Phosphorylation of the Antiviral Protein Interferon-inducible Transmembrane Protein 3 (IFITM3) Dually Regulates Its Endocytosis and Ubiquitination*

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Background: IFITM3 restricts the fusion of viruses within endolysosomes.
Results: Phosphorylation of IFITM3 on Tyr20 blocks IFITM3 endocytosis and ubiquitination.
Conclusion: Tyr20 of IFITM3 is part of a YXXΦ endocytosis signal and has a dual role in regulating IFITM3 ubiquitination.
Significance: Identifying mechanisms regulating IFITM3 trafficking and activity is crucial for understanding and manipulating IFITM3 biology for combating virus infections.

Interferon-inducible transmembrane protein 3 (IFITM3) is essential for innate defense against influenza virus in mice and humans. IFITM3 localizes to endolysosomes where it prevents virus fusion, although mechanisms controlling its trafficking to this cellular compartment are not fully understood. We determined that both mouse and human IFITM3 are phosphorylated by the protein-tyrosine kinase FYN on tyrosine 20 (Tyr20) and that mouse IFITM3 is also phosphorylated on the non-conserved Tyr27. Phosphorylation led to a cellular redistribution of IFITM3, including plasma membrane accumulation. Mutation of Tyr20 caused a similar redistribution of IFITM3 and resulted in decreased antiviral activity against influenza virus, whereas Tyr27 mutation of mouse IFITM3 showed minimal effects on localization or activity. Using FYN knockout cells, we also found that IFITM3 phosphorylation is not a requirement for its antiviral activity. Together, these results indicate that Tyr20 is part of an endocytosis signal that can be blocked by phosphorylation or by mutation of this residue. Further mutagenesis narrowed this endocytosis-controlling region to four residues conforming to a YXXΦ (where Φ is any amino acid and Φ is Val, Leu, or Ile) endocytic motif that, when transferred to CD4, resulted in its internalization from the cell surface. Additionally, we found that phosphorylation of IFITM3 by FYN and mutagenesis of Tyr20 both resulted in decreased IFITM3 ubiquitination. Overall, these results suggest that modification of Tyr20 may serve in a cellular checkpoint controlling IFITM3 trafficking and degradation and demonstrate the complexity of posttranslational regulation of IFITM3.

The interferon-inducible transmembrane proteins (IFITMs) inhibit cellular infection by a wide range of significant viral pathogens (1–9). IFITM3 is particularly important for the restriction of influenza virus. IFITM3 knockout mice succumb to sublethal doses of virus (10, 11), and a deleterious polymorphism in the human IFITM3 gene has been associated with increased severity of infection in at least three independent studies (10, 12, 13). Despite the clear importance of IFITM3 in restricting influenza virus, many questions remain regarding its mechanism of action, cellular trafficking patterns, and regulation by cellular enzymes.

IFITM3 localizes to acidic compartments, staining positively for endosomal and lysosomal markers (2, 4, 14), where it prevents viral fusion through an unknown mechanism (3, 14, 15). Experiments with pseudotyped viruses demonstrated that inhibition of viruses by IFITM3 is dependent upon the viral fusion glycoprotein used for cellular entry (1). Likewise, nearly all of the viruses shown to be inhibited by IFITM3 enter cells via endocytosis (16, 17). Conversely, Sendai virus, which fuses at the cell surface, is largely unaffected by IFITM3 (18). Similarly, exogenous protease treatment of severe acute respiratory syndrome-associated coronavirus allows it to fuse at the cell surface, thereby bypassing its pH-dependent activation in lysosomes and restriction by IFITM3 (2). Thus, endolysosomes appear to be the site of antiviral action by IFITM3, and the targeting signals that control IFITM3 trafficking to and from this compartment warrant further study.

