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# The Ubiquitin-Interacting Motifs Target the Endocytic Adaptor Protein Epsin for Ubiquitination

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## Summary

**The covalent attachment of ubiquitin to proteins is an evolutionarily conserved signal for rapid protein degradation. However, additional cellular functions for ubiquitination are now emerging, including regulation of protein trafficking and endocytosis [1]. For example, recent genetic studies suggested a role for ubiquitination in regulating epsin, a modular endocytic adaptor protein that functions in the assembly of clathrin-coated vesicles [2, 3]; however, biochemical evidence for this notion has been lacking. Epsin consists of an epsin $NH_2$-terminal homology (ENTH) domain that promotes the interaction with phospholipids [4], several AP2 binding sites [5], two clathrin binding sequences [6], and several Eps15 homology (EH) domain binding motifs [3]. Interestingly, epsin also possesses several recently described ubiquitin-interacting motifs (UIMs) that have been postulated to bind ubiquitin [7]. Here, we demonstrate that epsin is predominantly monoubiquitinated and resistant to proteasomal degradation. The UIMs are necessary for epsin ubiquitination but are not the site of ubiquitination. Finally, we demonstrate that the isolated UIMs from both epsin and an unrelated monoubiquitinated protein, Eps15 [8], are sufficient to promote ubiquitination of a chimeric glutathione-S-transferase (GST)-UIM fusion protein. Thus, our data suggest that UIMs may serve as a general signal for ubiquitination.**

## Results and Discussion

To test whether epsin is ubiquitinated in cells, we transiently coexpressed hemagglutinin (HA) epitope-tagged *Xenopus* epsin along with a Myc epitope-tagged ubiquitin (Myc-Ub). Western blot analysis of HA immunoprecipitates revealed a slower migrating species of epsin with an apparent increase in size of 10 kDa, consistent with the attachment of a single Myc-Ub (Figure 1, top gel). This slower migrating form also reacted with antibodies to Myc-Ub (Figure 1, bottom gel). Similar results were obtained using either a Myc epitope-tagged *Drosophila* epsin or a chimeric GST-epsin (*Xenopus*) in conjunction with HA-epitope-tagged Ub (HA-Ub) (see the Supplementary Material available with this article online). Given the conservation of epsin (40% amino acid identity, 54% similarity across species), these results suggest that ubiquitination is an evolutionarily conserved feature of epsin. Although epsin undergoes a cell cycle-dependent phosphorylation [9], the observed mobility shift in epsin is not attributable to phosphorylation, since phosphatase treatment of epsin immunoprecipitates did not alter the mobility shift (Supplementary Material). In contrast to the effect of phosphorylation on Eps15 monoubiquitination [8], we did not observe any effect of EGF on epsin ubiquitination (our unpublished data). Together, these results demonstrate that epsin is posttranslationally modified by ubiquitination.

Our results indicate that ubiquitinated epsin is not targeted for degradation. First, we did not observe any difference in the levels of ubiquitinated or nonubiquitinated epsin in the presence or absence of proteasome inhibitor (Figure 1 and Supplementary Material). Second, both HA- and Myc-tagged epsin were predominantly monoubiquitinated (as judged by mobility shift on Western blots) (Figure 1 and Supplementary Material). Although higher molecular weight, multiubiquitinated forms of GST-epsin were observed, we did not detect GST-Ub-epsin with greater than four ubiquitins, a signal for recognition by the proteasome [10]. We have not determined if these multiubiquitinated forms of epsin consist of polyubiquitin chains, multiple sites of monoubiquitination, or a combination of these two possibilities. However, additional experiments presented below suggest that GST-epsin is ubiquitinated at multiple sites.

