Fic-mediated deAMPylation is not dependent on homodimerization and rescues toxic AMPylation in flies

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Amanda K. Casey[‡], Andrew T. Moehlman[§], Junmei Zhang[‡¶], Kelly A. Servage[‡¶], Helmut Krämer[§], and Kim Orth[‡¶‖1]

From the [‡]Department of Molecular Biology, the [§]Department of Neuroscience, the [¶]Howard Hughes Medical Institute, and the [‖]Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9148

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Protein chaperones play a critical role in proteostasis. The activity of the major endoplasmic reticulum chaperone BiP (GRP78) is regulated by Fic-mediated AMPylation during resting states. By contrast, during times of stress, BiP is deAMPylated. Here, we show that excessive AMPylation by a constitutively active Fic^{E247G} mutant is lethal in *Drosophila*. This lethality is cell-autonomous, as directed expression of the mutant Fic^{E247G} to the fly eye does not kill the fly but rather results in a rough and reduced eye. Lethality and eye phenotypes are rescued by the deAMPylation activity of wild-type Fic. Consistent with Fic acting as a deAMPylation enzyme, its activity was both time- and concentration-dependent. Furthermore, Fic deAMPylation activity was sufficient to suppress the AMPylation activity mediated by the constitutively active Fic^{E247G} mutant in *Drosophila* S2 lysates. Further, we show that the dual enzymatic activity of Fic is, in part, regulated by Fic dimerization, as loss of this dimerization increases AMPylation and reduces deAMPylation of BiP.

AMPylation is a post-translational modification involving the covalent attachment of AMP to the hydroxyl group of a threonine, serine, or tyrosine residue. This modification was first discovered by Stadtman and colleagues (1) studying the regulation of glutamine synthetase by a post-translational modification with AMP. Interestingly, this modification can be reversed by a repeated catalytic site in the second domain of glutamine synthetase adenyltransferase (2, 3). Five decades later, AMPylation was rediscovered in studies with a bacterial virulence factor called VopS that encodes a Fic (filamentation-induced by cAMP) domain-containing protein (4). These identified AMPylators contain a Fic domain with a conserved HPF*X*(D/E)GN(G/K)R motif required for catalytic activity (5). Bacterial genomes appear to encode multiple Fic domain proteins with varied transferase activities (6, 7), whereas metazoan genomes encode only a single Fic domain–containing protein with a conserved topology and function.

In previous studies, we identified a *Drosophila* Fic domain-containing protein, Fic, that includes a single transmembrane domain upstream of conserved tetratricopeptide repeats (TPRs)[2] and a Fic domain. These domains are highly conserved in metazoan Fic domain proteins and are oriented with the TPR and Fic domains located within the ER lumen (8). Loss of *Drosophila* Fic results in defects in synaptic transmission in the visual system (8). We observed that the ER-localized *Drosophila* Fic is responsible for AMPylation of the chaperone BiP (GRP78) (9). During the unfolded protein response (UPR), BiP is both deAMPylated and transcriptionally up-regulated to help cells cope with ER stress (10). We found that BiP is AMPylated by Fic in resting, unstressed cells and deAMPylated by Fic in cells responding to ER stress. Since these initial studies, other groups have observed AMPylation of BiP by conserved metazoan Fic proteins, known as FicD or HYPE in mammals and Fic-1 in *Caenorhabditis elegans* (11–13). Thus, BiP, like other chaperones, is regulated by ATP and substrate binding but additionally by transcription and the conserved post-translational modification AMPylation (9).

One key factor to understanding the regulation of AMPylation by Fic is the role of a conserved autoinhibitory helix found in most Fic domain proteins. This inhibitory helix contains a conserved glutamate residue that is critical for the regulation of AMPylation activity of Fic domain proteins (9, 14, 15). This glutamate typically forms a salt bridge with a conserved arginine in the Fic domain active site. The salt bridge between these glutamate and arginine residues blocks the coordination of the γ phosphate of ATP in the active site, thus inhibiting the AMPylation activity of the Fic domain (14). Mutation of this conserved glutamate in Fic (Fic^{E247G} in *Drosophila*) and in other eukaryotic Fic proteins results in constitutively active AMPylating enzymes with apparent reduced substrate specificity (9, 12, 16). However, it is unclear how the inhibitory salt

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This article contains supplemental Table S1 and Figs. S1–S6.

[1] A Burroughs Wellcome Investigator in Pathogenesis of Infectious Disease, a Beckman Young Investigator, and a W. W. Caruth, Jr., Biomedical Scholar; holder of an Earl A. Forsythe Chair in Biomedical Science. To whom correspondence should be addressed: Dept. of Molecular Biology, University of Texas Southwestern Medical Center, 6000 Harry Hines Blvd., Rm. NA5.120F, Dallas, TX 75390-9148. Tel.: 214-648-1685; Fax: 214-648-1488; E-mail: Kim.Orth@UTSouthwestern.edu.

[2] The abbreviations used are: TPR, tetratricopeptide repeat; ER, endoplasmic reticulum; UPR, unfolded protein response; CHX, cyclohexamide; SEM, scanning electron microscopy; RIPA, radioimmune precipitation assay; UAS, upstream activating sequence.

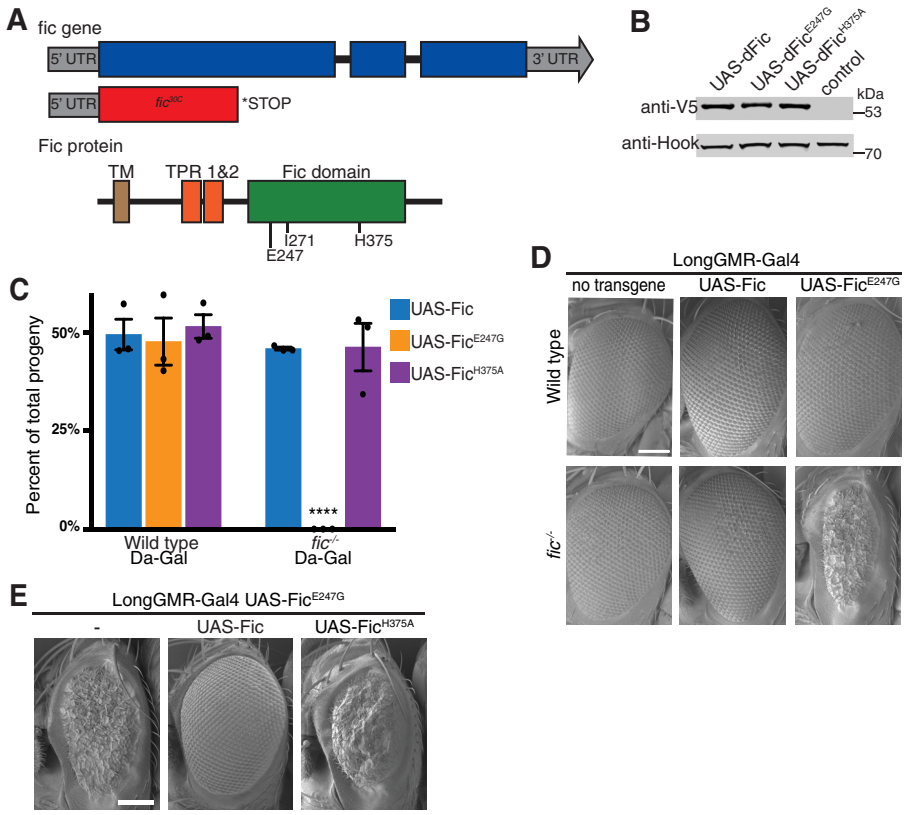

Figure 1. Recessive toxicity of Fic^{E247G} expression. *A*, structure of the *Drosophila fic* gene and protein with the regulatory Glu-247, dimerization Ile-271, and catalytic His-375 residues. The CRISPR/Cas9-induced *fic^{30C}* mutation induces a 101-base pair deletion, generating an early stop codon N-terminal to the conserved TPR domain. *B*, a representative Western blot shows expression of UAS-Fic transgenes (wild type, H375A, and E247G) under control of the ubiquitous Da-Gal4 driver in a wild-type background. Quantification of Western blots from three separate experiments showed no significant difference in expression of the UAS-Fic transgenes. *C*, *Da-Gal4* homozygous females were crossed to the indicated *UAS-Fic/TM6b, Hu* males either in a wild-type or *fic^{30C}* background. The graph represents percentage of progeny inheriting the indicated UAS-Fic transgene together with the Gal4 driver. The expected Mendelian percentage is 50%. *Bars*, mean of three independent crosses; *dots*, values for independent crosses; *error bars*, S.D. The total number of flies scored for each genotype was at least 150. *D*, representative SEM images of eyes expressing the indicated UAS-Fic transgene with LongGMR-Gal4 in a wild-type or *fic^{30C}* background. *Scale bar*, 100 μm. *E*, representative SEM images of eyes expressing the indicated UAS-Fic transgene along with LongGMR-Gal4, UAS-Fic^{E247G} in a *fic^{30C}* background. *Scale bar*, 100 μm.

bridge is regulated *in vivo* to permit AMPylation of substrates, including BiP. Structural studies have suggested that a dimerization event might be involved in the regulation of Fic-mediated AMPylation activity (12, 15). In humans and *C. elegans*, mutations in the predicted dimer interface of FicD and Fic-1 resulted in decreased auto-AMPylation, indicating dimerization may enhance AMPylation activity (12, 15).