IFITM3 is a highly regulated protein with at least four posttranslational modifications occurring on multiple residues reported to date. We first reported palmitoylation of IFITM3 occurring on three cysteines that are essential for proper membrane anchoring and antiviral activity (4, 19–21). We also reported ubiquitination of IFITM3 occurring on four lysines (4). This modification negatively regulates IFITM3 by targeting the protein away from endolysosomes for degradation (4). Set7-dependent methylation on a single lysine has also been

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3 The abbreviations used are: IFITM, interferon-inducible transmembrane protein; h, human; m, murine.
IFITM3 Phosphorylation Blocks Endocytosis and Ubiquitination

IFITM3 Phosphorylation by FYN Is Not Required for Anti-influenza Virus Activity—The previous finding that mutagenesis of the phosphorylated residue Tyr20 results in decreased antiviral activity of IFITM3 might suggest that phosphorylation is necessary for the function of IFITM3 (23). In addressing this hypothesis, we first sought to confirm that human (h) IFITM3 is phosphorylated and also examined whether this modification is conserved on murine (m) IFITM3, which possesses Tyr20 and also a non-conserved tyrosine nearby at amino acid position 27 (Fig. 1A). For these experiments, we utilized a murine fibroblast cell line deficient in SRC, YES, and FYN kinases (SYF cells) in which we observed nearly undetectable tyrosine phosphorylation of myc-tagged IFITM3 (Fig. 1, B and C). Upon FYN coexpression, tyrosine phosphorylation of both mIFITM3 and hIFITM3 could be detected, in agreement with the conclusion published previously showing that FYN can indeed phosphorylate hIFITM3 (23) (Fig. 1, B and C). Of note, sodium orthovanadate pretreatment of cells to inhibit phosphatases was required, as reported previously (23), to observe IFITM3 phosphorylation, indicating that IFITM3 phosphorylation is a dynamic and reversible process. Tyrosine mutagenesis indicated that hIFITM3 is phosphorylated solely on Tyr20 (Fig. 1B), whereas mIFITM3 phosphorylation can occur on both Tyr20 and Tyr27 (Fig. 1C). In these experiments, in which we utilized broad-spectrum phosphatase inhibitors in our lysis buffer, we reproducibly observed an upper band in anti-myc blots that likely represents phosphorylated IFITM3 (Fig. 1, B and C). However, this band does not correlate directly with anti-phospho-tyrosine blots, suggesting that additional phosphorylation sites on serine or threonine residues may exist on IFITM3. This should be a fruitful area for future investigation. Nonetheless,
tyrosine-specific phosphorylation clearly occurs primarily on Tyr\textsuperscript{20/27}.

Having confirmed tyrosine phosphorylation on both mIFITM3 and hIFITM3 by FYN, we sought to test whether IFITM3 is active in SYF cells, i.e. in cells in which IFITM3 phosphorylation is lacking. We and others (1, 4) have shown that murine fibroblast lines express a basal amount of IFITM3 that limits infection of these cells. Thus, we performed siRNA knockdown of IFITM3 in SYF cells (Fig. 2A) and, subsequently, examined infection by influenza virus. The percentage of infected SYF cells increased significantly upon IFITM3 knockdown, indicating that endogenous IFITM3 is active in these cells and that IFITM3 tyrosine phosphorylation by FYN is not a requirement for its antiviral function (Fig. 2B).

**IFITM3 Tyr\textsuperscript{20} Is Required for Complete Antiviral Activity**—We next examined several of our mIFITM3 and hIFITM3 tyrosine mutants for the ability to inhibit influenza virus compared with WT IFITM3. HEK293T cells are a commonly used cell line for the analysis of IFITM3 mutants because they express no detectable amount of endogenous IFITM3, are readily transfectable, and retain the ability to be highly infected with influenza virus even after transfection, allowing a large dynamic range for observations of differences in antiviral protective effects between various IFITM3 mutants (3, 4, 6, 15, 18, 19, 26). Tyr\textsuperscript{20} mutants of both mIFITM3 and hIFITM3 are expressed at levels similar to their respective WT proteins (Fig. 3A) but lose anti-influenza virus activity, as determined by an established flow cytometry assay that measures the percentage of cells infected in each condition (Fig. 3B) (4, 18, 19). Interestingly, mutation of Tyr\textsuperscript{27}, although phosphorylatable (Fig. 1C), has no apparent effect on antiviral activity of mIFITM3, and Tyr\textsuperscript{27} does not significantly compensate for mutation of Tyr\textsuperscript{20} (Fig. 3). These results demonstrate that Tyr\textsuperscript{20} is a critical amino acid for the anti-influenza virus activity of both mIFITM3 and hIFITM3.

