

p38 MAP kinase–dependent phosphorylation of the Gp78 E3 ubiquitin ligase controls ER–mitochondria association and mitochondria motility

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ABSTRACT Gp78 is an ERAD-associated E3 ubiquitin ligase that induces degradation of the mitofusin mitochondrial fusion proteins and mitochondrial fission. Gp78 is localized throughout the ER; however, the anti-Gp78 3F3A monoclonal antibody (mAb) recognizes Gp78 selectively in mitochondria-associated ER domains. Epitope mapping localized the epitope of 3F3A and a commercial anti-Gp78 mAb to an 8–amino acid motif (533–541) in mouse Gp78 isoform 2 that forms part of a highly conserved 41–amino acid region containing 14–3–3– and WW-binding domains and a p38 MAP kinase (p38 MAPK) consensus site on Ser-538 (S538). 3F3A binds selectively to nonphosphorylated S538 Gp78. Using 3F3A as a reporter, we induced Gp78 S538 phosphorylation by serum starvation and showed it to be mediated by p38 MAPK. Mass spectroscopy analysis of Gp78 phosphopeptides confirmed S538 as a major p38 MAPK phosphorylation site on Gp78. Gp78 S538 phosphorylation limited its ability to induce mitochondrial fission and degrade MFN1 and MFN2 but did not affect *in vitro* Gp78 ubiquitin E3 ligase activity. Phosphomimetic Gp78 S538D mutation prevented Gp78 promotion of ER–mitochondria interaction, and SB203580 inhibition of p38 MAPK increased ER–mitochondria association. p38 MAPK phosphorylation of Gp78 S538 therefore regulates Gp78-dependent ER–mitochondria association and mitochondria motility.

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INTRODUCTION

Gp78 is a key E3 ubiquitin ligase in the endoplasmic reticulum–associated degradation (ERAD) pathway (Christianson *et al.*, 2011) and targets misfolded and physiological substrates for ubiquitylation and subsequent degradation by the proteasome. Gp78 substrates include T-cell receptor subunit (CD3- δ , TCR α), ApoB lipopro-

tein, Insig-1, hydroxymethylglutaryl-CoA reductase, the Z variant of α 1-antitrypsin, mutant cystic fibrosis transmembrane conductance regulator (CFTR Δ 508), SOD1, ataxin 3, and the metastasis suppressor KAI1/CD82, linking Gp78 ERAD activity to lipid metabolism disorders, cystic fibrosis, neurodegenerative diseases, and cancer

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Abbreviations used: AMF, autocrine motility factor; ASK1, apoptosis signal-regulation kinase 1; ATF, activating transcription factor; CFTR, cystic fibrosis transmembrane conductance regulator; CHOP/GADD153, CCAAT/enhancer-binding protein (C/EBP)-homologous protein/growth arrest and DNA damage-inducible protein 153; CIAP, calf intestine alkaline phosphatase; CUE, coupling of ubiquitin conjugation to ERAD; DM, dimerization domain; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum–associated degradation; FRAP, fluorescence recovery after photobleaching; G2BR, Ube2g2-binding region; GFP, green fluorescent protein; Gp78, glycoprotein 78; GRP78, glucose-regulated protein 78; GST, glutathione S-transferase; IPTG, isopropyl- β -D-thiogalactoside; IRE1, inositol requiring 1; IRES, internal ribosome entry site; MAM, mitochondria-associated membrane; MAPK, mitogen-activated protein kinase;

MFN1, mitofusin 1; MFN2, mitofusin 2; MKK6, MAPK kinase 6; MKK6(CA), MAPK kinase 6 constitutively active; MKK6(DN), MAPK kinase 6 dominant negative; N.S., not significant; OxphosV, oxidative phosphorylation complex subunit V; PERK, PKR (RNA-dependent protein kinase)-like ER-kinase; PFA, paraformaldehyde; pOct-dsRed, mitochondrial dsRed-conjugated pre-ornithine carbamyl transferase; RING, really interesting new gene; RINGmut, Gp78 RING motif mutant; SER, smooth endoplasmic reticulum; STX17, syntaxin17; SUMO, small ubiquitin-like modifier; TRAF2, tumor necrosis factor receptor–associated factor 2; TRB3, tribbles-related protein 3; WT, wild type.

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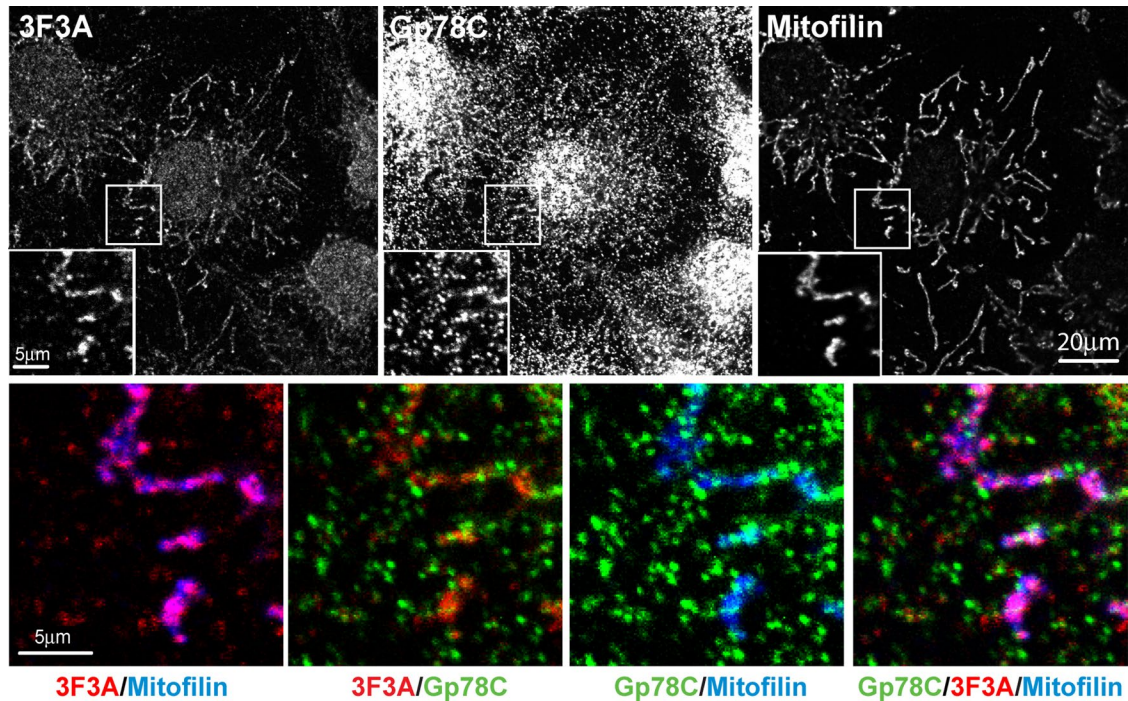


FIGURE 1: The 3F3A mAb selectively labels the mitochondria-associated ER. Cos7 cells were immunofluorescently labeled with the 3F3A and Gp78C anti-Gp78 antibodies, as well as with antibody to the mitofilin mitochondrial marker, followed by species-specific fluorescence-conjugated secondary antibodies. Individual confocal images in black and white and merged images of the boxed insets. Whereas Gp78C antibodies label Gp78 throughout the ER, the 3F3A only recognizes a subset of Gp78 localized to a mitochondria-associated ER domain.

(Fang *et al.*, 2001; Kostova *et al.*, 2007; Tsai *et al.*, 2007; Ying *et al.*, 2009). Increased expression of Gp78 and degradation of KAI1 is associated with preneoplastic hyperplasia in a transgenic breast cancer model (Joshi *et al.*, 2010) and sarcoma metastasis (Tsai *et al.*, 2007). More recently, we showed that Gp78 promoted degradation of the mitofusin mitochondrial fusion proteins, mitochondrial fission, and, upon mitochondrial depolarization, mitophagy (Fu *et al.*, 2013).

Gp78 was originally identified as a cancer-associated glycoprotein of 78 kDa and subsequently as the receptor for autocrine motility factor (AMF; Nabi and Raz, 1987, 1988; Nabi *et al.*, 1990; Silletti *et al.*, 1991). The 3F3A anti-Gp78 monoclonal antibody (3F3A mAb) was originally shown to stimulate tumor cell motility and metastasis (Nabi *et al.*, 1990). Immunolocalization studies with the 3F3A mAb localized Gp78 to both the cell surface and the smooth endoplasmic reticulum (SER; Benlimame *et al.*, 1995, 1998; Wang *et al.*, 1997). The Gp78-positive SER domain is closely associated with mitochondria, corresponding to a mitochondria-associated membrane (MAM) domain, and this interaction is regulated by cytosolic calcium (Wang *et al.*, 2000; Goetz *et al.*, 2007). However, immunolocalization with other anti-Gp78 antibodies or of epitope-tagged Gp78 shows that Gp78 is localized throughout the ER (Fang *et al.*, 2001). In FLAG-Gp78-transfected cells, the 3F3A mAb selectively recognizes FLAG-Gp78 in the peripheral ER, where Gp78-dependent ubiquitination is initiated, and not in the central ER (Registre *et al.*, 2004; St-Pierre *et al.*, 2012). Together with its selective labeling of endogenous Gp78 in MAM, this suggests that the 3F3A mAb recognizes a subpopulation of Gp78. Of importance, immunohistochemistry with the 3F3A mAb has been extensively used to define an association between Gp78 expression and tumor malignancy (Chiu *et al.*, 2008). However, the Gp78 motif recognized by the 3F3A mAb has yet to be identified.