To further investigate the regulation of Fic activity *in vivo*, we generated transgenes for the expression of wild-type and mutant Fic proteins. We found that expression of constitutively active Fic^{E247G} has no detectable phenotype in flies, as long as endogenous Fic is also present. By contrast, Fic^{E247G} overexpression in a *fic* null fly is lethal. Similarly, eye-specific expression of Fic^{E247G} caused a rough eye phenotype, but only in the absence of endogenous Fic. We hypothesize that the Fic^{E247G} toxicity in *fic* null flies is due to constitutive AMPylation without corresponding deAMPylation activity mediated by wild-type Fic. Supporting this hypothesis, we observed deAMPylation activity by wild-type Fic and found that this deAMPylation activity by wild-type Fic is active in the presence of the constitutive AMPylating activity of Fic^{E247G}. Furthermore, we found that dimerization of Fic was not essential for its deAMPylation activity, as a single mutation (I271D) in Fic that disrupts dimerization retains the ability to deAMPylate endogenous BiP. Interestingly, we found that disruption of Fic dimerization leads to increased AMPylation of recombinant BiP, indicating that dimerization could play a regulatory role in this enzymatic activity. In summary, we demonstrate that the balance of AMPylation and deAMPylation by Fic is essential for cell survival and is regulated, in part, by the dimerization state of Fic.

Results

Endogenous Fic expression is protective against the constitutive AMPylating activity of Fic^{E247G}

To study the effect of Fic in *Drosophila*, we used the CRISPR-Cas9 system to generate a *fic* null mutant, referred to as *fic^{30C}*. The CRISPR-Cas9 –induced *fic^{30C}* indel introduced a 101-base pair deletion resulting in a frameshift and early stop codon after leucine 155 of the coding sequence, creating a null *fic* allele (Fig. 1*A* and supplemental Fig. S1). As observed previously with other *fic* mutants, homozygous *fic^{30C}* flies are viable with no externally visible phenotypes (8). Next, to assess the effect of Fic expression, we generated transgenic flies with C-terminal V5 and His_6-tagged Fic proteins under control of the Gal4/UAS system.

All UAS-Fic transgenes were inserted at the same locus on chromosome 3. These flies, in contrast to flies previously generated with randomly inserted UAS-Fic transgenes (8), exhibit undistinguishable expression levels for all three Fic proteins (Fig. 1*B*).

We initially tested the effect of Fic transgene overexpression on the viability of wild-type flies. Flies homozygous for the ubiquitous Da-Gal4 driver on chromosome 3 (*Da-Gal4/Da-Gal4*) were crossed to flies containing the chromosome 3 balancer TM6B, Hu and one copy of a UAS-Fic transgene encoding either wild-type Fic, constitutively active Fic^{E247G}, or catalytically inactive Fic^{H375A}. From this cross, 50% of progeny were expected to be flies ubiquitously expressing the transgene. Viability of flies was determined by scoring adult flies for the presence or absence of a visible dominant chromosome 3 marker (*TM6B, Hu*). In these experiments, no difference was observed between the expected and observed numbers of flies expressing the wild-type UAS-Fic transgene compared with those with the balancer chromosome (Fig. 1*C*). Likewise, expression of either UAS-Fic^{E247G} or UAS-Fic^{H375A} transgenes under control of the Da-Gal4 driver did not affect viability of flies in a *fic* wild-type background.

Next, we assessed the effect of Fic transgene overexpression in *fic*^{30C} null flies. For this purpose, *fic*$^{30C/30C}$; *Da-Gal4/Da-Gal4* flies were crossed to *fic*$^{30C/30C}$; *UAS-Fic/TM6B* flies, *fic*$^{30C/30C}$; *UAS-Fic*^{H375A}*/TM6B* flies, or *fic*$^{30C/30C}$; *UAS-Fic*^{E247G}*/TM6B* flies. From these crosses, 50% of progeny were expected to be flies ubiquitously expressing the transgene. As observed in a *fic* wild-type background, we found no difference between the expected and observed viability of *fic*^{30C} flies expressing the UAS-Fic or UAS-Fic^{H375A} transgenes under Da-Gal4 control (Fig. 1*C*). However, expression of the UAS-Fic^{E247G} transgene under the ubiquitous Da-Gal4 promoter was lethal in the *fic*^{30C} null background. These data indicate that expression of the wild-type Fic protein from the endogenous *fic* locus is sufficient to suppress lethality caused by Fic^{E247G} overexpression.

Tissue-specific expression of Fic^{E247G} exhibits cell autonomous and recessive toxicity

To compare the effect of tissue-specific expression of the overactive Fic^{E247G} with wild-type Fic, we used the photoreceptor-specific LongGMR-Gal4 promoter for specific expression in the eye. This allowed us to detect eye-specific phenotypes in the surviving adult flies. Consistent with the results from ubiquitous expression, LongGMR-Gal4 – driven expression of either wild-type Fic or Fic^{E247G} transgenes resulted in no externally visible defects in a *fic* wild-type background (Fig. 1*D*). Expression of wild-type UAS-Fic in the *fic*^{30C} null flies did not interfere with normal eye development. However, in *fic*^{30C} null flies, expression of the overactive AMPylating Fic^{E247G} mutant resulted in flies with a severe eye phenotype characterized by smaller eyes with disturbed ommatidial structures. To confirm that this rough eye defect was caused by the loss of wild-type Fic in the *fic*^{30C} null flies, we tested the effect of LongGMR-Gal4, UAS-Fic^{E247G} expression in hemizygous *fic*^{30C}*/Df(2L)BSC296* flies (supplemental Fig. S1). As observed in *fic*^{30C} null flies, overexpression of UAS-Fic^{E247G} in the eye resulted in a rough eye phenotype.

Because the presence of endogenous Fic in flies appears to counterbalance the lethality and eye morphology defects of UAS-Fic^{E247G} expression, we next asked whether overexpression of UAS-Fic or UAS-Fic^{H375A} transgenes would rescue the rough eye phenotype of *fic*^{30C}*; LongGMR-Gal4, UAS-Fic*^{E247G} flies (Fig. 1*E*). As in flies expressing endogenous Fic, overexpression of the wild-type UAS-Fic transgene results in normal eye morphology. However, overexpression of the catalytically dead UAS-Fic^{H375A} transgene failed to rescue the rough eye phenotype.

We hypothesized that the lethality and rough eye phenotypes of UAS-Fic^{E247G} are caused by toxicity of this enzyme. To test this, TUNEL assays were performed on late third instar larval eye discs of wild-type and *fic*^{30C} null flies expressing UAS-Fic or UAS-Fic^{E247G} transgenes under the control of LongGMR-Gal4 (supplemental Fig. S2). As predicted, eye discs from *fic*^{30C} animals expressing UAS-Fic^{E247G} were heavily positive for TUNEL staining, indicating cell death. However, eye discs from wild-type and *fic*^{30C} animals in which wild-type UAS-Fic was expressed show no significant TUNEL staining.

These experiments indicate that expression of the overactive AMPylating Fic^{E247G} in the absence of wild-type Fic is toxic to cells. We have observed in previous *in vitro* studies that Fic^{E247G} is an enzyme with hyperactivity and reduced specificity (9). Indeed, other *in vitro* and structural studies have also found that mutations to the autoinhibitory helix of eukaryotic Fic enzymes result in overactive and promiscuous AMPylators (11, 12). Furthermore, the nature of the developmental eye defects under control of LongGMR-Gal4 suggests that the toxicity of dFic^{E247G} is cell-autonomous. Taken together, these data indicate that the catalytic activity of wild-type Fic protein counterbalances the unregulated AMPylating activity of the Fic^{E247G} mutant.

Recombinant Fic mediates deAMPylation of in vitro AMPylated BiP

Recently, it was found that FicD, the mammalian homolog of Fic, could both AMPylate and deAMPylate BiP and that disruption of the autoinhibitory α helix of FicD could inhibit the deAMPylation activity of FicD (17). Based on these recent findings and our fly studies, we hypothesized that both the AMPylation and deAMPylation activity of Fic are conserved functions for this enzyme and are essential for proper regulation and survival of a cell.

To address this hypothesis, we investigated whether an AMPylated BiP substrate could be deAMPylated by Fic. For these assays, recombinant [^{32}P]AMP-BiP^{T229A} was generated by *in vitro* AMPylation of purified His$_6$-BiP^{T229A}, a mutant with diminished ATP hydrolysis activity (18). We compared the *in vitro* deAMPylation activity of recombinant wild-type Fic and the catalytically dead Fic^{H375A} using [^{32}P]AMP-BiP^{T229A} as a substrate (Fig. 2*A*). The addition of recombinant wild-type Fic resulted in a concentration-dependent reduction of the [^{32}P]AMP signal from [^{32}P]AMP-BiP^{T229A} protein (Fig. 2*A*, *lanes 2–5*), whereas the addition of the catalytically dead Fic^{H375A} did not reduce this signal (Fig. 2*A*, *lanes 6–9*). This indicates that wild-type Fic, but not Fic^{H375A}, is able to deAMPylate recombinant AMPylated BiP.

