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Bifunctional Fatty Acid Chemical Reporter for Analyzing S-Palmitoylated Membrane Protein–Protein Interactions in Mammalian Cells

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Supporting Information

ABSTRACT: Studying the functions of S-palmitoylated proteins in cells can be challenging due to the membrane targeting property and dynamic nature of protein S-palmitoylation. New strategies are therefore needed to specifically capture S-palmitoylated protein complexes in cellular membranes for dissecting their functions *in vivo*. Here we present a bifunctional fatty acid chemical reporter, x-alk-16, which contains an alkyne and a diazirine, for metabolic labeling of S-palmitoylated proteins and photo-cross-linking of their involved protein complexes in mammalian cells. We demonstrate that x-alk-16 can be metabolically incorporated into known S-palmitoylated proteins such as H-Ras and IFITM3, a potent antiviral protein, and induce covalent cross-linking of IFITM3 oligomerization as well as its specific interactions with other membrane proteins upon in-cell photoactivation. Moreover, integration of x-alk-16-induced photo-cross-linking with label-free quantitative proteomics allows identification of new IFITM3 interacting proteins.

The discovery of many S-palmitoylated proteins by recent proteomic studies suggests broader roles of protein S-palmitoylation in regulating eukaryotic biology than previously appreciated.[1] As a reversible and dynamic posttranslational modification, S-palmitoylation controls the localization and protein–protein interactions of many membrane proteins.[2] Distinguishing the interacting partners of S-palmitoylation proteins from those of their unmodified forms is crucial for understanding the functions of this lipid modification in a variety of biological pathways. The analysis of S-palmitoylated membrane protein–protein interactions in cells, however, can be challenging compared to soluble proteins.[3] Membrane protein complexes may only be maintained under a native and unique lipid environment, which is often destroyed during cell lysis and difficult to reconstitute *in vitro*. Moreover, the hydrophobic and amphiphilic nature of membrane proteins makes classical techniques such as coimmunoprecipitation, two-hybrid systems, and native gel electrophoresis more difficult to implement compared to soluble proteins.[3a] These technical challenges are further exacerbated by the dynamic nature of S-palmitoylation. New tools are therefore needed to characterize S-palmitoylated membrane protein complexes in living cells for functional studies.

To facilitate the characterization of protein–protein interactions in cells, photo-cross-linking methods have provided powerful approaches by generating covalent bonds between protein interaction partners in response to light.[4] Owing to the newly introduced covalent bonds, weak and transient protein interactions can be trapped as covalent complexes for biochemical analysis even with harsh cell lysis and stringent protein purification conditions. Notably, photo-cross-linking can be conducted in live and intact cells to stabilize and capture native membrane protein–protein interactions. For instance, a variety of photo-cross-linking methods have been utilized to characterize protein–protein interactions,[5] glycan–protein interactions,[5b] and lipid–protein interactions.[6] Herein, we show that a bifunctional fatty acid chemical reporter, x-alk-16, which contains a clickable alkyne and a photoactivatable diazirine, can be metabolically incorporated into S-palmitoylated proteins and allows photo-cross-linking of their interacting partners in mammalian cells (Figure 1).

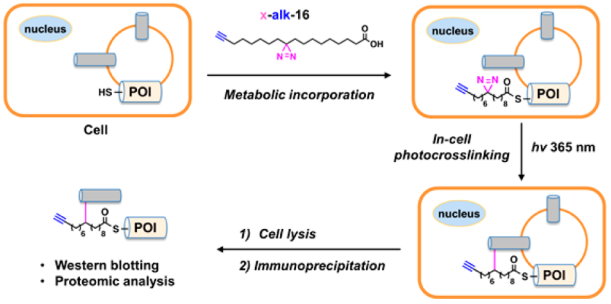

Figure 1. Strategy for characterizing S-palmitoylated membrane protein complexes in living cells. Metabolic incorporation of a bifunctional fatty acid chemical reporter, x-alk-16, equipped with an alkyne and a diazirine allows bioorthogonal detection of S-palmitoylated proteins and in-cell photo-cross-linking of S-palmitoylated protein complexes of interest (POI) for Western blotting and proteomic analysis after immunopurification.

