

1                   **Regulation of the Unfolded Protein Response by BiP AMPylation**  
2                   **protects photoreceptors from light-dependent degeneration.**

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## Abstract

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In response to environmental, developmental, and pathological stressors, cells engage homeostatic pathways to maintain their function. Among these pathways, the Unfolded Protein Response protects cells from the accumulation of misfolded proteins in the ER. Depending on ER stress levels, the ER-resident Fic protein catalyzes AMPylation or de-AMPylation of BiP, the major ER chaperone and regulator of the Unfolded Protein Response. This work elucidates the importance of the reversible AMPylation of BiP in maintaining the *Drosophila* visual system in response to stress. After 72 hours of constant light, photoreceptors of *fic*-null and AMPylation-resistant *BiP*<sup>T366A</sup> mutants, but not wild-type flies, display loss of synaptic function, disintegration of rhabdomeres, and excessive activation of ER stress reporters. Strikingly, this phenotype is reversible: photoreceptors regain their structure and function within 72 hours once returned to a standard light:dark cycle. These findings show that Fic-mediated AMPylation of BiP is required for neurons to adapt to transient stress demands.

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## Introduction

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Post-translational modifications (PTMs) of proteins are important for rapid responses to environmental challenges of cells. One such PTM is AMPylation, the reversible addition of adenosine monophosphate (AMP) to hydroxyl groups (also known as adenylylation) (Brown et al., 1971; Casey & Orth, 2018; Kingdon et al., 1967; Woolery et al.). AMPylation is catalyzed by at least two protein families, among them the conserved Fic-domain proteins (Casey & Orth, 2018; Harms et al., 2016). Eukaryotic Fic, an ER-resident type-II membrane protein (Rahman et al., 2012), AMPylates BiP (GRP78), a highly conserved and ubiquitous ER chaperone (Ham et

42 al., 2014; Preissler et al., 2015). Working together with a multitude of associated quality control  
43 proteins, BiP is critical for the translocation, folding, and secretion of proteins from the ER as  
44 well as for aiding in the clearing of misfolded ER aggregates and degradation of membrane-  
45 associated proteins (Hendershot et al., 1988; Kozutsumi et al., 1988; Meunier et al., 2002). BiP is  
46 both a mediator and transcriptional target of the Unfolded Protein Response (UPR), a  
47 coordinated cell signaling pathway that is activated during times of high misfolded protein levels  
48 in the ER. Like many protein chaperones, BiP depends on its ATPase activity to undergo a  
49 conformational change to bind to its substrates (Gaut & Hendershot, 1993). AMPylation locks  
50 BiP into a state resembling the ATP-bound conformation with high substrate off-rates, thereby  
51 inhibiting its chaperone function (Preissler, Rohland, et al., 2017; Witeska et al., 2017).

52 In agreement with this PTM's inhibitory role, BiP AMPylation levels are linked to  
53 protein homeostasis (Ham et al., 2014). Reduction of ER protein load promotes Fic-mediated  
54 AMPylation of BiP, whereas Fic catalyzes the deAMPylation of BiP under elevated ER stress  
55 conditions (Ham et al., 2014; Preissler et al., 2015). This switch in Fic's activity is linked to a  
56 key regulatory salt bridge in eukaryotic Fic. Mutations in Fic that disrupt this salt bridge result in  
57 an overactive AMPylator that lacks deAMPylation activity (Casey et al., 2017; Preissler, Rato, et  
58 al., 2017). Together, these studies suggest a model in which BiP is AMPylated in times of low  
59 ER stress, creating a reserve pool of inactive BiP that can be readily activated to respond to  
60 changes of ER homeostasis (**Figure 1A**). This reserve pool of BiP is proposed to act as a buffer  
61 to attenuate or shorten the need for a more dramatic activation of the transcriptional and  
62 translational arms of the UPR (Casey et al., 2017; Preissler, Rato, et al., 2017; Witeska et al.,  
63 2017). However, the physiological importance of endogenous Fic-mediated AMPylation for  
64 remains unclear.

