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ISG15 modification of filamin B negatively regulates the type I interferon-induced JNK signalling pathway

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Interferon (IFN)-induced signalling pathways have essential functions in innate immune responses. In response to type I IFNs, filamin B tethers RAC1 and a Jun N-terminal kinase (JNK)-specific mitogen-activated protein kinase (MAPK) module—MEKK1, MKK4 and JNK—and thereby promotes the activation of JNK and JNK-mediated apoptosis. Here, we show that type I IFNs induce the conjugation of filamin B by interferon-stimulated gene 15 (ISG15). ISGylation of filamin B led to the release of RAC1, MEKK1 and MKK4 from the scaffold protein and thus to the prevention of sequential activation of the JNK cascade. By contrast, blockade of filamin B ISGylation by substitution of Lys 2467 with arginine or by knockdown of ubiquitin-activating enzyme E1-like (UBEL1) prevented the release of the signalling molecules from filamin B, resulting in persistent promotion of JNK activation and JNK-mediated apoptosis. These results indicate that filamin B ISGylation acts as a negative feedback regulatory gate for the desensitization of type I IFN-induced JNK signalling.

Keywords: apoptosis; filamin B scaffold; ISG15; JNK signalling pathway; type I interferon

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INTRODUCTION

Interferons (IFNs) are cytokines that regulate cell proliferation and differentiation, and activate effector cells of the immune system (Platanias, 2005). The first signalling pathway shown to be activated by IFNs is the JAK–STAT pathway, but it has become evident that other signalling cascades are required for the generation of pleiotropic responses to IFNs. These include the p38 signalling cascade (Katsoulidis *et al*, 2005), pathways involving protein kinase C (PKC) isoforms (Uddin *et al*, 2002) or CRK proteins (Platanias, 2005), and the phosphoinositide-3 kinase (PI(3)K) pathway (Platanias, 2005; van Boxel-Dezaire *et al*, 2006). Recently, we have shown that type I IFNs activate a Jun N-terminal kinase (JNK)-specific signalling cascade—RAC1 → MEKK1 → MKK4 → JNK—and that filamin B facilitates type I IFN signalling by acting as a scaffold that tethers RAC1 and the JNK cascade members (Jeon *et al*, 2008).

Filamins are actin-binding proteins that comprise a family of three members: filamin A, B and C (Stossel *et al*, 2001; van der Flier & Sonnenberg, 2001). These filamin isoforms have a crucial function in crosslinking cortical actin filaments into a dynamic, three-dimensional structure. Filamins also interact with more than 30 cellular proteins of functional diversity (Stossel *et al*, 2001), suggesting that filamins function as molecular scaffolds by connecting and coordinating various cellular processes.

The interferon-stimulated gene 15 (ISG15) is the first reported ubiquitin-like protein (Haas *et al*, 1987) and its expression and conjugation to proteins are induced by type I IFNs (Der *et al*, 1998). The ubiquitin-activating enzyme E1-like (UBE1L) is an E1 ISG15-activating enzyme (Yuan & Krug, 2001); ubiquitin E2 enzymes, ubiquitin-conjugating enzyme in human (UBCH)6 and UBCH8, also function as ISG15-conjugating enzymes (Kim *et al*, 2004; Zhao *et al*, 2004); ubiquitin E3 ligases, HECT domain and RLD5 (HERC5) and estrogen-responsive finger protein (EFP), also act as ISG15 E3 ligases (Wong *et al*, 2006; Zou & Zhang, 2006); and ubiquitin-specific processing protease 43 (UBP43) acts as a deISGylating enzyme (Malakhov *et al*, 2002). Appropriately, all of the enzymes identified in the ISGylation pathway are induced in a coordinated manner by type I IFNs.

At least 200 putative ISG15 target proteins have been identified so far (Zhao *et al*, 2005). Many of them have crucial functions in the type I IFN response, and include JAK1, STAT1, RIG-I and the antiviral effector proteins MxA, PKR and RNase L (Zhao *et al*, 2005; Arimoto *et al*, 2008; Kim *et al*, 2008). ISG15 has been reported to prevent virus-mediated degradation of interferon regulatory factor 3 (IRF3), thereby increasing the induction of IFNβ expression (Lu *et al*, 2006). Other reports support a role for