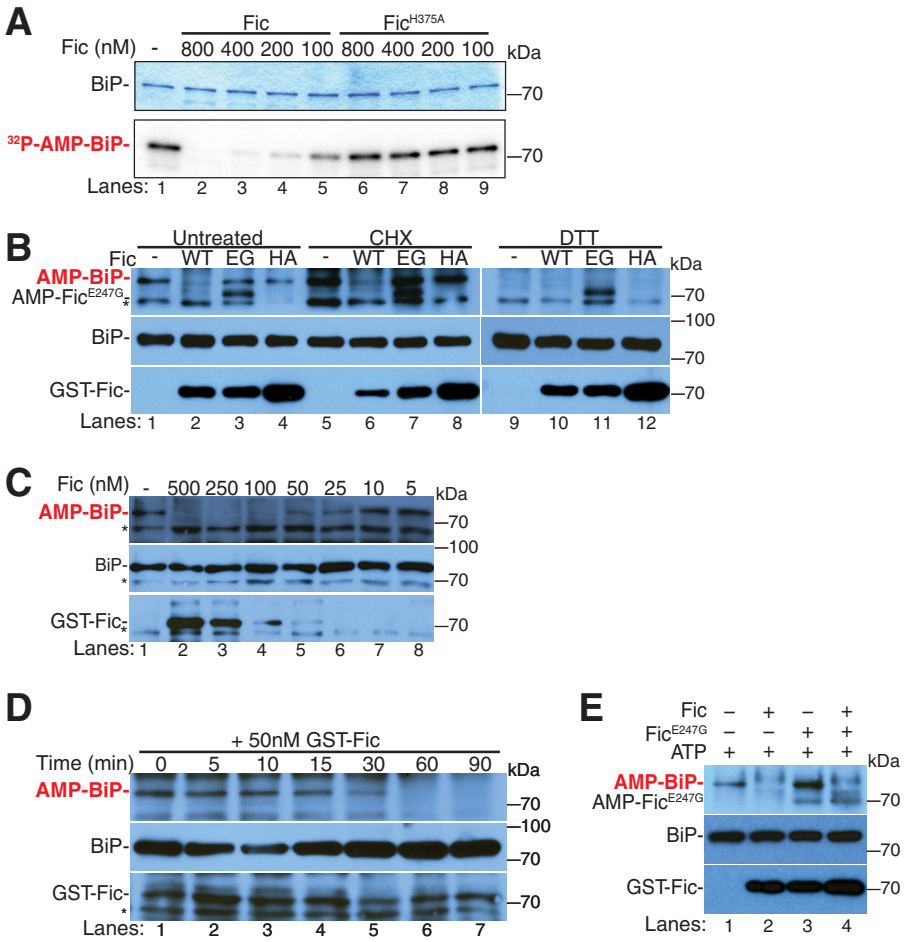

Figure 2. Fic-mediated deAMPylation of BiP. *A*, Fic-mediated deAMPylation of *in vitro* AMPylated BiP^{T229A} ([α-^{32}P]AMP) was assayed by autoradiograph. A representative Coomassie Blue gel and autoradiograph of concentration-dependent deAMPylation activity of HIS-FicΔ70 (*lanes 2–5*) and HIS-FicΔ70^{H375A} (*lanes 6–9*) are shown. *B*, Fic-mediated deAMPylation of endogenous BiP from S2 lysates was assayed by Western blotting. Shown is a representative Western blot of S2 lysates untreated (*lanes 1–4*), treated with CHX (*lanes 5–8*), or treated with DTT (*lanes 9–12*) for 4 h. S2 lysates were incubated with recombinant enzymes: GST-FicΔ70 (*lanes 2*, *6*, and *10*), GST-FicΔ70^{E247G} (*lanes 3*, *7*, and *11*), or GST-FicΔ70^{H375A} (*lanes 4*, *8*, and *12*). The *asterisk* indicates a nonspecific band. Blots were probed with anti-AMP-threonine, anti-BiP, and anti-GST antibodies. *C*, concentration-dependent deAMPylation activity of GST-FicΔ70 (*lanes 2–8*) was assayed by Western blotting. The *asterisk* indicates a nonspecific band. Blots were probed with anti-AMP-threonine, anti-BiP, and anti-GST antibodies. *D*, time-dependent deAMPylation activity of 50 nM GST-FicΔ70 (*lanes 1–7*) was assayed by Western blotting. Blots were probed with anti-AMP-threonine, anti-BiP, and anti-GST antibodies. *E*, representative Western blot of GST-FicΔ70-mediated deAMPylation of endogenous BiP in the presence of 250 nM ATP and GST-FicΔ70 (*lane 2*), GST-FicΔ70^{E247G} (*lane 3*), or equimolar GST-FicΔ70 and GST-FicΔ70^{E247G} (*lane 4*). Blots were probed with anti-AMP-threonine, anti-BiP, and anti-dFic antibodies.

Recombinant Fic mediates deAMPylation of AMPylated BiP in cell lysates

Next, we asked whether the addition of recombinant Fic could deAMPylate endogenous BiP from *Drosophila* Schneider 2 (S2) lysates. AMPylation of endogenous BiP was observed in S2 lysates by Western blot analysis using the anti-AMP-threonine antibody (19) (Fig. 2*B*). As observed previously, treating cells with cyclohexamide (CHX) increased levels of BiP AMPylation in S2 cells due to the fact that this compound reduces ER stress by halting protein translation (Fig. 2*B*, *lanes 5–8*) (9). By contrast, treatment of S2 cells with DTT, which reduces disulfide bonds, causes up-regulation of the UPR, resulting in the loss of BiP AMPylation (Fig. 2*B*, *lanes 9–12*) (9).

To validate our Western blot detection of AMPylation and to determine the site(s) on which endogenous BiP was AMPylated in S2 cells, we purified endogenous BiP using concanavalin A beads and performed LC-MS/MS analysis to identify AMPylated residues (supplemental Fig. S3). From this analysis, we found that endogenous BiP was AMPylated at a single site, Thr-518, a conserved threonine in the substrate-binding domain. This site has previously been identified in mammalian cells (13). In earlier studies, we identified Thr-366, a conserved residue in the ATPase domain of BiP, as a site of AMPylation (9); however, under our current conditions, only Thr-518 AMPylation was observed on endogenous BiP in these lysates. Of note, the Thr-518 AMPylation was detected in untreated and CHX-treated S2 cells but not found in DTT-treated S2 cells, consistent with our Western blot analysis of BiP AMPylation (9) (Fig. 2*B*).

To determine whether Fic has deAMPylation activity, we tested the ability of recombinant protein to remove AMPylation from endogenous BiP in *Drosophila* (S2) lysates. Recombinant proteins were added to solubilized organelle fractions of lysates and incubated for 1 h at 30 °C. In both untreated and CHX-treated cells, the addition of wild-type recombinant Fic resulted in the loss of BiP AMPylation, as indicated by Western

blot analysis using the anti-AMP-threonine antibody (Fig. 2*B*, *lanes 2* and *6*). No difference was observed in DTT-treated samples, as no AMPylated BiP was present to be modified (Fig. 2*B*, *lane 10*). The addition of recombinant overactive AMPylating Fic^{E247G} or the catalytically dead Fic^{H375A} mutant proteins did not alter the steady-state levels of BiP AMPylation in these lysates (Fig. 2*B*, *lanes 3*, *7*, and *11* and *lanes 4*, *8*, and *12*, respectively). We also tested the ability of recombinant Fic to deAMPylate BiP from lysates treated with tunicamycin, a UPR-inducing drug, and 4-phenylbutyrate, an ER stress inhibitor (supplemental Fig. S4). We found that the addition of recombinant wild-type Fic to lysates treated with 4-phenylbutyrate did result in loss of BiP AMPylation, whereas tunicamycin-treated lysates contained no observed AMPylated BiP to modify (supplemental Fig. S4).

Of note, the recombinant Fic^{E247G} remained auto-AMPylated when added to the lysate, indicating that endogenous Fic did not recognize Fic^{E247G} as a substrate for deAMPylation (Fig. 2*B*, *lanes 3*, *7*, and *11*). Another interesting observation is that recombinant Fic is able to efficiently deAMPylate BiP from CHX-treated lysates. Under CHX conditions, endogenous Fic becomes an AMPylator of BiP. This suggests that *in vivo* some other factor(s) control the enzymatic activity of Fic and the balance of BiP AMPylation. *In vitro*, the additional factor(s) may not be present or may be unable to efficiently regulate the recombinant Fic added to these lysates.

