

# SCF<sup>HOS</sup> ubiquitin ligase mediates the ligand-induced down-regulation of the interferon- $\alpha$ receptor

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**Down-regulation of activated signaling receptors in response to their ligands plays a key role in restricting the extent and duration of the signaling. Mechanisms underlying down-regulation of the type I interferon receptor consisting of IFNAR1 and IFNAR2 subunits remain largely unknown. Here we show that IFNAR1 interacts with the Homolog of Slimb (HOS) F-box protein in a phosphorylation-dependent manner, and that this interaction is promoted by interferon  $\alpha$  (IFN $\alpha$ ). IFNAR1 is ubiquitinated by the Skp1-Cullin1-HOS-Roc1 (SCF<sup>HOS</sup>) ubiquitin ligase *in vitro*. HOS expression and activities are required for IFN $\alpha$ -stimulated ubiquitination of IFNAR1, endocytosis of the type I interferon receptor, down-regulation of IFNAR1 levels, and IFNAR1 proteolysis via the lysosomal pathway. Furthermore, modulations of HOS activities affect the extent of Stat1 phosphorylation and Stat-mediated transcriptional activities as well as the extent of antiproliferative effects of type I interferons. These findings characterize SCF<sup>HOS</sup> as an E3 ubiquitin ligase that is essential for ubiquitination, proteolysis and down-regulation of IFNAR1, and implicate HOS in the regulation of cellular responses to IFN $\alpha$ .**

**Keywords:** F-box/interferon/receptor/Slimb/ubiquitin ligase

## Introduction

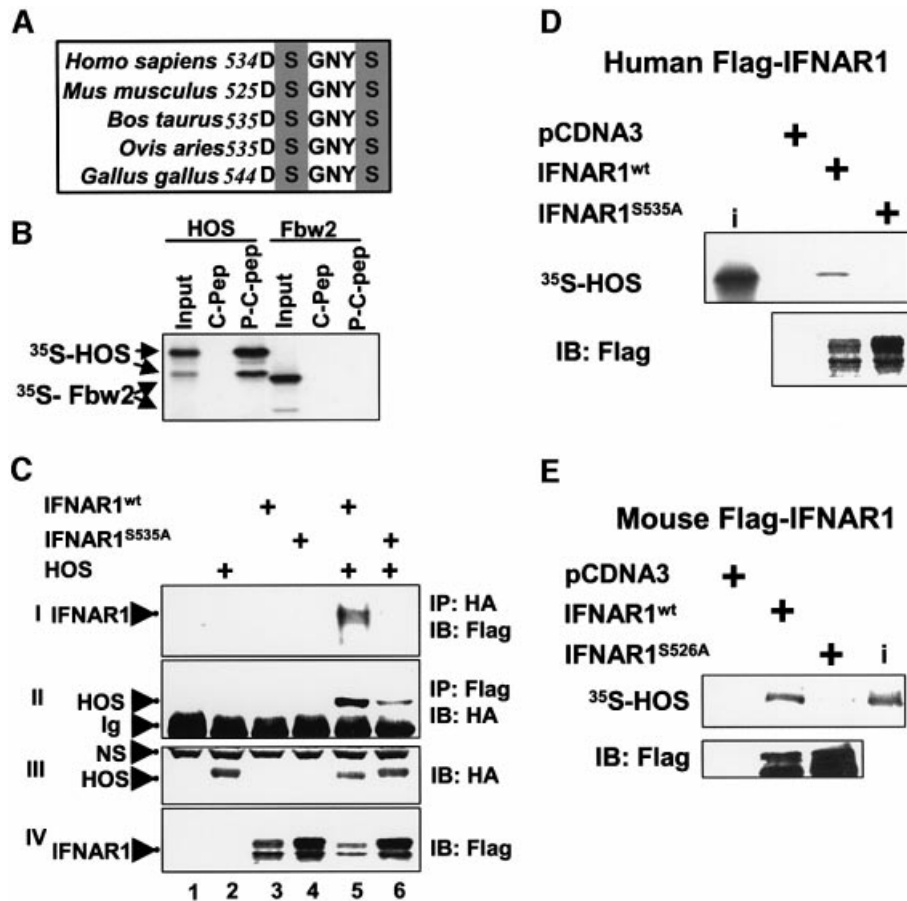
A key issue in the regulation of signal transduction has to do with how cells limit the duration and magnitude of signaling. Modification of signaling proteins via covalent attachment of ubiquitin polypeptide (ubiquitination) is an important mechanism that underlies the rapid changes in protein stability, localization and activity. Substrate specificity and timing of ubiquitin conjugation are determined by E3 ubiquitin protein ligases, which are the rate-limiting factors in the enzymatic cascade that also requires ubiquitin-activating (E1) and ubiquitin-conjugating (E2) enzymes. Proteins modified by lysine 48 polyubiquitin chains are efficiently degraded via a 26S proteasome-mediated pathway (reviewed in Weissman, 2001). Among E3 species that ubiquitinate proteasomal substrates, the

Skp1-Cullin1-F-box-Roc1 (SCF) family members represent the ligases, which specifically recognize and ubiquitinate phosphorylated substrates. Within SCF complexes, the ligase activity is mediated by the Cullin1 and Roc1 (also termed Rbx1 or Hrt1) complex, which is tethered via Skp1 to a specificity-conferring F-box protein. F-box proteins bind to Skp1 via the F-box motif and they interact with phosphorylated substrates through other protein-protein binding domains, including WD40 repeats (reviewed in Deshaies, 1999).

In addition to proteasomal degradation, ubiquitination plays a role in endocytosis of plasma membrane proteins followed by their sorting and lysosomal degradation. A few known ligases that mediate this ubiquitination (including c-Cbl/Hakai and Rsp5/NEDD4 family members) are important in regulating the magnitude and duration of various signal transduction pathways (Hicke, 1999; Katzmann *et al.*, 2001). However, the ubiquitin ligases that mediate degradation of many receptors, including receptors for interferons, remain to be identified.

Type I interferons (IFN) have become widely used therapeutic agents due to their potent anti-tumor, anti-viral and immunomodulatory activities (Biron, 2001; Kirkwood, 2002). These cytokines trigger their signaling by binding type I IFN receptor at the cell surface and inducing assembly of a receptor complex consisting of IFNAR1 and IFNAR2 subunits (Uze *et al.*, 1990; Novick *et al.*, 1994). Down-regulation and degradation of these subunits in response to the ligand are expected to play a key role in restricting the extent and duration of IFN signaling (Constantinescu *et al.*, 1994; Stark *et al.*, 1998). However, the mechanisms of ligand-induced down-regulation and degradation of type I IFN receptor have not been determined.

IFNAR1 is rapidly degraded upon treatment of cells with IFN $\alpha$  (Constantinescu *et al.*, 1994). Cellular responses to IFN $\alpha$  require an adequate expression of the IFNAR1 subunit and its genetic disruption in mice compromises innate immunity (Muller *et al.*, 1994; Hwang *et al.*, 1995). Conversely, IFNAR1 mutants that bear deletions in their C-terminus inhibit internalization of the type I IFN receptor, and these mutants mediate enhanced responses to IFN $\alpha$  (Gibbs *et al.*, 1996; Basu *et al.*, 1998). Among putative negative regulatory domains located within the distal cytoplasmic tail of IFNAR1 is a motif that may be recognized by the Homologue of Slimb (HOS) F-box-containing protein, which serves as a receptor for SCF<sup>HOS</sup> E3 ligase in ubiquitination of phosphorylated I $\kappa$ B and  $\beta$ -catenin (Fuchs *et al.*, 1999; Tan *et al.*, 1999). HOS (also termed  $\beta$ -TrCP2 or Fbw1b) recruits E3 activity via the F-box-dependent binding to Skp1 and recognizes phospho-motifs (DpSGXXpS) in its substrates via WD40 domains (Fuchs *et al.*, 1999; Suzuki *et al.*, 1999). Here we show that IFNAR1 undergoes



**Fig. 1.** Interaction between HOS and IFNAR1. (A) Multiple alignment of a putative HOS recognition motif in IFNAR1 of different species. Potentially phosphorylated serines are shaded. (B) Binding of an *in vitro*-translated (IVT) and <sup>35</sup>S-labeled HOS and Fbw2 to the beads coated with phosphorylated (P-C-Pep) or non-phosphorylated (C-Pep) IFNAR1-derived peptides. (C) Co-immunoprecipitation of HA-tagged HOS and human Flag-tagged IFNAR1 (wild type or S535A mutant) expressed in 293T cells as indicated. Ig, immunoglobulins; NS, non-specific band. (D-E) Binding of an IVT HOS to human (D) or mouse (E) Flag-IFNAR1 proteins expressed in 293T cells and immunopurified with Flag antibody. Aliquots of reactions were analyzed by autoradiography (upper panel) or immunoblotting with Flag antibody (lower panel). Input of radioactive HOS (10% of the amount added to the reactions) is also shown (i).

ubiquitination and degradation in a manner dependent on HOS and SCF<sup>HOS</sup> ligase activity, and that HOS contributes to the endocytosis of the type I IFN receptor and down-regulation of IFNAR1, as well as to regulation of cellular responses to IFN $\alpha$ .