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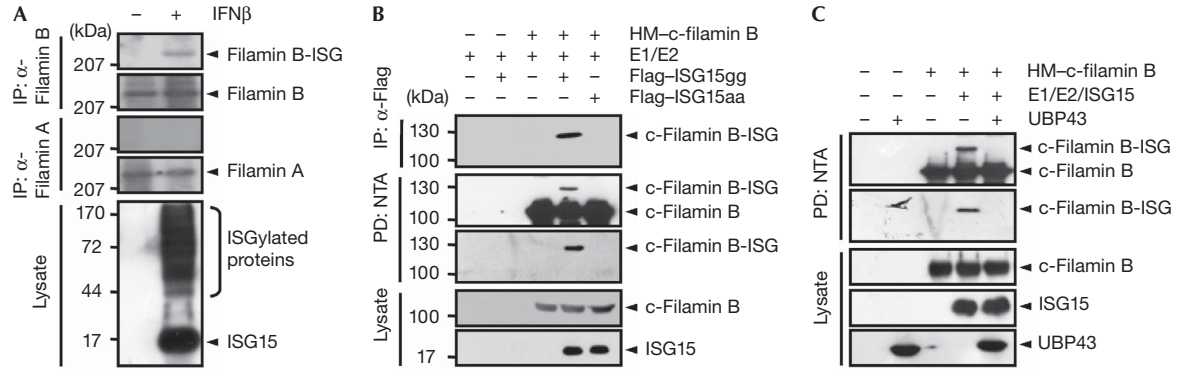

Fig 1 | Filamin B is a target for ISGylation. (**A**) A549 cells were incubated for 48 h with or without 1,000 U/ml of IFNβ. Cell lysates were immunoprecipitated (IP) with filamin B or filamin A antibodies, followed by immunoblot with ISG15 antibodies. They were also probed directly with ISG15 antibodies. (**B**) HisMax (HM)-c-filamin B were expressed with Flag–ISG15gg or Flag–ISG15aa. E1 and E2 were also expressed by co-transfection of cells with pcDNA-UBE1L and pcDNA-Myc–UBCH8, respectively. Cell lysates were immunoprecipitated with Flag antibodies, followed by immunoblot with Xpress antibodies. Cell lysates were also subjected to NTA pull-down (PD: NTA) under denaturing conditions followed by immunoblot with Xpress or Flag antibodies. (**C**) HM–c-filamin B, Flag–ISG15 and UBP43 were expressed with E1 and E2 as indicated. Cell lysates were then subjected to NTA pull-down as in (**B**). c-Filamin B, the carboxy-terminal H1–R24 region of filamin B; IFN, interferon; ISG, interferon-stimulated gene; NTA, nitrilotriacetic acid; UBC, ubiquitin-conjugating enzyme; UBE1L, ubiquitin-activating enzyme E1-like; UBP43, ubiquitin-specific processing protease 43.

ISG15 in mediating resistance to the Ebola virus through ISGylation of NEDD4 (Malakhova & Zhang, 2008; Okumura *et al*, 2008); however, it remains unknown how ISGylation of target proteins affects their cellular function in the control of IFN-mediated signalling pathway. Here, we show that filamin B is modified by ISG15 in response to type I IFNs and that this modification blocks its scaffold function, leading to the abrogation of the IFN-induced JNK signalling pathway. These results establish that ISGylation of filamin B acts as a negative feedback regulatory gate for the desensitization of type I IFN-induced JNK signalling.

RESULTS AND DISCUSSION

Filamin B is a target for ISGylation

Type I IFNs induce the accumulation of ISG15 and its conjugating enzyme system. In addition, filamin B has been identified as a putative target for ISGylation (Zhao *et al*, 2005). To determine whether filamin B could indeed be modified by ISG15, A549 cells were cultured with or without IFNβ. Immunoprecipitation analysis revealed that endogenous filamin B—but not filamin A—was ISGylated only when cells were treated with IFNβ or IFNα (Fig 1A; data not shown). Then, we examined whether overexpressed filamin B could also be ISGylated. The carboxy-terminal region from hinge-1 to repeat 24 (H1–R24; see Fig 2A) was expressed with ISG15 (Flag–ISG15gg) or its mutant form having the C-terminal Ala–Ala in place of Gly–Gly (Flag–ISG15aa). Henceforth, the C-terminal H1–R24 region of filamin B is referred to as c-filamin B. Expression of ISG15gg, but not ISG15aa, resulted in c-filamin B ISGylation (Fig 1B), indicating that the C-terminal glycine is required for filamin B ISGylation. In addition, coexpression of UBP43 led to deISGylation of c-filamin B (Fig 1C). Taken together, these results indicate that filamin B is an ISGylation target.

Lys 2467 of filamin B is the ISGylation site

To determine the ISGylation site, various deletions of filamin B were expressed in HeLa cells with Flag–ISG15 (Fig 2A). The mutants containing R22–24 were ISGylated, whereas H1–R21 and H2–R24 were not. Neither actin-binding domain–R7 (ABD–R7) nor R8–15 was ISGylated. These results indicate that the ISGylation site lies within R22–23. Each of the 13 lysine residues in the R22–23 region of HisMax-R22–24 was replaced by arginine. These mutants were expressed in HeLa cells with Flag–ISG15 followed by pull down with nitrilotriacetic acid (NTA) resins. Immunoblot of the precipitates with Flag antibodies revealed that the K2467R mutation, but not the other mutations, blocked the appearance of a 62-kDa band (indicated by R22–24-ISG), suggesting that Lys 2467 is the ISGylation site (Fig 2B). Immunoblot of the same precipitates with Xpress antibodies again showed that the K2467R mutation blocked the appearance of the 62-kDa band; however, it also blocked the appearance of an additional 55-kDa band (indicated by a dot), which was detected in the precipitates from cells expressing all other mutants and wild-type R22–24. To clarify whether Lys 2467 acts as the ISGylation site, we built the K2467R mutation into a full-length filamin B. Fig 2C shows that the K2467R mutation prevents the ISGylation of filamin B. Therefore, we concluded that Lys 2467 in filamin B is the ISG15 acceptor site, although the nature of the 55-kDa protein remains unknown.