65           In the fruit fly, *Drosophila melanogaster*, we previously demonstrated that *fic*-null  
66           mutants harbor a defect in visual signaling, as assessed by electroretinogram (ERG). The well-  
67           characterized Drosophila visual system has proven a valuable model for many fields, such as  
68           neuroscience (Borycz et al., 2002; Sugie et al., 2015), cell signaling (Dolph et al., 1993; Scott et  
69           al., 1995), protein trafficking (Akbar et al., 2009; Lee et al., 2003), and neurodegeneration  
70           (Johnson et al., 2002; Leonard et al., 1992; Ryoo et al., 2007). The specialized photoreceptor  
71           cells possess tightly packed microvilli-like membranes, termed rhabdomeres, that endow  
72           remarkable sensitivity to minute changes in light conditions (Montell, 2012). The ability to  
73           maintain this sensitivity is critical for flight behavior, foraging, and escape from predators. Thus,  
74           under a wide range of conditions, photoreceptors must maintain their light detection cascade,  
75           which requires the constant production, trafficking, and degradation of proteins through the  
76           endomembrane system (Colley et al., 1995; Kiselev et al., 2000; Rosenbaum et al., 2006).

77           We postulated that as a regulator of proteostasis and the UPR, BiP must be tightly  
78           regulated for proper maintenance of vision in the fly. Here we demonstrate that mutants lacking  
79           AMPylation of BiP at a specific residue, Thr366, possess the same ERG defect as *fic*-null  
80           animals, implicating BiP as the target of Fic necessary for visual signaling. We go on to find that  
81           application of an eye-specific stress, constant light, leads to exaggerated electrophysiology  
82           defects and the loss of photoreceptor rhabdomeres, akin to neurodegeneration. However, these  
83           defects exhibit a remarkable and unique reversibility: structural and functional phenotypes  
84           observed in light-stressed *fic*-null and AMPylation-resistant *BiPT<sup>366A</sup>* mutants are reversed after  
85           the flies are returned to a standard light/dark cycle. In addition, we identify changes in the  
86           regulation of UPR during constant light stress in these mutants, implicating ER dysregulation as  
87           the cause of the inability to adapt to altered light conditions.

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## Results

90 ***BiP*<sup>T366A</sup> rescues over-expression of constitutively active AMPylating *Fic*<sup>E247G</sup>**

91 To test the hypothesis that BiP is a critical target of Fic AMPylation *in vivo* (**Figure 1A**),  
92 we designed and generated transgenes expressing wild-type and AMPylation-resistant FLAG-  
93 tagged BiP proteins under control of the endogenous BiP promoter (**Figure 1- figure  
supplement 1A**). *BiP* null fly mutants die early in development; this lethality is rescued by  
94 including a copy of the genomic transgene expressing FLAG-BiP<sup>WT</sup> or the AMPylation-resistant  
95 FLAG-BiP<sup>T366A</sup> mutant (**Figure 1B**). We will refer to these rescued flies as *BiP*<sup>WT</sup> or *BiP*<sup>T366A</sup>,  
96 respectively. In contrast, a BiP transgene mutated at a second reported AMPylation site (Casey et  
97 al., 2017; Preissler et al., 2015), BiP<sup>T518A</sup>, did not rescue *BiP*<sup>-/-</sup> lethality. As *fic* null mutants that  
98 lack BiP AMPylation are viable, the lethality of the *BiP*<sup>T518A</sup> mutant is not likely to be due to the  
99 loss of AMPylation (Casey et al., 2017). Instead, these observations indicate an essential role for  
100 Thr<sup>518</sup> in BiP's chaperone activity. Consistent with this notion, the equivalent residue, Thr<sup>538</sup>, in  
101 the *S. cerevisiae* BiP homolog Kar2 is required for survival under heat stress even though yeast  
102 lack both Fic domain proteins and BiP AMPylation (**Figure 1- figure supplement 1B**).