**Phosphorylation of IFITM3 on Tyr\textsuperscript{20} Leads to Plasma Membrane Accumulation**—Previous imaging of hIFITM3 Tyr\textsuperscript{20} mutants demonstrated that mutation of this residue results in retention of IFITM3 at the plasma membrane (5, 23). However, these experiments did not address whether phosphorylation of Tyr\textsuperscript{20} or the Tyr\textsuperscript{20} residue itself is necessary for IFITM3 inter-

![FIGURE 1. IFITM3 phosphorylation by FYN. A, alignment of hIFITM3 and mIFITM3 amino acids 15–30. Tyrosines are highlighted with gray shading. B and C, SYF cells were cotransfected overnight with the indicated myc-tagged hIFITM3 (B) or mIFITM3 (C) constructs and either a vector control or a plasmid expressing FYN. Cells were then treated for 1 h with sodium orthovanadate. Cell lysates were subjected to anti-FYN and anti-actin Western blotting or anti-myc immunoprecipitation (IP) followed by blotting for anti-phospho-tyrosine (p-Tyr) and anti-myc. Blots are representative of at least three experiments.](https://jamanetwork.com/journals/jbc/fullarticle-2580872)

![FIGURE 2. IFITM3 is active in the absence of FYN. A and B, SYF cells were transfected for 18 h with control siRNA (siCont) or siRNA targeting IFITM3 (siIFITM3). A, cells were collected just prior to infection for confirmation of IFITM3 knockdown by anti-IFITM3 Western blotting. Anti-GAPDH blotting served as a loading control. B, following siRNA treatment, cells were infected with influenza virus strain PR8 at a multiplicity of infection of 5 for 24 h. Cells were then fixed and stained with anti-influenza nucleoprotein to measure the percentage of cells that were infected using flow cytometry. The results shown are an average of six samples from two independent experiments. The average percentage of infection of siCont cells was set to 1 for the calculation of relative fold infection. Error bars represent mean ± S.D. Student’s t test was used to calculate the indicated p value.](https://jamanetwork.com/journals/jbc/fullarticle-2580872)
IFITM3 Phosphorylation Blocks Endocytosis and Ubiquitation

Identification of a Putative IFITM3 Endocytosis Motif—Our imaging results suggest that Tyr20 may be part of an endocytosis signal that can be blocked by phosphorylation or perturbed by mutating Tyr20 (Fig. 4). Previous IFITM3 mutagenesis has shown that the 17PPN19 residues immediately prior to Tyr20 are dispensable for antiviral activity (Fig. 5A and Ref. 23). Likewise, we observed that mutation of Lys24 does not affect IFITM3 localization or activity (Fig. 5A and Ref. 4). This narrows the hIFITM3 endocytic motif to the region 20YEML23 (Fig. 5A).

Interestingly, this sequence conforms to the pattern of a YXXΦ motif (where X is any amino acid and Φ is Val, Leu, or Ile) that is involved in the adaptor protein complex-mediated endocytosis of a multitude of membrane proteins (27). Our experiments with mIFITM3 also support that this motif is an endocytosis signal because the 20YERI23 sequence of mIFITM3 also conforms to this pattern, whereas the 27YEVA30 motif involving Tyr27 does not (Fig. 5A), agreeing with our data that Tyr20, although phosphorylatable, does not play a major role in IFITM3 cellular distribution or antiviral activity (Figs. 3 and 4B).

To test the hypothesis that the 20YEML23 sequence in IFITM3 is an endocytosis signal, we made a conservative mutation of the Φ residue in hIFITM3 to valine (L23V) and a non-conservative mutation to the polar residue glutamine (L23Q). Imaging of these mutants revealed a similar localization of the L23V mutant, which preserves the canonical YXXΦ pattern, whereas the L23Q mutant was redistributed, including visible plasma membrane localization similar to what we saw previously with Tyr20 mutants (Figs. 4, A and B, and 5B).