Filamin B ISGylation blocks its scaffold function

Filamin B acts as a scaffold that tethers RAC1 and a JNK-specific mitogen-activated protein kinase (MAPK) module—MEKK1, MKK4 and JNK1—and thereby facilitates type I IFN-induced JNK activation (Jeon *et al*, 2008). To determine whether ISGylation of filamin B influences its function as a scaffold, RAC1 and the JNK cascade members were expressed in HeLa cells with c-filamin B or its K2467R mutant (henceforth referred to as c-K2467R). Coexpression of ISG15 prevented the interaction of RAC1 with c-filamin B, but not with c-K2467R (Fig 3A). In addition, ISG15aa expression showed little or no effect on the interaction of RAC1 with c-filamin B (Fig 3B). Similarly, the interaction of MEKK1 and MKK4 with c-filamin B, but not with c-K2467R, was markedly reduced by coexpression of

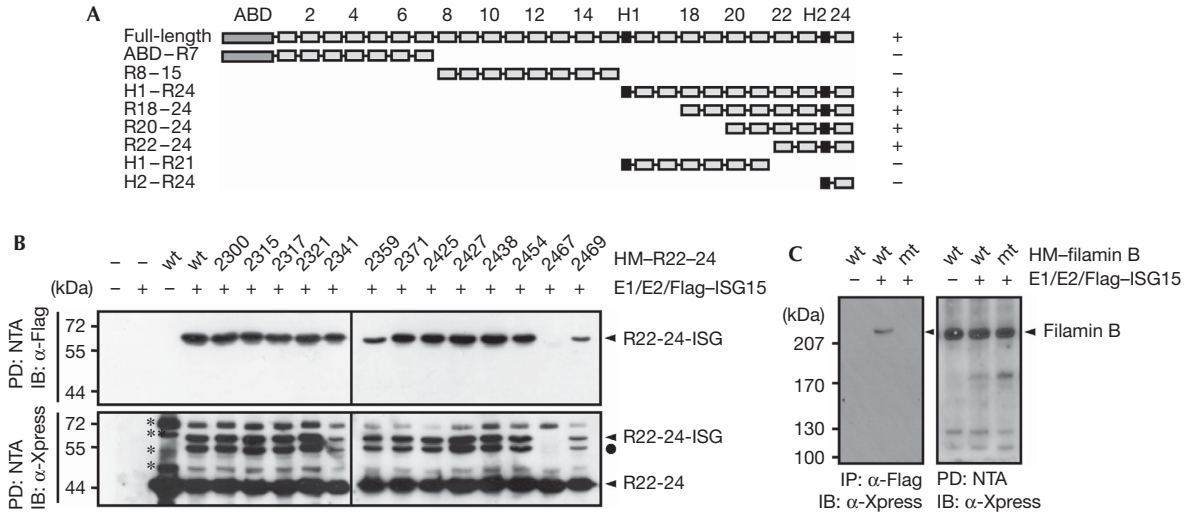

Fig 2 | Lys 2467 of filamin B is the ISGylation site. (**A**) All deletion constructs were tagged with HisMax (HM) to their amino termini. Each was expressed in HeLa cells with Flag–ISG15, E1 and E2. Cell lysates were subjected to NTA pull-down followed by immunoblot (IB) with Xpress antibodies. Whether each deletion was modified by ISG15 or not are marked as + or –. ABD indicates the actin-binding domain, and H1 and H2 are the hinge regions. The numerals show the numbers of IgG-like repeats. (**B**) The Lys-to-Arg mutants of HM–R22–24 were expressed in HeLa cells with Flag–ISG15, E1 and E2. Cell lysates were subjected to NTA pull-down (PD: NTA) followed by immunoblot with Flag or Xpress antibodies. The numerals indicate the positions of lysine in filamin B. The dot indicates a 55-kDa protein that can be stained with Xpress antibodies but not with Flag antibodies. The asterisks indicate nonspecific bands. (**C**) HM–filamin B (wt) or its K2467R mutant (mt) were expressed in HeLa cells with Flag–ISG15, E1 and E2. Cell lysates were subjected to SDS–PAGE in 6% gels followed by immunoprecipitation (IP) or NTA pull-down. Precipitates were immunoblotted with Xpress antibodies. ISG, interferon-stimulated gene; mt, mutant; NTA, nitrilotriacetic acid; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; wt, wild type.