Gp78 contains an N-terminal multitransmembrane domain and a C-terminal domain that contains a catalytic RING finger domain,

CUE motif, dimerization domain, and E2 ubiquitin conjugation enzyme (Ube2g2)- and p97-binding domains (Fang *et al.*, 2001; Chen *et al.*, 2006; Li *et al.*, 2009). Epitope mapping of the 3F3A antibody has now defined a novel p38-mitogen-activated protein kinase (MAPK) S538 phosphorylation site downstream of the CUE motif and upstream of the E2-binding domain of Gp78 that controls ER-mitochondria association and mitochondria motility.

RESULTS

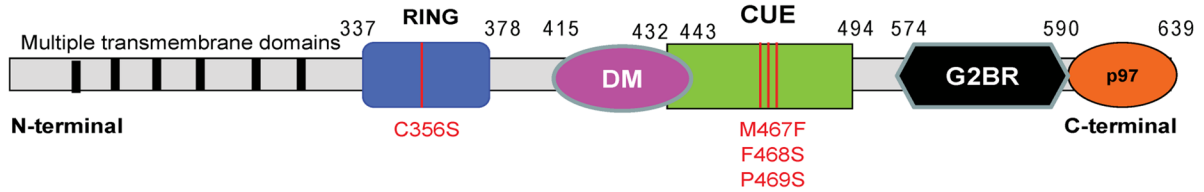
The 3F3A and anti-Gp78C mAbs label different populations of endogenous Gp78

Electron microscopy has localized 3F3A-labeled Gp78 to the smooth ER, including the mitochondria-associated ER, a distribution that is also evident by confocal microscopy (Benlimame *et al.*, 1995; Wang *et al.*, 1997, 2000; Benlimame *et al.*, 1998; Goetz *et al.*, 2007). However, other antibodies have localized Gp78 throughout the ER, a distribution that parallels that of transfected FLAG-tagged Gp78 (Fang *et al.*, 2001; Registre *et al.*, 2004). We therefore labeled Cos7 cells with both 3F3A anti-Gp78, as well as with a commercial monoclonal antibody raised against a Gp78 C-terminal peptide (referred to as anti-Gp78C). In Cos7 cells, 3F3A labeling was restricted to the mitochondria-associated ER, where it colocalized with anti-Gp78C labeling, which only partially overlaps with the mitochondria marker mitofilin (Figure 1). This differential labeling of endogenous Gp78 by 3F3A and Gp78C mAb prompted us to map the epitope of the two monoclonal antibodies.

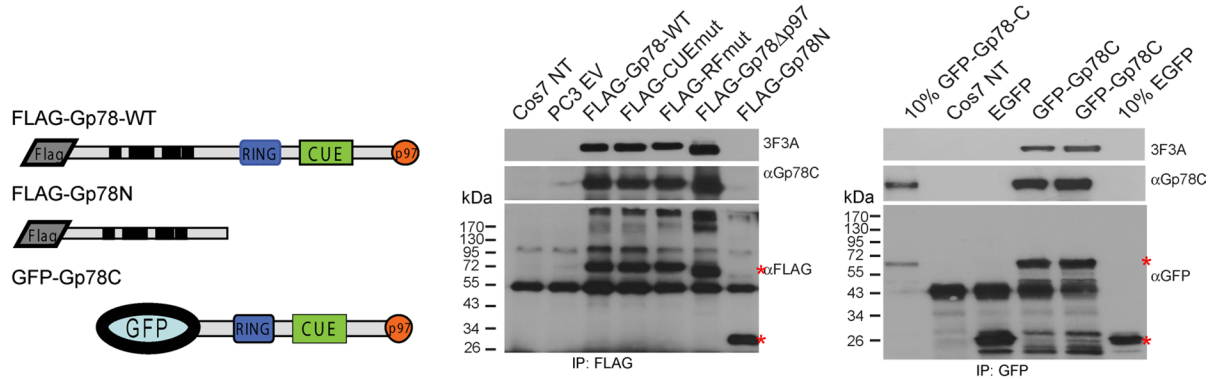
The 3F3A and anti-Gp78C epitopes map to a region between amino acids 533 and 541 of Gp78

Gp78 contains an N-terminal multitransmembrane domain and a C-terminal domain that contains a catalytic RING finger domain, CUE motif, dimerization domain, and E2 ubiquitin conjugation

A Gp78



B



C

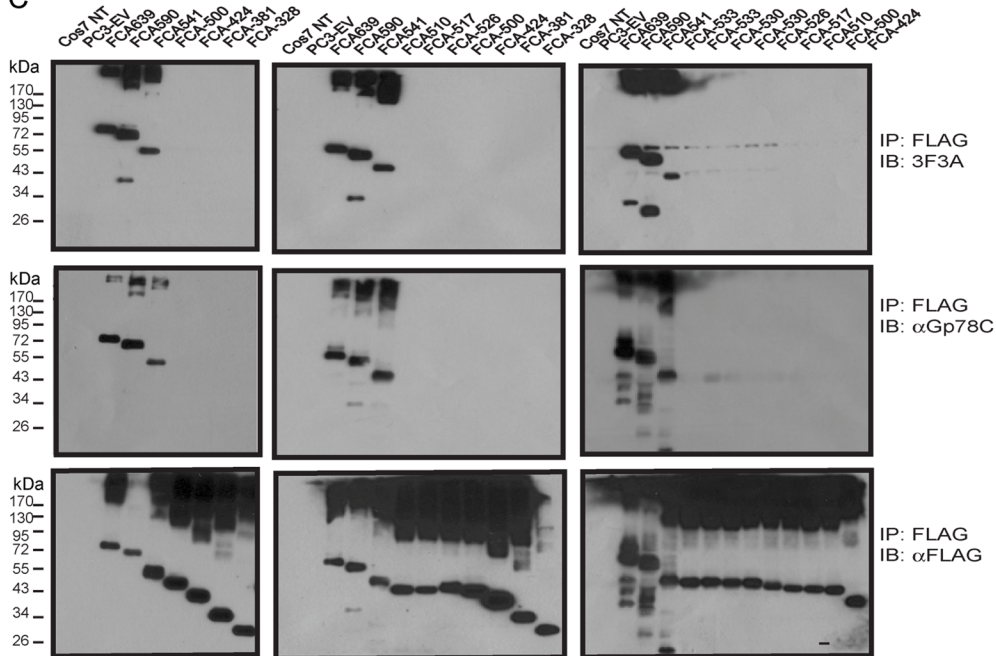


FIGURE 2: 3F3A and anti-Gp78C mAbs recognize the Gp78 C-terminal domain. (A) Schematic representation of the functional domain of Gp78. (B) Cos7 cells were untransfected (Cos7 NT) or transfected with empty vector (PC3 EV), FLAG-tagged Gp78 wild type (FLAG-Gp78-WT), CUE-domain mutant (FLAG-CUEmut), RING finger–domain mutant (FLAG-Gp78-RFmut), p97 binding–domain deletion mutant (FLAG-Gp78-Δp97), and C-terminal–deletion mutant (FLAG-Gp78N) and immunoprecipitated with FLAG beads and Western blotted with 3F3A, Gp78C, or FLAG mAbs. Alternatively, Cos7 cells were untransfected (Cos7 NT) or transfected with enhanced GFP empty vector (EGFP) or GFP-tagged Gp78 N-terminal deletion mutant (GFP-Gp78C) and immunoprecipitated with GFP Ab and probed with 3F3A mAb, Gp78C mAb, or GFP Ab. The 10% input of total cell lysates of Cos7 cells transfected with EGFP (10% EGFP) or GFP-Gp78C (10% GFP-Gp78C) are shown. (C) Cos7 cells were untransfected (Cos7 NT) or transfected with empty vector (PC3 EV), FLAG-tagged full-length Gp78 (FCA-639), or progressive C-terminal–deletion mutants of Gp78 (FCA-590, FCA-541, FCA-500, FCA-424, FCA-381, FCA-328, FCA-510, FCA-517, FCA-526, FCA-530, FCA-533) and immunoprecipitated with FLAG beads and Western blotted with 3F3A, Gp78C, and FLAG mAbs.

enzyme (Ube2g2)– and p97-binding domains (Figure 2A; Fang *et al.*, 2001; Chen *et al.*, 2006; Li *et al.*, 2009). To identify the epitope recognized by the 3F3A mAb, we first generated FLAG-tagged N-

terminal Gp78 comprising the multispanning membrane domains of Gp78 (FLAG-Gp78N) and green fluorescent protein (GFP)–tagged C-terminal Gp78 (GFP-Gp78C; Figure 2B). We then