To further confirm that the YEML sequence of hIFITM3 functions as an endocytosis signal, we transferred this tetrapeptide to the cytoplasmic C-terminal region of CD4, which normally localizes in part to the plasma membrane. Anti-CD4 staining of the cell surface of non-permeabilized cells showed outlining of cells transfected with a plasmid encoding WT myc-tagged CD4 (Fig. 6A). Under the same conditions, minimal staining of a CD4 construct containing the YEML peptide was observed (Fig. 6A), although this construct was expressed strongly and localized in internal compartments, as indicated by staining of permeabilized cells with anti-CD4 (Fig. 6A) or anti-myc (Fig. 6B). Overall, these results indicate that the YEML motif from hIFITM3 causes a robust internalization of CD4 (Fig. 6). Taken together, our results provide evidence that the hIFITM3 YEML motif functions as an endocytosis signal that can be regulated by FYN-mediated phosphorylation.

Tyrosine Phosphorylation Regulates IFITM3 Ubiquitination—Having previously discovered IFITM3 ubiquitination (4) and noting the often-observed cross-talk between phosphorylation and ubiquitination (28), we sought to investigate a potential link between these two modifications on IFITM3. In our previous studies of IFITM3, we found that, upon overexposure of IFITM3 Western blot analyses, bands above the expected IFITM3 molecular weight could be observed. We identified this banding pattern as ubiquitination through the use of mass spectrometry, anti-ubiquitin blotting, and lysine mutagenesis (4). Here we employed a straightforward assay whereby we transfected mIFITM3 and hIFITM3 into 293T cells with or without overexpression of FYN and examined the banding pattern of IFITM3 by Western blotting. Interestingly, bands that we identified previously as mono- and diubiquitinated IFITM3 (4) were diminished upon FYN overexpression for both mIFITM3 and hIFITM3, whereas the bands at the expected molecular weight were largely unaffected by FYN (Fig. 7A). We then compared the banding patterns for WT IFITM3 and tyrosine mutants. Tyr20 mutants of both mIFITM3 and hIFITM3 showed a decreased intensity of ubiquitinated bands, whereas a Tyr27 mutant of mIFITM3 was ubiquitinated similarly to WT mIFITM3 (Fig. 7B). A ubiquitination-deficient lysine-less mutant of mIFITM3 (termed UbΔ, note that the myc tag contains one lysine) we generated previously (4) was included as a control in this experiment to confirm that the higher molecular weight bands we observed indeed represent ubiquitination (Fig. 7B).

To further visualize IFITM3 ubiquitination, including polyubiquitinated IFITM3, and to be sure that the lysine residue present within the myc epitope tag was not altering our results,
we cotransfected HA-tagged mIFITM3 and tyrosine mutants with FLAG-ubiquitin and performed anti-HA immunoprecipitation followed by blotting for HA and FLAG. mIFITM3 constructs were chosen for this experiment because of the availability of the mIFITM3-Ub\(^{H904}\) construct, which served as a negative control, and because we also previously generated a palmitoylation-deficient mIFITM3 construct (Palm\(^{H904}\)) that does not have a defect in ubiquitination that served as an additional control (4). Ubiquitination patterns observed in this experiment agreed with our previous results in that Tyr\(^{20}\) mutants showed a partial defect in ubiquitination, whereas Tyr\(^{27}\) mutation had no effect compared with WT mIFITM3 (Fig. 7C). Overall, these data suggest that the phosphorylatable residue Tyr\(^{20}\) is involved in promoting IFITM3 ubiquitination in addition to its role in promoting endocytosis.

**DISCUSSION**

The posttranslational regulation of IFITM3 is rich with complexity. At least eight distinct amino acids within this small, 15-kDa protein are modified with at least four different post-translational modifications, including phosphorylation, palmitoylation, ubiquitination, and methylation (4, 19, 22, 23). We
demonstrate here, for the first time, that there is cross-regulation of posttranslational modifications on IFITM3, namely that phosphorylation of IFITM3 by FYN at Tyr20 decreases IFITM3 ubiquitination (Fig. 7). This is in contrast to the previously observed independent nature of IFITM3 ubiquitination with respect to its palmitoylation (4).