Given the proposed role of UIMs in ubiquitin binding [7], we hypothesized that this region might function in epsin ubiquitination. Indeed, deletion of the UIMs from HA-epsin (HA-epsinΔUIM) eliminated monoubiquitination (Figure 2A). In addition, deletion of the UIMs from GST-epsin (GST-epsinΔUIM; see Figure 3) dramatically reduced ubiquitination (Figure 2B). Using a series of epsin truncation mutants fused to GST (Figure 3), we demonstrated that all the UIM-containing fusion proteins were ubiquitinated (Figure 4A, left). Two UIMs were sufficient for ubiquitination, whereas expression of a single UIM led to a dramatic reduction in ubiquitination (Figure 4A, right). These results suggest that more than one UIM is required for efficient ubiquitination in vivo. GST, GST-ENTH, and GST-epsinΔUIM were not ubiquitinated, demonstrating specificity in our ubiquitination assays. Our data indicate that UIMs are both necessary and sufficient for epsin ubiquitination. The UIMs from Eps15 were also sufficient for ubiquitination of a chimeric fusion protein (Supplemental Materials), supporting a general role for UIMs in protein ubiquitination. Given the presence of UIMs in numerous proteins [7], additional studies will be needed to determine if these proteins are also ubiquitinated.

Although our data indicate that a single UIM is not sufficient to promote ubiquitination, in some circum-

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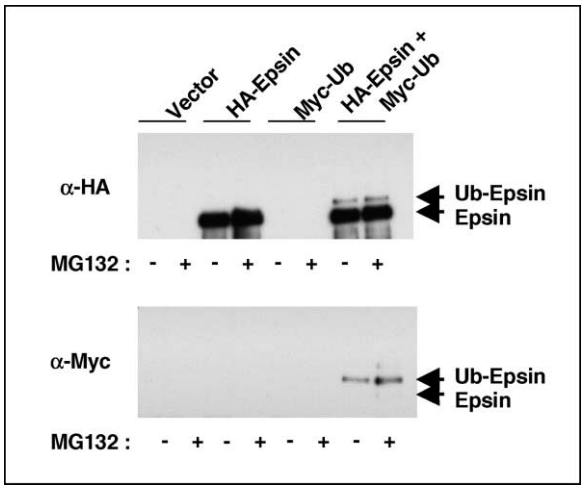


Figure 1. Epsin Is Ubiquitinated in 293T Cells

HA epitope-tagged *Xenopus* epsin was transiently coexpressed with Myc-tagged ubiquitin (Myc-Ub). Following immunoprecipitation with α-HA antibodies, Western blots of these immunoprecipitates were analyzed for total protein with α-HA (top gel) or for ubiquitin conjugation with α-Myc (bottom gel). Cells were incubated in the presence (+) or absence (−) of MG132 for 5 hr prior to lysis. Note that MG132 treatment does not have an appreciable effect on the levels of epsin or Ub-epsin.

stances, a single UIM does exhibit biochemical activity. The single UIM from the yeast S5a subunit was sufficient for ubiquitin binding [11]. Thus, UIMs may have several functions in ubiquitin metabolism, each of which may require different numbers of UIMs.

Our results suggest that the UIMs either function as ubiquitination sites or recruit specific ubiquitin-conjugating enzymes (E2s) or ubiquitin ligases (E3s) [12] to target ubiquitination distal to the UIMs. Mutation of the 6 Lys residues in UIM2-3 (UIM2-3ΔK) did not block ubiquitination (Figure 4A). Similarly, mutation of the two Lys in Eps15's UIMs did not inhibit ubiquitination (Supplementary Materials). These results support the notion that ubiquitination occurs distal to the UIMs. However, these mutations may lead to ubiquitination at a secondary site. To discriminate between these two possibilities, we purified the ubiquitinated GST fusion proteins from cells, cleaved the epsin portion from GST with thrombin, and then analyzed the resulting bead-bound GST and soluble epsin fragments for ubiquitination. GST was ubiquitinated when attached to any portion of epsin that contained the UIMs but was not modified when expressed alone or in combination with the ENTH domain or epsinΔUIM (Figures 2C and 4B). However, GST was most strongly ubiquitinated when fused to the COOH or UIM regions (Figure 4B). Furthermore, of the proteins that became ubiquitinated, GST derived from the Δ305 construct contained the least amount of ubiquitin (Figure 4B). In contrast, the soluble fragments derived from full-length epsin and Δ305 contained significant levels of HA-Ub, whereas the soluble COOH, UIM, and epsinΔUIM peptides did not (Figures 2D and 4C). These results indicate that the UIMs are not the actual site of ubiquitination but rather target ubiquitination to a site distal to the UIMs. Given the ubiquitination of the soluble Δ305 peptide and lack of ubiquitination of the soluble COOH and UIM peptides (Figure 4C), we conclude that the site(s) of epsin ubiquitination lies within the ENTH domain and selection of this site requires the presence of the UIMs.