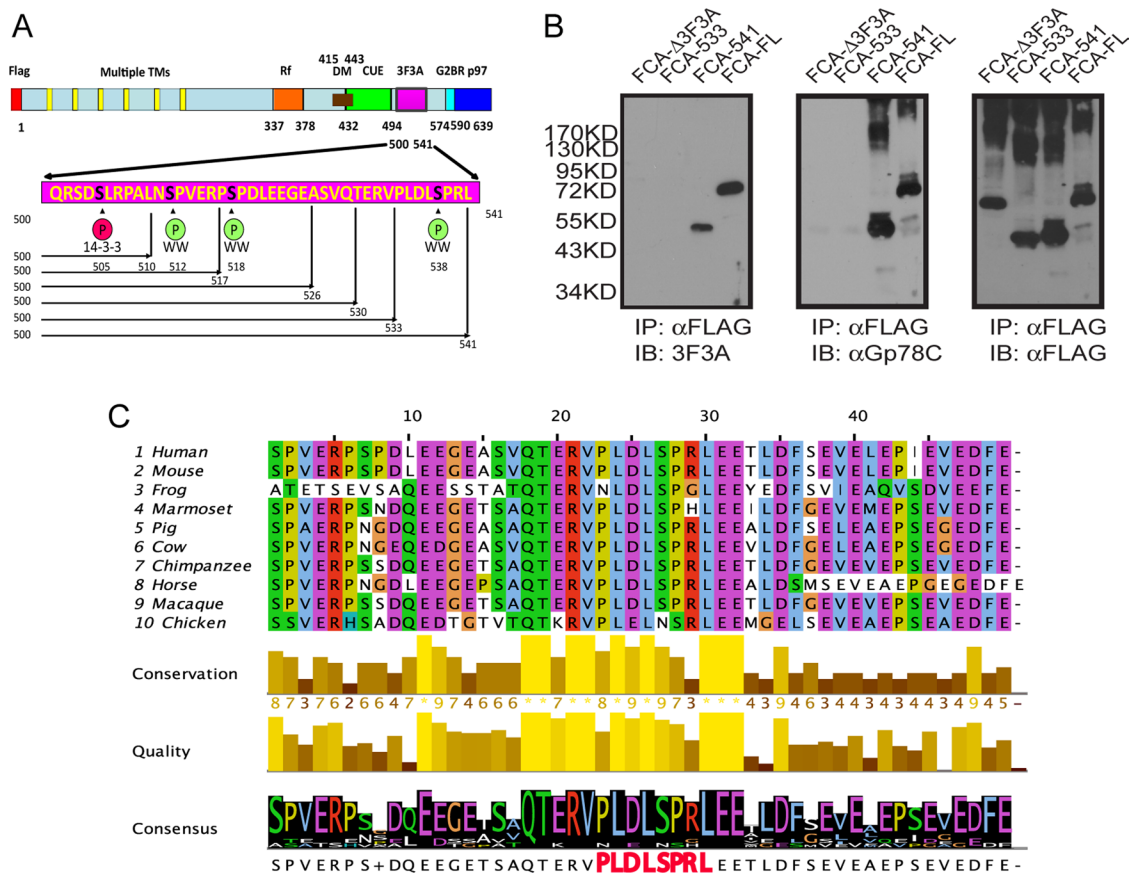


FIGURE 3: 3F3A and anti-Gp78C mAbs map to the same 8-amino acid epitope. (A) Schematic representation of the highly conserved 41-amino acid region containing 14-3-3- and WW-binding domains and putative phosphorylation sites. (B) Immunoprecipitation analysis showing that 3F3A mAb recognizes amino acids 533–541 of Gp78. Cos7 cells were transfected with FLAG-tagged full-length (FCA-639), C-terminal progressive deletion mutants of Gp78 (FCA-533, FCA-541), or amino acid 533–541 deletion mutant (FCA-Δ3F3A) and immunoprecipitated with FLAG beads and probed with 3F3A mAb or Gp78 C-terminal targeting mAb, Gp78C, or FLAG mAb. (C) Multialignment of different species of Gp78 showing the conservation of 3F3A binding site.

expressed full-length FLAG-Gp78 and FLAG-Gp78N, as well as RING finger (C356S; FLAG-RFmut), CUE domain (M467F, F468S, P469S; FLAG-CUEmut), and p97 deletion (FLAG-Gp78Δp97) mutants of Gp78 (St-Pierre *et al.*, 2012), in Cos7 cells. Both the 3F3A and anti-Gp78C mAbs recognized immunoprecipitated FLAG-Gp78, FLAG-RINGmut, FLAG-CUEmut, and FLAG-Δp97 but not FLAG-Gp78N (Figure 2B). After anti-GFP immunoprecipitation, both 3F3A and anti-Gp78C monoclonal antibodies recognized GFP-Gp78C but not free GFP (Figure 2B). The 3F3A epitope is therefore located in the C-terminal domain of Gp78.

We then generated progressive FLAG-tagged C-terminal deletion mutants of Gp78 (Figure 2C) and tested 3F3A and anti-Gp78C reactivity with the FLAG-immunoprecipitated proteins by immunoblot. C-terminal truncation of Gp78 to amino acid 541 but not 500 was recognized by both 3F3A and anti-Gp78C antibodies, mapping their epitopes to a region between amino acids 500 and 541 (Figure 2C), downstream of the CUE domain (amino acids 432–497) and upstream of the G2BR E2-binding domain (amino acids 574–590). This region contains four putative serine phosphorylation sites (S505, S512, S518, S538) identified by large-scale mass spectrometry serine phosphorylation analysis (Munton *et al.*, 2007; Dephoure *et al.*, 2008; Thingholm *et al.*, 2008; Trost *et al.*, 2009; Huttlin *et al.*, 2010) and predicted by PhosphoMotif finder (hprd.org/PhosphoMotif_finder) to be putative phosphorylation-regu-

lated 14-3-3- and WW-binding domains (Figure 3A). To further refine the antibody-binding motif, we generated additional deletion mutants between amino acids 510 and 541 based on those phosphorylation sites. This set of Gp78 C-terminal deletion mutants mapped the 3F3A- and anti-Gp78C mAb-binding region to a segment of eight amino acids between amino acids 533 and 541 (Figures 2C and 3B). An internal deletion mutant lacking residues 533–541 (Δ3F3A) abrogated binding by both the 3F3A and anti-Gp78C mAbs, localizing the epitope for these two Gp78 antibodies to the same eight-amino acid motif (Figure 3B). This suggests that the eight-amino acid segment recognized by both the 3F3A rat and anti-Gp78C mouse monoclonal antibodies represents an immunodominant motif in the Gp78 protein necessary for 3F3A and anti-Gp78C binding.

3F3A mAb binding to Gp78 is prevented by S538 phosphorylation

The binding region of both 3F3A and anti-Gp78C mAbs is a hydrophobic eight-amino acid motif, PLDLSRPL, containing one acidic (D), one basic (R), one hydrophilic (S), and five hydrophobic amino acids (P, L) and is predicted to form a coiled-coil structure and contains a putative serine phosphorylation-regulated WW-binding motif. The multialignment of Gp78 amino acid sequences from 10 different species showed that this motif is highly conserved and

