Spatial proteomics reveal that the protein phosphatase PTP1B interacts with and may modify tyrosine phosphorylation of the rhomboid protease RHBDL4

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Rhomboid-like proteins are evolutionarily conserved, ubiquitous polytopic membrane proteins, including the canonical rhomboid intramembrane serine proteases and also others that have lost protease activity during evolution. We still have much to learn about their cellular roles, and evidence suggests that some may have more than one function. For example, RHBDL4 (rhomboid-like protein 4) is an endoplasmic reticulum (ER)-resident protease that forms a ternary complex with ubiquitinated substrates and p97/VCP (valosin-containing protein), a major driver of ER-associated degradation (ERAD). RHBDL4 is required for ERAD of some substrates, such as the pre-T-cell receptor α chain (pTα) and has also been shown to cleave amyloid precursor protein to trigger its secretion. In another case, RHBDL4 enables the release of full-length transforming growth factor α in exosomes. Using the proximity proteomic method BioID, here we screened for proteins that interact with or are in close proximity to RHBDL4. Bioinformatics analyses revealed that BioID hits of RHBDL4 overlap with factors related to protein stress at the ER, including proteins that interact with p97/VCP. PTP1B (protein-tyrosine phosphatase nonreceptor type 1, also called PTPN1) was also identified as a potential proximity factor and interactor of RHBDL4. Analysis of RHBDL4 peptides highlighted the presence of tyrosine phosphorylation at the cytoplasmic RHBDL4 C terminus. Site-directed mutagenesis targeting these tyrosine residues revealed that their phosphorylation modifies binding of RHBDL4 to p97/VCP and Lys⁶⁰-linked ubiquitinated proteins. Our work lays a critical foundation for future mechanistic studies of the roles of RHBDL4 in ERAD and other important cellular pathways.

Rhomboids are evolutionarily conserved intramembrane serine proteases (1). They belong to the superfamily of rhomboid-like polytopic membrane proteases that includes active proteases and the enzymatically inactive pseudoproteases. Although the function is only known for a subset of rhomboid-like proteins, there is a clear theme that they control the fate of membrane proteins in the secretory pathway, in the mitochondria, and at the plasma membrane. To date, this role manifests in a variety of biological contexts including parasite infection, inflammatory responses, bacterial quorum sensing, growth factor secretion, mitochondria, and proteostasis (2–12). As membrane proteases, the rhomboid-like proteases have modular structures, with cytoplasmic, transmembrane, and luminal/extracellular domains, and there are emerging suggestions that some members of this superfamily might have more than one function.

The endoplasmic reticulum (ER)³ resident rhomboid protease RHBDL4, which is conserved in metazoans and plants (8), exemplifies this apparent multifunctionality. Recent reports indicate roles for RHBDL4 in canonical rhomboid-like release of a single pass TM protein, amyloid precursor protein (13), in secretion of transforming growth factor α (TGFα) as full length in exosomes (14); and, best understood, in controlling proteostasis by ER-associated degradation (ERAD) (8). RHBDL4 binds to p97/VCP, a AAA + type ATPase that powers ERAD, as well as binding to ubiquitinated substrates (8, 15). The assembly of this complex drives the cleavage and targeting to ERAD of the model substrate pTα, the α-chain of the progenitor form of the T-cell receptor (8, 16). Similarly, RHBDL4 determines ERAD targeting of a major adhesion molecule of the peripheral nervous system, myelin protein zero (8). More recently, RHBDL4 has been reported to trigger the release of the epithelial growth factor (EGF) receptor ligand pro-TGFα in a process that is distinct from canonical rhomboid transmembrane domain cleavage of growth factors (14). In this case, full-length pro-TGFα is released associated with exosomes from cells, by a mechanism that is currently unknown, although its traffic and secretion dynamics is likely controlled by RHBDL4 catalytic residues. Finally, in another context, RHBDL4 behaves as a sheddase-like rhomboid protease, cleaving and thereby releasing the extracel-
lular domain of the amyloid precursor protein (APP), and its close homologues, amyloid precursor like protein 1 (APLP1) and 2 (APLP2), in a cholesterol-regulated manner (13, 17). This event can be influenced by the primary sequence of the substrate transmembrane region (18), is distinct from the one performed by α-secretase, and might avoid subsequent toxic processing by β- and γ-secretases. All these diverse cases depend on the conserved catalytic residues Ser144 and His195 that comprise the rhomboid catalytic dyad. However, despite this dependence on protease activity, the molecular mechanisms underlying these functions remain unknown and appear to be very different from each other, perhaps representing distinct molecular roles of RHBDL4.