ISG15 (Fig 3D,E). By contrast, the interaction of c-filamin B with JNK1 was not affected regardless of ISG15 coexpression (Fig 3C). These results indicate that ISGylation of filamin B prevents its ability to interact with the upstream activators of the JNK cascade.

To confirm whether type I IFN-induced filamin B ISGylation blocks the interaction between filamin B and JNK cascade members, M2 cells, having a negligible amount of filamin B (Jeon *et al*, 2008), were complemented with HisMax-tagged full-length filamin B or K2467R followed by IFNα treatment. The interaction of filamin B with endogenous RAC1, MEKK1 and MKK4 increased until about 12 h after IFNα treatment and gradually decreased thereafter, concomitant with an increase in the level of ISGylated filamin B (Fig 3F). However, K2467R persistently interacted with the JNK cascade members even when ISGylated cellular proteins reached a maximal level. To confirm whether ISGylation of filamin B is responsible for the prevention of its interaction with the JNK activators, an UBE1L-specific short hairpin RNA (shUBE1L) was transfected with M2 cells. shUBE1L, but not a control vector (shControl), abolished the negative effect of filamin B ISGylation on IFNα-induced interaction of filamin B with RAC1, MEKK1 and MKK4 (Fig 3G). These results indicate that the ISGylation of filamin B abrogates its scaffold function.

Filamin B ISGylation inhibits IFNα-induced JNK signalling

As ISGylation abrogates the scaffold function of filamin B, we examined its effect on type I IFN-induced JNK activation. Coexpression of ISG15 led to a marked decrease in the activation of JNK in M2 cells complemented with c-filamin B, but not in c-K2467R-complemented cells (Fig 4A), indicating that ISGylation of filamin B abrogates its ability to promote type I IFN-induced JNK activation. In addition, ISG15 expression strongly inhibited the ability of c-filamin B, but not of c-K2467R, in the promotion of IFNα-induced RAC1 activation (Fig 4B). These results indicate that ISGylation of filamin B blocks its ability to promote IFNα-induced RAC1 activation. Next, we examined the effect of c-filamin B ISGylation on sequential activation of the JNK cascade. MEKK1-mediated phosphorylation of MKK4 was enhanced by c-filamin B or c-K2467R (Fig 4C); however, coexpression of ISG15 blocked the promotion of MKK4 activation by c-filamin B, but not by c-K2467R. Similarly, MKK4-mediated activation of JNK1 was increased by c-filamin B or c-K2467R, and coexpression of ISG15 abolished the stimulatory effect of c-filamin B, but not of c-K2467R (Fig 4D). In addition, c-filamin B or c-K2467R could enhance MEKK1-mediated activation of JNK1, and the stimulatory effect of c-filamin B, but not of c-K2467R, was prevented by ISG15 coexpression (Fig 4E). Taken together, these results indicate that ISGylation of filamin B prevents its ability to promote the sequential activation of the JNK cascade—MEKK1→MKK4→JNK1—by blocking its scaffold function.

As filamin B accelerates IFNα-induced apoptosis through the activation of JNK (Jeon *et al*, 2008), we examined the effect of filamin B ISGylation on JNK-mediated apoptosis. Coexpression of ISG15 led to a decrease in the levels of TRAIL-R1 (tumour necrosis factor-related apoptosis-inducing ligand) and in the cleavage of PARP (poly (ADP-ribose) polymerase) in M2 cells complemented with c-filamin B, but not in c-K2467R-complemented cells (Fig 4F), indicating that ISGylation of filamin B blocks its ability to promote IFNα-induced apoptosis. To confirm this finding, M2