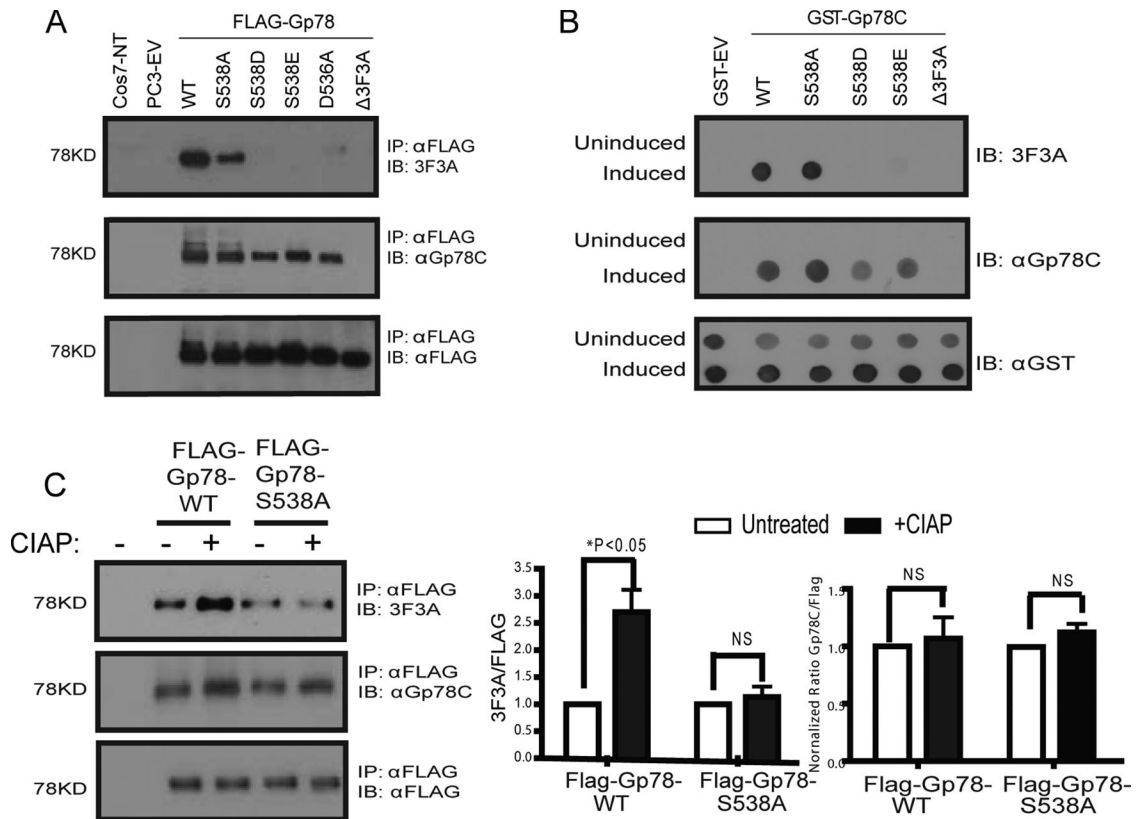


FIGURE 4: 3F3A mAb, but not anti-Gp78C, selectively recognizes dephosphorylated Gp78. (A) Cos7 cells were untransfected (Cos7 NT) or transfected with empty vector (PC3 EV), FLAG-tagged wild type (WT), S538A, phosphomimetic S538D or S538E, D536A mutant, or amino acid 533–541 deletion mutant (Δ 3F3A) of Gp78. Cell lysates were immunoprecipitated with FLAG beads and probed with 3F3A, Gp78C, or FLAG mAbs. (B) Bacteria transformed with GST-tagged empty vector (GST-EV) or Gp78 C-terminal (GST-Gp78C) wild type (WT), S538A, S538D, S538E mutants, or amino acid 533–541 deletion mutant (Δ 3F3A) were uninduced or induced by IPTG. Total bacterial lysates were dot blotted onto membranes and probed with 3F3A, Gp78C, or GST mAbs. (C) FLAG-tagged wild type of Gp78 (FLAG-Gp78-WT) or phosphorylation dominant-negative mutant (FLAG-Gp78 S538A) from Cos7 cells were immunoprecipitated and treated or not with CIAP and probed with 3F3A mAb, Gp78C, or FLAG mAb (\pm SEM; $n = 3$).

contains a putative phosphorylation site at Ser-538 of Gp78 (Figure 3C). The antigenic epitope prediction program (immunax.dfc.harvard.edu/Tools/) identified Ser-538 and Asp-536 as amino acids critical for antigenicity within this motif. On the basis of this prediction, we generated nonphosphorylatable S538A and phosphomimetic S538D and S538E mutants, as well as a D536A mutant. The anti-Gp78C mAb recognizes S538A, S538D, S538E, and D536A mutants; however, 3F3A recognizes only S538A and not D536A, S538D, or S538E (Figure 4A). This suggests that 3F3A binding, but not that of anti-Gp78C, is selectively prevented by phosphorylation of Ser-538 (S538). To validate further the binding difference between the two antibodies, we glutathione S-transferase (GST) tagged the Gp78 C-terminal region (GST-Gp78C) containing the 3F3A deletion (GST-Gp78C Δ 3F3A) and Ser-538 phosphorylation mutants (GST-Gp78C-S538A, Gp78C-S538D, and S538E). Dot blot analysis showed that both antibodies recognized GST-Gp78C and GST-Gp78C-S538A but not free GST or Gp78C- Δ 3F3A. Recognition of phosphomimetic Gp78C-S538D and S538E mutants was selectively lost for 3F3A and retained for anti-Gp78C mAb (Figure 4B).

These results suggest that binding of the 3F3A mAb, but not anti-Gp78C mAb, is selectively prevented by Gp78 phosphorylation at Ser-538. To confirm these findings, we used *in vitro* alkaline phosphatase treatment of FLAG-Gp78 immunoprecipitates. Treatment

with calf intestine alkaline phosphatase (CIAP) resulted in a 2.5-fold increase in the 3F3A/FLAG ratio of wild-type FLAG-Gp78 compared with untreated samples and did not significantly change the extent of 3F3A recognition of the FLAG-Gp78S538A mutant (Figure 4C). This confirms that the 3F3A mAb selectively recognizes Gp78 when S538 is not phosphorylated.

Serum deprivation stimulates S538 phosphorylation of Gp78 via p38 MAPK signaling

As seen in Figure 5A, serum starvation of Cos7 cells dramatically reduced 3F3A recognition of transfected FLAG-Gp78, indicative of increased S538 phosphorylation. Treatment of the cells with NaF, a broad serine phosphatase inhibitor, suppressed the elevated 3F3A/FLAG ratio in the presence of serum, confirming the sensitivity of 3F3A binding to Gp78 phosphorylation. Serum starvation activates the cellular stress response and p38 MAPK signaling, and treatment of cells with the p38 MAPK inhibitor SB203580 restored the elevated 3F3A/FLAG ratio in the absence of serum. Cotransfection of FLAG-Gp78 with constitutively active MKK6 (MKK6(CA)), a p38 MAPK signaling activator, decreased the 3F3A/FLAG ratio, which was restored by SB203580 treatment (Figure 5B). These results suggest that phosphorylation of Gp78 at Ser-538 occurs via p38 MAPK signaling.