Post-translational modifications diversify the functions of proteins. Regulatory modifications such as phosphorylation may therefore be responsible for determining the appropriate action of multifunctional proteins. For example, phosphorylation triggered by G protein–coupled receptor stimulation with agonists such as phorbol 13-myristate 12-acetate was found to alter activity of members of the pseudoprotease iRhom2. This facilitates the trafficking of tumor necrosis factor α–converting enzyme (TACE/ADAM17) in a serine/threonine phosphorylation–regulated manner, with the help of the phosphosite–binding 14–3–3 protein (5, 19–21). Similarly, phorbol 13-myristate 12-acetate treatment can synergize with RHBDL4 activity to increase the secretion of TGFα (14). Until now there have been no reports of whether RHBDL4 is regulated by any post-translational modification that could affect its molecular functions.

A powerful approach to uncovering the functional mechanisms of uncharacterized proteins is to identify their binding partners. BioID is a spatial proteomic approach in which a promiscuous R118G mutant of the Escherichia coli biotin ligase BirA (BirA*) is used to label proteins within the radius of ~10 nm from the bait (22–25). Because of a biotin-streptavidin–based isolation strategy combined with stringent washes with 2.0% SDS, BioID provides a restricted list of candidate neighbors. Unlike more classical co-immunoprecipitation approaches, BioID can identify very weak and transient interactions that may nevertheless be functionally important including, for example, enzymes such as kinases or E3 ligases and their substrates (26, 27). To begin to understand how RHBDL4 can play its distinct roles, we conducted a comparative spatial proteomic study using BioID. We performed a BioID screen with the protease RHBDL4 and, as a negative control to assess the specificity of RHBDL4 hits, with iRhom2, a nonprotease rhomboid-like protein located predominantly in the ER. WT human RHBDL4 was tagged at the cytoplasmic C terminus with a flexible arm of seven serines followed by BirA* (RHBDL4mycBirA*). Likewise, human iRhom2 was tagged at its cytoplasmic N terminus with mycBirA* followed by seven serines (mycBirA*iRhom2). We generated HeLa cells stably expressing mycBirA*, RHBDL4mycBirA*, and mycBirA*iRhom2. Additionally, we made HEK293 cells stably expressing RHBDL4mycBirA*. The expected sizes of the tagged proteins were measured by Western blotting as mycBirA* c30 kDa, RHBDL4mycBirA* c70 kDa, and mycBirA*iRhom2 c130 kDa (Fig. 1A). These molecular masses are consistent with those of BirA (35 kDa), RHBDL4 (93 kDa), or iRhom2 (93 kDa). Treatment with 50 μM biotin for 18 h had no impact on these bands (Fig. 1A), but when neutavidin-HRP was used to detect cellular biotinylation, the same lysates showed intense labeling in biotin-treated cells that were expressing mycBirA*, RHBDL4mycBirA*, and mycBirA*iRhom2 (Fig. 1B). As expected, endogenous mitochondrial carboxylases that contain biotin as covalently bound co-factor are visible in all cells at 130 kDa and between 100 and 70 kDa. In immunofluorescent micrographs, RHBDL4mycBirA* and mycBirA*iRhom2 were primarily localized in the ER, which is consistent with the localization of RHBDL4 and iRhom2 (8, 28) (Fig. 1C and Fig. S1). mycBirA*iRhom2 but not RHBDL4mycBirA* also showed occasional nuclear localization when localized with anti-BirA* antibody (Fig. S1). Upon treatment with biotin, RHBDL4mycBirA*–dependent biotinylation was detected with fluorescent streptavidin–Alexa 488 conjugate, and comparing it with the mitochondrially localized carboxylases that were labeled by anti-cytochrome c oxidase 4 (COX4) antibody (Fig. 1C, top row). Interestingly, biotinylation was also detected toward the periphery of the cell, not overlapping with the ER marker, phosphodiester isomerase (PDI), or RHBDL4mycBirA*, which was localized with anti-BirA antibody. We concluded that the localization of RHBDL4mycBirA* and mycBirA*iRhom2 is consistent with the literature, and the biotinylation levels induced upon treatment with biotin were compatible with proteomic use.

Bioinformatic analysis of BioID show enrichment in protein-stress related factors

Biotinylated proteins in extracts from cells expressing mycBirA* alone, RHBDL4mycBirA*, and mycBirA*iRhom2 were captured by affinity purification and analyzed by MS. Results from two independent replicates in HEK293 cells and three replicates of HeLa cells were compared. We excluded any protein with less than three weighted spectral counts in all lists. We also ignored any protein that was found in the mycBirA* alone condition, because we considered them non-specific hits of the mutant biotin ligase (R118G). In HEK293 cells, peptides mapped reproducibly to more than 146 proteins for RHBDL4 that were absent in the BioID of BirA* alone (Fig.
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2A, domains a and ab, and Table S1). BioID was repeated in HeLa cells, where ~60 reproducible candidates were identified for RHBDL4 (Fig. 2A, domains ab and b, and Table S1). By intersecting the BioID of RHBDL4 from HEK293 and HeLa, 43 proteins were identified as common and high priority hits (Fig. 2A, domain ab, and Table S1). Similarly, ~28 candidates specific for iRhom2 were identified after exclusion of proteins that are in common with the BioID of myc-BirA* or RHBDL4 (Table S1). Approximately 80 proteins were common hits between RHBDL4 and iRhom2 in the BioIDs that were performed in HEK293 (Table S1).