To photo-cross-link S-palmitoylated protein complexes, we synthesized a bifunctionalized fatty acid chemical reporter with a terminal alkyne and an internal diazirine, x-alk-16 (Figure 1 and Supporting Information (SI) Scheme S1). We envisioned that insertion of an internal diazirine into a previously described chemical reporter for S-palmitoylation, alk-16,[7] would not significantly change the properties of the long-chain fatty acid, so the resulting palmitic acid analogue could still be utilized by

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native enzymes for S-palmitoylation in mammalian cells. Once incorporated into S-palmitoylated proteins, the diazirine group could facilitate covalent cross-linking of x-alk-16-modified proteins with their interacting proteins upon photoactivation (Figure 1).

We first tested whether x-alk-16 could serve as a chemical reporter for S-palmitoylation in mammalian cells. Human embryonic kidney (HEK) 293T cells were incubated with 50 μM alk-16 or x-alk-16 for 6 h and harvested. Cell lysates were prepared, reacted with azido-rhodamine (az-rho), and analyzed by in-gel fluorescence (Figure 2a). Like alk-16, incubation of cells

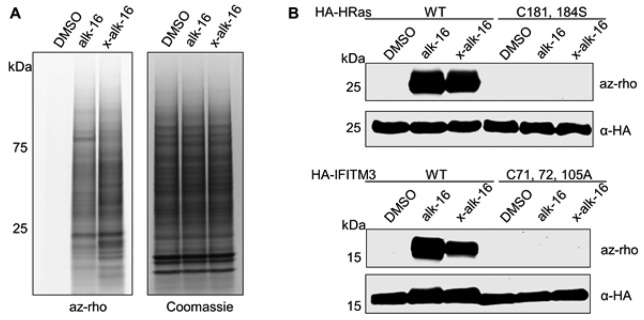

Figure 2. Analysis of x-alk-16 incorporation into S-palmitoylated proteins in mammalian cells. (A) HEK293T cells were metabolically labeled with 50 μM alk-16, x-alk-16, or DMSO and lysed. Cell lysates were reacted with azido-rhodamine (az-rho) and analyzed by in-gel fluorescence. (B) Known S-palmitoylated proteins, HA-tagged H-Ras and IFITM3, can be metabolically labeled with x-alk-16 on key Cys residues as judged by in-gel fluorescence detection. Coomassie blue staining in (A) and anti-HA Western blots in (B) are included as protein loading controls.

with x-alk-16 afforded robust protein labeling relative to DMSO control (Figure 2a). In addition, protein labeling with x-alk-16 in living cells was dose- and time-dependent (SI Figure S1), indicating that active cellular metabolism is required for its incorporation. Comparative proteomic analysis of alk-16 and x-alk-16 labeled proteins after click reaction with azido-biotin and streptavidin enrichment indicated over 70% overlap of labeling between these two fatty acid chemical reporters, including many known S-palmitoylated proteins (SI Figure S2 and Table S1).

To further confirm that x-alk-16 is able to specifically label S-palmitoylated proteins, we investigated two well-characterized S-palmitoylated proteins, H-Ras and IFITM3.[8] HEK293T cells were transfected with plasmids encoding HA-tagged wild-type proteins or S-palmitoylation-deficient mutants and labeled with alk-16 or x-alk-16. The proteins were then immunoprecipitated, reacted with az-rho, and analyzed by in-gel fluorescence. Wild-type HA-tagged H-Ras and IFITM3 were efficiently labeled with both alk-16 and x-alk-16, whereas S-palmitoylation-deficient mutants of HA-H-Ras (C181, 184S) and HA-IFITM3 (C71, 72, 105A) showed no fluorescence labeling with either reporter (Figure 2b), indicating that x-alk-16, like alk-16, is specifically incorporated into key S-palmitoylation sites of both H-Ras and IFITM3.

We then examined whether the incorporation of x-alk-16 could induce cross-linking of S-palmitoylated protein complexes in cells. For these studies, we initially focused on IFITM3 photo-cross-linking with its interacting proteins as part of ongoing efforts in our laboratory to characterize the antiviral mechanisms of this protein.[8] HEK293T cells were thus transfected with HA-IFITM3, labeled with fatty acid reporters, and then irradiated with UV light at 365 nm. Cell lysates were prepared and analyzed by Western blot. Apparent higher molecular weight complexes were observed for HA-IFITM3 only in the x-alk-16 labeled and UV-irradiated sample (Figure 3a). By contrast, no cross-linking