Cross-talk between phosphorylation and ubiquitination has been documented extensively for multiple other proteins (28). Particularly, phosphorylation often serves as a signal for modification by E3 ubiquitin ligases. For example, CBL family E3 ubiquitin ligases recognize phosphorylated tyrosines on their substrates (29), which, coincidentally, include FYN (30). Thus, it may have been expected that IFITM3 phosphorylation would promote ubiquitination. However, the opposite was observed in that IFITM3 phosphorylation by FYN led to a defect in ubiquitination, and mutating the Tyr20 phosphorylation site resulted in the same effect (Fig. 7).

The observed decrease in ubiquitination of IFITM3 upon phosphorylation may have at least two possible explanations. First, IFITM3 endocytosis may be required for ubiquitination to occur. We found that phosphorylation blocks an endocytosis motif that is necessary for proper localization and full antiviral activity of IFITM3 (Figs. 1–6). Our imaging data indicate that IFITM3 traffics to the plasma membrane and is either retained there upon phosphorylation or is internalized (Figs. 4 and 5). During the writing of this manuscript, a second group also reported identification of the 20YEML23 motif of hIFITM3 as a critical determinant for its endocytosis (31), thus validating and complementing our observations regarding phosphorylation of this motif. Thus, if ubiquitination takes place at the endolysosome or another cellular compartment, it may be inhibited by retention of IFITM3 at the plasma membrane upon Tyr20 mutation or phosphorylation. Second, Tyr20 may be part of an additional motif recognized by ubiquitin ligases, again explaining the similar results we obtained when either blocking Tyr20 by phosphorylation or by mutating Tyr20 (Fig. 7). The sequence 17PPNY20 in both mIFITM3 and hIFITM3 presents a potential E3 ligase interaction motif because it conforms to the PPX pattern recognized by HECT E3 ubiquitin ligases (Fig. 5A) (32).

Both of these aforementioned possibilities are currently under active investigation in our laboratory, and our findings should aid in the future identification of the IFITM3 ubiquitin ligase(s) responsible for modifying IFITM3.

It remains to be determined what effect relocalization of IFITM3 upon Tyr20 mutation or upon increasing FYN activity would have on its range of viral restrictions. For instance, IFITM1, which lacks a YYXX/H motif and localizes in part to the plasma membrane, has been described to have an overlapping but somewhat distinct specificity for different viruses compared with IFITM3 (2, 8, 17). Likewise, it will be interesting to examine what effect drugs that inhibit SRC family tyrosine kinases such as FYN would have on IFITM3 activity because phosphorylation of IFITM3 by FYN has both potentially negative and positive effects through modulating endocytosis and

FIGURE 7. Unmodified Tyr-20 is necessary for proper IFITM3 ubiquitination. A, 293T cells were cotransfected overnight with the indicated myc-tagged IFITM3 constructs and a vector control or a plasmid expressing FYN. Anti-myc and anti-fyn blotting were performed. B, 293T cells were transfected overnight with the indicated myc-tagged IFITM3 constructs, and anti-myc blotting was performed. A and B, anti-myc blots were overexposed to allow visualization of ubiquitinated IFITM3. Single arrows and double-headed arrows indicate monoubiquitinated and diubiquitinated IFITM3, respectively. IFITM3 at the expected molecular mass of 15 kDa serves as a loading control. C, 293T cells were cotransfected overnight with the indicated HA-tagged IFITM3 constructs and a plasmid expressing FLAG-tagged ubiquitin (Ub). Anti-HA immunoprecipitation was performed, followed by anti-HA and anti-FLAG blotting. The results in A–C are representative of at least three experiments.
ubiquitination, respectively. Overall, the continued study of IFITM3 posttranslational modifications, their cross-talk, and their mechanisms of regulating antiviral activity will be important for understanding and controlling IFITM3 biology for combating influenza and other viruses.

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