GeneMANIA is an algorithm that compares available proteomic, transcriptomic, genetic, shared domain, and predicted interactions to any given list of proteins to draw possible interaction networks (29–31). We used GeneMANIA application in the data analysis software Cytoscape 3.6.1 that enables further analysis of networks. We constructed a gene network using the 146 identifications of the RHBDL4 HEK293 BioID and compared this to previously published proteomic and genetic studies (32–38) (Fig. S2 and Table S2). Of these 146 identifications, 104 were available on GeneMANIA; 35 of 146 proteins could be linked to p97/VCP using all the search criteria of the GeneMANIA networks (Fig. 2B, domain ab1), whereas 10 were related to p97/VCP via evidence of direct interactions in proteomic or candidate-based studies (Fig. 2B, domain ab2). In addition, among the hits of BioID of RHBDL4 in HEK293 and HeLa cells, there are known interactors of VCP/p97. For instance, the ERAD factor UBXN4 interacts with p97/VCP to reduce misfolded proteins in Caenorhabditis elegans and Homo sapiens (39). The B-cell chronic lymphocytic leukemia-related E3 ubiquitin-ligase TRIM13 (TRI13) is an interactor of p97/VCP (40); similarly the deubiquitinase involved in postmitotic Golgi reassembly VCPIP1 interacts with VCP/p97 and the Golgi SNARE co-factor STX5 (41–43).
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In a complementary approach to predicting gene function, we used the Gene Ontology (GO) PANTHER classification system (44–46). GO includes the molecular functions, cellular components, and biological processes associated with genes by the GO Consortium. PANTHER searches through GO terms associated with a given protein and provides an estimation for the enrichment of specific GO terms within a list of proteins, with respect to a randomized control list. From the list of BioID hits of RHBDL4 in HEK293 cells, the algorithm identified ~144 proteins with mostly ER-related functions as enriched by at least 2-fold with respect to expected frequency (Fig. 2C and Table S3). Proteins associated with membrane localization and insertion, response to unfolded proteins or topologically incorrect proteins, and ERAD were clearly enriched over 10-fold compared with expected frequency (Fig. 2C). Similarly, this enrichment was maintained, with at least a 30-fold increase in frequency, when the 43 reproducible common hits of the BioIDs of RHBDL4 in HeLa and HEK293 cells were searched (Fig. 2D). By comparison, the 28 reproducible candidates in the BioID of iRhom2 scored highest for functions related to multivesicular bodies and EGF signaling with at least 60-fold increase frequency and no predicted enrichment for ER functions (Fig. 2E). Importantly, both BioID of RHBDL4 and iRhom2 contained previously described interactors. For example, p97/VCP is found consistently in the BioID of RHBDL4, and TACE/ADAM17 is in the BioID of iRhom2 (Table S1).

**RHBDL4 is substrate of the nonreceptor type tyrosine phosphatase PTP1B, also called PTPN1**

The PANTHER search on the BioID of RHBDL4 in HEK293 cells highlighted roles in topology-related stress functions. We analyzed the predicted topology of the BioID candidate list for RHBDL4 in HEK293 and HeLa cells and compared with the BioID of iRhom2 in HeLa cells. RHBDL4 BioID in HEK and HeLa cells contained 10–20% of proteins with C-terminal TM domain proteins). As expected, we found increased presence of TM domain proteins (~60%) in all BioID of RHBDL4. Among the 146 proteins that are found only in the BioID of RHBDL4 in HEK cells, 16 contain a single TM domain at the N terminus and 21 at the C-terminal domain (25%). In the BioID of RHBDL4 in HeLa cells, proteins with terminal TM domain are 21 (34%). On the contrary, no protein with terminal TM domain was in the list of proteins exclusive to the BioID of iRhom2 in HEK cells (Fig. S3A and Table S1). Among the 43 common candidates identified in RHBDL4 BioID in both HEK293 and HeLa cells, there are 18 protein (40%) with TM at either N or C terminus. Among these, 7 are tail anchor (TA)
The tyrosine phosphatase PTP1B (also called PTPN1) is an ER resident TA protein with roles in ER stress and recycling of receptors (47). PTP1B is common to the BioID of RHBDL4 in both HEK293 cells and HeLa cells and is a lower hit in the BioID of iRhom2 in HEK293 cells (Fig. 3A). PTP1B is a clinically relevant treatment target for cancer and diabetes with roles in insulin and EGF signaling and anxiety (48–51). In GeneMANIA, 21 genes in the BioID of RHBDL4 in HEK293 cells had predicted direct connections with PTP1B. In particular, ITGB1 are direct physical interactors of both PTP1B and p97/VCP (Table S2).