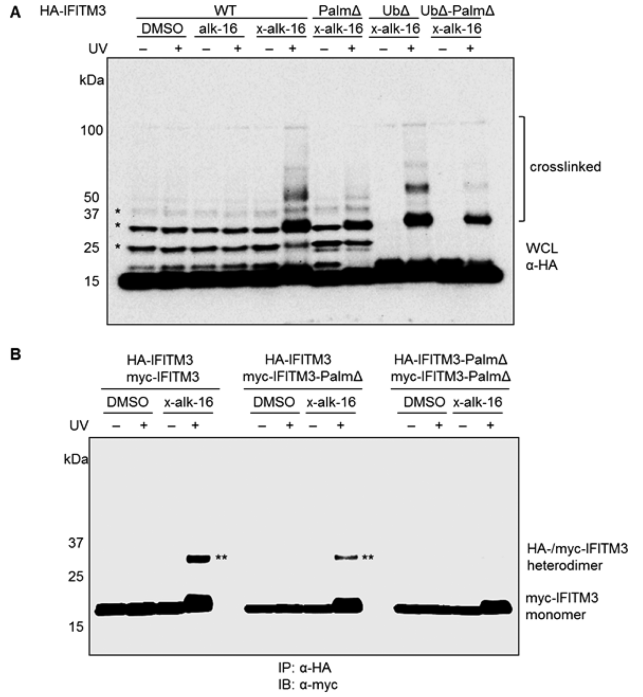

Figure 3. Photo-cross-linking of IFITM3 induced by x-alk-16 incorporation. (A) Detection of x-alk-16-induced IFITM3 photo-cross-linking complexes in whole cell lysate (WCL) by anti-HA Western blot. IFITM3 ubiquitination bands are marked with asterisks. (B) Validation of x-alk-16-induced photo-cross-linking of IFITM3 dimerization (double asterisk) by immunoprecipitation and Western blot.

occurred for HA-IFITM3 in DMSO, alk-16 labeled samples, or in the x-alk-16 labeled nonirradiated sample (Figure 3a). Photo-cross-linking conditions, including x-alk-16 dose, labeling time, and irradiation time, were then optimized for HA-IFITM3 (SI Figure S3). Cells were generally incubated with 50 μM x-alk-16 for 2 h, and then UV irradiated for 5 min to minimize the cytotoxic effects of UV light, which is sufficient to achieve nearly maximal photo-cross-linking. The cell lysates were also reacted with azido-biotin and enriched with streptavidin beads before elution for Western blotting analysis. Cross-linking complexes of HA-IFITM3 were only detected in x-alk-16 labeled and UV-irradiated sample after clicked with azido-biotin (SI Figure S4). Together, these results demonstrate that the cross-linking is dependent on UV irradiation and mediated by x-alk-16.

Photo-cross-linking induced by x-alk-16 was not limited to overexpressed IFITM3. Other HA-tagged S-palmitoylated membrane proteins (CD9 and CD81) were also photo-cross-linked to form higher molecular weight complexes when cells were labeled with x-alk-16 and irradiated with UV (SI Figure S5a). In addition, x-alk-16-induced photo-cross-linking was observed for endogenously expressed S-palmitoylated proteins (IFITM3, CAV1, and CANX) (SI Figure S5b). By contrast, under the same conditions, no photo-cross-linking occurred for nonpalmitoylated proteins, such as p53 and green fluorescent protein (GFP) (SI Figure S6). It is noteworthy that overexpressed H-Ras was not photo-cross-linked so obviously as those shown above (SI Figure S5a). We reasoned that the low steady-

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state S-palmitoylation level of H-Ras[9] may affect its photo-cross-linking efficiency and that the abundance of H-Ras photo-cross-linking complexes might be too low for detection with Western blotting.