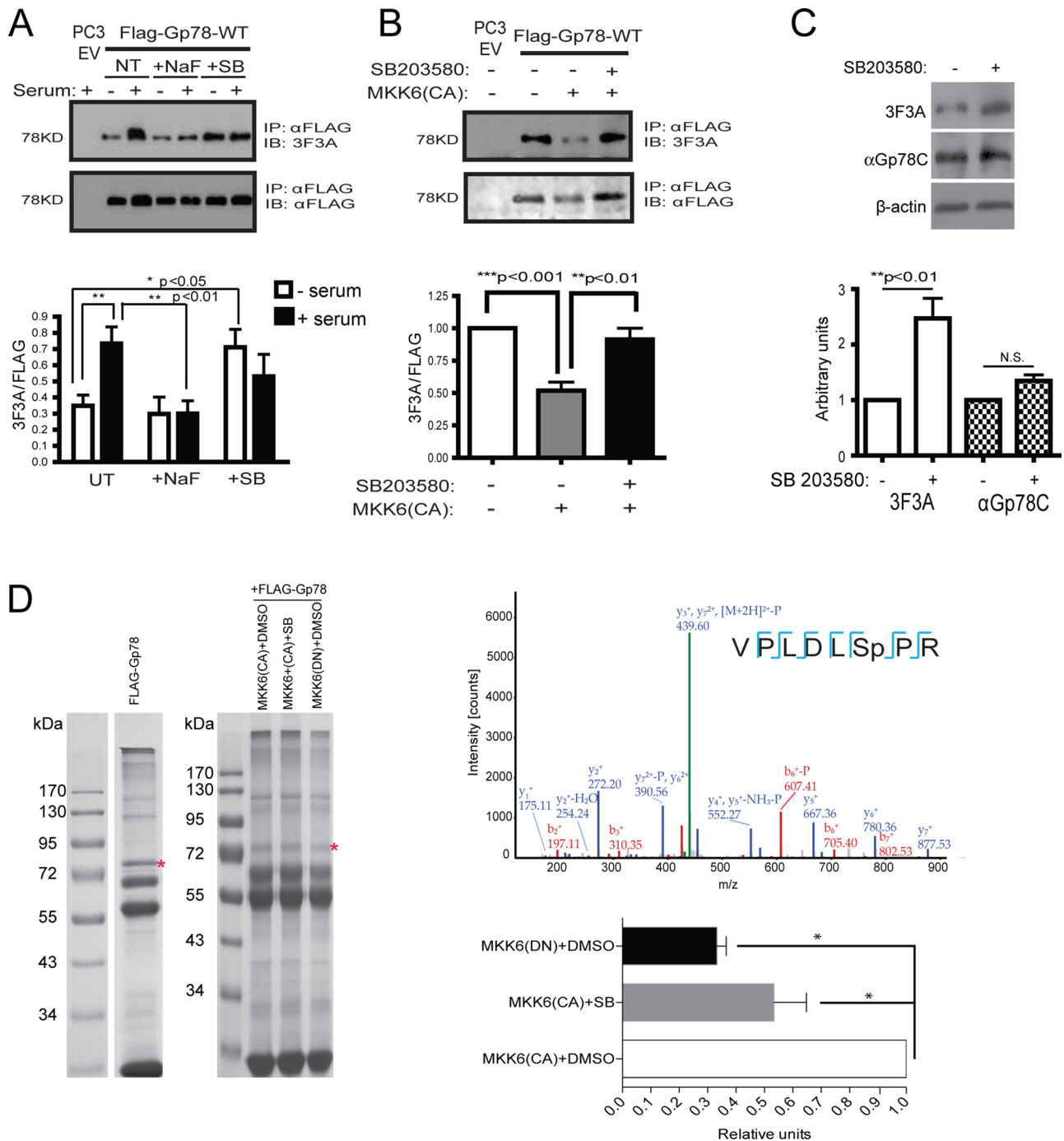


FIGURE 5: Serum deprivation stimulates p38 MAPK-dependent Gp78 S538 phosphorylation. (A) Cos7 cells were transfected with FLAG-tagged wild-type Gp78 for 14.5 h, followed by nontreatment (NT) or treatment with 5 mM NaF or 30 μ M SB203580 (SB) for 4 h in the presence or absence of serum. FLAG-Gp78 was immunoprecipitated with FLAG beads and probed with 3F3A or FLAG mAb (\pm SEM; $n = 4$). (B) Cos7 cells were transfected with FLAG-tagged wild-type Gp78 alone or cotransfected with constitutively active MKK6(CA) for 14.5 h, followed by treatment with 30 μ M SB203580 (SB) for 4 h in the presence of serum and then processed as in A (\pm SEM; $n = 3$). (C) To test whether p38 MAPK affects 3F3A binding of endogenous Gp78, HT-1080 cell were treated with or without 30 μ M SB203580 for 4 h and then Western blotted for 3F3A, α Gp78C, and β -actin (\pm SEM; $n = 3$). (D) Cos7 cells were transfected with FLAG-Gp78 or cotransfected with FLAG-Gp78 and MKK6(CA) or MKK6(DN) and treated with 30 μ M SB203580 (SB) or DMSO for 4 h before cell lysis. FLAG-Gp78 was immunoprecipitated, run on SDS-PAGE, and stained by blue-silver. Asterisk indicates FLAG-Gp78 bands. In-gel digestion was applied to the incised bands, and the samples were processed by mass spectrometry. Phosphorylation of S538 in VPLDLSpPR was identified and the abundance of this peptide quantified. For quantification, the extracted ion chromatograms of precursors of labeled VPLDLSpPR were taken and ratios were calculated (\pm SEM; $n = 3$).

To test whether endogenous Gp78 is also phosphorylated by p38 MAPK on S538, we treated HT-1080 cells, which express elevated level of endogenous Gp78 (St-Pierre *et al.*, 2012), with the p38 MAPK inhibitor SB203580 and Western blotted lysates for 3F3A and anti-Gp78C. As seen in Figure 5C, SB203580 inhibition of p38 MAPK increased recognition of endogenous Gp78 by 3F3A but not by anti-Gp78C. From large-scale immunoprecipitation of FLAG-Gp78 from transfected Cos7 cells, we identified a 78-kDa band by Coomassie staining (Figure 5D). We then performed mass spectrometry phosphopeptide analysis of immunoprecipitated FLAG-Gp78 from Cos7 cells cotransfected with constitutively active MKK6 (MKK6(CA)), which activates p38 MAPK, relative to cells cotransfected with MKK6(CA) and treated with SB203580 or cells cotransfected with dominant-negative MKK6 (MKK6(DN)). The major mGp78 phosphopeptide detected was S538, which showed increased abundance in MKK6(CA)-cotransfected cells (Figure 5D).

Gp78 S538A limits mitochondrial motility and promotes mitochondrial fission, mitofusin degradation, and ER-mitochondria association

Gp78 expression induces mitochondrial fission (Fu *et al.*, 2013), and to test whether Gp78 S538 phosphorylation affected mitochondrial shape and motility, we cotransfected Cos7 cells with wild-type FLAG-Gp78 or S538 mutants. As seen in Figure 6A, transfection of Gp78 WT and S538A, but not S538D, induces mitochondrial clustering around the nucleus; further, anti-FLAG labeling indicates that transfected FLAG-Gp78 and Gp78 S538A show increased colocalization with mitochondria relative to Gp78 S538D. Cos7 cells were then cotransfected with FLAG-Gp78-IRES-GFP and mitochondrial dsRed-conjugated pre-ornithine carbamyl transferase (pOct-dsRed), and the recovery of mitochondrial fluorescence after photobleaching was measured (Figure 6B). Wild-type and S538A Gp78 both dramatically restricted mitochondrial motility, whereas mitochondrial motility of Gp78 S538D-transfected cells did not differ from that of the control (Figure 6, C and D). We then tested whether Gp78 S538 phosphorylation affects Gp78-dependent proteasomal degradation of the mitochondrial fusion proteins mitofusin 1 (MFN1) and MFN2 (Fu *et al.*, 2013; Shankar *et al.*, 2013). Expression of Gp78 S538A reduced MFN1 and MFN2 levels, whereas the S538D mutant did not affect mitofusin expression (Figure 7A). Furthermore, serum starvation reduced Gp78-dependent loss of the mitofusins, an effect that was reversed by SB203580 inhibition of p38 MAPK (Figure 7B). Serum starvation and/or p38 MAPK inhibition did not affect mitofusin levels in untransfected or Gp78 S538A-transfected cells. Gp78-dependent degradation of the mitofusins (Fu *et al.*, 2013) is therefore inhibited by Gp78 S538 phosphorylation (Figure 7B).

However, S538 mutation did not affect the ability of Gp78 to interact with myc-tagged MFN1 or MFN2 after coimmunoprecipitation of cotransfected Cos7 cells (Figure 7C). We then tested the *in vitro* ubiquitin ligase activity of recombinant N-terminally histidine (His)-tagged human Gp78C (hGp78 amino acids 309–643) purified from bacterial lysates containing S542A and S542D mutations, human S542 corresponding to mouse isoform 2 S538. As for S538 mutation of mouse Gp78, 3F3A did not recognize the human Gp78 S542D mutant (Figure 7D). Gp78 *in vitro* E3 ubiquitin ligase activity assay was initially assayed by dose response of His-hGp78C; enzymatic activity plateaued at 100 nM His-hGp78C (Figure 6E). Using a concentration of 100 nM for His-hGp78C wild type, S542A, and S542D as well as His-Gp78C RF mutant as a negative control, we found that Gp78C S542A or Gp78C S542D mutations did not inhibit Gp78 *in vitro* ubiquitin ligase activity.

3F3A labeling localizes Gp78 to the mitochondria-associated ER or MAM (Wang *et al.*, 2000; Goetz *et al.*, 2007), and we therefore tested whether S538 phosphorylation affected ER proximity to mitochondria. Cos7 cells were cotransfected with pOct-dsRed and FLAG-Gp78, Gp78 S538 mutants, or Gp78 RFmut and then labeled for the MAM marker syntaxin17 (Hamasaki *et al.*, 2013) and, to identify transfected cells, anti-FLAG. Three-dimensional confocal stacks were acquired, and overlap of the syntaxin17-labeled MAM and total mitochondria was quantified. Relative to control pcDNA3-transfected cells, both wild-type Gp78- and Gp78 S538A-transfected cells increased the extent of ER-mitochondria association and reduced mitochondrial size, the latter consistent with their ability to degrade the mitofusins. Neither Gp78 S538D nor Gp78 RFmut affected ER-mitochondria association or mitochondrial size (Figure 8, A and B). Expression levels of the transfected FLAG-tagged Gp78 constructs were equivalent and did not affect syntaxin17 levels (Figure 8C). To test whether p38 MAPK activity affected the extent of ER-mitochondria contacts, we treated Cos7 and HT-1080 cells with SB203580 and quantified overlap of the syntaxin17-labeled MAM and mitochondria. SB203580 inhibition of p38 MAPK increased ER-mitochondria association (Figure 8D). The 3F3A binding site is therefore identified as a p38 MAPK serine phosphorylation site that is a critical regulator of Gp78-dependent ER-mitochondria association and thereby mitofusin degradation and mitochondrial motility.

DISCUSSION

The ER is characterized as a continuous membrane system, executing diverse cellular functions, including calcium homeostasis, lipid and protein synthesis, and metabolism (Lynes and Simmen, 2011). MAM is a distinct ER subdomain closely associated with mitochondria, where the close contact site between ER and mitochondria is ~10–25 nm (Perkins *et al.*, 1997; Csordas *et al.*, 2006; Hayashi-Nishino *et al.*, 2009). The physical linkage of the ER and mitochondria interface ensures fast, non-vesicular-mediated lipid transfer and calcium exchange between the two organelles and hence functions as a major cellular signaling hub that controls cellular metabolism and cell death (de Brito and Scorrano, 2010; Grimm, 2012; Rowland and Voeltz, 2012).

By electron microscopy, the 3F3A mAb labels endogenous Gp78 localized in smooth ER tubules (Benlimame *et al.*, 1995, 1998; Wang *et al.*, 1997). Gp78-positive smooth ER tubules are closely associated with mitochondria, and this interaction is regulated by cytosolic calcium (Wang *et al.*, 2000; Goetz *et al.*, 2007). Gp78 has recently been shown to promote ER-mitochondria association and calcium coupling, effects that are prevented by its extracellular ligand, AMF (Wang *et al.*, 2015). The close association of 3F3A-labeled Gp78 to mitochondria-associated ER tubules contrasts with the distribution of Gp78 throughout the ER (Figure 1; Fang *et al.*, 2001). Similarly, upon overexpression of FLAG-Gp78, the 3F3A mAb selectively labels the reticulon-positive peripheral ER and is excluded from the central ER (St-Pierre *et al.*, 2012). This suggests that 3F3A recognizes a conformation-specific form of Gp78. Indeed, we show here that 3F3A mAb binding to Gp78 is prevented by phosphorylation of S538. Of importance, the anti-Gp78C antibody, which maps to the same epitope as the 3F3A mAb and recognizes both phosphorylated and nonphosphorylated Gp78C S538, labels Gp78 throughout the ER. This suggests that dephosphorylation of Gp78 S538 is a critical determinant of the MAM localization of Gp78.