Both the HA- and Myc-tagged epsins were predominantly monoubiquitinated. Our mapping studies with the GST-epsin fusion proteins suggest that ubiquitination occurs within the ENTH domain and requires the presence of the UIMs to target this domain for modification. Given the ability of the ENTH domain to bind $PI_{4,5}P_2$ [4] and the transcription factor PLZF [13], one possibility is that ubiquitination of epsin alters its interaction with these two targets. Although inhibition of $PI_{4,5}P_2$ binding would disrupt epsin's endocytic activity [4], the consequences of altered PLZF binding are currently unknown. Deubiquitination of epsin might then restore interaction with these or possibly other targets. This model is consistent with genetic studies in *Drosophila* suggesting that epsin is regulated by the deubiquitinating enzyme Faf [2]. Our results, however, are not consistent with the model of proteasomal degradation of epsin as suggested from the genetic interaction between Faf, *Drosophila* epsin, or Lqf, and the proteasome [2] but rather suggest an alternative interpretation. The suppression of Faf and Lqf mutant phenotypes by a proteasome mutation may stem from increased levels of an additional negative regulator of this developmental pathway that is normally degraded by the proteasome. Increased levels of this protein may counteract the decrease in Lqf and Faf activities. Given the complexity of photoreceptor development, this alternative interpretation is also possible.

UIMs were first identified through an iterative database search for proteins with similarity to the ubiquitin binding region of the S5a subunit of the proteasome [7]. Although several studies report ubiquitin binding by UIM-containing proteins [11, 14–16], ataxin-3's UIMs do not bind ubiquitin [17]. Our results indicate that epsin's UIMs do not directly bind ubiquitin. First, we were unable to detect specific binding of either monoubiquitin or tetraubiquitin to GST-UIM as compared to GST alone using surface plasmon resonance (data not shown) [18]. The difference in ubiquitin binding between epsin and Eps15 [16] may reflect real differences in ubiquitin recognition by Eps15's UIMs versus epsin's UIMs. Alternatively, there are significant differences in the experimental approaches that may also contribute to this difference (Supplemental Materials). Second, in vitro binding experiments with bacterially expressed GST fusion proteins of epsin (similar to those shown in Figure 3) were repeatedly negative for binding HA-Ub from cell lysates. Third, affinity purification of the GST-UIM fusion proteins from cell lysates overexpressing HA-Ub did not result in copurification of either HA-Ub or additional ubiquitinated proteins (see Figure 4A, bottom). Fourth, addition of ubiquitin to an NMR sample of $^{15}N$-labeled UIM1-3 (lacking GST) did not alter the NMR spectrum of the UIMs (our unpublished data). These data suggest that the UIMs from epsin do not directly bind ubiquitin.

Biophysical analysis of epsin indicated that the protein exists in an unstructured state with the exception of the ENTH domain [19]. These findings suggest that