103 Previously, we reported that over-expression of the constitutively active AMPylating  
104 *Fic*<sup>E247G</sup> was lethal in a *fic*-null fly background (*fic*<sup>30C</sup>) because it lacks the essential  
105 deAMPylation activity (Casey et al., 2017). We tested whether flies expressing the AMPylation-  
106 resistant BiP<sup>T366A</sup> could survive this lethality. Consistent with previous findings, we observe  
107 over-expression of the mutant UAS-*Fic*<sup>E247G</sup> transgene using the ubiquitous *Da-Gal4* driver was  
108 lethal in an otherwise *fic*-null animal (**Figure 1C**). Lethality of the constitutively active  
109 AMPylating *Fic*<sup>E247G</sup> was suppressed in flies expressing the AMPylation-resistant BiP<sup>T366A</sup> but

111 not BiP<sup>WT</sup> (**Figure 1C**). These results indicate that Thr<sup>366</sup> of BiP is a physiologically relevant  
112 AMPylation target *in vivo*.

113

114 **The UPR protects eyes from overactive AMPylation**

115 To test the interaction between Fic-mediated AMPylation and the UPR, we employed an  
116 eye-specific Fic gain-of-function model. Eye-specific expression of the constitutively active  
117 AMPylating UAS-Fic<sup>E247G</sup> transgene using a LongGMR-Gal4 driver in otherwise *fic*-null  
118 animals results in a severe rough-eye defect (Casey et al., 2017). However, in a *fic* heterozygous  
119 background, eye-specific expression of constitutively active AMPylating Fic<sup>E247G</sup> yields only a  
120 mildly rough eye (**Figure 2A**). We used this intermediate phenotype to assess genetic  
121 interactions between Fic<sup>E247G</sup> and components of the UPR with a candidate-based targeted RNAi  
122 screen (**Table 1**). Fic<sup>E247G</sup>-induced eye roughness was significantly enhanced by knockdown of  
123 the key UPR components *Perk*, *Atf4*, and *Ire1* (**Figure 2C-E** and **Table 1**), but not *ATF6* (**Figure**  
124 **2B**). Furthermore, *Xbp1* knockdown in conjunction with Fic<sup>E247G</sup> expression was lethal (**Figure**  
125 **2F**). By contrast, knockdown of these UPR genes in the absence of Fic<sup>E247G</sup> did not influence  
126 eye phenotype or fly survival (**Figure 2A'-F'**). These genetic interactions suggest a role for UPR  
127 signaling in mitigating cellular stress imposed by expressing the constitutively active  
128 AMPylating Fic<sup>E247G</sup> in the eye.

129

130 **AMPylation of BiP is necessary for maintaining vision**

131 The findings that BiP is a target of Fic *in vivo* and that silencing UPR pathway  
132 components enhances the severity of the constitutively active AMPylating Fic<sup>E247G</sup>-induced  
133 rough-eye phenotype prompted us to assay the physiological effects of cellular stress in flies

134 lacking BiP AMPylation. To do this we utilized flies that are either null for *fic* (*fic*<sup>30C</sup>) or express  
135 the AMPylation-resistant BiP<sup>T366A</sup> instead of wild-type BiP. By using this strategy, we are able to  
136 discern BiP AMPylation-specific changes from other potential changes that are due to as-yet-  
137 unknown targets of Fic AMPylation.