Fic-mediated deAMPylation is enzymatic

The fact that the catalytic histidine His-375 is required for deAMPylation of BiP strongly suggests that the deAMPylation of BiP is an enzymatic process rather than a block of Fic AMPylation activity. To determine whether the deAMPylation activity of Fic is enzymatic, deAMPylation of endogenous BiP was assayed over a range of concentrations and times. The levels of observed endogenous BiP AMPylation decreased with the addition of higher concentrations of recombinant Fic (Fig. 2*C*). Furthermore, when a moderate concentration of Fic (50 nM) was added to the lysate and incubated over an extended period of time, the levels of BiP AMPylation decreased in a time-dependent manner (Fig. 2*D*). These data show a time and concentration dependence of the deAMPylation activity mediated by Fic, thereby supporting our proposal that Fic is an enzyme that deAMPylates the modified BiP substrate.

Fic deAMPylation can compete with unregulated Fic^{E247G} AMPylation in cell lysates

Interestingly, no increased BiP AMPylation was observed in any of the lysates incubated with Fic^{E247G} (Fig. 2*B*, *lanes 3*, *7*, and *11*). We predict that the lack of additional AMPylation could be explained by the lack of additional sources of ATP and the presence of low levels of deAMPylation activity by endogenous Fic. This would be consistent with our observations in wild-type flies, where the activity of overexpressed exogenous Fic^{E247G} is overcome by low levels of endogenous wild-type Fic (Fig. 1). Because these lysates contain very low levels of Fic (9), a small amount of Fic could indeed counterbalance the unregulated AMPylating activity of Fic^{E247G} without altering the appropriate levels of BiP AMPylation in the cell, as observed in flies overexpressing the UAS-Fic^{E247G} transgene.

We predicted that the addition of an exogenous ATP source may alter the balance of AMPylation and deAMPylation activity when Fic^{E247G} is added to S2 lysate. Consistent with this hypothesis, when ATP was added to S2 lysates, the addition of recombinant Fic^{E247G} resulted in an observed increase in BiP AMPylation (Fig. 2*E*, *lane 3*), whereas the addition of recombinant Fic was still able to efficiently deAMPylate BiP (Fig. 2*E*, *lane 2*). To determine whether recombinant Fic could also compete with the AMPylation activity of recombinant Fic^{E247G}, equal amounts of recombinant wild-type Fic and Fic^{E247G} were incubated in S2 lysates supplemented with ATP (Fig. 2*E*, *lane 4*). The addition of Fic together with Fic^{E247G} to the S2 lysate resulted in deAMPylation of endogenous BiP, indicating that recombinant wild-type Fic can outcompete Fic^{E247G}. Because Fic^{E247G} retains high AMPylating activity, the ability of Fic to counterbalance Fic^{E247G} enzymatic activity indicates that the deAMPylation activity of Fic is also quite high in S2 lysates. Furthermore, the apparent increased efficacy of endogenous Fic also suggests that additional factors or modifications to endogenous Fic must occur in the ER to control the activity of this bifunctional enzyme.

We also investigated whether competition between wild-type Fic and constitutively active Fic^{E247G} occurs during *in vitro* AMPylation of a substrate. For these assays, purified recombinant BiP ATPase domain, His_6-BiP(27–407), was used as a substrate for Fic-mediated AMPylation, as this was previously shown to be modified (9). AMPylation assays were performed by incubating Fic^{E247G} with BiP(27–407) in AMPylation buffer with ATP at 30 °C for 1 h. After incubation with 100 nM Fic^{E247G}, AMPylation of 4 μM BiP(27–407) was detected by Western blot analysis using the anti-AMP-Thr antibody (supplemental Fig. S5 (*A* and *B*), *lane 3*). To validate our Western blot detection of BiP(27–407) AMPylation, we performed LC-MS/MS analysis on BiP(27–407) from these AMPylation assays. LC-MS/MS analysis confirmed that BiP(27–407) is predominantly AMPylated at Ser-365/Thr-366 (supplemental Fig. S5*C*) with other, less abundant AMPylation sites also identified in the sample (supplemental Table S1). This is consistent with our previously published observations on Fic *in vitro* AMPylation of BiP (9).

To determine whether recombinant Fic could compete with Fic^{E247G} for BiP(27–407) *in vitro*, increasing concentrations (from 25 to 800 nM) of Fic were added to AMPylation reactions containing 100 nM Fic^{E247G} and 4 μM BiP(27–407) (supplemental Fig. S5*A*, *lanes 4–9*). The addition of increasing amounts of Fic to the reaction resulted in decreasing BiP(27–407) AMPylation (supplemental Fig. S5*A*, *lanes 4–9*). As a control, we observed no effect on the *in vitro* AMPylation of BiP(27–407) treated with increasing concentrations of Fic^{H375A} (supplemental Fig. S5*B*, *lanes 4–9*).

Appreciably more Fic was required to completely counteract the AMPylation of BiP(27–407) by Fic^{E247G} *in vitro*. One possible explanation for this is that Fic^{E247G} is a more efficient enzyme than wild-type Fic *in vitro*. This somewhat contradicts our observation that endogenous Fic can counterbalance the toxic effects of transgenic expression of Fic^{E247G} *in vivo*. Of note, in previous studies, we observed that the truncated form of BiP is a better *in vitro* substrate for AMPylation than full-

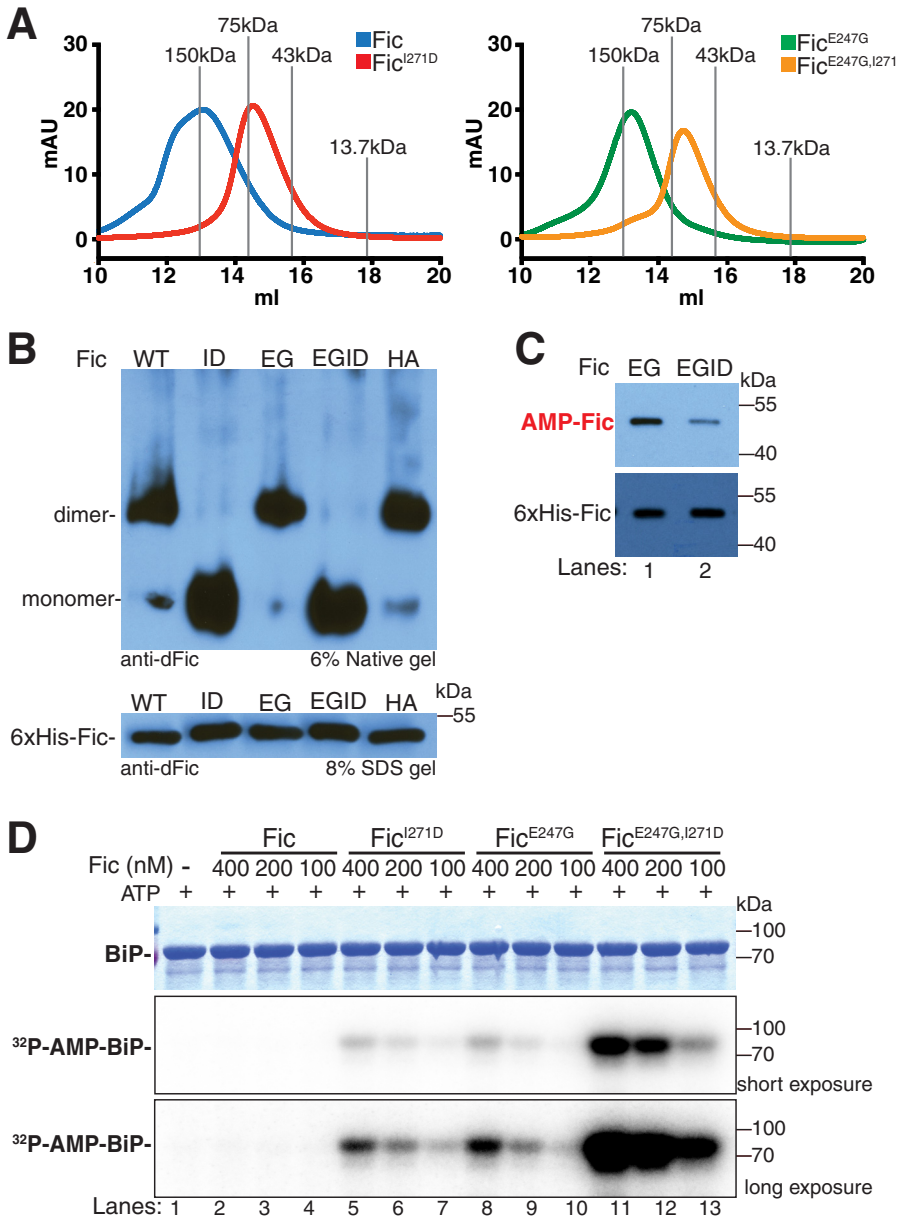

Figure 3. Fic AMPylation of full-length BiP is inhibited by dimerization. *A*, representative traces of FPLC elution of His_6-FicΔ70, His_6-FicΔ70^{I271D}, His_6-FicΔ70^{E247G}, and His_6-FicΔ70^{E247G,I271D}. Molecular mass standards alcohol dehydrogenase (150 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), and ribonuclease (13.7 kDa) are *labeled* in *gray*. *B*, representative immunoblots of His_6-FicΔ70, His_6-FicΔ70^{I271D}, His_6-FicΔ70^{E247G}, His_6-FicΔ70^{E247G,I271D}, and His_6-FicΔ70^{H375A} from 6% native polyacrylamide gel and 8% SDS-polyacrylamide gel. Blots were probed with anti-dFic antibody. *C*, representative immunoblots of auto-AMPylation of His_6-FicΔ70^{E247G} (*EG*, *lane 1*) and His_6-FicΔ70^{E247G,I271D} (*EGID*, *lane 2*). Blots were probed with anti-AMP-threonine and anti-dFic antibodies. *D*, representative Coomassie Blue gel and autoradiographs of AMPylation assays in which 4 μM His_6-BiP^{T229A} was used as a substrate for varying concentrations of His_6-FicΔ70 (*lanes 2–4*), His_6-FicΔ70^{I271D} (*lanes 5–7*), His_6-FicΔ70^{E247G} (*lanes 8–10*), and His_6-FicΔ70^{E247G,I271D} (*lanes 11–13*). Short and long exposures of autoradiographs are shown to display dynamic range of each enzyme activity.