Functional validation of the MAM localization of Gp78 was obtained through studies with S538 mutants of Gp78. Gp78 ubiquitin ligase activity targets the mitofusins for proteasome degradation

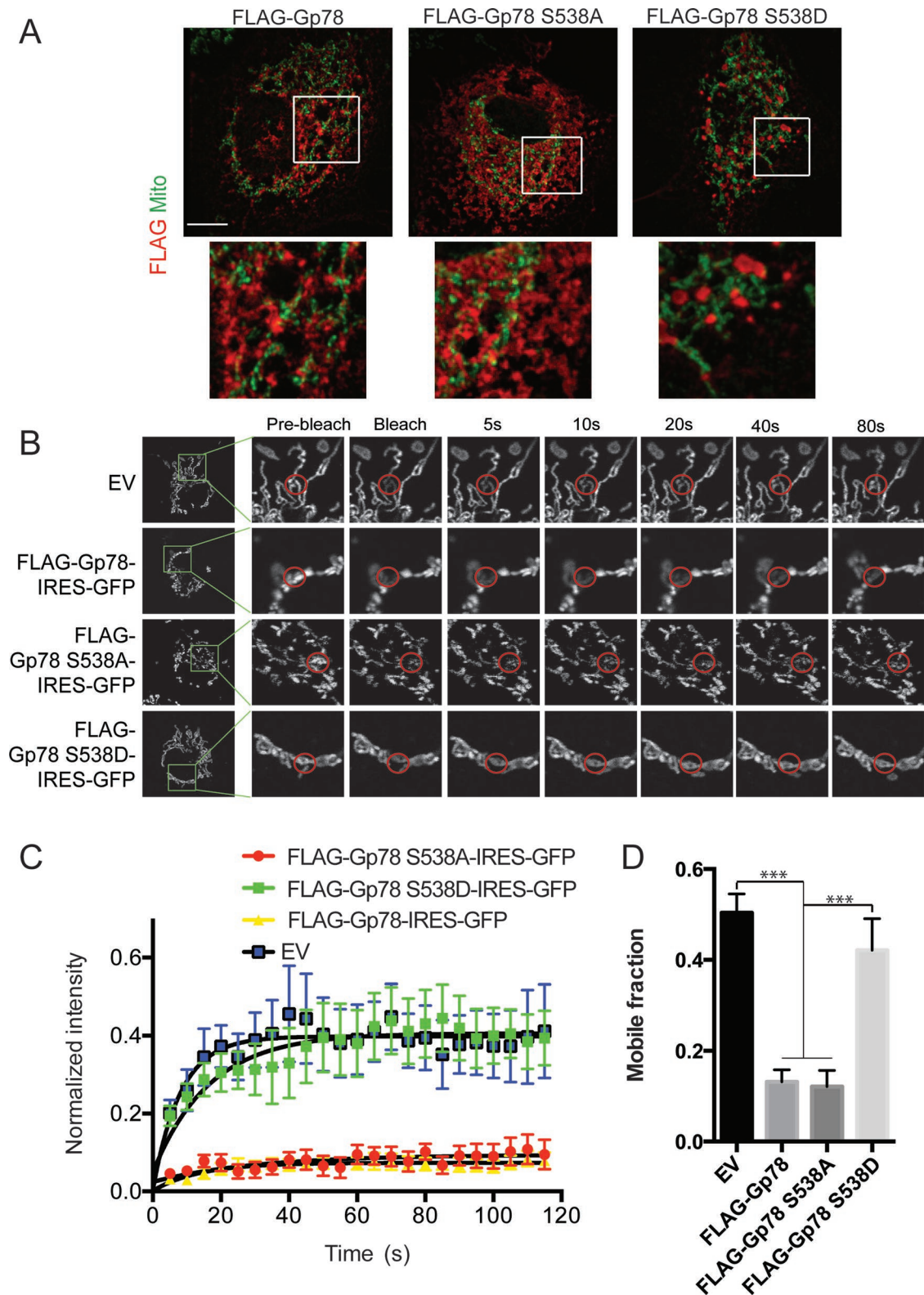


FIGURE 6: S538 phosphorylation regulates Gp78-dependent mitochondrial motility. (A) Cos7 cells were transfected with FLAG-Gp78, FLAG-Gp78 S538A, or FLAG-Gp78 S538D and fluorescently labeled with anti-FLAG and anti-mitofilin antibodies (bar, 10 μ M). (B) FRAP analysis of Cos7 cells cotransfected with mitochondrial-targeted pOct-dsRed and empty vector (EV), FLAG-Gp78, FLAG-Gp78 S538A, or FLAG-Gp78 S538D IRES-GFP plasmids and transfected cells identified by their GFP fluorescence. pOct-dsRed mitochondria were photobleached (bleach region shown with a red circle) and recovery followed over 2 min. Images are shown prebleach, immediately after bleach, and 5, 10, 20, 40, and 80 s after bleach. (C) Recovery of pOct-dsRed fluorescence in the bleached region of cells transfected as in A (percentage recovery \pm SEM; 8–10 cells/experiment). (D) Graph of the mobile fraction of pOct-dsRed for cells transfected as in A (\pm SEM; *** p < 0.001; n = 3).