## Results

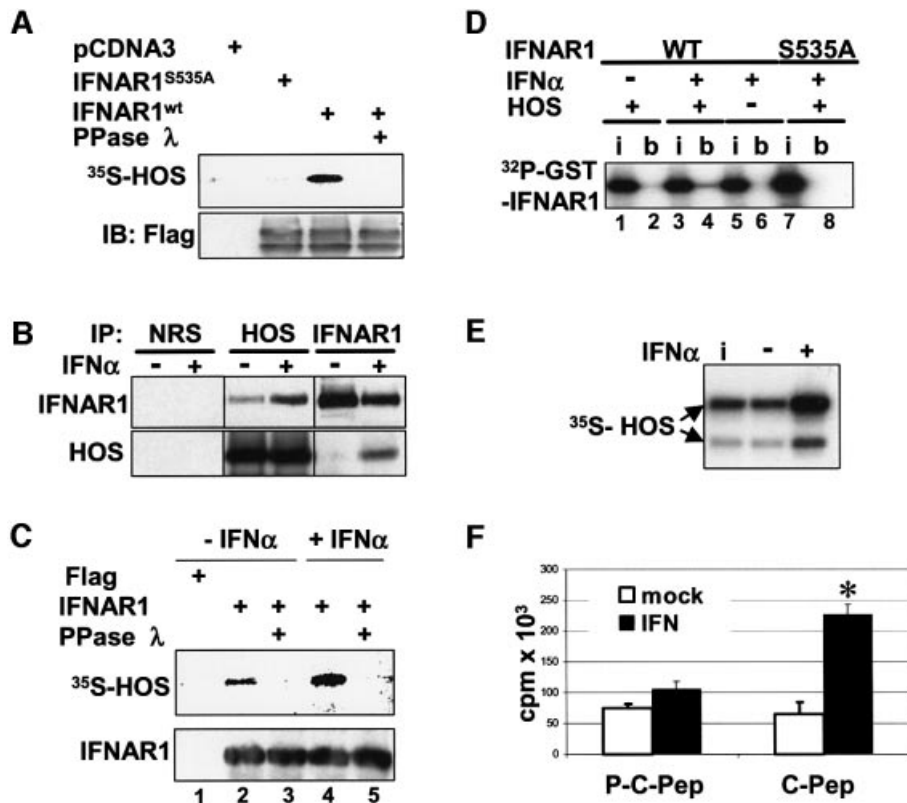
### **HOS interacts with IFNAR1 in a phosphorylation-dependent manner**

A putative HOS recognition motif within the C-terminus of IFNAR1 is conserved among different vertebrate species (Figure 1A). HOS interacted with synthetic doubly phosphorylated peptide encompassing this motif (P-C-Pep), but not with the non-phosphorylated peptide (C-Pep) *in vitro* (Figure 1B). Another F-box/WD40 repeat-containing protein Fbw2 (Cenciarelli *et al.*, 1999; Winston *et al.*, 1999a) did not associate with phospho-peptide, suggesting that the interaction of HOS with P-C-Pep was highly specific. Recombinant HA-tagged HOS and Flag-tagged IFNAR1 expressed in 293T cells were co-immunoprecipitated with respective antibodies (Figure 1C). Mutation of potentially phosphorylated serine

535 within HOS recognition motif rendered IFNAR1<sup>S535A</sup> resistant to down-regulation in response to HOS co-expression (Figure 1C, panel IV, lane 3 compared with lane 5, and lane 4 compared with lane 6) and noticeably inhibited IFNAR1 binding to HOS (panels I and II, lane 5 compared with lane 6). A residual binding of HOS to IFNAR1<sup>S535A</sup> mutant (lane 6) may be attributed to recruitment of HOS by endogenous proteins that partake in plasma membrane complexing with IFNAR1<sup>S535A</sup>. Furthermore, human and mouse Flag-IFNAR1<sup>wt</sup> interacted with HOS *in vitro*, and this interaction was not observed with either human IFNAR1<sup>S535A</sup> or murine IFNAR1<sup>S526A</sup> mutant (Figure 1D and E). These data indicate that IFNAR1 interacts with HOS *in vivo* and *in vitro* depending on the integrity of HOS recognition motif, which likely requires phosphorylation of IFNAR1 within this motif.

### **Phosphorylation of IFNAR1 and its ability to interact with HOS is promoted by IFN $\alpha$**

We further tested a role of IFNAR1 phosphorylation in binding to HOS. Phosphatase treatment of Flag-IFNAR1 decreased its ability to bind HOS *in vitro* (Figure 2A), indicating that specific phosphorylation of IFNAR1 was