length BiP (9). Therefore, deAMPylation of this truncated form of BiP could be somewhat compromised. Alternatively, Fic deAMPylation activity may be enhanced by other factors *in vivo*, such as changes in post-translational modifications or effects on the dimerization state of the Fic enzyme that are not present in this *in vitro* reaction.

Fic AMPylation of BiP is attenuated by Fic dimerization

Because the AMPylation activity of human FicD and *C. elegans* Fic-1 was shown to be reduced in dimerization mutants (12, 15), we wanted to know whether the AMPylation and deAMPylation activity of *Drosophila* Fic was also affected by dimerization. To test this, we mutated an isoleucine (Ile-271) in the predicted dimer interface of Fic shown to be critical for dimerization in *C. elegans* Fic-1 and human FicD (12, 15). To prevent dimerization artifacts from the GST tag, we utilized an N-terminal His_6-tagged Fic expression system. As predicted, purified Fic and Fic^{I271D} eluted by size exclusion chromatography as a dimer and monomer, respectively (Fig. 3*A*). This was also true for purified Fic^{E247G} and Fic^{E247G,I271D}. Furthermore, native gel migration of purified Fic^{I271D} and Fic^{E247G,I271D} was significantly faster than Fic, Fic^{E247G}, and Fic^{H375A}, consistent

with a change in the oligomeric state of these mutants (Fig. 3*B*).

Initially, we tested the AMPylation activity of our dimer mutant. We hypothesized that, as observed in human and *C. elegans*, disruption of Fic dimerization would lead to decreased AMPylation activity of the enzyme. Because wild-type Fic exhibits very low AMPylation activity, we compared the AMPylation activity of the constitutively active AMPylating mutant Fic^{E247G} with its corresponding dimerization mutant Fic^{E247G,I271D}. We observed that auto-AMPylation levels of recombinant Fic^{E247G,I271D} were markedly lower than recombinant Fic^{E247G} by Western blot analysis (Fig. 3*C*). To determine whether dimerization of Fic alters its AMPylation activity for the substrate BiP, AMPylation assays were performed in the presence of [α-^{32}P]ATP. Several concentrations (100–400 nM) of recombinant Fic, Fic^{I271D}, Fic^{E247G}, and Fic^{E247G,I271D} were added to 4 μM His$_6$-BiP^{T229A} and incubated for 30 min at 30 °C (Fig. 3*D*). Incubation of BiP without Fic enzyme or with wild-type Fic showed no [^{32}P]BiP AMPylation signal by autoradiograph (Fig. 3*D*, *lanes 1–4*). However, incubation of BiP with Fic^{E247G} showed a clear [^{32}P]BiP AMPylation signal (Fig. 3*D*, *lanes 8–10*), indicating that Fic^{E247G} is an active AMPylating enzyme. Strikingly, the addition of Fic^{I271D} to BiP also resulted in [^{32}P]BiP AMPylation signal (Fig. 3*D*, *lanes 5–7*), indicating that Fic^{I271D} is also an active AMPylating enzyme. Furthermore, the addition of the Fic^{E247G,I271D} mutant resulted in a substantially stronger [^{32}P]BiP signal than either Fic^{E247G} or Fic^{I271D} (Fig. 3*D*, *lanes 11–13*), suggesting that the AMPylating activity of the double mutant is significantly higher than that of either mutant alone. This also suggests that the regulatory effects of the autoinhibitory helix salt bridge and the dimerization interface have independent but synergistic effects on Fic AMPylation activity.

Fic deAMPylation activity does not require dimerization

Next, we tested the deAMPylation activity of our dimer mutant on AMPylated BiP. Because both enzymatic activities of Fic use the same active site, we predicted that, like AMPylation, the deAMPylation activity of Fic would also be altered by the disruption of dimerization. To determine whether dimerization is required for deAMPylation activity, we performed *in vitro* deAMPylation assays using recombinant [^{32}P]AMP-BiP^{T229A} as a substrate as described above. Recombinant Fic, Fic^{I271D}, Fic^{E247G}, Fic^{E247G,I271D}, and Fic^{H375A} were each added to [^{32}P]AMP-BiP^{T229A} and incubated for 60 min in the absence of ATP (Fig. 4*A*). In the samples treated with wild-type Fic or the monomeric Fic^{I271D}, the [^{32}P]AMP signal from [^{32}P]AMP-BiP^{T229A} was greatly reduced compared with the no-enzyme control (Fig. 4*A*, *lanes 1–3*), whereas the Fic^{E247G}-, Fic^{E247G,I271D}-, and Fic^{H375A}-treated samples showed no reduction in [^{32}P]AMP signal of BiP^{T229A} (Fig. 4*A*, *lanes 4–6*). Both wild-type Fic and monomeric Fic^{I271D} were further tested in a concentration-dependent manner, and we observed that the addition of these enzymes resulted in a concentration-dependent reduction of the [^{32}P]AMP signal from [^{32}P]AMP-BiP^{T229A} protein (Fig. 4*B*). However, the enzymatic activity of monomeric Fic^{I271D} appeared to be slightly compromised, as more enzyme was required to fully deAMPylate the substrate

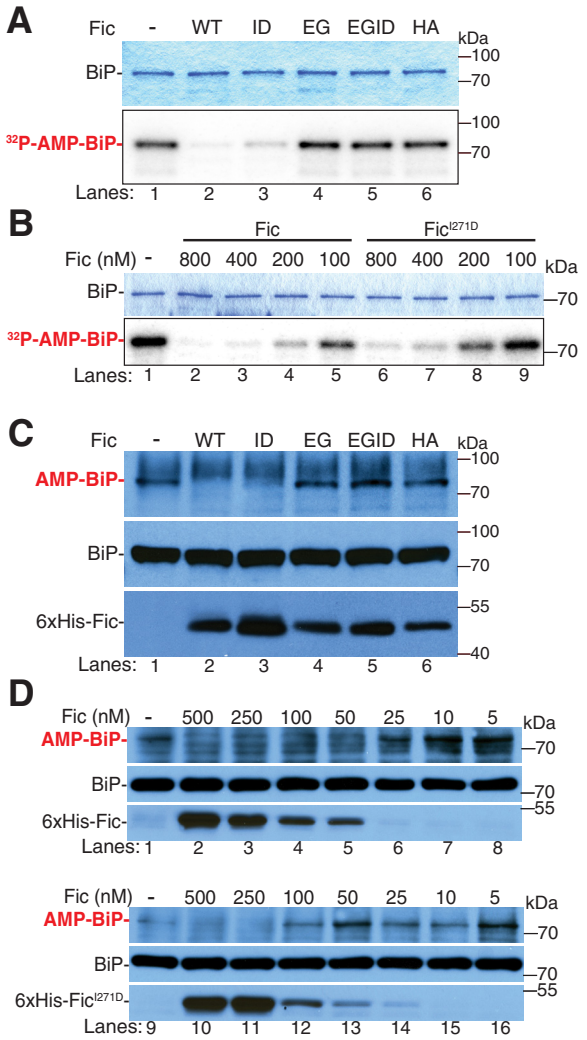

Figure 4. Fic deAMPylation is independent of dimerization. *A*, representative Coomassie Blue gel and autoradiographs of deAMPylation assays in which *in vitro* AMPylated BiP ([α-^{32}P]AMP) was incubated with recombinant enzymes: His$_6$-FicΔ70 (*WT*, *lane 2*), His$_6$-FicΔ70^{I271D} (*ID*, *lane 3*), His$_6$-FicΔ70^{E247G} (*EG*, *lane 4*), His$_6$-FicΔ70^{E247G,I271D} (*EGID*, *lane 5*), or GST-FicΔ70^{H365A} (*HA*, *lane 6*). *B*, representative Coomassie Blue gel and autoradiographs of deAMPylation assays in which *in vitro* AMPylated BiP ([α-^{32}P]AMP) was used as a substrate for varying concentrations of His$_6$-FicΔ70 (*lanes 2–5*) and His$_6$-FicΔ70^{I271D} (*lanes 6–9*). *C*, representative Western blot of S2 lysates incubated with recombinant enzymes: His$_6$-FicΔ70 (*WT*, *lane 2*), His$_6$-FicΔ70^{I271D} (*ID*, *lane 3*), His$_6$-FicΔ70^{E247G} (*EG*, *lane 4*), His$_6$-FicΔ70^{E247G,I271D} (*EGID*, *lane 5*), or GST-FicΔ70^{H365A} (*HA*, *lane 6*). Blots were probed with anti-AMP-threonine, anti-BiP, and anti-dFic antibodies. *D*, concentration-dependent deAMPylation activities of His$_6$-FicΔ70 (*lanes 2–8*) and His$_6$-FicΔ70^{I271D} (*lanes 10–16*) were assayed by Western blotting. Blots were probed with anti-AMP-threonine, anti-BiP, and anti-dFic antibodies.