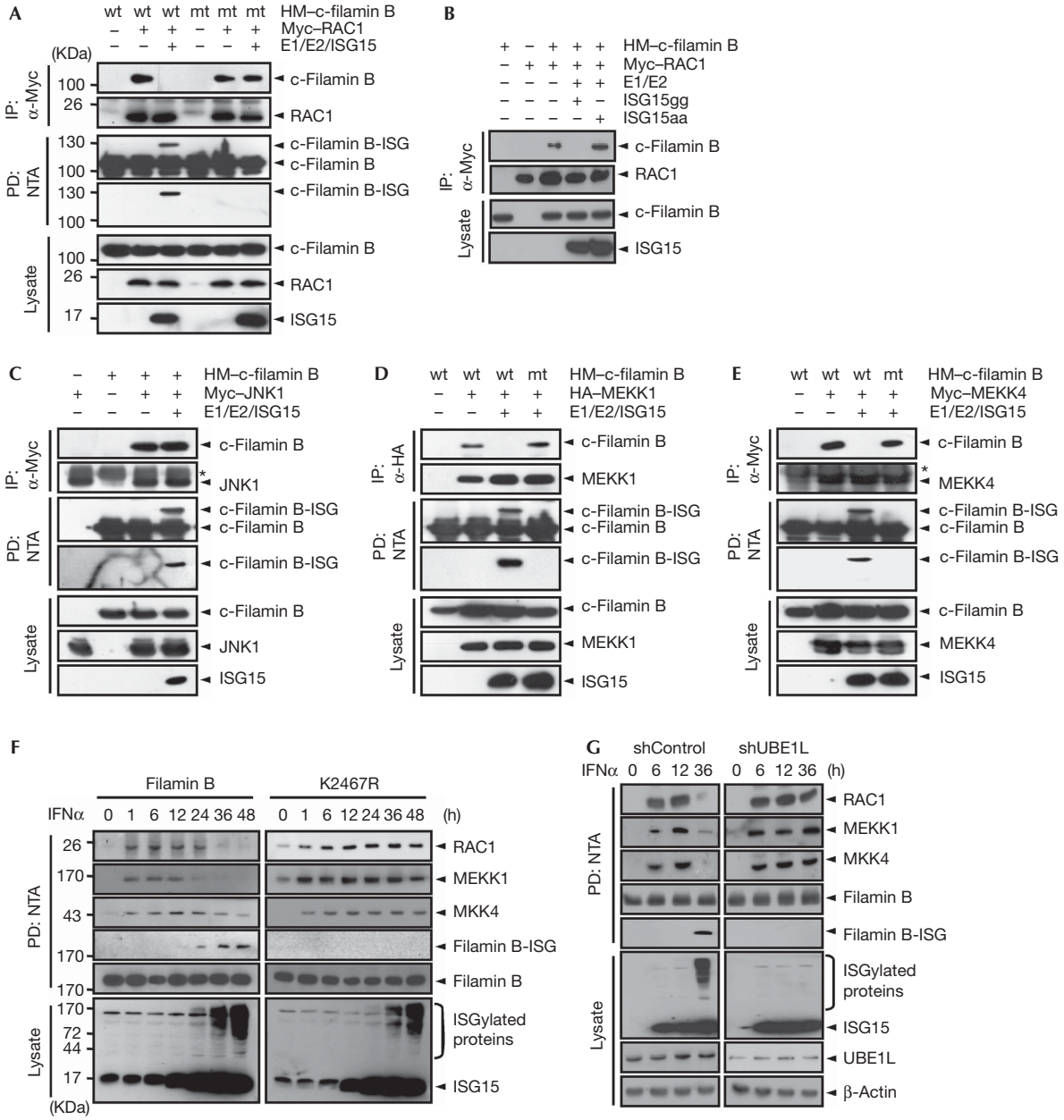

Fig 3 | ISGylation of filamin B blocks its interaction with RAC1 and JNK cascade members. (**A**) HM–c-filamin B (wt) or HM–c-K2467R mutant (mt), Myc–RAC1, Flag–ISG15, E1 and E2 were expressed in HeLa cells as indicated. Cell lysates were immunoprecipitated (IP) with Myc antibodies, followed by immunoblot with Xpress and Myc antibodies; they were also subjected to NTA pull-down (PD: NTA) followed by immunoblot with Xpress or Flag antibodies. (**B**) HM–c-Filamin B and Myc–RAC1 were expressed in HeLa cells with Flag–ISG15gg or Flag–ISG15aa, E1 and E2. Cell lysates were analysed as above. (**C–E**) HM–c-filamin B (wt), HM–c-K2467R (mt), Flag–ISG15, E1 and E2 were expressed in HeLa cells with (**C**) Myc–JNK1, (**D**) HA–MEKK1 or (**E**) Myc–MKK4 as indicated. Cell lysates were immunoprecipitated with HA or Myc antibodies, followed by immunoblot with Xpress, HA or Myc antibodies. The asterisks in (**C,E**) indicate IgG heavy chain. (**F**) M2 cells complemented with HM–filamin B (left panel) or HM–K2467R (right panel) were treated with IFNα. Cell lysates were subjected to NTA pull-down followed by immunoblot analysis. They were also probed directly with ISG15 antibodies. (**G**) M2 cells transfected with shControl or shUBE1L were complemented with HM–filamin B. After incubation with IFNα, cell lysates were subjected to NTA pull-down followed by immunoblot analysis. They were also probed directly with ISG15 or UBE1L antibodies. c-Filamin B, the carboxy-terminal H1–R24 region of filamin B; HA, haemagglutinin; HM, HisMax; IFN, interferon; IgG, immunoglobulin G; ISG, interferon-stimulated gene; JNK, Jun N-terminal kinase; NTA, nitrilotriacetic acid; Sh, Short hairpin; UBE1L, ubiquitin-activating enzyme E1-like; wt, wild type.