Next we focused on the characterization of x-alk-16-induced photo-cross-linking of IFITM3. We performed additional experiments with IFITM3 S-palmitoylation-deficient mutant HA-IFITM3-PalmΔ. Photo-cross-linked bands with higher molecular weights were also observed for HA-IFITM3-PalmΔ, however, much weaker than those observed for wild-type protein (Figure 3a). To eliminate the interference from IFITM3 lysine ubiquitination (marked by asterisks) previously reported by our laboratory,[8b] we also examined x-alk-16 induced photo-cross-linking on ubiquitination-deficient mutants of IFITM3, HA-IFITM3-UbΔ and HA-IFITM3-UbΔ-PalmΔ. UV- and x-alk-16-dependent molecular weight shifts on these mutants are more evident (Figure 3a). Interestingly, HA-IFITM3-UbΔ-PalmΔ still exhibited a detectable, albeit much weaker, amount of UV- and x-alk-16-dependent cross-linking in the ∼30 kDa region (Figure 3a), probably resulted from photo-cross-linking with x-alk-16-modified endogenous proteins or phospholipid-mediated photo-cross-linking of membrane proteins, as diazirine-containing fatty acid analogues have been shown to be metabolized into cellular phospholipids.[6a,f]

The apparent molecular weights of photo-cross-linked complexes of IFITM3 suggest that IFITM3 dimers or oligomers might be captured by x-alk-16 photo-cross-linking. To examine higher order IFITM3 complexes in cells and eliminate the interference from photo-cross-linking of x-alk-16-modified endogenous proteins, HEK293T cells coexpressing HA- and myc-tagged IFITM3 were labeled with x-alk-16 and UV-irradiated. Immunoprecipitation was then performed using anti-HA-coupled beads, and the eluted samples were analyzed for the presence of myc-IFITM3 by Western blotting with an anti-myc antibody. Myc-IFITM3 at 15 kDa was detected in all samples coexpressing HA-IFITM3, while not in HA-vector samples, regardless of whether the samples were labeled with x-alk-16 or irradiated with UV, indicating that myc-IFITM3 was coimmunoprecipitated with HA-IFITM3 (SI Figure S7a). This result is consistent with a recent report suggesting intermolecular interaction of IFITM3.[10] A ∼30 kDa complex, twice the IFITM3 molecular weight, appeared only in the sample labeled with x-alk-16 and treated with UV (Figures 3b and SI S7a). An identical complex was detected in a reciprocal experiment (SI Figure S7b) in which immunoprecipitation was performed using anti-myc-coupled beads, followed by Western blotting using an anti-HA antibody, suggesting that x-alk-16 enables capture of IFITM3 dimerization upon UV irradiation. Interestingly, much weaker or no photo-cross-linked dimer band was observed in samples coexpressing HA-IFITM3/myc-IFITM3-PalmΔ or HA-IFITM3-PalmΔ/myc-IFITM3-PalmΔ (Figure 3b). Taken together, these results demonstrate that IFITM3 oligomerization revealed by x-alk-16 photo-cross-linking is largely dependent on IFITM3 S-palmitoylation.

To examine whether x-alk-16 can capture the interaction of IFITM3 with its known interacting partners, we focused on vesicle-membrane-protein-associated protein A (VAPA), a protein involved in intracellular cholesterol homeostasis and recently reported to interact with IFITM3.[11] HEK293T cells coexpressing HA-IFITM3 and myc-VAPA were labeled with x-alk-16, irradiated, and then lysed for immunoprecipitation with anti-HA-coupled beads. The immunoprecipitates were analyzed by Western blotting with an anti-myc antibody. As expected, coimmunoprecipitated myc-VAPA at 30 kDa was detected in all samples coexpressing HA-IFITM3 and myc-VAPA, whereas new bands at 45 kDa and 60 kDa appeared only in x-alk-16 photo-cross-linking sample (SI Figure S8a), demonstrating the formation of IFITM3-VAPA and likely dimeric IFITM3-VAPA complexes, respectively. These photo-cross-linked complexes were also detected in the reciprocal immunoprecipitation experiment (SI Figure S8b). More importantly, a VAPA truncation mutant, VAPA$^{1-227}$, lacking the key domain for interaction with IFITM3[11] did not coimmunoprecipitate and cross-link with IFITM3 (SI Figure S8a), suggesting protein interaction domains are required for x-alk-16-induced photo-cross-linking complex formation. In addition, although myc-IFITM3 coimmunoprecipitated with CAV1-HA and HA-CD9 (SI Figure S9), two unrelated S-palmitoylated membrane proteins, neither of them photo-cross-linked with myc-IFITM3 to form high molecular weight complexes (Figure S9), further confirming the specificity of x-alk-16-induced photo-cross-linking of IFITM3. These results demonstrate that, in addition to IFITM3 homotypic interactions, x-alk-16 also enables specific photo-cross-linking of IFITM3 with other interacting proteins.