Overall, PTP1B is a well-connected protein to VCP/p97 and other hits within the BioID of RHBDL4, and we hypothesized that it could be a significant member of the RHBDL4 protein network.

To test the possibility that RHBDL4 is a substrate of PTP1B and is therefore itself tyrosine-phosphorylated, we used substrate-trapping mutants of the phosphatase. The enzymatic mechanism of PTP1B is well-characterized and a substrate-trapping mutant (D181A) as well as a super-trapping mutant (D181A/Y46F) with even higher affinity to substrates have been previously described (52–55). Such mutants are needed to identify possible substrates because the interaction between a WT enzyme and its substrate is typically too transient.
co-transfected RHBDL4, C-terminally tagged with myc and FLAG (RHBDL4mycFLAG). In co-immunoprecipitations using anti-FLAG beads, mCherry-PTP1B WT and mutants D181A and D181A/Y46F were detected by anti-mCherry antibody, weakly for WT PTP1B, more strongly for the D181A mutant, and most strongly for the super-trapping D181A/Y46F (Fig. 3B). This interaction was inhibited by the tyrosine phosphatase inhibitor, Na$_2$VO$_4$ at a concentration of 4 mM. When this competitive inhibitor was present in washes, we observed reduced binding of mCherry-PTP1B to RHBDL4mycFLAG (Fig. 3C). These results suggest that RHBDL4 could be a substrate of the tyrosine phosphatase PTP1B.

**Proteomic identification of phosphorylations on RHBDL4**

Our observation of RHBDL4 binding to PTP1B (also called PTPN1) substrate-trapping mutants led us to investigate directly whether RHBDL4 is itself tyrosine-phosphorylated. The analysis of the last 110 amino acid residues of the C terminus of RHBDL4, which is its major cytoplasmic domain, contains 10 tyrosine residues that are organized into eight sites, where two sites are tyrosine doublets (Fig. 4A). An additional tyrosine, Tyr$^{295}$, is predicted to be part of the last transmembrane domain. The tyrosine residues of the C terminus of RHBDL4 are close to important interaction motifs capable of binding to ubiquitin and p97/VCP (8). Tyrosine frequency in the proteome is ~3%, compared with ~10% in this RHBDL4 cytoplasmic domain (206–315). Similar enrichment of tyrosine residues exists in the C terminus of RHBDL4 homologues of different vertebrates including *Mus musculus*, *Gallus gallus*, *Xenopus tropicalis*, and *Danio rerio* (Fig. S4). Consistent with the conservation of these tyrosine residues, MS analysis of the BioID data for RHBDL4 revealed phosphorylated peptides in the C terminus of RHBDL4. Specifically, residues Thr$^{263}$/Tyr$^{264}$/Thr$^{265}$, Ser$^{269}$, Thr$^{289}$, Ser$^{291}$, Tyr$^{295}$, and Ser$^{300}$ (Fig. 4, A and B, and Fig. S5B) were predicted to include phosphate groups. For example, in the peptide 296NSPYYGFHLSPEEMR$^{305}$, Tyr$^{295}$ was identified with gain of mass +79.97 compatible with a phosphorylation detected between fragments b10 and y11 ($q \leq 0.01$) (Fig. 4B). In other cases, 296NSPYYGFHLSPEEMR$^{305}$ was phosphorylated at Tyr$^{295}$ in 21 of 123 peptides with $q \leq 0.01$, whereas 259NYDTYTAGLSEEQLR$^{276}$ was phosphorylated in residue Thr$^{263}$, Tyr$^{264}$, or Thr$^{265}$ in 11 of 32 peptides with $q \leq 0.01$.

**Tyrosine residues of the C terminus of RHBDL4 are important for interaction with p97/VCP and Lys$^{63}$-linked ubiquitin**

We tested whether these tyrosines influence the binding of p97/VCP and ubiquitin. The mutants of individual sites or a mutant in which all tyrosines are replaced by phenylalanines (Ally→F) did not affect cellular distribution, and we detected no major effect on cellular morphology when observed by confocal light microscopy; all mutants showed similar expression levels and localization (data not shown). The variants of RHBDL4mycFLAG were affinity-captured using anti-FLAG beads and compared with WT RHBDL4. Conversely, the mutant Ally→F showed reduced binding to p97/VCP when co-immunoprecipitates were probed with anti-p97/VCP antibody (Fig. 5, A, panel a, and B, panel a). This mutant runs faster on a gel than the WT or mutants for individual sites (Fig. 5A, panel d). Interestingly, the site 7 mutant (Y261F) appears to show slightly increased binding to p97/VCP. In addition to the altered interactions with p97/VCP, the Ally→F mutant showed strongly reduced binding to endogenous ubiquitin (Fig. 5A, panel b) and to Lys$^{63}$-linked polyubiquitin (Fig. 5, A, panel c, and B, panel b). The site 7 (Y261F) mutant might have altered binding of endogenous ubiquitin but was undistinguishable concerning the binding of Lys$^{63}$-linked ubiquitin (Fig. 5A, panels b–d). Overall, these results show that C-terminal tyrosine residues of RHBDL4 are important for binding RHBDL4 to ubiquitin, Lys$^{63}$-linked ubiquitin and p97/VCP.