(Fig. 4*B*, *lanes 6–9*) compared with wild-type Fic (Fig. 4*B*, *lanes 2–5*).

To determine whether dimerization is required for deAMPylation of endogenous BiP, recombinant His$_6$-Fic proteins were incubated with organelle fractions of *Drosophila* S2 cell lysates for 1 h at 30 °C (Fig. 4*C*). Consistent with the observed deAMPylation activity toward recombinant BiP, both wild-type Fic and the monomeric Fic^{I271D} proteins were able to deAMPylate endogenous BiP, whereas Fic^{E247G}, Fic^{E247G,I271D}, and Fic^{H375A} were not able to deAMPylate BiP. Again, compared with wild-type Fic, the deAMPylation activity of Fic^{I271D} is slightly

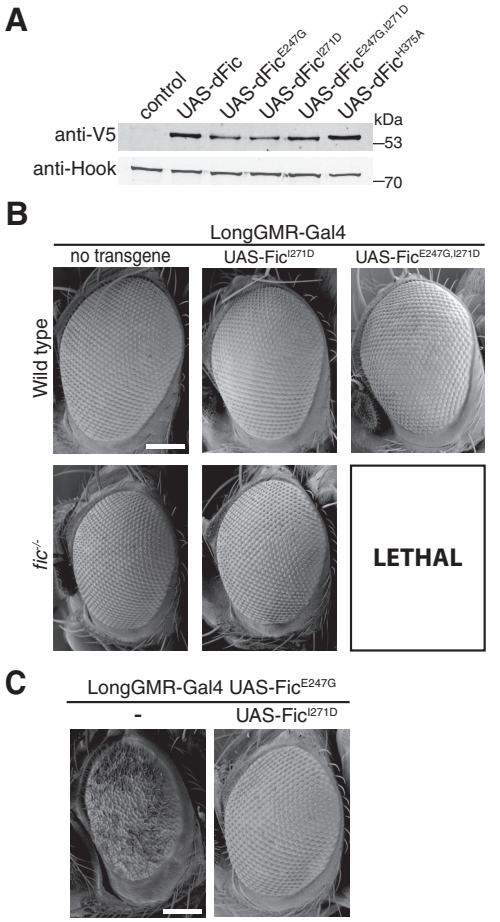

Figure 5. Fic dimerization is not required to rescue Fic^{E247G} toxicity. *A*, a representative Western blot shows expression of UAS-Fic transgenes (Fic, Fic^{E247G}, Fic^{I271D}, Fic^{E247G,271D}, Fic^{H375A}) under control of the ubiquitous Da-Gal4 driver in a wild-type background. *B*, representative SEM images of eyes expressing the indicated UAS-Fic transgene with LongGMR-Gal4 in a wild-type or *fic^{30C}* background. *Scale bar*, 100 μm. *C*, representative SEM images of eyes expressing the indicated UAS-Fic transgene along with LongGMR-Gal4, UAS-Fic^{E247G} in a *fic^{30C}* background. *Scale bar*, 100 μm.

reduced, as higher concentrations of Fic^{I271D} enzyme are required to completely deAMPylate endogenous BiP (Fig. 4*D*). When a limiting concentration of Fic^{I271D} (50 nM) is added to the lysate and incubated for 90 min, the levels of BiP AMPylation decreased in a time-dependent manner (supplemental Fig. S6, *lanes 8–14*); however, deAMPylation of BiP is not taken to completion as was observed with wild-type Fic (supplemental Fig. S6, *lanes 1–7*). Taken together, these data indicate that Fic^{I271D} retains its deAMPylation activity despite also having increased AMPylation activity.

Fic dimerization is not required to rescue Fic^{E247G} toxicity

Because Fic^{E247G} and Fic^{E247G,I271D} both display increased AMPylation activity, we wanted to know the effects of these mutant proteins *in vivo*. We generated UAS-Fic^{I271D} and UAS-Fic^{E247G,I271D} transgenic flies with C-terminal V5 and His$_6$ tags as described above. As with UAS-Fic, UAS-Fic^{E247G}, and UAS-Fic^{H375A}, Western analysis of the monomeric UAS-Fic^{I271D} and UAS-Fic^{E247G,I271D} proteins indicates stable expression of these transgenes in flies (Fig. 5*A*).

Next, we assessed the effect of Fic transgene overexpression in *fic^{30C}* null fly eyes. Consistent with the results from previous experiments, LongGMR-Gal4–driven expression of either Fic^{E247G} or Fic^{E247G,I271D} transgenes resulted in no externally visible defects in a *fic* wild-type background (Fig. 5*B*). Furthermore, expression of the monomeric Fic^{I271D} in *fic^{30C}* null flies did not interfere with normal eye development. However, expression of the UAS-Fic^{E247G,I271D} transgene under the LongGMR-Gal4 promoter was lethal in the *fic^{30C}* null background. Overexpression of toxic proteins under the LongGMR-Gal4 promoter has previously been shown to cause lethality, most likely due to LongGMR-Gal4 expression in larval and pupal neurons outside of the visual system (20). The lethality of UAS-Fic^{E247G,I271D} under the LongGMR-Gal4 promoter suggests that the observed increase in *in vitro* AMPylation is retained *in vivo* because corresponding UAS-Fic^{E247G} flies are viable in the same genetic background. Furthermore, these data indicate that expression of the wild-type Fic protein from the endogenous *fic* locus is sufficient to suppress lethality caused by Fic^{E247G,I271D} overexpression, supporting the hypothesis that both deAMPylation and AMPylation are required for cell survival.

Next, we asked whether overexpression of the UAS-Fic^{I271D} transgene would rescue the rough eye phenotype of *fic^{30C}*; LongGMR-Gal4, UAS-Fic^{E247G} flies (Fig. 5*C*). Again, as with the UAS-Fic transgene (Fig. 1*E*), overexpression of the monomeric UAS-Fic^{I271D} transgene suppressed the UAS-Fic^{E247G}–induced eye phenotype. Taken together, these data suggest that the remaining deAMPylation activity of Fic^{I271D} is sufficient to counterbalance the enhanced AMPylation activity of this enzyme *in vivo*.

Discussion

Overall, our studies have shown that uncontrolled Fic-mediated AMPylation is lethal, and endogenous wild-type Fic can rescue flies from this lethality. Additionally, we have demonstrated that deAMPylation is likely to be the default activity of wild-type Fic and that this enzyme must change in a way so that net AMPylation of a substrate can occur. In these studies, we have also found that Fic dimerization attenuates the AMPylation activity of Fic toward its substrate BiP but is not critical for the deAMPylation activity of Fic.

Wild-type Fic appears to principally exhibit deAMPylation activity. During low ER stress, the activity of Fic appears as an AMPylator, thereby promoting the AMPylation of a portion of the available BiP and generating a subpopulation of inactive BiP that is held in reserve in the ER. When ER stress is elevated, Fic-mediated deAMPylation of BiP during ER stress would then increase the pool of active BiP, allowing for a more rapid and more acute response to stress. It is important to note that BiP resides in multiple conformational states (21) that are regulated by ER stress, which may also impact its accessibility as a Fic substrate.