Finally, we sought to identify new IFITM3-interacting proteins using our photo-cross-linking strategy coupled with label-free quantitative proteomic analysis. Briefly, HEK 293T cells were transfected with HA-IFITM3, labeled with x-alk-16, and UV-irradiated in the photo-cross-linking sample group with four biological replicates. In the non-photo-cross-linking control group, HA-IFITM3-transfected cells were either not labeled with x-alk-16 or not irradiated with UV, each experimental design having three biological replicates. All samples were processed in parallel, including anti-HA immunoprecipitation, in-solution trypsin digestion, and LC−MS/MS analysis (SI Figure S10a). Proteins were then identified and quantified with the label-free MaxLFQ algorithm in MaxQuant software suite,[12] which revealed 12 proteins that were enriched in x-alk-16 photo-cross-linking samples versus non-photo-cross-linking controls and are therefore IFITM3-interacting candidate proteins (Figure 4a and SI Table S2). Notably, 9 of the 12 proteins appear to be membrane-associated proteins. Functional annotation revealed an enrichment of these proteins involved in ER membrane

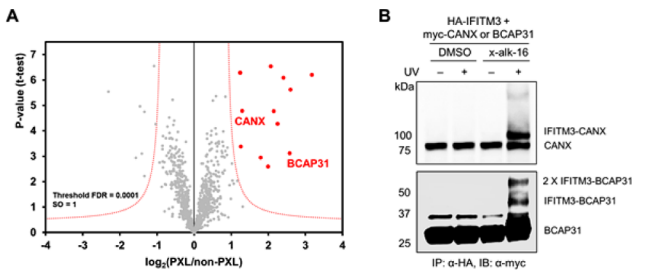

Figure 4. Proteomic analysis of S-palmitoylated IFITM3-interacting partners captured by x-alk-16 photo-cross-linking in mammalian cells. (A) Volcano plot representing results of the label-free quantitative proteomic analysis of IFITM3 pull-downs. The logarithmic ratios of protein intensities in photo-cross-linking sample group (PXL) over non-photo-cross-linking control group (non-PXL) were plotted against negative logarithmic *p*-values of the *t* test performed from multiple replicates. A hyperbolic curve (red dotted line) separates specific IFITM3-cross-linking proteins (red dots) from background (gray dots). (B) Validation of x-alk-16-induced photo-cross-linking of IFITM3-CANX and IFITM3-BCAP31 complexes by anti-HA immunoprecipitation and anti-myc Western blotting.

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protein quality control and sterol metabolism (SI Figure S10b, c). To confirm the reliability and robustness of our photo-cross-linking proteomics data set, we picked two hits, CANX and BCAP31, for further validation. HA-IFITM3 and myc-tagged candidates were introduced into HEK293T cells, which were then subjected to photo-cross-linking, immunoprecipitated, and analyzed by Western blotting. As shown in Figure 4b, CANX and BCAP31 indeed interact with IFITM3 as revealed by coimmunoprecipitation. More importantly, photo-cross-linking complexes of IFITM3-CANX and IFITM3-BCAP31 with high apparent molecular weights were observed only in the photo-cross-linking samples. Together, these results demonstrate that our x-alk-16 photo-cross-linking strategy coupled with quantitative proteomic analysis allows identification of new IFITM3-interacting proteins.

In summary, we present a bifunctional fatty acid reporter x-alk-16 with a bioorthogonal alkyne detection tag and a photoactivatable diazirine for studying membrane protein interactions of S-palmitoylated proteins. We show that x-alk-16 is readily incorporated into S-palmitoylated proteins by metabolic labeling and enables covalent cross-linking of S-palmitoylated proteins with their interacting partners upon photoactivation in intact mammalian cells. Using this photo-cross-linkable fatty acid chemical reporter, we have demonstrated the oligomerization of IFITM3 and its specific interaction with VAPA, both of which are important for the full antiviral activity of IFITM3. Integration of x-alk-16-enabled photo-cross-linking with label-free quantitative proteomic studies also revealed new candidate IFITM3 interacting proteins that may uncover key cellular factors important for host resistance to virus infection in the future. This bifunctional lipid chemical reporter and photo-cross-linking proteomics should afford new opportunities to characterize S-palmitoylated membrane protein complexes.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis of x-alk-16, experimental methods, and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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