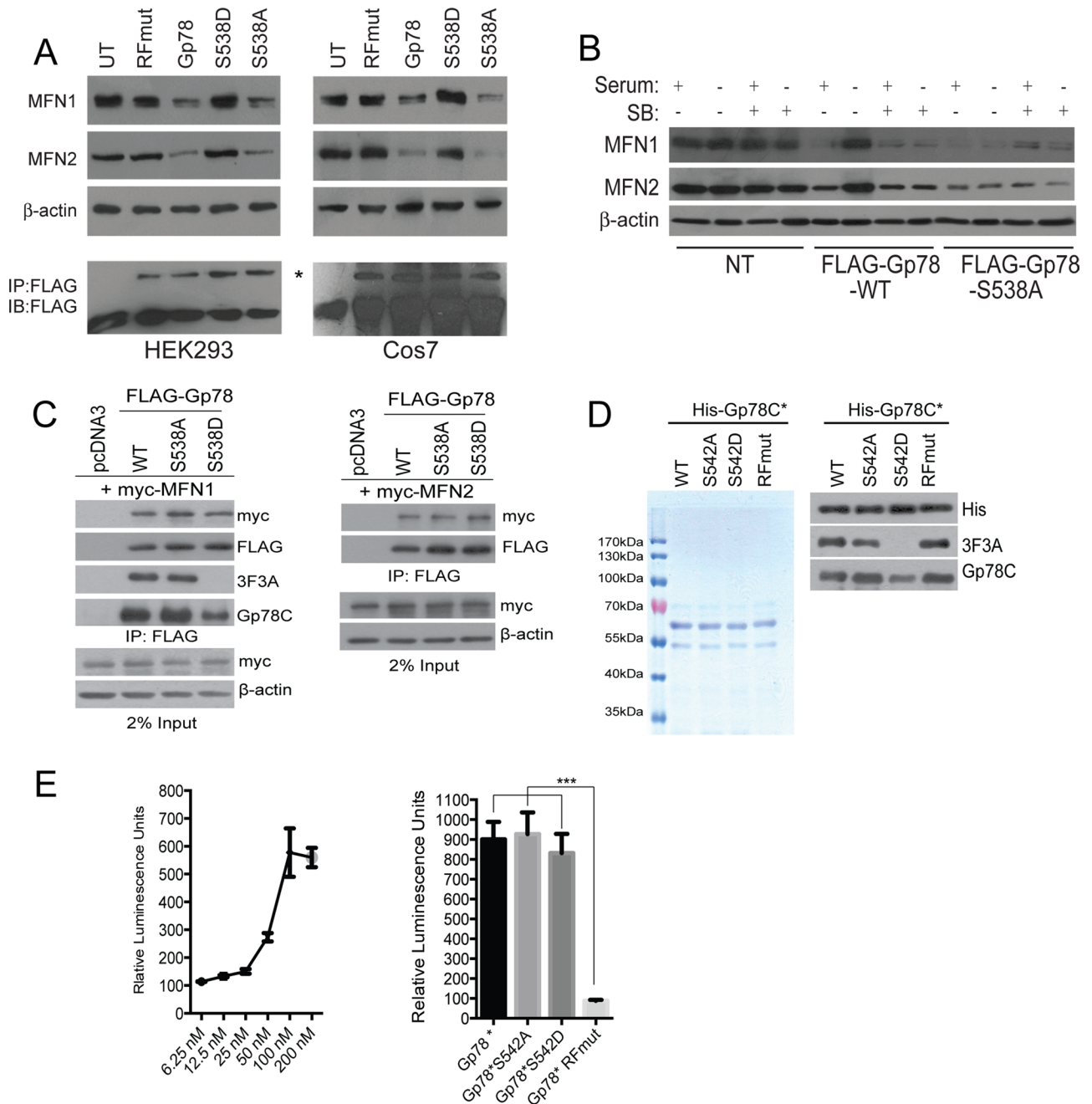


FIGURE 7: S538 phosphorylation prevents the Gp78-dependent decrease in mitofusin levels. (A) HEK-293 and Cos7 cells were transfected with empty vector (EV), FLAG-Gp78, FLAG-Gp78 RFmut, FLAG-Gp78 S538A, and FLAG-Gp78 S538D. Cell lysates were analyzed by Western blot for MFN1, MFN2, and β -actin expression or immunoprecipitated with anti-FLAG beads and blotted with anti-FLAG mAb. (B) Lysates of Cos7 cells untransfected (NT) or transfected with FLAG-Gp78 or FLAG-Gp78 S538A for 14.5 h and either untreated or treated with 30 μ M SB203580 (SB) for 4 h in the presence or absence of serum were analyzed by Western blot for MFN1, MFN2, and β -actin. (C) Cos7 cells were cotransfected with myc-MFN1 (left) or myc-MFN2 (right) and empty vector (pcDNA3), FLAG-Gp78-WT, S538A, or S538D. Cell lysates were immunoprecipitated with anti-FLAG antibodies and FLAG immunoprecipitates and input lysates probed with myc, FLAG, 3F3A, and Gp78C antibodies, as indicated. (D) His-Gp78C WT, S542A, S542D, and RFmut were purified from bacterial lysates by Ni chromatography. Coomassie staining and Western blots probed with His, 3F3A, and Gp78C antibodies. (E) Dose dependence of the *in vitro* ubiquitinase activity of His-Gp78 (left) and His-Gp78C WT, S542A, S542D, and RFmut (right) at 100 nM (\pm SEM; *** p < 0.001; n = 3).

and induces mitochondrial fission (Fu *et al.*, 2013). Phosphomimetic S538D prevented Gp78-dependent mitochondrial fission and the decrease in mitofusin levels observed in the presence of wild-type and S538A Gp78 (Figures 6 and 7). In view of our previous work (Fu

et al., 2013), we conclude that phosphorylation of Gp78 prevents mitofusin degradation. Intriguingly, Gp78 was found to enhance association of the MAM marker syntaxin17 with mitochondria, and this, as for mitofusin degradation and mitochondrial fission, was

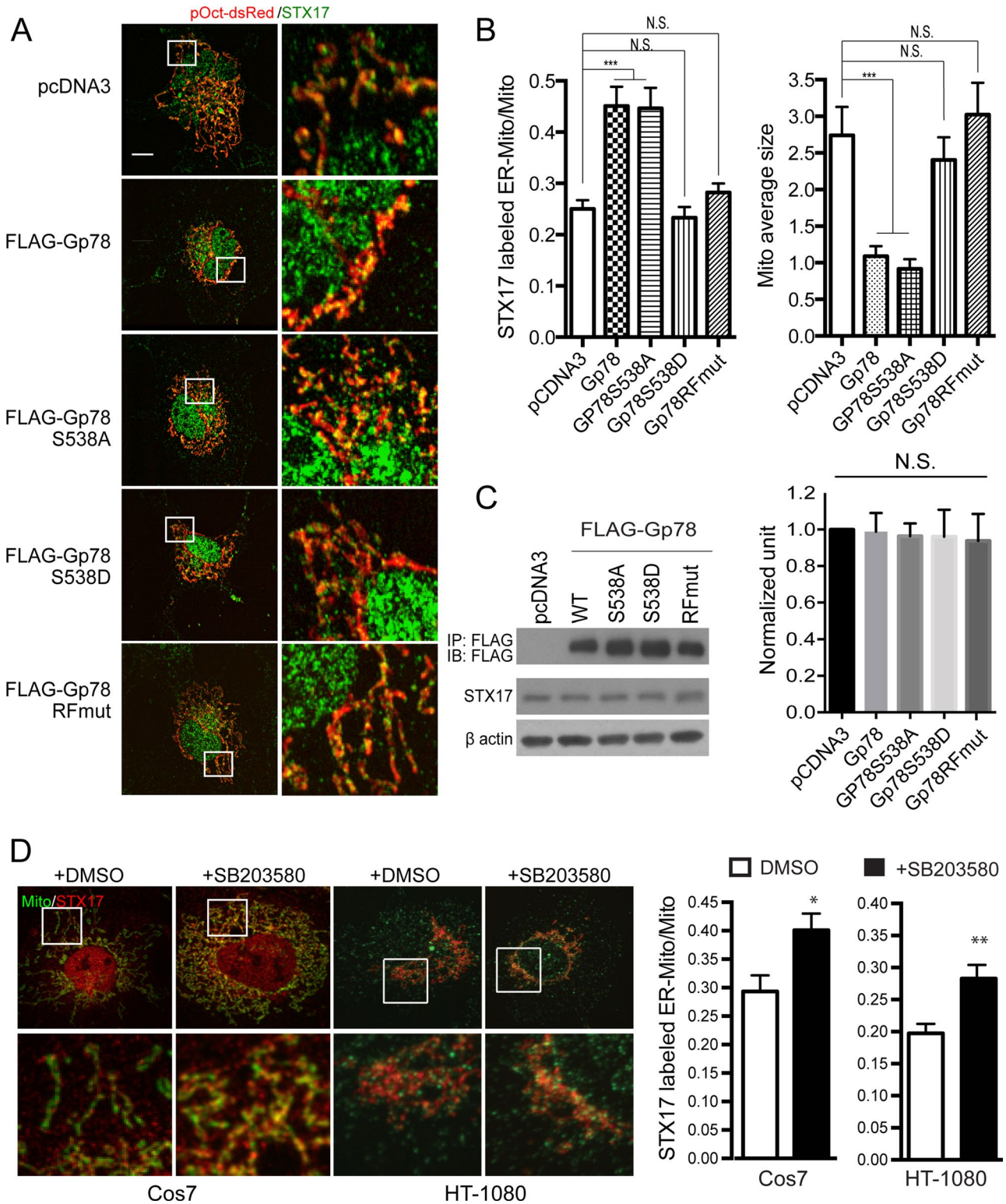


FIGURE 8: S538 phosphorylation regulates Gp78-dependent ER-mitochondria association. (A) 3D reconstructed images of Cos7 cells cotransfected with empty vector (pcDNA3), FLAG-Gp78, FLAG-Gp78 S538A, FLAG-Gp78 S538D, or FLAG-Gp78 RFmut and pOct-dsRed (red) and labeled for syntaxin17 (green; bar, 10 μ m). (B) Bar graphs show percentage of mitochondrial volume overlapped by syntaxin17 (left) and average mitochondrial volume (right) for the cells indicated in A (mean \pm SEM; $n = 3$; *** $p < 0.001$). (C) FLAG immunoprecipitation and Western blot of cell lysates for syntaxin17 and β -actin for the cells indicated in A. Bar graph shows densitometry of syntaxin17 relative to β -actin (\pm SEM; $n = 3$; N.S., not significant). (D) 3D reconstructed immunofluorescence images of Cos7 (left) and HT-1080 (right) cells untreated or treated with 30 μ M SB203580 for 4 h and labeled with syntaxin17 (green) and OxphosV (mito; red) antibodies. Bar graph shows percentage of mitochondrial volume overlapped by syntaxin17 (mean \pm SEM; $n = 3$; * $p < 0.05$, ** $p < 0.01$).

prevented by phosphomimetic S538D Gp78. The Gp78 S538A mutant retained the ability to degrade the mitofusins, induce mitochondrial fission, and promote ER–mitochondria association. Together with the MAM localization of the 3F3A mAb, this suggests that nonphosphorylated Gp78 S538 is localized to MAM and that 3F3A is a MAM marker.

Gp78 S538 phosphorylation was found to be p38 MAPK mediated. It was promoted by MKK6 activation of p38 MAPK and prevented by SB203580 inhibition of p38 MAPK. Reduced 3F3A recognition of Gp78 upon serum starvation was reversed by SB203580, linking Gp78 S538 phosphorylation to stress-activated p38 MAPK activation. Of importance, SB203580 inhibition of p38 MAPK increased association between the syntaxin17-labeled ER and mitochondria. This identifies p38 MAPK as a novel regulator of ER–mitochondria interaction.

p38 MAPK is involved in ER stress–induced apoptosis through IRE1-TRAF2-ASK1-MKK6 signaling (Wang and Ron, 1996; Maytin et al., 2001; Oyadomari and Mori, 2004; De Chiara et al., 2006). On the other hand, p38 MAPK is activated downstream of the ER stress response element PERK (Liang et al., 2006; Zhang et al., 2010) and mediates the protective response against ER stress through a number of mechanisms, including activation of the ER stress sensor ATF6 α , up-regulation of Bip/GRP78, activation of Akt, and down-regulation of TRB3 expression, probably by phosphorylation and decreased transactivation activity of proapoptotic transcription factor CHOP/GADD153 (Ranganathan et al., 2006; Seimon et al., 2009; Zou et al., 2009; Egawa et al., 2010). Perturbing ER–mitochondria contacts by depleting tethering complexes leads to delayed apoptosis progression due to decreased transfer of calcium from the ER to mitochondria (Pinton et al., 2008; Akl and Bultynck, 2012; Grimm, 2012). Whether Gp78 S538 phosphorylation represents an additional p38 MAPK target protective against ER stress–induced cell death remains to be determined.