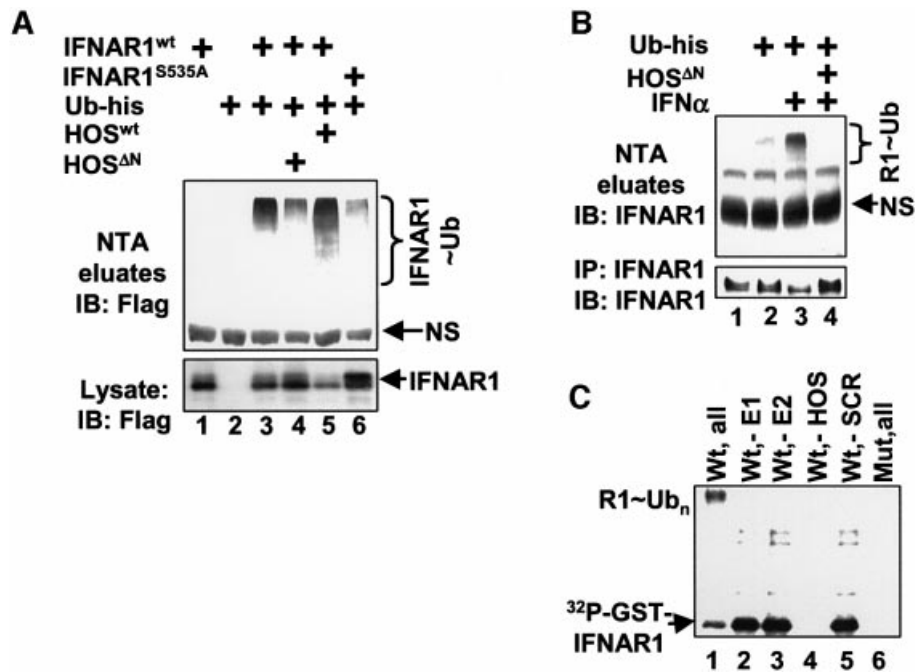


**Fig. 2.** Treatment with IFN $\alpha$  promotes phosphorylation-dependent binding of HOS to IFNAR1. (A) Binding of an IVT HOS to human Flag-IFNAR1 proteins expressed in 293T cells and immunopurified with Flag antibody before or after treatment with protein phosphatase  $\lambda$ . Aliquots of reactions were analyzed by autoradiography (upper panel) or immunoblotting with Flag antibody (lower panel). (B) Co-immunoprecipitation of endogenous HOS and IFNAR1 from 293T cells (8 mg of lysates) either treated or not with IFN $\alpha$  (4000 IU/ml for 30 min) using naïve rabbit serum (NRS) or antibodies against HOS or IFNAR1 (4B1). Immunoblotting analysis with antibodies against HOS or IFNAR1 (SC) is shown. (C) Binding of an IVT HOS to endogenous IFNAR1 immunopurified from 293T cells (treated or not with IFN $\alpha$ , 1000 IU for 30 min) with 4B1 antibody before or after treatment with protein phosphatase  $\lambda$ . Aliquots of reactions were analyzed by autoradiography (upper panel) or immunoblotting with IFNAR1 antibody (SC, lower panel). Control immunoprecipitation with Flag antibody is also shown. (D) Binding of GST-IFNAR1 proteins phosphorylated *in vitro* in the presence of [<sup>32</sup>P] $\gamma$ -ATP by extracts from 293T cells (pre-treated with IFN $\alpha$  as indicated) with SCF<sup>HOS</sup> complexes immobilized on HA-agarose. Input of GST-IFNAR1 proteins (100%, 'i') and their amount bound to SCF<sup>HOS</sup> complexes and retained on the beads after washing was analyzed by autoradiography. (E) Binding of an IVT HOS to the beads coated with C-Pep IFNAR1-derived peptide pre-phosphorylated by the extracts from 293T cells (either pre-treated or not with IFN $\alpha$ , as indicated). Reactions were analyzed by autoradiography. Input of radioactive HOS (10% of the amount added to the reactions) is also shown ('i'). (F) Phosphorylation of IFNAR1-derived peptides by the extracts from 293T cells, which were either pre-treated with IFN $\alpha$  (black bars) or not (white bars) in the presence of [<sup>32</sup>P] $\gamma$ -ATP, was analyzed by scintillation counter. A representative of two independent experiments (each in triplicate) is shown. \**P* < 0.05 (Student's *t*-test) (compared with extracts from non-treated cells).

essential for its affinity to HOS. This result also indicates that recombinant Flag-IFNAR1<sup>wt</sup> overexpressed in 293T cells is already phosphorylated and capable of binding to HOS in the absence of added ligand. This does not rule out a role for IFN $\alpha$  in HOS-IFNAR1 interaction since mammalian cells often secrete type I IFN (Pestka, 2000) and overexpression of cytokine receptors alone (e.g. tumor necrosis factor  $\alpha$  receptor; Heller *et al.*, 1992) is known to mediate signaling events. Indeed, treatment of cells with IFN $\alpha$  promoted the interaction of endogenous IFNAR1 and HOS in 293T (Figure 2B) or HeLa (data not shown) cells. Furthermore, endogenous IFNAR1 purified from the cells treated with the ligand exhibited a higher capacity for binding HOS *in vitro* compared with IFNAR1 from untreated cells. This interaction was abolished by pre-treatment of IFNAR1 with phosphatase  $\lambda$  (Figure 2C), indicating that phosphorylation of IFNAR1 is required for its association with HOS.

These results also imply that IFN $\alpha$  induces phosphorylation of IFNAR1 within the HOS recognition motif. Thus,

we tested the ability of the glutathione *S*-transferase (GST)-IFNAR1 (cytoplasmic tail) fusion protein, phosphorylated *in vitro* by extracts from 293T or HeLa cells, to bind to an immobilized SCF<sup>HOS</sup> complex. Consistent with previous findings (Colamonici *et al.*, 1994), GST-IFNAR1 proteins were efficiently phosphorylated by cell extracts in an IFN $\alpha$ -independent manner (Figure 2D, odd lanes). However, binding of SCF<sup>HOS</sup> to GST-IFNAR1 phosphorylated by extracts from the cells pre-treated with the ligand was increased compared with the extracts from untreated cells (lane 4 versus 2). Omitting HOS from the SCF<sup>HOS</sup> complex or mutating Ser535 to alanine within GST-IFNAR1 abrogated this binding (Figure 2D, lanes 6 and 8). Furthermore, whereas IFNAR1-derived non-phosphorylated C-Pep peptide failed to interact with HOS *in vitro* (Figure 1B), phosphorylation of C-Pep by cell extracts enabled this binding, and its efficiency was increased when the extracts were from the ligand-treated cells (Figure 2E). Additionally, a kinase activity assay (with cell extracts as a source of a kinase and C-Pep as a



**Fig. 3.** IFNAR1 is ubiquitinated by SCF<sup>HOS</sup> E3 ubiquitin protein ligase. **(A)** *In vivo* ubiquitination of Flag-IFNAR1 in 293T cells co-transfected with His-tagged ubiquitin and HOS constructs as indicated. Cell lysates (lower panel) and ubiquitinated proteins purified on Ni-NTA agarose under denaturing conditions (upper panel) were analyzed by immunoblotting with Flag antibody. Ubiquitinated Flag-IFNAR1 species (IFNAR1~Ub) are indicated. **(B)** *In vivo* ubiquitination of endogenous IFNAR1 in 293T cells transfected with His-tagged ubiquitin and HOS<sup>ΔN</sup> constructs and treated with IFN $\alpha$  as indicated. Endogenous IFNAR1 immunoprecipitated with 4B1 antibody (from ~10% of harvested cells, lower panel) and ubiquitinated proteins purified on Ni-NTA agarose under denaturing conditions (from ~90% of harvested cells, upper panel) were analyzed by immunoblotting with IFNAR1 antibody (SC). Ubiquitinated endogenous IFNAR1 species (R1~Ub) are indicated. **(C)** *In vitro* ubiquitination of GST-IFNAR1 proteins (wild type or S535A, 'mut') phosphorylated in the presence of [<sup>32</sup>P]γ-ATP by the extracts from 293T cells (pre-treated with IFN $\alpha$ ) and pre-bound to SCF<sup>HOS</sup> complexes or HOS only ('-SCR') immobilized on HA-agarose. After washing away the non-bound proteins, the ubiquitination reaction was carried out in the presence of ubiquitin, ATP and E1/E2 (as indicated), and analyzed by autoradiography. Ubiquitinated GST-IFNAR1 species (R1~Ub) are indicated.

substrate) showed that treating cells with IFN $\alpha$  induced the ability of cell extracts to phosphorylate C-Pep (Figure 2F). This induction was less evident when using P-C-Pep (in which Ser535 and Ser539 are pre-phosphorylated and, thus, protected from incorporation of <sup>32</sup>P) as a substrate, indicating that IFN $\alpha$  induced phosphorylation of one or both of these serines (Figure 2F). These results collectively suggest that IFN $\alpha$  induces a kinase activity that phosphorylates IFNAR1 within the HOS recognition motif and enables HOS recruitment.

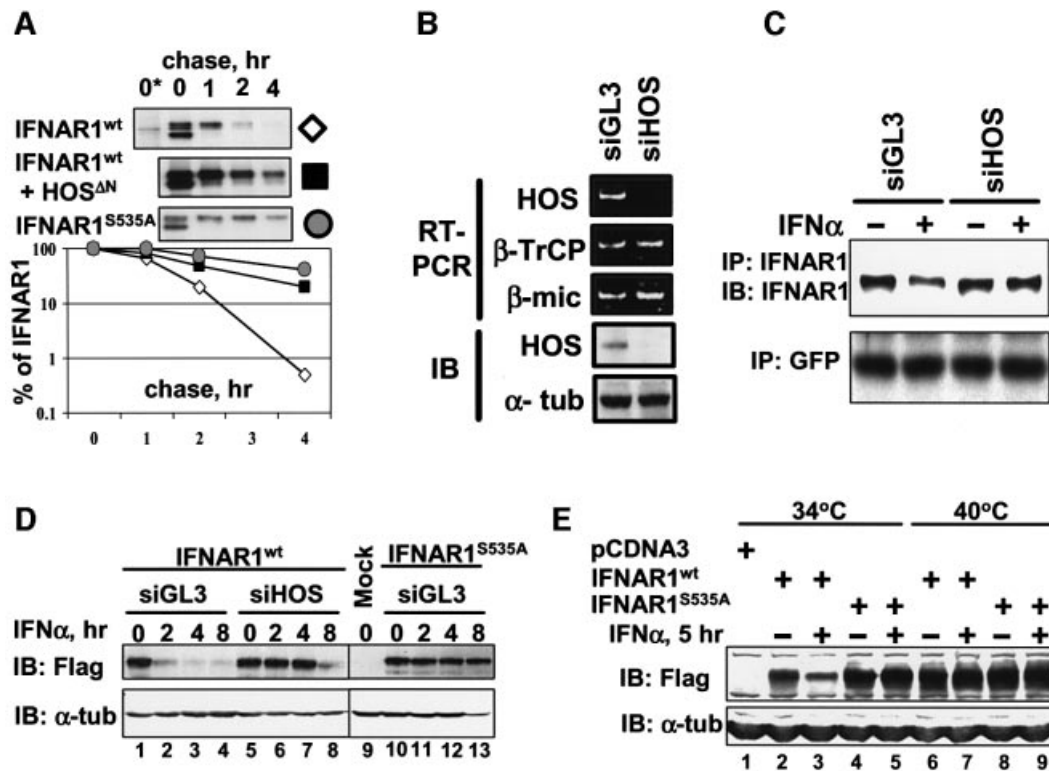
#### Ubiquitination of IFNAR1 by SCF<sup>HOS</sup> E3 ubiquitin protein ligase