**Discussion**

In this work, we present the first spatial proteomics performed on a member of the rhomboid-like protein superfamily. Using spatial proteomics approaches like BioID, it is possible to detect both strong interactions that can be found by conventional methods but also weaker and more transient proximal proteins, which may nevertheless be functionally important. Using BioID, we probed the protein neighborhood of RHBDL4, comparing it with iRhom2 as a control for the specificity of any detected interactions. GeneMANIA network analysis indicated that the RHBDL4 BioID hits partially overlapped with candidate interaction lists for p97/VCP and PTP1B (also called PTPN1) from previously performed proteomic interaction screens. These earlier studies were done using affinity purification–based strategies, and the presence of common candidates helps to validate our BioID screen on RHBDL4. TMMHM analyses showed that BioID of RHBDL4 contains TA proteins, and intriguingly, PTP1B is a TA protein too. It will be interesting to investigate in the future the potential interplay between PTP1B, other identified TA proteins, and RHBDL4. For instance, Vamp-associated protein (VAP) homologues are present in the BioID of RHBDL4. VAPA and VAPB are TA proteins and important adaptor proteins of the ER with roles at ER contact sides and has recently been associated with PTP1B activity in regulating receptor recycling (56–59).

PANTHER Gene Ontology analysis indicates enrichment in the BioID of RHBDL4 for terms related to ERAD, protein stress at the ER, and protein localization. Conversely, terms related to EGF signaling are enriched in the BioID of iRhom2. This is consistent with the current view of RHBDL4 as an ERAD determinant (8), with roles as a secretase enzyme (14, 60), whereas iRhom2 regulates EGF signaling by regulating TACE/ADAM17, a major sheddase of EGF receptor ligands (28). Similarly, the enrichments for protein localization GO terms in the BioID of RHBDL4 are consistent with the roles in the control of traffic dynamics that have been suggested (14). From this point of view, it is intriguing that BioID of RHBDL4 identified PTP1B, a tyrosine phosphatase related to ER stress and recycling of signaling receptors (47).

The identification of the tyrosine phosphatase PTP1B led us to discover that the C terminus of RHBDL4 is indeed tyrosine-phosphorylated. Moreover, we have shown that the binding of p97/VCP and ubiquitin is affected by mutations targeting these tyrosines, providing the first indication that RHBDL4 may be
regulated by tyrosine kinases. This will be an important area for future exploration. We also found that RHBDL4 binds to Lys63-linked polyubiquitin and that this interaction is also reduced when all tyrosine residues in the C terminus are mutated to phenylalanines. Unlike Lys48-linked ubiquitin, which is more often related to proteasome functions (61), Lys63-linked ubiquitin has been related to autophagy, protein traffic and signaling (61, 62). This result provides another platform for future investigation of RHBDL4 function. An E3 ubiquitin ligase, TRIM13, was also identified in the RHBDL4 BioID hits.

Figure 4. Mapping of identified post translational modifications to the C terminus of RHBDL4.

A, plasticine model of RHBDL4 interactions with mapping of phosphopeptides to the primary sequence of the C terminus (209–315) of RHBDL4. In red are identified phosphorylations, and in bold type are the 11 tyrosine residues. Site 1 (Tyr205) is predicted to be in the transmembrane domain and was ignored. Site 2 (Tyr227), site 3 (Y233F/Y234F), site 4 (Y242F), site 5 (Y245F/Y246F), site 6 (Y254F), site 7 (Tyr261), site 8 (Tyr264), and site 9 (Tyr295) are represented in bold type.

B, analysis of the fragmentation of the peptide GNTRNSPPYGFHLSPEEMR encompassing Tyr295 and the filtered fragment mass error (q value < 0.01).
TRIM13 is an ER-localized E3 ubiquitin ligase that can facilitate the degradation of CD3δ (40). Additionally, a role for TRIM13 in ER-stress induced autophagy has been suggested (63, 64). On the other hand, overexpression of this E3 ligase correlates to increased Lys29-linked polyubiquitin (65), indicating that it is premature to build functional hypotheses on this possible interaction, which will need further investigation.

In the past, BioID had been successful in identifying novel candidates that are hard to detect by more conventional strategies such as affinity purification. The BioID of RHBDL4 further strengthens the value of this technology, although our list of proteins might be skewed by the expression of additional copies of RHBDL4. We report here many interesting candidates for functional partners of RHBDL4, and our work provides the foundations for targeted mechanistic investigations of how these putative interactors may act with RHBDL4 in its emerging roles in cellular quality control, protein traffic, and signaling. Furthermore, the identifica-
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tion of phosphosites at the C terminus of RHBDL4 and tyrosine residues that are important for p97/VCOP and ubiquitin binding suggests new regulatory mechanisms. For instance, RHBDL4 is required for G protein–coupled receptor–stimulated TGFα release in exosomes (14). Finally, this work validates the use of BioID as a tool to investigate functionally relevant proteins that interact with the rhomboid-like proteins more generally, as well as other factors in the secretory pathway.