138 As previously reported in ERG recordings, *fic*-null flies display a reduction of the initial  
139 response (termed the ON Transient, Figure 3A) to a light pulse compared to wild-type controls.  
140 Interestingly, *BiP*<sup>T366A</sup>, but not *BiP*<sup>WT</sup> flies, exhibited the same defect in ON Transients as *fic*<sup>30C</sup>  
141 mutants, consistent with BiP being the primary target of Fic AMPylation required for proper  
142 visual neurotransmission (**Figure 1- figure supplement 2**). Of note, we used an eye-specific  
143 RNAi construct against *white* to minimize any effect of the *mini-white* gene used as a marker in  
144 these BiP transgenes. When we compared ERG traces of *fic*<sup>30C</sup> and *BiP*<sup>T366A</sup> flies in *white*<sup>+</sup> (red  
145 eyed) backgrounds, the reductions in ON transients were no longer detectable (**Figure 1- figure**  
146 **supplement 3**). This is likely due to the previously established protective effect provided by the  
147 red pigment in *white*<sup>+</sup> flies. Indeed, a similar *white*-dependent phenotype has been reported for  
148 photoreceptor synaptic plasticity after prolonged light exposure (Damulewicz et al., 2017; Sugie  
149 et al., 2015), consistent with previous observations that a functional *white* gene masks some  
150 degenerative phenotypes in the retina (Lee & Montell, 2004; Soukup et al., 2013). Therefore, we  
151 used the *white*-eyed flies (referred to as *w*<sup>1118</sup>) to test whether AMPylation may play a role in this  
152 type of photoreceptor plasticity, which is induced by rearing flies in uninterrupted low light for  
153 72 hours (Damulewicz et al., 2017; Sugie et al., 2015).

154 We conducted ERG recordings under different light conditions with four fly lines, *w*<sup>1118</sup>,  
155 *fic*<sup>30C</sup>, *BiP*<sup>WT</sup> and *BiP*<sup>T366A</sup> (**Figure 3B**). Compared to age-matched siblings reared under the  
156 standard 12 hr Light:12 hr Dark (LD) treatment, *fic*<sup>30C</sup> and *BiP*<sup>T366A</sup> flies reared for three days

157 under continuous light (LL) at 500 lux exhibited severe ERG defects. This included reduction in  
158 the sustained negative potential (SNP), a measure of photoreceptor activation, and loss of ON  
159 and OFF transients, which reflect synaptic transmission to downstream L1/L2 lamina neurons  
160 (**Figure 3C & D**). Wild-type controls maintained healthy OFF transients following LL, but ON  
161 transients were reduced, reflecting the sensitivity of this component to various light conditions  
162 (**Figure 3D**). To test for behavioral consequences, we assayed  $w^{1118}$  and  $fic^{30C}$  flies after 72 hours  
163 of LD or LL treatment for light-induced startle behavior using single-fly activity chambers (Ni et  
164 al., 2017). Following a 5-min light pulse, LD-reared  $fic^{30C}$  flies exhibited a startle response  
165 indistinguishable from control  $w^{1118}$  flies, whereas  $fic^{30C}$  flies, but not  $w^{1118}$  flies, failed to  
166 respond to the light pulse after 72 hours of LL (**Figure 3- figure supplement 1**). Thus, Fic-  
167 mediated AMPylation is required to maintain vision acuity under LL conditions.

168 We next designed experiments to test whether these functional ERG changes in flies  
169 lacking AMPylation reflected light-induced neurodegeneration or a failure to adapt to constant  
170 stimulation. First, we asked if the LL-induced ERG defects of  $BiP^{T366A}$  and  $fic^{30C}$  flies were  
171 reversible. We reared mutant and control flies for 72 hours in LL followed by 72 hours of  
172 recovery in LD (referred to as “Rec”; **Figure 3B**). This recovery period was sufficient to restore  
173 both healthy OFF transients and SNPs in  $BiP^{T366A}$  and  $fic^{30C}$  flies (**Figure 3C & D**). Second, we  
174 asked if the intensity of the light would exaggerate the defects of  $BiP^{T366A}$  and  $fic^{30C}$  flies.  
175 Exposure of mutant or control flies with 5000 lux, instead of 500 lux, did not alter the severity of  
176 ERG defects, indicating the changes were not simply a reflection of the increased amount of total  
177 light exposure during LL treatment (**Figure 3- figure supplement 2**). Third, we asked if  
178 prolonging the LL stress would alter the reversibility of these defects. Mutant flies reared under  
179 LL for ten days retained the capability to recover healthy ERG traces after only three days on

180 LD, indicating that photoreceptors are not dying but maintained during prolonged light stress  
181 (**Figure 3- figure supplement 3**). Together, these data support a model for a dysregulated  
182 adaptive response, rather than phototoxicity, inducing the reversible loss of OFF transients and  
183 reduced SNPs.