Co-transfection of Flag-tagged IFNAR1 with His-tagged ubiquitin in 293T cells, followed by purification of ubiquitinated proteins on nickel resins under stringent denaturing conditions, allowed detection of ubiquitinated Flag-IFNAR1 *in vivo* (Figure 3A). Whereas the overall levels of Flag-IFNAR1 were reduced by expression of HOS (most likely due to IFNAR1 degradation), the extent of ubiquitin-conjugated Flag-IFNAR1 was noticeably increased (Figure 3A, lane 3 versus lane 5). Expression of the dominant-negative HOS<sup>ΔN</sup> mutant, which is known to compete with endogenous HOS for substrates and to inhibit the activities of SCF<sup>HOS</sup> ubiquitin ligase (Fuchs *et al.*, 1999; Suzuki *et al.*, 1999), elevated Flag-IFNAR1 levels and decreased the extent of its ubiquitination (Figure 3A, lanes 3 and 4). Compared with Flag-

IFNAR1<sup>wt</sup>, mutant IFNAR1<sup>S535A</sup> protein exhibited less ubiquitination (Figure 3A, lane 6 versus lane 3) and its extent was not affected by expression of HOS<sup>ΔN</sup> (Supplementary figure 1, available at *The EMBO Journal* Online).

Ubiquitination of endogenous IFNAR1 in 293T cells transfected with His-ubiquitin was promoted by the treatment of cells with IFN $\alpha$ , concurrent with a decrease in the levels of IFNAR1 (Figure 3B). Expression of HOS<sup>ΔN</sup> inhibited ubiquitination and prevented down-regulation of endogenous IFNAR1. These results indicate that ubiquitination of IFNAR1 *in vivo* is promoted by the ligand and mediated by HOS, although the putative contribution of other ubiquitin ligase(s) to this process cannot be ruled out. These data also suggest that HOS activities are required for down-regulation of IFNAR1 in response to IFN $\alpha$ .

The extracts from 293T cells pre-treated with IFN $\alpha$  were used to phosphorylate GST-IFNAR1 fusion protein captured on immobilized recombinant SCF<sup>HOS</sup> ubiquitin ligase (Tan *et al.*, 1999) for recapitulation of IFNAR1 ubiquitination *in vitro*. This reaction yielded a high molecular weight smear characteristic of ubiquitinated proteins (Figure 3C, lane 1). Omission of either E1 or E2 severely reduced the intensity of this smear (lanes 2 and 3), indicating that it represented *in vitro* ubiquitinated phospho-GST-IFNAR1. The extent of ubiquitination was decreased when Skp1, Cullin1 and Roc1 were omitted



**Fig. 4.** SCF<sup>HOS</sup> regulates IFNAR1 stability and levels. (A) Pulse-chase analysis of human Flag-IFNAR1 proteins expressed in 293T cells (metabolically labeled with [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine) and immunoprecipitated with Flag antibody, and analyzed by autoradiography. Asterisk denotes the mock-transfected cells. A representative of four independent experiments is shown. (B) Inhibition of HOS expression by siRNA against HOS (siHOS) or luciferase (siGL3) transfected into HeLa cells. Depicted are the levels of HOS, and β-TrCP1 and β-microglobulin mRNA (measured by RT-PCR, three upper panels), and HOS and α-tubulin proteins (measured by immunoblotting, two lower panels). (C) Effect of IFNα on the levels of endogenous IFNAR1 in 293T cells co-transfected with the indicated siRNA oligonucleotides and GFP plasmid. IFNAR1 was immunoprecipitated and analyzed by immunoblotting (with 4B1 antibody and SC antibody, respectively). Levels of GFP analyzed by immunoblotting are also shown. (D) Effect of IFNα (2000 IU/ml) on the levels of human Flag-IFNAR1 proteins co-transfected with the indicated siRNA oligonucleotides and β-gal construct in HeLa cells. Loading was normalized per β-gal activity and the levels of proteins were analyzed by immunoblotting with Flag antibody (upper panel) or α-tubulin antibody (lower panel). (E) Effect of IFNα (1000 IU/ml for 5 h) on the levels of human Flag-IFNAR1 proteins expressed in hamster ts41 cells grown at the indicated temperatures. Immunoblotting with Flag antibody (upper panel) or α-tubulin antibody (lower panel) is depicted.

from the immobilized material (Figure 3C, lane 5). Consistent with the data shown in Figure 2D, omitting HOS from immobilized complexes or the use of the mutant GST-IFNAR1<sup>S535A</sup> prevented capturing phospho-GST-IFNAR1 (lanes 4 and 6). These observations provide biochemical evidence suggesting that SCF<sup>HOS</sup> is capable of serving as an E3 protein ubiquitin ligase for phosphorylated IFNAR1.

#### HOS is required for IFNAR1 degradation

We next investigated whether HOS regulates proteolysis of IFNAR1. As analysis of the half-life of endogenous IFNAR1 is technically challenging (Gauzzi *et al.*, 1997; data not shown), we determined the half-life of Flag-IFNAR1 expressed in 293T cells, which interacted with HOS (Figure 1C and D) and underwent ubiquitination (Figure 3A) in the absence of exogenously added IFNα. Overexpressed IFNAR1 appeared in the form of a doublet (Figures 1C–E and 3A), which was consistent with observations of another group who clearly demonstrated that a slower migrating form of IFNAR1 represents the mature IFNAR1 species (Ragimbeau *et al.*, 2003). As evident from the pulse-chase experiments (Figure 4A), Flag-IFNAR1<sup>S535A</sup> mutant exhibited a substantially longer half-life than Flag-IFNAR1<sup>wt</sup>, which might reflect poor

ability of this mutant to interact with HOS (Figure 1C and D) and undergo ubiquitination (Figure 3A). Moreover, degradation of Flag-IFNAR1<sup>wt</sup> was delayed by co-expression of HOS<sup>ΔN</sup> (Figure 4A). These results suggest that HOS plays a major role in regulating IFNAR1 proteolysis.

#### HOS levels and Cullin1 activity are essential for down-regulation of IFNAR1 in response to IFNα

We further examined the role of HOS in down-regulation of IFNAR1 in response to its ligand using short inhibitory RNA (siRNA) technology. Transfection of HeLa (Figure 4B) or 293T cells (data not shown) with siRNA against HOS (siHOS) decreased the steady-state levels of HOS mRNA and protein (without affecting the expression of closely related β-TrCP1 gene) compared with the control siGL3. Treatment of cells with IFNα decreased the levels of endogenous IFNAR1, and this decrease was abrogated by siHOS in 293T cells (Figure 4C) and in HeLa (not shown) cells. These data, together with our findings that the HOS<sup>ΔN</sup> mutant inhibits IFNα-induced down-regulation of endogenous IFNAR1 (Figure 3B), suggest that HOS expression and activities are essential for this down-regulation.