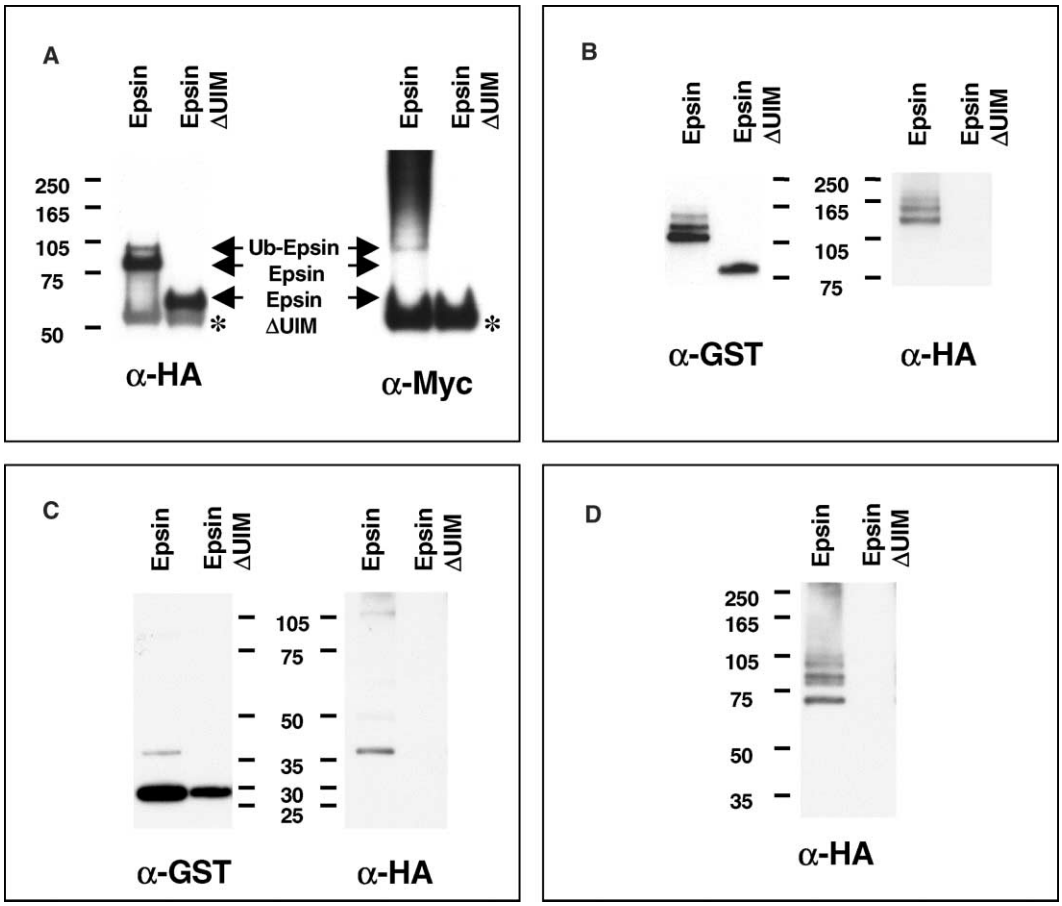


Figure 2. Deletion of the UIMs from Epsin Prevents Ubiquitination

(A) HA-epitope-tagged epsin or epsinΔUIM were cotransfected into cells along with Myc-Ub. Samples were immunoprecipitated with α-HA antibodies and then probed with either α-HA (left) or α-Myc (right) antibodies. *, indicate the position of the immunoglobulin heavy chain.

(B–D) GST-Epsin or GST-EpsinΔUIM was coexpressed in cells along with HA-Ub. Following purification, the fusion proteins were fractionated on gels (B), and filters were probed with either α-GST (left) or α-HA (right).

(C) Samples from (B) were treated with thrombin to cleave the epsin portions from GST. Proteins remaining attached to the GSH beads were fractionated on gels, and filters were probed with either α-GST (left) or α-HA (right).

(D) The soluble epsin fragments recovered from the supernatants following thrombin cleavage of samples from (B) were fractionated on gels and analyzed for the presence of HA-ubiquitin. The ~70 kDa HA-reactive band in the epsin lane corresponds to a nonspecific thrombin cleavage product of epsin resulting from thrombin digestion.

the UIMs may function as extended peptide recognition sequences that recruit specific E2s and E3s. Indeed, we are able to detect association of epsin with a ubiquitin ligase complex that is dependent on the presence of the UIMs (our unpublished data). These data suggest that epsin's UIMs may facilitate ubiquitin binding indirectly through recruitment of E2s and E3s.

Identification of the E3 that targets epsin ubiquitination and characterization of its activity will help to uncover the mechanism for epsin ubiquitination and the role of this modification in epsin function. Genetic screens in *Drosophila* have uncovered a common pathway regulated by Faf and the E3 Highwire, suggesting that these two proteins regulate a similar substrate [20–22]. Given the genetic interaction between Faf and epsin [2], epsin ubiquitination may be controlled in part by Highwire. Additionally, epsin interacts with intersectin which forms a complex with the E3 c-Cbl raising the possibility that c-Cbl may regulate epsin ubiquitination [23]. Finally, Nedd4 ubiquitinates Eps15 in a UIM-dependent manner and may therefore also regulate epsin [16].