184

185 **Constant light induces severe but reversible morphological defects in AMPylation mutants**

186 To determine if the underlying eye substructures were being altered in these AMPylation  
187 deficient mutants, we performed TEM on ultrathin transverse eye sections. Under LD conditions,  
188 *fic*<sup>30C</sup> and *BiP*<sup>T366A</sup> mutant and wild-type controls appeared indistinguishable (**Figure 4A**).  
189 However, following 72 hours of LL (500 lux), *fic*<sup>30C</sup> and *BiP*<sup>T366A</sup> mutants, but not *w*<sup>1118</sup> and  
190 *BiP*<sup>WT</sup> controls, displayed severe defects in the integrity of rhabdomeres, the microvilli-like  
191 membrane structures that house the phototransduction cascade (**Figure 4B**). After a three-day  
192 recovery at LD, the rhabdomeres were nearly restored in both AMPylation-deficient mutants  
193 (**Figure 4C**). To quantify these structural changes in large cohorts of flies, we assessed flies for  
194 the presence of wild-type “deep pseudopupils” (DPP) (**Figure 4D**). Visualization of the DPP  
195 affords an assessment of rhabdomere structural integrity in living flies (Franceschini &  
196 Kirschfeld, 1971). Consistent with the TEM data, 3 days of LL caused loss of DPP in *fic*<sup>30C</sup> and  
197 *BiP*<sup>T366A</sup>, and DPPs returned after a 3-day recovery (**Figure 4D**). This suggests that proper  
198 regulation of BiP through AMPylation is required for maintaining both function and structure of  
199 photoreceptor cells.

200

201 **Fic regulates ER stress signaling in the retina and lamina neuropil**

202 Given the unique role of Fic in both AMPylating and deAMPylyating BiP to modulate its  
203 chaperone activity and maintaining ER homeostasis, we evaluated *fic*<sup>30C</sup> flies for changes in the  
204 UPR under LD, LL, and Rec conditions. First, we performed immunohistochemistry against BiP,  
205 a transcriptional target of the UPR, which is upregulated during states of ER stress (Gardner et  
206 al., 2013; Ham et al., 2014). After 3 days of LL, sections of *fic*<sup>30C</sup> revealed increased levels of  
207 BiP in retinas and in the lamina neuropils where photoreceptor axons form synapses onto lamina  
208 neurons. BiP levels returned to control levels following three days of recovery (**Figure 5A & B**).  
209 To further assess UPR signaling in these tissues, we utilized a sensor for Ire1-mediated Xbp1  
210 splicing (Sone et al., 2013) and an Atf4 translational reporter which serves as a proxy for Perk-  
211 mediated phosphorylation of eIF-2a (Kang et al., 2015). In wild-type flies, Xbp1-GFP was  
212 slightly induced in the lamina after 24 hours of LL in wild-type flies and the signal decreased  
213 over time (**Figure 5C, top row, & 5D**). However, in *fic*<sup>30C</sup> flies, the Xbp1-GFP signal in the  
214 lamina continued to increase after 48 hours of LL and remained elevated after 72 hours (**Figure**  
215 **bottom row, & 5D**). In the retina, control flies showed little to no increase of Xbp1-GFP levels,  
216 while *fic*<sup>30C</sup> flies showed a significant transient increase after one and two days LL. With the  
217 Atf4-DsRed reporter, we observed a significant increase of signal in both the retina and lamina  
218 of wild-type flies after one day, but no difference in *fic*<sup>30C</sup> mutants at one or two days LL when  
219 compared to LD controls (**Figure 5E & F**). However, by three days of LL, Atf4-DsRed reporter  
220 activity in the wild-type flies returned to control levels, while the *fic*<sup>30C</sup> mutants showed a  
221 significant increase in both the retina and lamina neuropil (**Figure 5E & F**). These changes in  
222 UPR signaling were reversible as each of the reporters returned to near control intensity after  
223 72 hours of LD recovery (**Figure 5A, C & E, last columns**). The elevated UPR response in

224 *fic*<sup>30C</sup> mutants correlated with the timing of the observed severe defects in the integrity of  
225 rhabdomeres (**Figure 4B**). Together, these data identify a crucial role for Fic-mediated BiP  
226 AMPylation in regulating UPR signaling during homeostatic responses of photoreceptor  
227 neurons.