Treatment with IFNα decreased the levels of IFNAR1<sup>wt</sup> (but not of IFNAR1<sup>S535A</sup> mutant) co-transfected with

siGL3, whereas co-transfection with siHOS (Figure 4D), but not with si $\beta$ TrCP (directed against  $\beta$ -TrCP1; Supplementary figure 2), substantially delayed IFNAR1<sup>wt</sup> down-regulation in HeLa cells. These results together with the data shown in Figure 4A indicate that down-regulation of overall levels of IFNAR1 in response to IFN $\alpha$  parallels degradation of IFNAR1. These observations provide genetic evidence suggesting that HOS is essential for degradation of IFNAR1 in response to its ligand.

Stability of SCF<sup>HOS</sup> substrates depends on activity of Cullin1 (Fuchs *et al.*, 1999; Tan *et al.*, 1999), which requires its modification by ubiquitin-like protein NEDD8 (Read *et al.*, 2000; Wu *et al.*, 2000). The recent finding that the NEDD8 conjugating system is impaired in hamster ts41 cells at a non-permissive temperature (40°C) (Chen *et al.*, 2000) makes these cells a convenient model for studying Cullin-dependent protein degradation (Ohh *et al.*, 2002). Levels of Flag-IFNAR1<sup>wt</sup> were down-regulated by IFN $\alpha$  in ts41 cells at a permissive temperature (34°C). However, shifting the temperature to 40°C led to the accumulation of IFNAR1<sup>wt</sup> and rendered it insensitive to the ligand (Figure 4E), although IFN $\alpha$  activity was not impaired in ts41 cells [as assessed by the induction of interferon-stimulated response element (ISRE)-luciferase reporter; data not shown]. Neither temperature shift nor IFN $\alpha$  treatment affected the levels of IFNAR1<sup>S535A</sup> mutant, indicating that alterations in the levels of IFNAR1<sup>wt</sup> most likely resulted from changes in the rate of proteolysis. These data indirectly point to a role for Cullin proteins in IFNAR1 down-regulation and (together with other findings) implicate SCF<sup>HOS</sup> in regulating the stability of IFNAR1.

### **HOS regulates internalization of the type I IFN receptor and cell surface levels of IFNAR1**

Ubiquitination of cell surface receptors is implicated in their endocytosis and degradation via the lysosomal pathway (Hicke, 1999; Katzmann *et al.*, 2001). Treatment of cells with methylamine HCl, which inhibits the lysosomal pathway (Reijngoud *et al.*, 1976), delayed the degradation of endogenous IFNAR1 (Figure 5A) and transiently expressed Flag-IFNAR1<sup>wt</sup> (Figure 5B) in cells treated with IFN $\alpha$ . Methylamine HCl (as well as with chloroquine) also prolonged the half-life of Flag-IFNAR1<sup>wt</sup> expressed in 293T cells (Supplementary figure 3). Inhibition of proteasomes by lactacystin led to the accumulation of c-Jun (Figure 5A), but did not affect degradation of IFNAR1 (Figure 5A and B). These results indicate that IFNAR1 is mainly degraded via the lysosomal pathway, although the role of proteasomes cannot be entirely ruled out.

Inhibiting HOS activities by expression of HOS<sup>ΔN</sup> prevented IFN $\alpha$ -mediated down-regulation of endogenous cell surface IFNAR1 (Figure 5C). We next assessed a role of HOS in the endocytosis of type I IFN receptor by determining the initial rates of its internalization. Expression of HOS<sup>ΔN</sup> significantly inhibited internalization of endogenous receptor in 293T cells (Figure 5D). Moreover, a slower rate of type I IFN receptor internalization was detected in mouse embryo fibroblasts (MEF) from IFNAR1-null cells transfected with mouse IFNAR1<sup>S526A</sup> mutant (which did not interact with HOS;

Figure 1E) compared with MEF transfected with IFNAR1<sup>wt</sup> (Figure 5E). This result is in line with the failure of IFN $\alpha$  to down-regulate the levels of surface receptor in cells expressing IFNAR1<sup>Δ525–557</sup> mutant (Basu *et al.*, 1998). These data collectively indicate that HOS is required for down-regulation of cell surface IFNAR1 and internalization of the entire type I IFN receptor in response to IFN $\alpha$ .

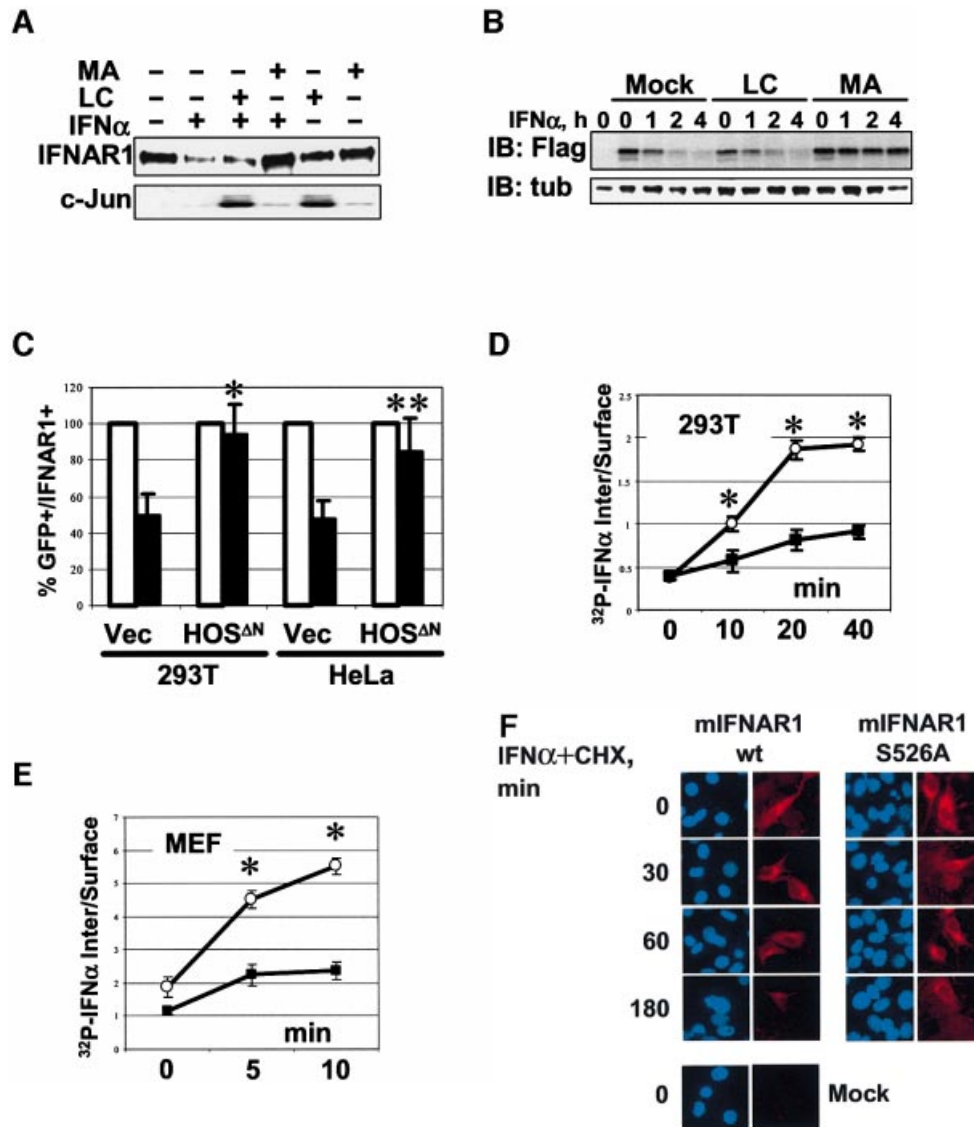
Mouse Flag-tagged IFNAR1 proteins were detected at the plasma membrane and were distributed throughout the cell (Figure 5F). Treatment of cells with IFN $\alpha$  and cycloheximide (to inhibit *de novo* protein synthesis) led to a noticeable re-distribution of the wild-type Flag-IFNAR1 from the cell surface to the perinuclear compartments within 30–60 min, followed by a drastic decrease in overall IFNAR1 levels. In contrast, mouse Flag-IFNAR1<sup>S526A</sup> protein exhibited a prominent decoration of the cell surface up to 60 min after treatment, and a higher level of expression compared with the wild-type protein (Figure 5F). These data indicate that down-regulation of IFNAR1 is likely to depend on the ability of IFNAR1 to interact with HOS.