MATERIALS AND METHODS

Antibodies and chemicals

The rat immunoglobulin M (IgM) 3F3A mAb against Gp78 was as described (Nabi et al., 1990). Antibodies to FLAG tag, syntaxin17, and β -actin were from Sigma-Aldrich (St. Louis, MO), to Gp78C and mitofusin 1 from Abcam (Cambridge, MA), to His tag and GST from Cell Signaling (Littleton, CO), to GFP from Synaptic Systems, to Myc tag from EMD Millipore (Darmstadt, Germany), to mitofusin 2 from Santa Cruz Biotechnology (Santa Cruz, CA), to mitofilin from Novus (Littleton, CO), and to OxPhosV from Life Technologies (Carlsbad, CA). Horseradish peroxidase (HRP)–conjugated secondary antibodies to mouse and rabbit IgG were from Sigma-Aldrich. Alexa Fluor 647 goat anti-rat IgM and HRP-conjugated anti-rat IgM plus IgG were from Jackson ImmunoResearch (West Grove, PA). All other fluorescent secondary antibodies were purchased from Invitrogen (Carlsbad, CA). SB203580 was from EMD Biosciences, Darmstadt, Germany. NaF and other reagents were from Sigma-Aldrich.

Cell culture and transfection

Cos7 and HEK293 cells were grown in DMEM and HT-1080 cells in RPMI 1640 supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer at 37°C in a humidified atmosphere (5% CO₂ and 95% air). Cells were transfected using Lipofectamine (Invitrogen) or Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. For drug treatment, cells were incubated with 5 mM NaF or 30 μ M

SB203580 for 4 h in the presence or absence of serum, as indicated.

Constructs and site-directed mutagenesis

FLAG-Gp78 and FLAG-Gp78-IRES-GFP constructs were as described (Registre et al., 2004; St-Pierre et al., 2012). GST-fused C-terminal of Gp78 was prepared using pGEX-4T backbone plasmid from GE Healthcare. Briefly, a full-length FLAG-tagged mouse Gp78 cloned in pCDNA-3.1 (used as a template) and a set of primers (GST-cAMFR-BH1-F, 5'GC GGA TCC CCG CCC CCG ATC CGT CGA CAC AAG AAT TAT TTT 3'; GST-cAMFR-NotI-R, 5'AGT CGG CCG CTC CTA GGT TGT CCG TTG CCT CTG 3') were used to PCR generate C-terminal Gp78 (encoding for amino acids 309–639). The amplified DNA product was restriction digested and ligated into pGEX-4T plasmid at *Bam*HI-*Not*I sites, sequence verified, and transformed into *Escherichia coli* BL21 (DE3) *plyS* strain in order to produce GST-fused Gp78C recombinant protein. GST-Gp78C protein expression was induced by 1 mM isopropyl- β -D-thiogalactoside (IPTG) at 37°C for 4 h. pE-SUMO His-Gp78 was purchased from LifeSensors and the His-hGp78C induced with 0.4 mM IPTG at 16°C overnight and purified with His60 Ni Superflow Resin (Clontech). pcDNA3-Flag dominant-negative MKK6(K82A) and constitutively active MKK6 (Glu) were gifts from Roger Davis (University of Massachusetts, Worcester, MA; Addgene plasmids 13519 and 13518; Raingeaud et al., 1996). MFN1-Myc and MFN2-Myc were gifts from David Chen (California Institute of Technology, Pasadena, CA; Addgene plasmids 23212 and 23213; Chen et al., 2003). pOct-dsRed plasmid was a kind gift from Heidi McBride (McGill University, Montreal, Canada). Site-directed mutagenesis was carried out according to the protocol recommended in the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Western blots and dot blots

For dot blots, bacterial lysates were prepared in 1 \times loading buffer (50 mM Tris, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% [vol/vol] glycerol, 100 mM β -mercaptoethanol), and then equal amounts of GST-Gp78 bacterial lysates were blotted onto nitrocellulose membrane and probed with 3F3A, anti-Gp78C, or anti-GST antibodies. Chemiluminescence was revealed using ECL (GE Healthcare, Piscataway, NJ) and densitometry performed using Scion software. Western blots were as described (Shankar et al., 2013).

Immunoprecipitation

Cells were solubilized for 1 h with lysis buffer containing 20 mM Tris, pH 7.4, 1% Triton X-100, 125 mM NaCl, 3 mM EDTA, 3 mM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol, 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.1% SDS, 3.0 mM Na₃VO₄, 2.5 mM NaF, 2 mM tetra-Na pyrophosphate, and 25 mM β -glycerophosphate disodium salt hydrate supplemented with protease inhibitor and PhosSTOP phosphatase inhibitor cocktail tablets (Roche). Cell lysates were passed through a syringe at least 15 times to disrupt the pellets, cleared at 16,100 \times g for 10 min at 4°C and incubated with FLAG M2 beads (Sigma-Aldrich) overnight at 4°C. FLAG beads were washed once with lysis buffer, three times with 150 mM NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, and 0.2% bovine serum albumin (BSA), three times with 500 mM NaCl, 20 mM Tris, pH 8.0, 0.5% Triton X-100, 0.2% BSA, and once with 50 mM Tris, pH 8.0. All of the washing buffers contained 2.5 mM NaF and 0.1 mM Na₃VO₄, except for the CIAP assay as indicated. FLAG-Gp78 immunoprecipitates were incubated with 50 U of calf intestine alkaline phosphatase (New England Biolabs) at 37°C for 1 h.

Phosphoproteomics

Cos7 cells were cotransfected with FLAG-Gp78 and FLAG-MKK6(CA) or FLAG-MKK6(DN). Cells were starved for 4 h and treated with dimethyl sulfoxide (DMSO) or SB203580. Cell lysates were separated on SDS-PAGE, and the gel was fixed (40% ethanol and 10% acetic acid) overnight, washed in H₂O twice (5 min/wash), and stained for 60 min in staining solution (0.12% Coomassie G250, 10% ammonium sulfate, 10% *o*-phosphoric acid, and 20% methanol). Destaining was done by successive washes with H₂O. The target band was excised and followed by in-gel digestion of protein bands and analysis of peptides by mass spectrometry.

Gp78 in vitro E3 ubiquitin ligase activity

His-hGp78C-, His-hGp78C S542A-, His-hGp78C S542D-, and His-hGp78C C356G H361A-transformed (Liu *et al.*, 2014) bacteria were induced with 0.4 mM IPTG at 16°C overnight and purified with His60 Ni Superflow Resin (Clontech). E3 LITE Customizable Ubiquitin Ligase Kit with Ube2g2 and ubiquitin-WT was purchased from LifeSensors, and assay solutions were prepared according to the manufacturer's instruction. Gp78 in vitro E3 ubiquitin ligase activity assay was conducted following the manufacturer's suggested protocol except for the extended enzymatic reaction (90 min) after adding ATP starting solution. The signal was read with a SpectraMax Microplate Reader from Molecular Devices.

Immunofluorescence and confocal microscopy

For 3F3A and Gp78C mAb labeling, Cos7 cells grown on glass coverslips were fixed 24 h posttransfection with precooled (−80°C) methanol:acetone (80:20% [vol/vol]) for 15 min at −20°C. To measure mitochondria-associated ER, Cos7 cells were grown on glass coverslips, cotransfected with FLAG-Gp78 plasmids, pOct-dsRed fixed with prewarmed 3% paraformaldehyde after 24 h transfection, and then labeled with antibodies to FLAG and syntaxin17. After fixation, the cells were washed with PBS-CM (PBS supplemented with 1 mM CaCl₂ and 10 mM MgCl₂), blocked with 0.5% BSA in PBS-CM, and then incubated with primary antibodies in blocking solution, washed, and incubated with the corresponding species-specific, fluorescently conjugated secondary antibodies. Images of 3F3A-Gp78C-labeled cells were acquired with the 100× Plan Apochromatic objective of a FluoView 1000 confocal laser scanning microscope (Olympus, Markham, Canada). Three-dimensional (3D) deconvolved image stacks of syntaxin17- and pOct-dsRed- or Flag-Gp78- and OxPhosV-labeled Cos7 cells labels were obtained with the 100× (numerical aperture 1.4) Zeiss PlanApochromat objective of an Intelligent Imaging Innovations spinning disk confocal microscope. To quantify mitochondria-associated ER, we generated a 3D mask of the mitochondria label and quantified the percentage of syntaxin17 label that overlapped with mitochondria, using SlideBook image analysis software (Intelligent Imaging Innovations).

Fluorescence recovery after photobleaching

Cells were grown on glass cover slides and cotransfected with FLAG-Gp78-IRES-GFP plasmids and pOct-dsRed. After 24 h of transfection, cover slides were transferred to a ChamSlide chamber containing FluoroBrite DMEM (Life Technologies) supplemented with 10% FBS and 4 mM L-glutamine. A region of interest (ROI) of pOct-dsRed-labeled mitochondria was bleached with the 488-nm laser of an Olympus FluoView 1000 confocal microscope, and images were acquired every 5 s over 2 min. DsRed intensity in the ROI was calculated from each image and quantified using GraphPad.

Statistical analyses

Data are presented as mean ± SEM ($n \geq 3$). One-way analysis of variance and Student's *t* test were used for statistical analysis. Differences were considered statistically significant when $p < 0.05$.

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