Experimental procedures

Cloning and generation of BioID cell lines

cDNA of RHBDL4 and iRhom2 were PCR-amplified from fibroblast cDNA library “Basinger preparation” kindly provided by Chris Norbury. 7 serine flexible arm (7S) was introduced between mycBirA* and the bait protein by overlap PCR. The cDNAs of RHBDL4–7S-mycBirA* and mycBirA*-7S-iRhom2 so generated were introduced by gateway cloning (Thermo Fisher Scientific) into FU-tetO-Gateway (Addgene, 43914) via DONR221, a plasmid kindly provided by Dragana Ahel. HeLa and HEK293 cells were transfected using Lipofectamine and selected with 400 μg of Zeocin initially and then kept at 200 μg/ml.

Western blots

NuPAGE precast gradient 4–12% polyacrylamide (Thermo Fisher Scientific) was used for anti-BirA blot. All other blots are either 10% or 12.5% polyacrylamlid:bis (37.5:1) gels. The membranes were incubated in 5% milk in PBS with Tween or Nonidet P-40 at 0.2%. Anti-RHBDL4 (Sigma, HPA013972) was used at 1:200. Anti-K63 linkage–specific (Abcam, EPR8590-448), anti-p97 (Thermo Scientific Pierce), anti-mCherry (GeneTex, ab14002), and 1:800 for streptavidin-488 (Thermo Fisher Scientific) were all used at 1:1000. Anti–FLAG-HRP (Sigma–Aldrich A5450) was used at 1:2000 whenever indicated. Neutravidin blots were done after blocking 3% BSA in PBS, with 0.2% Tween 20; neutravidin–HRP conjugate (Thermo Fisher Scientific) was used at 1:4000 in 3% BSA in PBS. 0.1% Ponceau S stain in 1% acetic acid was used to stain nitrocellulose membranes.

Immunofluorescence

The cells on cover glasses were rinsed in PBS three times prior to fixation in paraformaldehyde 4% in PBS for 20 min. The fixative was substituted twice with 200 mM PIPES with 100 mM glycerine and incubated for 40 min. Fixed cells were extracted in 0.2% Triton X-100 in PBS for 20 min. The cover glasses were postfixed in methanol at −20 °C for 45 min and then rehydrated in 3% BSA in PBS at 4 °C overnight. The next day, the cover glasses were incubated with primary antibodies diluted in 3% BSA with 0.2% Triton X-100 in PBS for 2 h. Dilution of the primary antibodies is 1:100 for PDI (Cell Signaling, 3501), 1:500 for COX4 (Cell Signaling, E311), 1:800 for anti-BirA (Abcam, ab14002), and 1:800 for streptavidin–488 (Thermo Fisher Scientific). The cover glasses were washed in 3% BSA three times. Anti-mouse, -rabbit, or -chicken goat antibody 568 was used at 1:2000 in 3% BSA for 1 h as secondary labeling. Cover glasses were mounted on slides on 4’,6’-diamino-2-phenylindole mount (ProLong™ Diamond antifade mountant; Thermo Fisher Scientific) and then sealed with nail polish and air-dried. Confocal light micrographs were taken at an Olympus FV1000 at 63× or 100× magnification.

Image analysis

Quantifications of intensities of signals from Western blotting or images of Ponceau red stains were done using Nikon D3200 camera and ImageQuant (GE Healthcare). All p values are calculated by using Student’s t tests on values from three independent biological replicates. The error bars are the standard deviation from the mean. Models were made with plasticine (Play-Doh, Hasbro), then photographed with MotoG5 cell phone camera, and edited with Photoshop (Adobe). All immunomicrographs were processed with Fiji/ImageJ and postprocessed in Photoshop (Adobe).