### **HOS is involved in the regulation of cellular responses to IFN $\alpha$**

The extent of cellular responses to IFN $\alpha$  depends on the dose of IFNAR1 gene (Hwang *et al.*, 1995), suggesting that HOS-mediated alteration of IFNAR1 levels via changes in its stability is expected to influence the outcome of IFN $\alpha$  signaling. Indeed, phosphorylation of Stat1 at Tyr701 in IFN $\alpha$ -treated cells was moderately decreased by HOS overexpression (at higher doses of IFN $\alpha$ ), but augmented by the expression of HOS<sup>ΔN</sup> (especially at lower doses of IFN $\alpha$ ; Figure 6A). In IFNAR1-null MEF, re-expression of mouse Flag-IFNAR1 proteins (although undetectable by Flag immunoblotting) allowed analysis of Stat1 phosphorylation in response to IFN $\alpha$ , and the IFNAR1<sup>S526A</sup> mutant was more efficient in this assay than IFNAR1<sup>wt</sup> (Figure 6B). Moreover, whereas co-expression of HOS noticeably decreased the ability of IFNAR1<sup>wt</sup> to transduce signals, resulting in Stat1 phosphorylation, the effects of the IFNAR1<sup>S526A</sup> mutant were hardly affected. These data suggest that HOS contributes to the regulation of the extent of IFN $\alpha$  signaling, most likely by controlling IFNAR1 stability and abundance.

We next determined the effects of HOS on transcriptional activity of the Stat1–Stat2–p48<sup>IRF9</sup> complex (ISGF3) measured by ISRE-driven luciferase reporter activity. Expression of HOS significantly inhibited the induction of this activity by IFN $\alpha$  in 293T cells, whereas HOS<sup>ΔN</sup> mutant produced an opposite result (Figure 6C). Furthermore, the experiments with re-expression of mouse IFNAR1 in IFNAR1-null MEF showed that mouse Flag-IFNAR1<sup>S526A</sup> mutant was more efficient in activation of ISGF3 than the wild-type mouse IFNAR1 (Figure 6D). These data collectively suggest that regulation of IFNAR1 stability by HOS contributes to the outcome of cellular responses to IFN $\alpha$ .

In the absence of the ligand, expression of IFNAR1 inhibited the proliferation of K562 human leukemia cells (Colamonici *et al.*, 1994), and the growth rate of K562 cells stably transfected with IFNAR1<sup>S535A</sup> underwent more

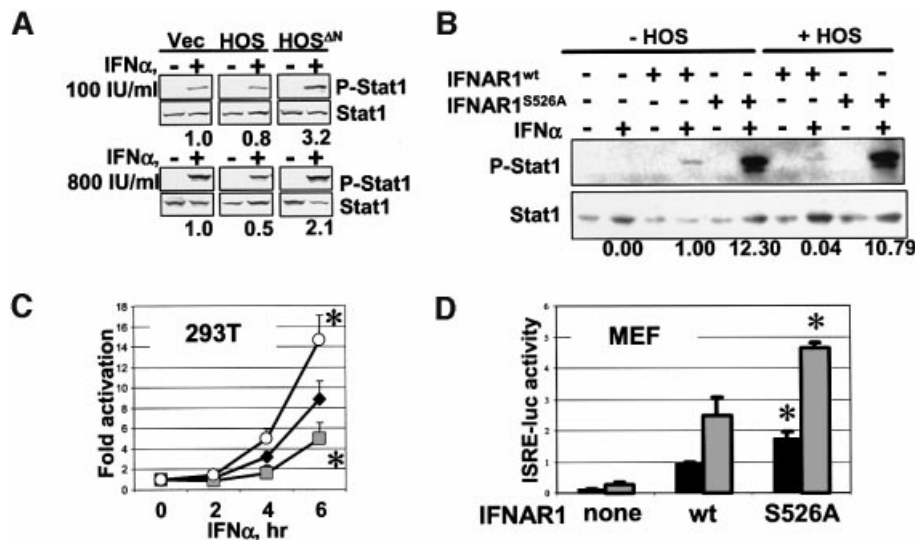


**Fig. 5.** Down-regulation of IFNAR1 cell surface levels and endocytosis of type I IFN receptor in response to IFN $\alpha$  depends on HOS. (A) Inhibition of lysosomal pathway prevents down-regulation of endogenous IFNAR1 by IFN $\alpha$  (1000 IU/ml for 2 h) in 293T cells. Cells were treated with IFN $\alpha$  with or without adding either proteasome inhibitor lactacystin (LC, 50  $\mu$ M) or lysosomal pathway inhibitor methylamine HCl (MA, 20 mM), as indicated. Immunoblotting analyses of IFNAR1 (immunoprecipitated with 4B1 antibody), as well as *c-Jun* are shown. (B) Inhibition of lysosomal pathway prevents down-regulation of human Flag-IFNAR1 expressed in HeLa cells together with  $\beta$ -gal construct. Cells were treated with IFN $\alpha$  (1000 IU/ml) and either lactacystin or methylamine HCl (as in Figure 5A), as indicated. Lane 1 represents mock-transfected cells. Loading was normalized per  $\beta$ -gal activity and the levels of proteins were analyzed by immunoblotting with Flag antibody (upper panel) or  $\alpha$ -tubulin antibody (lower panel). (C) The levels of endogenous IFNAR1 on the surface of HeLa and 293T cells transfected with GFP-expressing plasmid and either HOS $\Delta$ N or empty vector ('Vec'), and treated with IFN $\alpha$  (1000 IU/ml for 2 h) for the times indicated. Average data of five experiments are shown. Depicted are the percentages of GFP-positive and IFNAR1-positive cells (analyzed by FACS with AA3 monoclonal antibody against the extracellular domain of IFNAR1) in the absence (white bars) or presence (black bars) of IFN $\alpha$ . \* $P$  < 0.01 and \*\* $P$  < 0.05 compared with empty vector (Student's *t*-test). (D) Rate of the type I IFN receptor endocytosis as measured by internalization of  $^{32}$ P-labeled IFN $\alpha$  in 293T cells transfected with either HOS $\Delta$ N (closed squares) or empty vector (open circles). Ratio of acid-resistant internal radioactivity to cell surface radioactivity was assessed in two independent experiments (each in triplicate). \* $P$  < 0.01 (Student's *t*-test). (E) Rate of type I IFN receptor endocytosis (measured as in Figure 5D) in IFNAR1-null MEF transfected with mouse IFNAR1 constructs (wild type, open circles; S526A mutant, closed squares). \* $P$  < 0.01 (Student's *t*-test). (F) The levels of flag-tagged murine IFNAR1<sup>wt</sup> and IFNAR1<sup>S526A</sup> proteins expressed in NIH 3T3 cells treated with IFN $\alpha$  (1000 IU/ml) and cycloheximide (50  $\mu$ g/ml) for the indicated times, fixed, permeabilized and analyzed by immunocytochemistry with M2 Flag antibody (red) and counterstaining with DAPI (blue) for detection of nuclei. Results representative of two independent experiments are shown.

pronounced inhibition compared with IFNAR1<sup>wt</sup> (Figure 7A). Since IFNAR1<sup>S535A</sup> mutant is insensitive to HOS-mediated ubiquitination and degradation (Figures 3 and 4), this finding indicates that the stability of IFNAR1 contributes to the extent of its antiproliferative potential. Furthermore, expression of HOS $\Delta$ N sensitized WM115

human melanoma cells to growth inhibition by IFN $\alpha$  (Figure 7B) or IFN $\beta$  (Figure 7C), presumably via the combination of augmenting ISGF3 transcriptional activity (Figure 6C and D) and inhibiting NF- $\kappa$ B transcriptional activity (Fuchs *et al.*, 1999), which is otherwise increased by IFN $\alpha$  (Yang *et al.*, 2000).