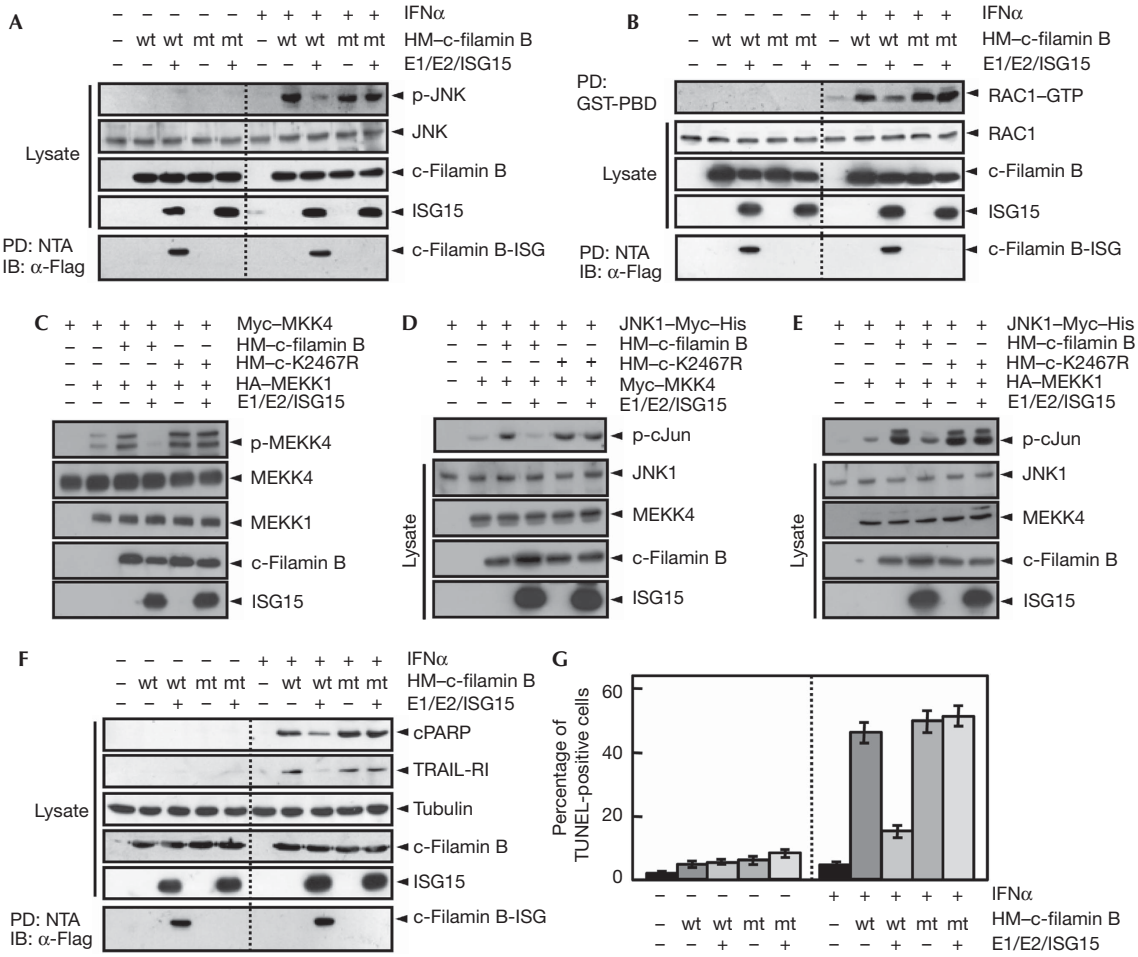

Fig 4 | ISGylation of filamin B inhibits IFNα-induced JNK signalling. (**A**) HM–c-filamin B (wt) or HM–c-K2467R (mt) was expressed in M2 cells with or without Flag–ISG15, E1 and E2. After incubation with or without IFNα for 1 h, cell lysates were subjected to immunoblot (IB) analysis. They were also subjected to NTA pull-down (PD: NTA) followed by Flag antibodies. (**B**) HM–c-filamin B (wt) or HM–c-K2467R (mt) was expressed in M2 cells with or without ISG15, E1 and E2. After incubation with or without IFNα for 30 min, cell lysates were assayed for the activation of RAC1. (**C**) Myc–MKK4 and either HM–c-filamin B or HM–c-K2467R were expressed in HeLa cells with HA–MEKK1, Flag–ISG15, E1 and E2 as indicated. Cell lysates were immunoblotted with p-MKK4 antibodies. (**D**) JNK1–Myc–His and either HM–c-filamin B or HM–c-K2467R were expressed in HeLa cells with Myc–MKK4, Flag–ISG15, E1 and E2. Cell lysates were subjected to *in vitro* kinase assay for JNK using recombinant cJun as a substrate followed by immunoblot with p-cJun antibodies. (**E**) JNK1–Myc–His and either HM–c-filamin B or HM–c-K2467R were expressed in HeLa cells with HA–MEKK1, Flag–ISG15, E1 and E2. Cell lysates were subjected to *in vitro* kinase assay for JNK. (**F**) HM–c-filamin B (wt) or HM–c-K2467R (mt) was expressed in M2 cells with or without Flag–ISG15, E1 and E2. After incubation with or without IFNα for 12 h, cell lysates were immunoblotted. (**G**) M2 cells prepared as in (**F**) were incubated with or without IFNα for 12 h followed by TUNEL assay. Error bars indicate the mean ± s.d. c-Filamin B, the carboxy-terminal H1–R24 region of filamin B; HM, HisMax; IFN, interferon; ISG, interferon-stimulated gene; JNK, Jun N-terminal kinase; mt, mutant; NTA, nitrilotriacetic acid; TUNEL, TdT-mediated dUTP nick-end labelling; wt, wild type.