228

229 **Discussion**

230 Here we demonstrate that BiP is a critical *in-vivo* target of Fic-mediated AMPylation, as  
231 an AMPylation-resistant BiP blocks over-expressed constitutively active AMPylating Fic<sup>E247G</sup>  
232 lethality and recapitulates *fic*-null vision defects. This work also sheds light on a novel  
233 physiological role for AMPylation/deAMPylation control of BiP: fine-tuning UPR signaling to  
234 allow for visual system adaptation. We observe genetic interactions with the constitutively active  
235 AMPylating Fic<sup>E247G</sup> and the UPR sensors Ire1 and PERK as well as their effectors, perhaps due  
236 to the critical role of BiP as both a regulator (Amin-Wetzel et al., 2017; Bertolotti et al., 2000;  
237 Carrara et al., 2015; Shen et al., 2005) and downstream transcriptional target of the UPR (Ham et  
238 al., 2014; Kozutsumi et al., 1988). Indeed, we hypothesize that unregulated Fic<sup>E247G</sup>, in the  
239 absence of deAMPylation activity, AMPylates excess BiP, excluding it from its normal  
240 chaperone role and leading to cell death (Casey et al., 2017; Truttmann et al., 2017). In support  
241 of this hypothesis, the developmental defects due to excessive unregulated AMPylation are  
242 suppressed by AMPylation-resistant BiP<sup>T366A</sup>. Furthermore, the enhancement of the rough-eye  
243 Fic<sup>E247G</sup> phenotype by knockdown of the Ire1 and PERK pathways suggest a protective role for  
244 the UPR, perhaps through the known effects on translation by Ire1-mediated decay of mRNA,  
245 Xbp1-driven transcription or PERK-mediated phosphorylation of eIF-2a (Gardner et al., 2013).

246 Our work supports an *in-vivo* requirement for Fic-mediated AMPylation of BiP<sup>T366</sup> in the  
247 context of long-term adaptation to prolonged light exposure. BiP<sup>T366</sup> replacement mutants  
248 phenocopy *fic*-null flies in both the light-induced blindness and the unexpected recovery from  
249 this phenotype. These functional changes are mirrored in the structural changes of photoreceptor  
250 rhabdomeres. Rhabdomere appearance of AMPylation deficient flies was reminiscent of retinal  
251 degeneration mutants (Ryoo et al.; Smith et al., 1991), however the remarkable recovery of the  
252 tissue structure in three days is unlike any reported retinal degeneration phenotype. Together,  
253 this work demonstrates a seminal role for Fic-mediated AMPylation of BiP *in vivo*: enabling  
254 photoreceptors to adapt and maintain both structural and functionality integrity during periods of  
255 prolonged stress due to uninterrupted light stimulation. The exact mechanism through which  
256 these defects in *fic* mutants arise remains undetermined, but previous studies have demonstrated  
257 a requirement for maintaining normal ER folding and trafficking of transmembrane visual  
258 signaling components, such as Rhodopsin (Colley et al., 1995; Rosenbaum et al., 2006). This  
259 intense demand for proper ER stress regulation sensitizes the eye to otherwise mild defects in *fic*  
260 mutants, and the additional demands posed by the stress of constant light stimulation.