**Fig. 6.** HOS regulates the IFN $\alpha$  signaling pathway. (A) Immunoblotting analysis of phospho-Stat1/Stat1 in 293T cells transfected and treated with IFN $\alpha$  (100 IU/ml, upper panel or 800 IU/ml, lower panel) for 30 min as indicated. Relative ratio between phospho-Stat1 and Stat1 densitometric values is depicted in numbers below. Vec, empty vector. (B) Immunoblotting analysis of phospho-Stat1/Stat1 in IFNAR1-null MEF transfected with either mIFNAR1<sup>wt</sup> or mIFNAR1<sup>S526A</sup> mutant (with or without HOS) as indicated. Cells were treated with IFN $\alpha$  (1000 IU/ml for 30 min) as indicated. Relative ratio between phospho-Stat1 and Stat1 densitometric values is depicted in numbers below. (C) IFN $\alpha$ -dependent activation of 5 $\times$  ISRE-luc reporter in 293T cells transfected with either vector (black diamonds) or HOS (gray squares), or HOS<sup>AN</sup> (white circles) from three independent experiments (each in triplicate). Depicted is the fold activation of normalized luciferase activity values (over values in cells not treated with IFN $\alpha$ ). \* $P$  < 0.05 compared with cells transfected with vector (Student's  $t$ -test). (D) 5 $\times$  ISRE-luc reporter activity in IFNAR1-null MEF transfected with either mIFNAR1<sup>wt</sup> or mIFNAR1<sup>S526A</sup> mutant as indicated. Depicted (in arbitrary units) are the normalized luciferase values from the cells treated with IFN $\alpha$  (500 IU for 6 h, gray bars) and from untreated cells (black bars). \* $P$  < 0.05 compared with mIFNAR1<sup>wt</sup> (Student's  $t$ -test).

## Discussion

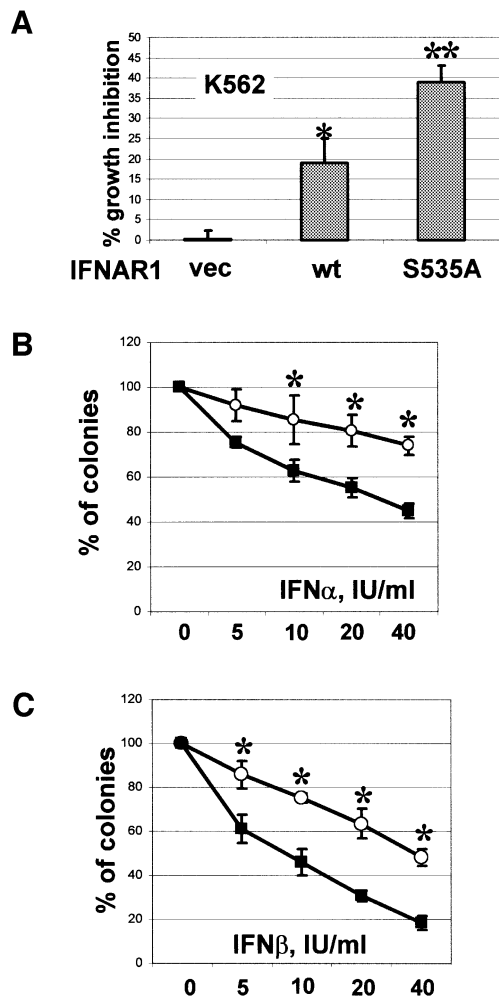
The data presented here collectively delineate the mechanisms underlying the ligand-induced down-regulation of IFNAR1 that include phosphorylation-dependent binding (Figures 1 and 2) and ubiquitination (Figure 3) of IFNAR1 by SCF<sup>HOS</sup> E3 ubiquitin protein ligase, followed by internalization of the cell surface receptor and degradation of IFNAR1 via the lysosomal pathway (Figures 4 and 5). Our findings also implicate SCF<sup>HOS</sup> ubiquitin ligase in the regulation of the extent of IFN $\alpha$  signaling (Figure 6) and cellular responses to type I interferons (Figure 7). SCF<sup>HOS</sup>-dependent regulation of IFNAR1 is likely to represent an important mechanism that contributes to negative regulation of cytokine signaling among other such mechanisms, including suppressor of cytokine signaling- and phosphatase-mediated inhibition of Janus kinase levels and activity, and the effects of protein inhibitors of activated STAT (reviewed in Greenhalgh and Hilton, 2001). As a conserved HOS recognition motif is also found within the intracellular domains of some other cytokine receptor subunits that are related to IFNAR1 (e.g. interleukin-10 receptor  $\alpha$  chain), future studies will show whether SCF<sup>HOS</sup>-dependent ubiquitination may represent a common mechanism in the regulation of stability of these receptors. Further detailed studies are also required to identify and characterize an IFN $\alpha$ -inducible kinase(s) that phosphorylates IFNAR1 at the HOS recognition site.

SCF<sup>HOS</sup> E3 ubiquitin ligase mediates ubiquitination and degradation of I $\kappa$ B and  $\beta$ -catenin (Fuchs *et al.*, 1999; Suzuki *et al.*, 1999). Here we identify IFNAR1 as a novel substrate for this ligase. Another F-box/WD40 domain-containing protein  $\beta$ -TrCP1 (Margottin *et al.*,

1998), which is closely related to HOS, also recognizes phosphorylated DpSGXXp motifs in I $\kappa$ B and  $\beta$ -catenin (Yaron *et al.*, 1998; Spencer *et al.*, 1999; Winston *et al.*, 1999b), and is capable of binding to the IFNAR1-derived phospho-peptide (data not shown). Evidence that specific inhibition of HOS (but not  $\beta$ -TrCP1) expression by siRNA suffices to block IFNAR1 down-regulation (Figure 4 and Supplementary figure 2), together with the findings of other groups that  $\beta$ -TrCP1 is primarily localized to the nucleus (Sadot *et al.*, 2000; Lassot *et al.*, 2001; Davis *et al.*, 2002) and no apparent alterations of IFN $\alpha$  signaling are observed in  $\beta$ -TrCP1-knockout mice (Guardavaccaro *et al.*, 2003; Nakayama *et al.*, 2003), suggest that HOS plays a predominant role in regulating IFNAR1 stability. Nevertheless, a potential contribution of SCF <sup>$\beta$ -TrCP1</sup> to IFNAR1 ubiquitination and degradation cannot be ruled out, especially under conditions that robustly increase  $\beta$ -TrCP1 levels (e.g. activation of Wnt signaling; Spiegelman *et al.*, 2000). Ubiquitin ligases of the SCF family are involved in control of stability of many substrates, including cell cycle regulators, transcription factors and signaling proteins. Whereas the role of these E3s in proteasomal degradation of these substrates has been well established (Deshaies, 1999), this report implicates SCF ligases in regulating endocytosis and lysosomal proteolysis of a cytokine receptor.

Our data show that HOS regulates an initial rate of internalization of the type I IFN receptor (Figure 5). It remains to be determined how SCF<sup>HOS</sup>-mediated ubiquitination of IFNAR1 targets this receptor for its degradation via the lysosomal pathway. It is conceivable that, similar to the effect of ubiquitination on the yeast





**Fig. 7.** HOS regulates cellular responses to IFN $\alpha$ . (A) Proliferation of K562 cells stably transfected with either vector ('vec') or IFNAR1 (wild type or S535A mutant), as assessed 3 days after plating with the aid of the WST-1 Cell Proliferation kit. Depicted is the percentage of growth inhibition (compared with the cells transfected with vector) as average data from five experiments. \* $P < 0.05$  compared with vector, and \*\* $P < 0.05$  compared with IFNAR1<sup>wt</sup> (Student's *t*-test). (B–C) Colony-forming efficiency of WM115 human melanoma cells co-transfected with pBABE-puro and either vector (white circles) or HOS<sup>ΔN</sup> (black squares). Cells were grown in the presence of puromycin and either IFN $\alpha$  (B) or IFN $\beta$  (C), and the number of colonies was scored. Average data (in percentage of cells grown in the absence of IFN) obtained from three independent experiments (in triplicates) are depicted. \* $P < 0.05$  compared with cells transfected with vector (Student's *t*-test).