cells were subjected to TdT-mediated dUTP nick-end labelling (TUNEL) staining. In the presence of IFNα, unlike in its absence, the number of TUNEL-positive cells was increased on complementation of either c-filamin B or c-K2467R (Fig 4G). Coexpression of ISG15 caused a decrease in the number of TUNEL-stained cells on complementation with c-filamin B but not with c-K2467R, again indicating that ISGylation of filamin B blocks its ability to promote IFNα-induced apoptosis. The images of TUNEL-stained cells are shown in supplementary Fig S1 online. These results indicate that ISGylation of filamin B negatively regulates the IFNα-induced JNK signalling.

An important question is how ISGylation of a small fraction of filamin B could inhibit its ability to promote the type I IFN-induced JNK pathway. However, if the small fraction of ISGylated filamin B is localized to a functionally unique subcellular site, the inhibitory mechanism could operate efficiently. Filamin B sequesters RAC1 and JNK cascade members in membrane ruffles for facilitating type I IFN signalling (Jeon *et al*, 2008); therefore, we examined whether filamin B could also recruit UBCH8, an E2 for ISG15, to membrane ruffles for facilitating the ISGylation of filamin B. In HeLa cells, UBCH8 was present throughout the cytoplasm and the nucleus; however, on filamin B coexpression a significant portion of UBCH8

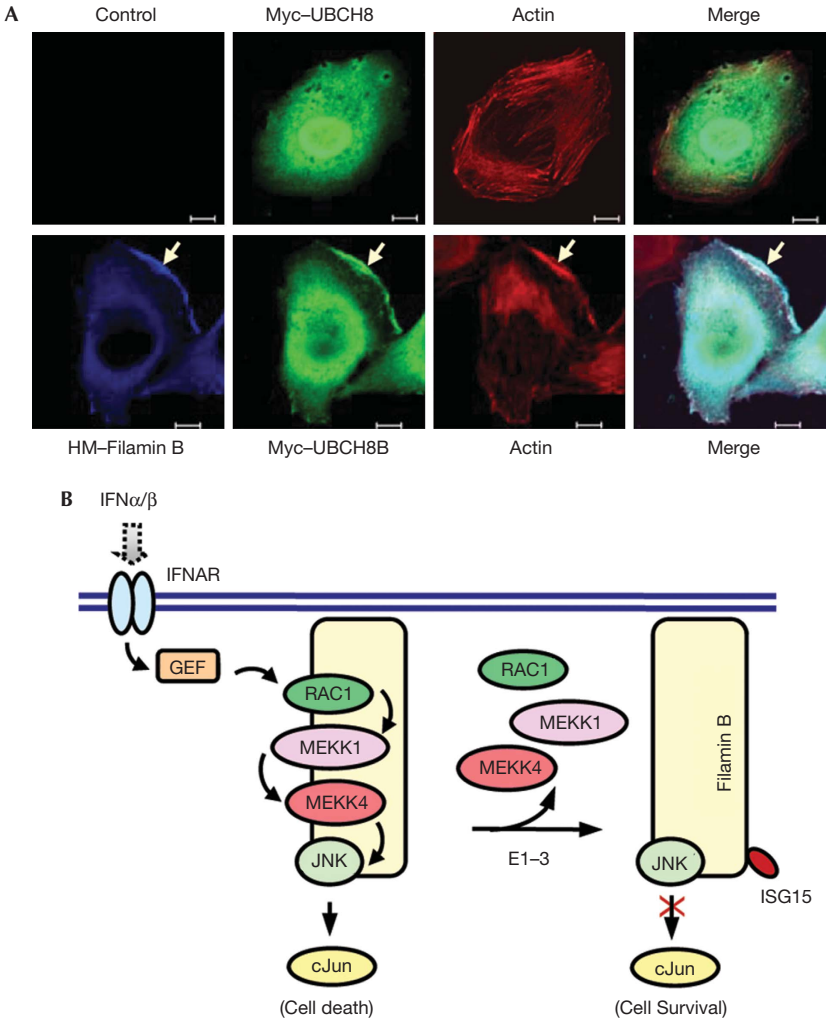

Fig 5 | Colocalization of UBCH8 and filamin B in membrane ruffles, and a model for the control of type I IFN-induced JNK signalling by ISGylation of filamin B. (**A**) Myc–UBCH8 was expressed in HeLa cells with or without HM–filamin B. Cells were stained with Xpress or Myc antibodies or phalloidin. The arrows indicate membrane ruffles; scale bar, 10 μm. (**B**) Early IFN response leads to the promotion of filamin B scaffold function and thus to the activation of cJun; late IFN response leads to the ISGylation of filamin B, dissociation of RAC1, MEKK1 and MKK4 from filamin B, and termination of the early response. GEF indicates a putative guanine nucleotide exchange factor that links type I IFN signal to RAC1. HM, HisMax; IFN, interferon; IFNAR, type I IFN receptor; ISG, interferon-stimulated gene; JNK, Jun N-terminal kinase; UBC, ubiquitin-conjugating enzyme.

