripheral ER might occur selectively in response to enhanced ERAD activity. Finally, multiple E3 ligases contribute to ERAD (Vembar and Brodsky, 2008); however, how differential, and potentially competitive, interactions among ligases, substrates and ubiquitin supply impact on gp78 function in ERAD remains to be determined.

Materials and Methods

Antibodies

Anti-FLAG, anti-β-actin, anti-Derlin-1 and anti-Derlin-2 antibodies were purchased from Sigma-Aldrich, anti-p97 from Abcam and anti-HA from Neomarker. The 3F3A monoclonal antibody was as previously described (Nabi et al., 1990) and mouse monoclonal anti-gp78 antibody was purchased from Abcam. The anti-KAI1 antibody was purchased from Santa-Cruz Biotechnology. All fluorescent secondary antibodies were purchased from Invitrogen and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch.

Plasmids and constructs

HA–Ubwt and HA–Ubmono expression plasmids were obtained from Tony Morielli (University of Vermont) and Myc-Rtn4a and GFP–Sec61β from Gia Voeltz (University of Colorado). FLAG–gp78wt was inserted into pcDNA3.1 (+) as previously described (Registre et al., 2004). FLAG–CUEmut (M467F, F468S, P469S) and FLAG–RINGmut (C356S) were generated by point mutation using the Quickchange mutagenesis kit (Stratagene). FLAG–Δp97 was generated by deletion of the 49 amino acid C terminus and recloned in pcDNA 3.1 (+). For live cell visualization, a tetracysteine tag (4c) was added in frame (FLNCCPGCCMEP) by PCR extension to the N-terminal extremity and, in a second step, a FLAG tag was added to the N terminus of the 4c tag to prevent palmitoylation of the 4c tag (Martin et al., 2005).

Cell culture and transfection

Cos7 and HEK293t cells were maintained in DMEM containing 10% fetal bovine serum (FBS, Medicorp), and supplemented with 0.2 mM L-Glutamine, 10 μg/ml penicillin, 10 μg/ml streptomycin, vitamins and non-essential amino acids. HT-1080 cells were maintained in RPMI containing the same supplements (all supplements and media were purchased from Invitrogen). Cells were transfected using Effectene (Qiagen). Where indicated, cells were treated with MG132 (Sigma-Aldrich) for 8 hours at a concentration of 30 μM.

Fluorescence recovery after photobleaching (FRAP)

Cos7 cells were transfected 24 hours after plating in chamber slides (Ibidi) and stained with FlAsH dye (Invitrogen) according to the manufacturer’s instructions. Briefly, cells were rinsed twice with Opti-MEM media (Invitrogen) and incubated for 30 minutes with FlAsH diluted in Opti-MEM media (1:800) and then rinsed twice with Opti-MEM + 1X BAL buffer (0.25 mM 2,3-dimercapto-1-propanol, Invitrogen). FRAP measurements were conducted immediately after staining at 37℃ with a Fluoview 1000 laser-scanning confocal microscope (Olympus). After the first frame was taken, a small region in the central or peripheral ER was bleached with the 405-nm laser of a SIM scanner (Olympus).

Immunofluorescence labeling

Cells grown on glass cover slips were fixed 24-hour post-transfection with pre-cooled (−80℃) methanol:acetone (80:20) for 15 minutes at −20℃, a fixation procedure that liberates free cytosolic proteins, such as GFP and ubiquitin. The cells were then washed with PBS supplemented with 1 mM $CaCl_2$ and 10 mM $MgCl_2$ (PBS-CM). Cells were blocked with PBS-CM containing 1% BSA blocking solution for 15 minutes, incubated with primary antibodies for 30 minutes, rinsed three times with blocking solution, and then further incubated with the appropriate secondary antibody for an additional 30 minutes. The cells were rinsed three times for 10 minutes with blocking solution. Cover slips were mounted and imaged with the 100× Planapochromatic objective of a Fluoview 1000 confocal laser-scanning microscope (Olympus). Quantification of the percentage of ubiquitylated substrates localized to the central ER was obtained by measure the HA–Ubwt-labeling intensity co-localizing with a mask of GFP–Sec61β (threshold 5000–65,535) over the total ubiquitin intensity signal. All fluorescence quantification experiments, including Pearson’s coefficients, were determined from images obtained from at least three independent experiments.

shRNA silencing of gp78

Short-hairpin (sh)RNA–gp78 plasmids were purchased from Molecular Biosystems and transfected with a third-generation viral encapsidation in HEK239t following the manufacturer’s guideline. The viruses were harvested and used to infect HT-1080 cells. Infected cells were selected with 2 μg/ml puromycin and FACS sorted after 2 weeks in selection media. We used semi-quantitative RT-PCR to assess gp78 knockdown at the transcript level, as previously described (Joshi et al., 2010). Briefly, total RNA was extracted by the TRIzol method, total cDNAs generated using SuperScript III reverse transcriptase (Invitrogen) and transcript levels determined from total cDNAs by PCR using primer sets for gp78 (5′-cca tgc cgc tgc tct tcc tc-3′; 5′-gct gag gcc cgt gta ggt gcg-3′) and GAPDH (5′-ggt cgg agt caa cgg att tgg tcg-3′;5′-cct ccg acg cct gct tca cca c-3′) (25 cycles each).

Western blot and immunoprecipitation

For western blot analysis, cells were manually collected in cold PBS, pelleted and resuspended in lysis buffer [25 mM Tris-HCl pH 8.0, 20 mM N-ethylmaleimide, 2 mM EDTA, 0.5 mM PMSF, 140 mM NaCl, 1% TX-100 and protease inhibitor cocktail (Roche)]. Lysates were clarified at 15,000 rpm and supernatant and insoluble fractions were run on 8% SDS-PAGE gels and transferred onto PVDF membranes (Millipore) using a semi-dry system (0.8 mA/cm^2). Membranes were blocked overnight in 5% milk and then probed with the indicated antibodies. For anti-FLAG and anti-HA immunoprecipitation, cells were rinsed and harvested in PBS and cell pellets resuspended in lysis buffer [25 mM Tris pH 8.0, 20 mM N-ethylmaleimide, 2 mM EDTA, 1% Triton X-100, 0.5 mM PMSF and protease inhibitor tablet (Roche)], passed through a 25G syringe five times and incubated for 30 minutes on ice. Supernatants were incubated with anti-FLAG- or anti-HA-coupled agar beads (Sigma-Aldrich) pre-blocked with 1% BSA, then washed once with lysis buffer, three times with rinsing buffer A (Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% TX-100, 0.1% SDS and 0.2% BSA), three times with rinsing buffer B (500 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.5% TX-100 and 0.2% BSA) and once with 50 mM Tris-HCl pH 8.0. Proteins were eluted from beads by boiling in sample buffer before analysis by western blot.

Funding

This study was supported by a grant from the Canadian Institutes for Health Research (CIHR) [grant number MT-15132]. P.S.P. is the recipient of a CIHR Frederick Banting and Charles Best Canada Graduate Scholarship.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.096396/-/DC1

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