$\alpha$ -factor receptor Ste2 (Terrell *et al.*, 1998) or mammalian growth factor receptors (Haglund *et al.*, 2003), conjugation of ubiquitin within the intracellular tail of IFNAR1 creates an internalization signal, which is recognized by the endocytosis machinery. IFNAR2 is likely to co-internalize within the context of the entire receptor, and our data (Figure 5D and E) indirectly support this possibility. In addition, ubiquitination of IFNAR1 may also affect post-internalization sorting/recycling of IFNAR1 in a manner similar to the mechanism that contributes to the degradation of the epidermal growth factor receptor ubiquitinated by c-Cbl (Levkowitz *et al.*, 1998).

Until recently, little was known about the mechanisms of type I IFN receptor down-regulation. It has been reported that IFNAR1 protein levels are decreased in human (Gauzzi *et al.*, 1997) but not murine (Karaghiosoff *et al.*, 2000; Shimoda *et al.*, 2000) cells, which lack Tyk2 kinase expression. Recent evidence indicates that restoration of Tyk2 expression maintains IFNAR1 cell surface levels and inhibits basal internalization and degradation of IFNAR1 proteins, including an IFNAR1 deletion mutant that lacks an HOS recognition motif (Ragimbeau *et al.*, 2003). Our data point to the role of the SCF<sup>HOS</sup> ubiquitin ligase in the ligand-dependent degradation of IFNAR1 in human and murine cells with no apparent defects in Tyk2. Future studies will determine physiological or pathological conditions under which the Tyk2-dependent and HOS-dependent mechanisms of IFNAR1 down-regulation and degradation may overlap.

Our data show that inhibition of HOS sensitizes human melanoma cells to the anti-proliferative effects of type I IFN. Considering a wide use of these cytokines in the therapy of human tumors (Kirkwood, 2002), chronic viral infections (Davis, 2001) and multiple sclerosis (Khan *et al.*, 2002), our findings imply that targeting HOS-mediated IFNAR1 proteolysis may provide novel means for optimization of these therapeutic efforts.

## Materials and methods

A detailed description of the materials and methods is provided as Supplementary data (available at *The EMBO Journal* Online).

### Materials

Recombinant IFN $\alpha$  A/D that is active in human and rodent cells was purchased from Sigma. Recombinant IFN $\alpha$  A/D P1 containing a phosphorylation site is described elsewhere (Wang *et al.*, 1994). Double phosphorylated [P-C-Pep; EDHKKYSSQTSQDS-(PO<sub>3</sub>)-GNYS-(PO<sub>3</sub>)-NEDESES] or non-phosphorylated (C-Pep) IFNAR1-derived synthetic peptides were immobilized on agarose beads.

### Plasmids

Human or mouse IFNAR1 cDNA (kind gifts from J.Krolewski and G.Uze), with the addition of a 3 $\times$  Flag tag encoding sequence at the C-terminus, were subcloned into pCDNA3 vector (Invitrogen) via PCR, and mutations were introduced by site-directed mutagenesis. Constructs for the expression of HA-HOS and HOS<sup>ΔN</sup> (Fuchs *et al.*, 1999), His-ubiquitin (Treier *et al.*, 1994), Fbw2 (Cenciarelli *et al.*, 1999), Cul1 and Skp1 (Ohta *et al.*, 1999), Roc1 (Tan *et al.*, 1999) and 5 $\times$  ISRE-luciferase reporter (Parisien *et al.*, 2002) were described previously. Chemically synthesized siRNA duplexes against luciferase (siGL3), HOS (siHOS) and  $\beta$ -TrCP1 (si $\beta$ TrCP) were purchased from Dharmacon.

### Tissue culture and transfections

293T, HeLa, NIH 3T3 and WM115 cells were kindly provided by Z.Ronai. Hamster ts41 cells (Chen *et al.*, 2000) and mouse embryo fibroblasts (MEF) from IFNAR1-knockout mice (Muller *et al.*, 1994) were generous gifts from R.Neve and S.Hemmi. Cells were grown in DMEM or DMEM/F-12 (1:1) in the presence of 10% fetal bovine serum (FBS) and antibiotics at 37°C and 5% CO<sub>2</sub>. Hamster ts41 cells were grown in DMEM supplemented with 10% calf serum, antibiotics and fungizone at 34°C and 5% CO<sub>2</sub>. These cells were shifted to 40°C 24 h before harvesting, where indicated. Transfections were performed using calcium phosphate procedure or lipofection (with Lipofectamine Plus or Lipofectamine 2000; Invitrogen) 24–48 h before harvesting.

### Antibodies and immunotechniques

4B1 (Constantinescu *et al.*, 1994) and AA3 (Goldman *et al.*, 1999) (a kind gift of Dr M.Zafari) monoclonal antibodies against the extracellular domain of IFNAR1 have been described previously. Antibodies against HA (Roche), Flag (M2, Sigma), GFP (Clontech), c-Jun and IFNAR1 (against the intracellular domain; Santa Cruz), as well as against phospho-

Stat1 and Stat1 (Cell Signaling Technology) were purchased. Antibody against HOS, as well as immunoprecipitation, immunocytochemistry and immunoblotting procedures, have been described previously (Spiegelman *et al.*, 2002).

#### **In vitro binding assays**

Recombinant IFNAR1 expressed in 293T cells or endogenous IFNAR1 proteins were immunopurified with Flag or 4B1 antibodies, respectively, and protein A beads, and stringently washed. IFNAR1 proteins or IFNAR1-derived synthetic peptides immobilized on the beads were incubated with *in vitro*-translated and  $^{35}\text{S}$ -labeled HOS, followed by washing and analysis by SDS-PAGE and autoradiography.

#### **Ubiquitination and degradation assays**

*In vivo* ubiquitination assays were carried out as described previously (Treier *et al.*, 1994). For *in vitro* ubiquitination assay, GST-IFNAR1 proteins were expressed in bacteria and phosphorylated by the extracts from IFN $\alpha$ -treated cells in the presence of [ $^{32}\text{P}$ ] $\gamma$ -ATP. Proteins were captured on immobilized SCF<sup>HOS</sup> (Tan *et al.*, 1999), washed and incubated with ubiquitin, E1, Cdc34 and ATP at 37°C for 60 min followed by SDS-PAGE and autoradiography. Pulse-chase analysis was carried out on 293T cells transfected with indicated constructs as described elsewhere (Fuchs *et al.*, 1999). Briefly, cells were grown in 100 mm dishes and transfected with indicated plasmids. After metabolic labeling with  $^{35}\text{S}$ [methionine]/ $^{35}\text{S}$ [S-cysteine] mixture, cells from one-quarter of the sector of each plate were harvested at each time point of the chase. Harvested cells were lysed and IFNAR1 proteins were immunoprecipitated with Flag antibody, separated on SDS-PAGE and analyzed by autoradiography.

#### **Endocytosis assays**

Bacterially expressed IFN $\alpha$  A/D P1 was phosphorylated by cAMP-dependent kinase in the presence of [ $^{32}\text{P}$ ] $\gamma$ -ATP as described elsewhere (Wang *et al.*, 1994). 293T cells or IFNAR1-null MEF (transfected with indicated plasmids) were incubated with labelled IFN $\alpha$  A/D P1 on ice, washed and incubated at 37°C for different time points to allow ligand internalization. Cells were washed, the cell surface-bound IFN $\alpha$  was removed by acid wash, and the cells were lysed to release the internalized IFN $\alpha$ . Amounts of radioactivity in both fractions were determined using a scintillation counter.

#### **Luciferase reporter assays**

293T cells were transfected with 5 $\times$  ISRE-luc and pBABE-puro plasmids, and selected in media supplemented with puromycin (1  $\mu\text{g}/\text{ml}$ ) for 5 days. Then the cells were co-transfected with renilla luciferase vector and HOS plasmids or empty vector and treated with IFN $\alpha$  (500 IU/ml) for the indicated periods of time before harvesting. IFNAR1-null MEF were co-transfected with 5 $\times$  ISRE-luc and renilla luciferase vector and treated with IFN $\alpha$  (1000 IU/ml for 6 h). Luciferase activities were determined using the Dual Luciferase Assay Kit (Promega).

#### **Supplementary data**

Supplementary data are available at *The EMBO Journal* Online.

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