In this study, we demonstrated that epsin is modified by monoubiquitination in a UIM-dependent manner. In contrast to polyubiquitination, we demonstrated that Ub-epsin is not targeted for proteasomal degradation. We speculate that these motifs are responsible for assembly of a ubiquitination complex on epsin and may play a general role in targeting proteins for ubiquitin conjugation. Given that epsin and Eps15 are both monoubiquitinated, it is tempting to speculate that UIMs may represent a specific targeting signal important for monoubiquitination.

**Supplementary Material**

Supplementary Material including Experimental Procedures and figures [Figure S1, Both *Drosophila* Epsin and GST-Epsin (*Xenopus*) Are Ubiquitinated in 293T Cells; Figure S2, The Mobility Shift in Epsin Is Not Due to Phosphorylation; and Figure S3, The UIMs of Eps15

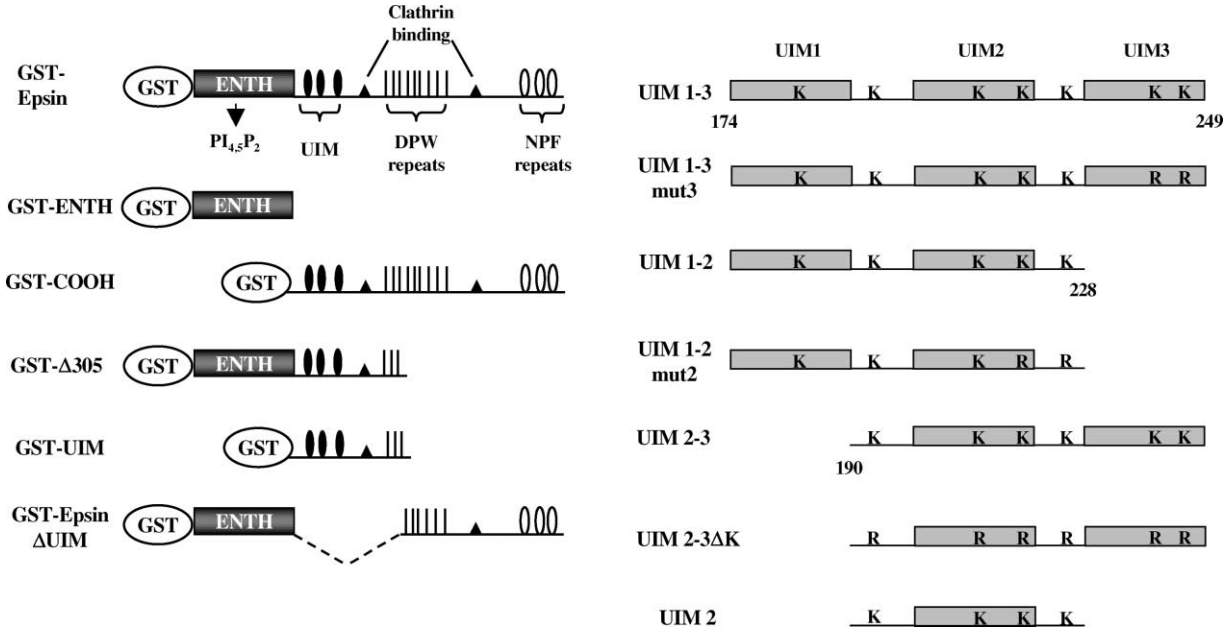


Figure 3. *Xenopus* Epsin Expression Constructs

Each construct was subcloned into the mammalian expression vector pEFG, resulting in fusion of GST to the amino terminus of the protein. GST-ENTH, amino acids 1–167; GST-COOH, amino acids 167–608 of epsin; GST-Δ305, amino acids 1–305; GST-UIM, amino acids 167–305, which includes a clathrin binding site and three AP2 binding sites. Additional UIM constructs encompassing only the UIMs were made and are shown on the right. Indicated Lys were mutated to Arg to determine their role in ubiquitination. In UIM2-3ΔK, all six Lys in UIM2-3 were mutated to Arg; however, the GST portion still contains several Lys residues.