Exactly how this activity is switched *in vivo* has yet to be identified; however, there are two independent mutations in Fic that alter its AMPylation and deAMPylation activity on BiP (Fig. 6). The first mutation, Fic^{E247G}, is a well-characterized overactive AMPylating mutant. Mutation of this conserved glutamate results in the disruption of an inhibitory salt bridge in the active site that promotes the proper coordination of ATP in

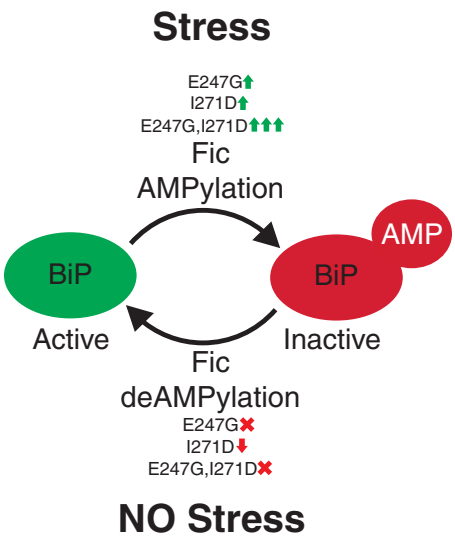

Figure 6. Model of Fic AMPylation and deAMPylation activity. See "Discussion" for details.

the binding pocket and AMPylation activity (9, 14, 15). Importantly, disruption of this salt bridge also completely disrupts deAMPylation activity of the enzyme. The second mutation, Fic^{I271D}, has been observed in this study and by others to disrupt homodimerization of Fic (12, 15). Disruption of Fic dimerization increases AMPylation activity for its substrate BiP while reducing Fic auto-AMPylation and without significantly attenuating Fic's deAMPylation activity. Interestingly, despite the increased AMPylation activity of Fic^{I271D}, flies overexpressing this mutant do not display the toxicity associated with Fic^{E247G}. This observation further supports the hypothesis that both deAMPylation and AMPylation are required for cell survival. Indeed, these two mutations appear to work by distinct molecular mechanisms, as combining these two mutations resulted in a synergistic effect on the AMPylation activity of $Fic^{E247G,I71D}$.

It is intriguing that dimerization would inhibit the AMPylation activity of Fic but not significantly alter the deAMPylation activity of this enzyme. It is tempting to speculate that the dimerization state of Fic could be a key regulatory factor working with another unknown component or modification *in vivo*. Future studies on how the bimodal activities of Fic are regulated within the ER will be critical to understanding how Fic regulation of BiP is controlled in the ER.

Experimental procedures

Cell culture and fly work

Drosophila melanogaster Schneider 2 (S2) cells were cultured with standard protocols and grown at 27 °C in Schneider's medium supplemented with 10% fetal bovine serum and antibiotics (22). Flies were maintained using standard conditions. The Bloomington Stock Center provided Da-Gal4 (BS 55850) and LongGMR-Gal4 (BS 8121) driver lines as well as the *fic* deficiency stock Df(fic) = Df(2L)BSC296 and OreR.

Fly stocks

OreR, fic^{30C}, Df(2L)BSC296 (Bloomington Stock #25720), *w;; Da-Gal4* (Bloomington Stock #55850), *w;; LongGMR-Gal4* (Bloomington Stock #8121), *w;; UAS-V5-*$Fic^{WT\_attP\text{-}B3}$*/TM6B,hu*, *w;; UAS-V5-*$Fic^{E247G\_attP\text{-}B3}$*/TM6B,hu*, *w;; UAS-V5-*$Fic^{H375A\_attP\text{-}B3}$*/TM6B,hu*, *w;; UAS-V5-*$Fic^{I271D\_attP\text{-}B3}$*/TM6B,hu*, *w;; UAS-V5-*$Fic^{E247G,I271D\_attP\text{-}B3}$*/TM6B,hu*, *w*; fic^{30C}; *Da-Gal4*, *w*; fic^{30C}; *LongGMR-Gal4*, *w*;fic^{30C}; *UAS-V5-*$Fic^{WT\_attP\text{-}B3}$*/TM6B,hu*, *w*; fic^{30C}; *UAS-V5-*$Fic^{E247G\_attP\text{-}B3}$*/TM6B,hu*, *w*; fic^{30C}; *UAS-V5-*$Fic^{H375A\_attP\text{-}B3}$*/TM6B,hu*, *w*; fic^{30C}; *UAS-V5-*$Fic^{I271D\_attP\text{-}B3}$*/TM6B,hu*, *w*; fic^{30C}*/CyO*; *UAS-V5-*$Fic^{E247G,I271D\_attP\text{-}B3}$*/TM6B,hu*, *w;; LongGMR-Gal4,UAS-V5-*$Fic^{WT\_attP\text{-}B3}$*/TM6B,hu*, *w;; LongGMR-Gal4,UAS-V5-*$Fic^{E247G\_attP\text{-}B3}$*/TM6B,hu*, *w*; fic^{30C}; *LongGMR-Gal4,UAS-V5-*$Fic^{WT\_attP\text{-}B3}$*/TM6B,hu*, *w*; fic^{30C}; *LongGMR-Gal4,UAS-V5-*$Fic^{E247G\_attP\text{-}B3}$*/TM6B,hu*, *w*; fic^{30C}/Df(2L)BSC296; LongGMR-Gal4,UAS-V5-$Fic^{E247G\_attP\text{-}B3}$*/TM6B,hu*.

Generation of fic^{30C} null mutation

CRISPR-mediated mutagenesis (23) was performed with tools and the protocol available from the O'Connor-Giles, Wildonger, and Harrison laboratories (23). Mutagenesis was performed in *ywv;;Nanos-Cas9* using two gRNAs targeting *fic*-Exon1 (*gRNA1*, CCGGAAGTCCTACTGCGCTA; *gRNA2*, ACCGTCAGCGCACGGCAGA). Injections were performed by Rainbow Transgenic Services (Camarillo, CA). The surviving F1 progenies were crossed to balancer stocks, and subsequently, potential founder F2 males were individually crossed to the balancer stock and screened for CRISPR-induced indels. Mutations were detected by PCR amplification of the target region, reannealing, T7 endonuclease treatment, and polyacrylamide gel electrophoresis analysis of potential cleavage products. Sanger sequencing of PCR products detected the precise sequence of candidate deletions.

Generation of UAS-V5-Fic constructs

Fic cDNA sequences (Fic^{WT}, Fic^{E247G}, and Fic^{H375A}) were subcloned into a pUASt vector modified by insertion of an AttB sequence, sequence-verified, and injected into embryos (BestGene, Chino Hills, CA) at the 62E1 landing site (24). To generate the Fic^{I271D} and $Fic^{E247G,I271D}$ mutants, gBlocks (IDT, Coralville, IA) containing the respective mutant sequences were synthesized, subcloned into the pUASt-V5-Fic-AttB vector via the HiFi Assembly Kit (New England Biolabs, Ipswich, MA), and injected into embryos at the same site. Da-Gal4–driven expression levels of all constructs were determined by Western blotting. In brief, five flies were homogenized in 200 μl of lysis buffer (10% SDS, 6 M urea, and 50 mM Tris-HCl, pH 6.8, plus 10% DTT), boiled for 2 min, and spun for 10 min at 20,000 $\times g$ to remove debris. 10 μl were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with anti-V5 (1:3000; R960-25 Invitrogen) and anti-Hook (1:8000 (25)) and detected using fluorescence-labeled antibodies and an Odyssey scanner (LI-COR Biosciences, Lincoln, NE).

Survival analysis of flies expressing Fic^{E247G}

To assess the lethality of unregulated Fic, we expressed C-terminally V5-His_6-tagged UAS-Fic, UAS-Fic^{H375A}, or UAS-Fic^{E247G} via the ubiquitous Da-Gal4 driver in either wild-type or fic^{30C} null flies. Offspring were scored, and the number of adults with both Da-Gal4 and the UAS-Fic variant were compared with the number of heterozygous sibling controls. Per-

cent of expected was calculated from the ratio of recovered flies of the relevant genotypes compared with half the total number (number observed/(total number/2)). Crosses were repeated three times. The total number of flies scored was at least 150 for all crosses.

Electron microscopy

SEM of fly eyes were obtained as described previously (26, 27). In short, eyes were fixed in 2% paraformaldehyde, 2% glutaraldehyde, 0.2% Tween 20, and 0.1 M cacodylate buffer, pH 7.4, for 2 h. Samples were washed four times with increasing ethanol (25–100%) for 12 h each followed by a series of hexamethyldisilazane washes (25–100% in ethanol) for 1 h each. Flies were air-dried overnight and mounted on SEM stubs, and the bodies were coated in fast-drying silver paint. Flies were sputter-coated with a gold/pallidum mixture for 60 s and imaged at ×900 magnification, with extra high tension set at 3.0 kV on a scanning electron microscope (SIGMA, Zeiss). Stereoscopic light images were acquired on a V12 stereoscope (Zeiss) at ×100 zoom. Stacks of ~30 × 10-μm optical sections were merged and filtered with CZFocus software (Zeiss).

TUNEL staining

Cell death was detected with the TUNEL method as described previously (28). Briefly, eye discs from wandering third instar larvae were dissected into PBS, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS (PBST), and incubated in TUNEL reaction mixture (In Situ Cell Death Detection Kit-TMR Red, Roche Applied Science) for 2 h. Tissues were mounted in Vectashield with DAPI (Vectorlabs, Burlingame, CA). Stacks of 20 × 1-μM confocal sections were acquired with an LSM 710 confocal microscope (Carl Zeiss) and equally processed for brightness and contrast using ImageJ (National Institutes of Health).