BioID and MS

Stably expressing BioID cell lines were treated with biotin 50 μM for 24 h prior to lysis in ice-cold radioimmuneprecipitation assay buffer with Complete protease inhibitor (Roche). The sample was then added with 2× final modified Laemmli’s buffer (120 mM Tris-HCl, pH 6.8, 4% SDS, 0.8% 2-mercaptoethanol, and 20 μM 1,4-DTT, 4% glycerol, 0.05% bromphenol) supplemented with biotin 50 μM and boiled for 10–15 min at 95 °C. For proteomic submission, cell culture was scaled to two 15-cm plates per condition. Biotinylated proteins were isolated using original Roux-buffer system for BioID (22); Only modification has been the use of 1–2.5 kU of Benzonase (Sigma–Aldrich), prior to addition of immunomagnetic MyOne streptavidin C1 beads (Lifesciences). Moreover, biotinylated proteins were eluted from beads in modified Laemmli’s buffer just mentioned, without glycerol and bromphenol but supplemented with biotin 50 μM at 95 °C for 9.5 min. 10% of eluates were kept for later neutravidin–blot analysis, prior to submission for MS analysis. Remaining 90% was processed by filter-aided sample preparation (66). The sample was washed 5–13 times in 8 μm urea in 100 mM triethylammonium bicarbonate (TEAB), pH 8.5, before reduction by 10 mM tris(2-carboxyethyl)phosphine in 8 μm urea in 100 mM TEAB, pH 8.5. Tris(2-carboxyethyl)phosphine was removed, and the sample was alkylated with 10 mM chloroaceticamide in 8 μm urea for 30 min. Alkylated material was washed with 8 μm urea in 50 mM TEAB, pH 8.5, and resuspended into 1 μm urea in 50 mM TEAB, pH 8.5. The sample was tryptically digested at a 1:5 enzymes:substrate ratio at 37 °C overnight and then blocked with formic acid 1%. Peptides were eluted in 0.1% TCA and 50% acetonitrile in 0.1% TCA. These peptides were speed vacuumed to dryness, prefiltered from participate with 100% acetonitril in 0.1% TCA equilibrated handmade C18 custom columns, and eluted in 50% acetonitrile in 0.1% TCA. Finally, the sample was resuspended in 0.1% formic acid in 2% acetonitrile and sonicated in ultrasonic bath. For injection purposes, the peptides were resuspended in 5% formic acid and 5% DMSO and then trapped on a C18 PepMap100 pre-column (300-μm inner diameter × 5 mm, 100 Å; Thermo Fisher Scientific) with 0.1% formic acid, at 26 bar and separated on an Ultimate 3000 UHPLC system (Thermo Fisher Scientific) coupled...
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Bioinformatic analyses of BioID

The results were analyzed via Central Proteomics Facilities Pipeline (67) and triple searched in MASCOT (68) Server (Matrix Sciences), X!Tandem (69) kscore (GPM), and Open Mass Spectrometry Search Algorithm (70). These triple-searched peptides were matched against UPR_HomoSapiens. Searches were conducted with one maximum missed trypsin translational modifications. Precursor tolerance was at 20 ppm, and fragment tolerance was at 0.1 Da, with 1+, 2+, and 3+ default charge states. Weighted spectral counts were quantified with SINQ (71) with q value of ≤0.01. Identifications with less than three spectral counts were manually discarded and then ranked according to the sum of spectral counts of biological replicates. Common candidates from different experiments were searched via a custom-made Python coded application. From so-obtained lists of reproducible candidates, gene networks were generated using GeneMANIA and Cytoscape 3.6.1 (29–31); we compared the candidate list of BioIDs against the previously published proteomes as in Refs. 30 and 32–37. Direct interactions were selected using Cytoscape → MANIA tools → physical interaction → select first neighbors and then table panel → export table. GeneMANIA-predicted-missing-link candidates were removed manually. Circular disposition of results was chosen to represent Fig. S2. Potential biological functions of identified candidates were searched using the Gene Ontology PANTHER Classification System (44–46) (http://pantherdb.org/),4 and GO terms with more than 2-fold increase in frequencies are reported in the results. Prediction of the topology of the BioID hits were performed by batch retrieval of the primary sequences of protein in FASTA format via Uniprot (https://www.uniprot.org/batch/).4 Sequences so obtained were batch uploaded on TMHMM server 2.0 (www.cbs.dtu.dk/services/TMHMM/).4 We scored manually the presence of predicted TM domain with in 50AA from either N or C termini of proteins. Finally, we used the definition of TA or C termini of proteins. From Borgese et al. (72) to score their presence.

Site-directed mutagenesis and transfections

All point mutations were introduced by site-directed mutagenesis using Clonetech HiFi PCR premix (Clontech). pCMV6 RHBDL4mycFLAG was purchased from Origene (RC210708). C termini of Aliy→F mutant was designed with Genearth (Thermo Fisher Scientific) and used as long primers to PCR using WT RHBDL4 as template. The PCR product was cloned into SgfI/XhoI–excised RC210708 plasmid, by Gibson assembly (NEB). mCherry-PTP1B D181A was obtained from Addgene (40270). mCherry-PTP1B WT was generated by reverting the Ala181 codon back to aspartate, whereas super-trapping mutant D181A/Y46F was generated by site-directed mutagenesis of Tyr46 to Phe on the D181A mutant (Addgene, 40270).