Are Sufficient for Ubiquitination] is available online at http://images.cellpress.com/supmat/supmatin.htm.

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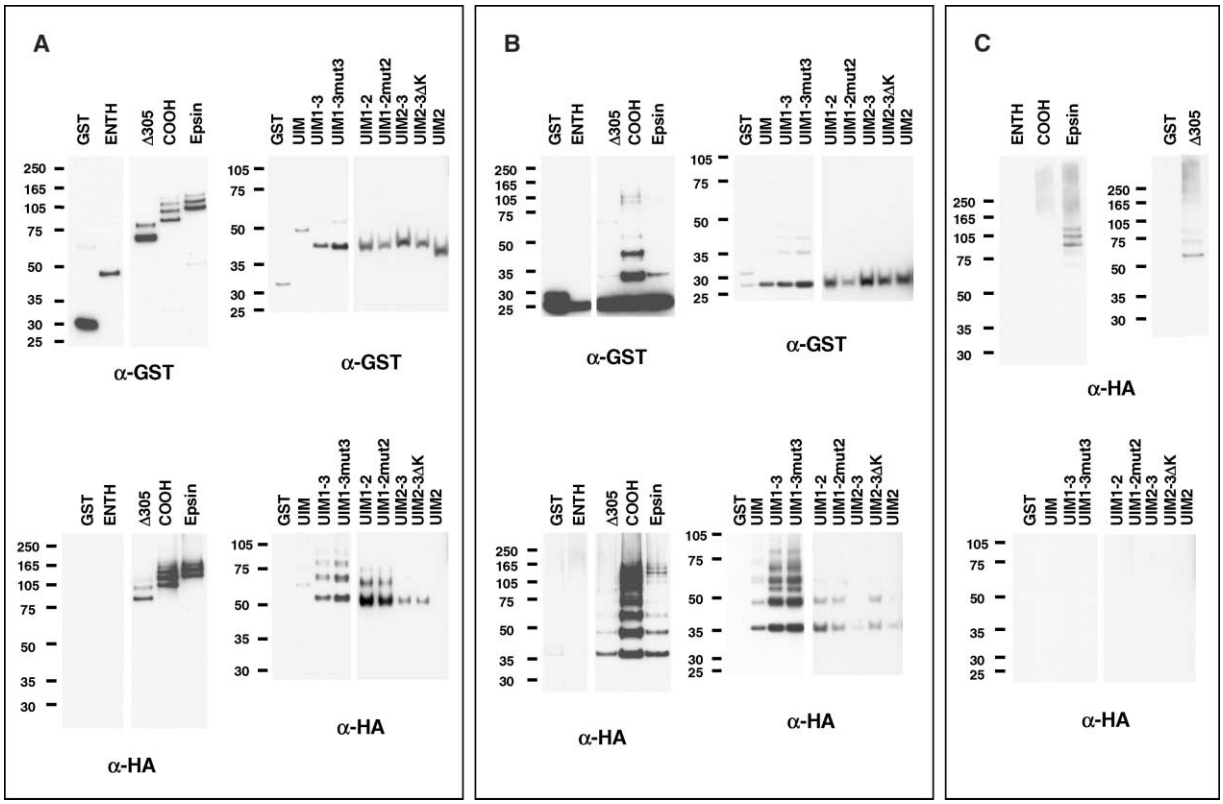


Figure 4. The Ubiquitin-Interacting Motifs of Epsin Are Sufficient for Ubiquitination

(A) The indicated GST fusion proteins were purified from cells coexpressing HA-Ub and analyzed for total protein levels (α-GST, top gels) or ubiquitinated protein (α-HA, bottom gels). Longer exposures of the UIM2 sample revealed weak reactivity with α-HA.

(B) GST undergoes ubiquitination when fused with fragments of epsin containing the UIMs. Following thrombin cleavage of the GST-epsin fusion proteins attached to beads, the GSH beads were fractionated on gels and analyzed for GST (top gels) and HA-ubiquitin (bottom gels).

(C) Both full-length epsin and Δ305 undergo ubiquitination. The soluble epsin fragments recovered from the supernatants following thrombin cleavage were fractionated on gels and analyzed for the presence of HA-ubiquitin. These results are representative of at least three independent experiments.

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