Plasmid construction

Plasmids for bacterial expression of His_6-Δ26BiP, His_6-BiP(27–407), GST-FicΔ70, GST-FicΔ70^{E247G}, and GST-FicΔ70^{H375A} were generated previously (9). Plasmids for bacterial expression of His_6-FicΔ70, His_6-FicΔ70^{E247G}, and His_6-FicΔ70^{H375A} were generated by subcloning the Fic genes from GST-FicΔ70, GST-FicΔ70^{E247G}, and GST-FicΔ70^{H375A}, respectively, into the pet28A vector using BamHI and NotI sites. Plasmids for bacterial expression of His_6-FicΔ70^{I271D} and His_6-FicΔ70^{E247G,I271D} were generated by PCR amplification and ligation of His_6-FicΔ70 and His_6-FicΔ70^{E247G} using primers 5′-GACGGCAAATCCGATGACGAGCACAACGAGATCTTGGGCATGGATCTGGC-3′ and 5′-TACGGCCATGCGGGTCTCTAGG-3′. Plasmids for bacterial expression of His_6-Δ26BiP^{T229A} were generated by PCR amplification and ligation of His_6-Δ26BiP using primers 5′-GCCTTCGATGTCTCCCTGCTGACC-3′ and 5′-GCCGCCGCCCAAATCGAAC-3′.

Purification of recombinant proteins

For protein purification, plasmids for expression of His_6-Δ26BiP^{T229A}, His_6-BiP(27–407), His_6-FicΔ70, His_6-FicΔ70^{E247G}, His_6-FicΔ70^{H375A}, His_6-FicΔ70^{I271D}, and His_6-FicΔ70^{E247G,I271D} were transformed into Rosetta cells, grown to mid-log phase (A_{600} ~0.5), and induced with 400 μM isopropyl β-D-1-thiogalactopyranoside at 23 °C for 4 h. Plasmids for expression of GST, GST-FicΔ70, GST-FicΔ70^{E247G}, and GST-FicΔ70^{H375A} were transformed into Rosetta cells, grown to mid-log phase (A_{600} ~0.5), and induced with 400 μM isopropyl β-D-1-thiogalactopyranoside at 37 °C for 2 h. Cells were lysed using a cell disrupter (Emulsiflex-C3), and proteins were purified using standard nickel-affinity (Thermo Scientific) and GST-affinity (Thermo Scientific) purification protocols. FPLC of His_6-FicΔ70, His_6-FicΔ70^{E247G}, His_6-FicΔ70^{I271D}, and His_6-FicΔ70^{E247G,I271D} was performed on a Superdex 200 10/300 column (GE Healthcare).

AMPylation and deAMPylation of BiP ATPase domain

In vitro AMPylation and deAMPylation assays of purified His_6-BiP(27–407) were performed as described previously (9). Recombinant His_6-BiP(27–407) proteins and recombinant GST-FicΔ70, GST-FicΔ70^{E247G}, and GST-FicΔ70^{H375A} were used as substrates and enzymes, respectively. Reactions were performed in AMPylation buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM $CaCl_2$, 250 μM ATP) at 30 °C for 1 h. Reactions were stopped by the addition of SDS sample buffer. Samples were analyzed by Western blot analysis using the anti-AMP-Thr antibody (19) and anti-BiP antibody (Abcam MAC143). Detection was performed by standard HRP methods.

In vitro AMPylation and deAMPylation of endogenous BiP

In vitro deAMPylation of endogenous BiP was performed on ER/mitochondrial fractions of S2 cells. To induce ER stress, S2 cells were treated with 5 mM DTT for 4 h. To reduce ER stress, S2 cells were treated with 100 μg/ml CHX for 4 h. S2 cells were harvested with cell scrapers, suspended in a HNMEK lysis buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM EDTA, 10 mM KCl, 20 mM NaF, 50 nM EGTA, protease inhibitors), and lysed using a Dounce homogenizer. Lysates were centrifuged at 500 × *g* for 10 min at 4 °C to remove nuclei and cellular debris. The supernatant was collected and centrifuged at 10,000 × *g* for 10 min to pellet ER and mitochondrial membranes. ER/mitochondrial fractions were washed once in HNMEK lysis buffer and centrifuged at 10,000 × *g* for 10 min. ER/mitochondrial pellets were suspended in deAMPylation buffer (50 mM HEPES, pH 7.4, 5 mM MgCl, 100 mM KCl, 1 mM $CaCl_2$, 0.1% Triton). Recombinant GST-FicΔ70, GST-FicΔ70^{E247G}, GST-FicΔ70^{H375A}, His_6-FicΔ70, His_6-FicΔ70^{E247G}, His_6-FicΔ70^{H375A}, His_6-FicΔ70^{I271D}, and His_6-FicΔ70^{E247G,I271D} were used as enzymes. Reactions were performed at a final concentration of ~4 mg/ml of total cellular protein at 30 °C for 1 h. For *in vitro* AMPylation of endogenous BiP, reactions were performed in deAMPylation buffer supplemented with 250 μM ATP.

In vitro AMPylation and deAMPylation of recombinant BiP

In vitro AMPylation of recombinant His_6-Δ26BiP^{T229A} was performed as follows. Recombinant His_6-Δ26BiP^{T229A} was used as a substrate for the enzymes His_6-FicΔ70, His_6-FicΔ70^{E247G}, His_6-FicΔ70^{H375A}, His_6-FicΔ70^{I271D}, and His_6-

FicΔ70^{E247G,I271D}. Reactions were performed in deAMPylation buffer supplemented with 250 mM cold ATP and ~0.5 μCi of [α-^{32}P]ATP at 30 °C for 30 min. Reactions were stopped by the addition of SDS sample buffer. Samples were analyzed by SDS-PAGE stained with Coomassie Brilliant Blue, and ^{32}P radioactive signal was detected with a Typhoon FLA 7000 (GE Healthcare) after ~18-h exposure to storage phosphor screens.

For *in vitro* deAMPylation of recombinant [^{32}P]AMP-BiP^{T229A}, His$_6$-Δ26BiP^{T229A} was first *in vitro* AMPylated and repurified as described previously (17). Briefly, 10 μg of recombinant His$_6$-Δ26BiP^{T229A} preincubated with 15 μM cold ATP was added to 1 μg of GST-FicΔ70^{E247G} and 20 μCi of [α-^{32}P]ATP in deAMPylation buffer and incubated at room temperature for 1 h. Reactions were stopped by the addition of 400 μl of deAMPylation buffer supplemented with 500 mM KCl. The AMPylated His$_6$-Δ26BiP^{T229A} was repurified using a standard nickel-affinity (Thermo Scientific) and GST-affinity (Thermo Scientific) purification protocols and buffer-exchanged in an Amicon Ultra 0.5-ml 30-kDa centrifugal filter (Millipore Sigma). For *in vitro* deAMPylation reactions, recombinant His$_6$-FicΔ70, His$_6$-FicΔ70^{E247G}, His$_6$-FicΔ70^{H375A}, His$_6$-FicΔ70^{I271D}, and His$_6$-FicΔ70^{E247G,I271D} were used as enzymes. Reactions were performed at a final substrate concentration of 800 nM cold AMP-BiP^{T229A} and 80 nM radiolabeled [^{32}P]AMP-BiP^{T229A} in each reaction. Reactions were performed in deAMPylation buffer at room temperature for 1 h. Samples were analyzed by SDS-PAGE stained with Coomassie Blue, and ^{32}P radioactive signal was detected with a Typhoon FLA 7000 (GE Healthcare) after ~18-h exposure to storage phosphor screens.

Mass spectrometry analysis

Samples were run on SDS-polyacrylamide gels and stained with Coomassie Blue dye to isolate proteins before analysis by mass spectrometry. Sample preparation included reduction and alkylation of proteins using DTT and iodoacetamide, overnight digestion with trypsin, and desalting via solid-phase extraction. LC-MS/MS experiments were carried out on a Thermo Scientific EASY-nLC 1200 liquid chromatography system coupled to a Thermo Scientific Orbitrap Fusion Lumos mass spectrometer. MS1 spectra were acquired in the Orbitrap (resolution = 120,000). Peptide fragmentation subsequently took place via high-energy collision-induced dissociation, and MS2 spectra were acquired in the ion trap. Resulting MS/MS spectral data were searched using the Mascot search engine (Matrix Science) for peptide identification. Additionally, AMPylation PTM assignments were manually verified from fragmentation spectra. The spectral count totals displayed in supplemental Table S1 refer to the number of MS/MS spectra identified with a Mascot score above a threshold of 15.

Concanavalin A purification of BiP for mass spectrometry

Concanavalin A pulldowns were performed on ER/mitochondrial fractions of S2 cells prepared as described above. ER/mitochondrial fractions were suspended in RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 5 mM $MgCl_2$, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM PMSF, protease inhibitor mixture) for 30 min on ice and centrifuged at 10,000 $\times$ *g* for 10 min at 4 °C. The soluble lysate was incubated with equilibrated concanavalin A beads overnight at 4 °C. The beads were washed three times with RIPA buffer. To elute endogenous BiP from the concanavalin A beads, the pulldowns were incubated with RIPA buffer + 5 mM ATP at room temperature for 15 min. Eluate was transferred to a new tube, TCA-precipitated, and suspended in SDS loading buffer for SDS-PAGE and Western blot analysis.

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