Co-immunoprecipitations

HEK293 cells were grown to 70% confluency on 15-cm dishes. The cells were transfected with 2 μg of DNA for all constructs using Lipofectamine Ltx at 1% with plus reagent at 0.5% (Thermo Fisher Scientific). After 8.5 h, fresh medium was provided. After 24 h, the cells were lysed in ice-cold 1% Triton in 50 mM Tris, pH 7.4, with 150 mM NaCl, Complete protease inhibitors, 2 mM sodium pervanadate, and 1 mM sodium fluoride. Sodium pervanadate was prepared by repeated heating and adjustment of alkalinity to pH 10 with NaOH. Lysates were centrifuged for 10 min at 1000 × g at 4 °C. Supernatants were collected and provided with 250 units of benzonase before addition to pre-equilibrated immunomagnetic anti-FLAG M2 beads (Sigma–Aldrich). Lysates were incubated with beads for 90 min and then washed in same lysis buffer four times for 5 min for mCherry-PTP1B variants. In washout experiments, Na3VO4 was added to the wash buffer at 4 mM final. Additional 100 mM NaCl was provided to the wash buffer to co-immunoprecipitates that were eluted in above mentioned modified Laemmli’s buffer for later probing with anti-p97VCP or anti-ubiquitin antibodies.

Author contributions—K. N. I. and M. F. conceptualization; K. N. I. and M. F. resources; K. N. I. data curation; K. N. I. software; K. N. I. validation; K. N. I. investigation; K. N. I. writing-review and editing; K. N. I. all experimental work.

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Spatial proteomics reveal that the protein phosphatase PTP1B interacts with and may modify tyrosine phosphorylation of the rhomboid protease RHBDL4
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Supporting Information 1 HeLa cells stably expressing mycBirA*Rhom2 were prepared for immunofluorescence and stained with anti-BirA, Streptavidin-488, anti-PDI, anti-COX4, and DAPI prior to imaging with confocal microscopy. Size markers represent 20μm in anti-BirA immunofluorescent micrographs (a-f). For Streptavidin-488 biotin localisation micrographs (g-o), size bar is 32 μm.
**Supporting Information 2** GeneMANIA-specified network of genetic (green) and physical (red) interactions of the BioID of RHBDL4 in HEK293 cells. Large circles are actual BioID hits, while small circles are GeneMANIA predicted missing links among proteins.
Supporting Information 3  Predicted topology of candidates in the BioID of RHBDL4 and iRhom2 according to TMHMM. A) Percentage of proteins found in BioID of RHBDL4 in HEK293, HeLa, HEK293 and HeLa, or of iRhom2 in HEK293 cells. The graph shows the percentages of proteins with predicted no TM (black), N-terminal TM (white) and TA (red) and other transmembrane (grey).
B) Probability of transmembrane region (posterior probability) along the primary sequence of the predicted TA proteins found in both the BioID of RHBDL4 in HEK293 and HeLa.
Supporting Information 4  Alignment of the C-terminus of RHBDL4 homologues from *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus tropicalis*, *Danio rerio*, *Arabidopsis thaliana*, and *Oryza sativa*. End of the 6th transmembrane domain and beginning of the C-terminus domain is indicated by ”V”. **Bolded** are tyrosine residues.
A

Protein coverage for sp|Q8TEB9|RHBL4_HUMAN

Identified in SUB8791 - HEK293 BioID R4 iR2 / MSS11056 - HEK293 BioID R4 iR2 (Meta)

sp|Q8TEB9|RHBL4_HUMAN - Rhomboid-related protein 4 OS=Homo sapiens GN=RHBDD1 PE=1 SV=1

Percent Coverage: 52.1%

MQRRSRGINT GLILLSQIF HVGINNIPPV TLATLALNW FFLNPQXPLY SSCLSVEKY QOKDWORLLI SPLHADDWJH 80
LYFNMASAILK XGINLERRLGC SRWFAYVIITA FSVLTGVVYL LLOQAVAEMM DEPDKRSACA VGFSGVLFAK KVLNNHYPGPD 160
GFVNLGFPV PNRFACWVEL VAIHLSPGT SFAGHLAGIL VGLMYTQGPL KXIMEACAGG FSSSVGYGPR QYYFNSSGSS 240
GYQDYYPGHGR PDHYTEEPRN YDTYTAGLSE EEOERLQA SLWDRGNTRN SPPPYFGHLS PEEMRRQRLH RFDSQ

Click here to view PTM summary report for this protein ID.

B

NYDTY(-79.97)TAGLSSEEQLER

1 x Phospho (Y)

Identified in MSS11093 with qvalue 0.00
Charge: 2, Exp. m/z: 1049.940, Calc. m/z: 1049.437
Data File: Qex03_SVH_150624_kyo_k2, Scans: 32745 - 32745

Filtered Fragment Mass Errors (Mean: 6.33 ppm SD: 15.01 ppm)
Supporting Information 5 MS coverage and fragmentation of the peptides mapping to RHBDL4. A) Peptides cover 52% of RHBDL4. B) Fragmentation gallery of phospho-peptides mapping to RHBDL4. All identification are q value <0.01.