was recruited to membrane ruffles where filamin B is also concentrated with actin (Fig 5A). These results suggest that filamin B ISGylation occurs in membrane ruffles, which should result in the prevention of filamin B scaffold function.

Fig 5B shows a model for the regulatory role of filamin B ISGylation in the type I IFN-induced JNK signalling pathway. Binding of IFNα/β to the type I IFN receptor (IFNAR) induces a successive activation of the RAC1- and JNK-specific cascade through phospho-relay reactions, resulting in JNK activation and thus in JNK-mediated apoptosis. On the accumulation of ISG15 and its conjugation system as a late response to type I IFNs, ISGylation of filamin B proceeds and blocks its role as a scaffold in tethering RAC1 and JNK cascade members, thus desensitizing type I IFN-induced JNK signalling.

Apoptosis acts as a crucial mechanism for the killing of host cells on viral infection. IFNs promote not only apoptosis but also cell survival against various proapoptotic stimuli such as viral infection. Thus, the antiviral action induced by IFNs could be due to the protection of uninfected cells against virus-induced apoptosis, as well as to the direct killing of infected cells. For example, IFNs promote the survival of activated T cells (Marrack *et al*, 1999), protect $CD4^+$ cells from human immunodeficiency virus (HIV)-induced cell death (Cremer *et al*, 1999) and protect lymphoblastoid cells from cell death induced by viral infection (Einhorn & Grander, 1996). In addition, the IFNβ transduction of peripheral blood lymphocytes from uninfected or HIV-infected donors has been shown to inhibit viral replication and increase the survival of $CD4^+$ cells (Vieillard *et al*, 1997). In this respect, we suggest that the control of JNK-mediated apoptosis by ISGylation of filamin B in response to type I IFNs could be a crucial mechanism for the survival of uninfected bystander cells and thus for antiviral action.

METHODS

RAC1 activation assay. RAC1 activation was assayed as described previously (Benard *et al*, 1999). Briefly, the GTPase-binding domain of human PAK1 (p21-activated kinase 1) was expressed in *Escherichia coli* as a glutathione *S*-transferase (GST) fusion. Cells were serum-starved for 3 h, treated with 10,000 U/ml of IFNα for 0.5 h and lysed. Cell lysates were incubated for 1 h with 5 μg of GST–PBD, followed by a pull-down with glutathione-Sepharose. Precipitates were subjected to immunoblot with RAC1 antibodies.

Immunoprecipitation and pull-down analysis. For immunoprecipitation, cells were lysed in 50 mM Tris–HCl (pH 8), 150 mM NaCl, 1% Triton X-100 or 0.5% NP-40, 1 mM PMSF and 1 × protease inhibitor cocktail (Roche, Mannheim, Germany). Cell lysates were incubated with the appropriate antibodies for 2 h at 4 °C and then with 50 μl of 50% slurry of protein A-Sepharose for 1 h. Cell lysates prepared as above were also subjected to pull-down with NTA resins. For pull-down analysis under denaturing conditions, cell lysates were prepared in 0.1 M NaH_2PO_4/Na_2HPO_4 buffer (pH 7.4) containing 8 M urea and 5 mM imidazole. After incubation with NTA resins, precipitates were washed with the same buffer containing 5 mM imidazole followed by SDS–PAGE.

***In vitro* kinase assay.** For assaying JNK activity, cell lysates were incubated for 5 h with GST–cJun bound to glutathione-agarose. Precipitates were washed twice with 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM PMSF. They were again washed with buffer-A consisting of 25 mM Tris–HCl (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4 and 10 mM $MgCl_2$. After washing, the precipitates were incubated in buffer-A containing 0.2 mM ATP for 30 min at 30 °C. The samples were resolved by SDS–PAGE, and phosphoproteins were visualized by immunoblot with the p-cJun antibody.

For assaying MEKK1 activity, cell lysates were immunoprecipitated with the MEKK1 antibody. Precipitates were incubated with 2 μg GST–MKK4 as a substrate in buffer-A containing 0.2 mM ATP for 30 min at 30 °C. The samples were then resolved by SDS–PAGE, and phosphoproteins were visualized by immunoblot with the p-MKK4 antibody.

For other methods, see the supplementary information online. **Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org)

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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