261 We also observed that loss of BiP AMPylation deregulates, but does not block, the UPR  
262 to this physiological stress. This observation supports previous claims that AMPylation and  
263 deAMPylation of BiP aids in maintaining ER homeostasis (**Figure 1A**) by establishing a reserve  
264 pool of BiP that can readily be deAMPylated in response to acute ER insults (Casey et al., 2017;  
265 Ham et al., 2014; Preissler, Rato, et al., 2017). This regulation would allow for fine-tuning of the  
266 UPR response under specific contexts, comparable to findings in *C. elegans* in which *fic-1* and  
267 *hsp3* (a BiP homologue) mutants are sensitive to bacterial infection (Truttmann et al., 2016). We  
268 speculate that the eye requires tight control of BiP activity and suppression of UPR signaling, to

269 facilitate adaptation of the vision signaling cascade. Under standard LD conditions, only slight  
270 differences are observed, presumably because ER stress is low (**Figure 5C & D, 1<sup>st</sup> column**).  
271 However, exposure to constant light results in ER stress and UPR signaling, which wild-type  
272 flies can clear, presumably because there is a reserve pool of AMPylated BiP to rapidly respond  
273 to the stress. In *fic* mutants, we speculate, loss of the reserve BiP results in the UPR  
274 dysregulation revealed by the Ire1 and PERK activity reporters (**Figure 5C & D**) as a larger  
275 proportion of BiP would be previously engaged and not able to respond to the extra stress.  
276 Additionally, the prolonged UPR response in the eyes with dysregulated AMPylation may result  
277 in increased expression of UPR-regulated proteins while blocking production of the visual  
278 signaling components necessary for adapting to transient stress.

279 BiP expression is subject to multiple levels of feedback regulation and is induced when  
280 the UPR is activated (Kozutsumi et al., 1988; Ma & Hendershot, 2003). However, in a negative  
281 feedback loop, BiP also inhibits activation of the UPR sensors Ire1, PERK, and Atf6, through  
282 direct binding (Bertolotti et al., 2000; Shen et al., 2005). It remains unknown how AMPylation  
283 affects the interactions of BiP with these proteins *in vivo*; however, *in-vitro* work suggests that  
284 AMPylation of BiP abolishes its inhibitory effect on Ire1 dimerization and activation (Amin-  
285 Wetzel et al., 2017). We speculate that the loss of BiP AMPylation/deAMPylation cycle in a *fic*  
286 null affects both the ability of BiP to quickly respond to misfolded protein aggregates and to  
287 regulate UPR activation. Future studies on the dynamic role of reversible BiP AMPylation and  
288 its interaction with downstream UPR sensors should provide unique insight into neuronal  
289 plasticity and regeneration.

290

291 **Materials and Methods**

292 **Fly stocks and genetics**

293 Bloomington Stock Center provided  $w^{1118}$  (BS# 3605),  $BiP^{G0102}/FM7c$  (BS#11815),  $Da-$   
294  $Gal4$  (BS#55850),  $LongGMR-Gal4$  (BS#8121) stocks. The  $fic^{30C}$  allele and UAS-Fic<sup>E247G</sup> flies  
295 was previously described (Casey et al., 2017). Lines used in the RNAi screen are described in  
296 Table S1 and were obtained from Bloomington Stock Center and the Vienna Drosophila  
297 Resource Center (Dietzl et al., 2007). The  $Atf4^{5'UTR}$ -dsRed (Kang et al., 2015) and the  $Xbp1-$   
298 GFP (Sone et al., 2013) lines were a gift from Dr. Don Ryoo (NYU) and were recombined with  
299 the  $fic^{30C}$  allele. We generated the  $p[gen3xFLAG-BiP^{WT}]^{AttP-89E11}$ ,  $p[gen3xFLAG-BiP^{T366A}]^{AttP-}$   
300  $89E11$ ,  $p[gen3xFLAG-BiP^{T518A}]^{AttP-89E11}$  and  $p[GMR-dsRNA^{white}]$  alleles using the Phi30C  
301 integrase strategy (Venken et al., 2006).  $p[GMR-dsRNA^{white}]$  was recombined with the  $fic^{30C}$   
302 allele and white-eyed candidates were screened for the  $fic$  allele by PCR.  $BiP^{G0102};;$   
303  $p[gen3xFLAG-BiP^{WT}]^{AttP-89E11}$  and  $BiP^{G0102};;p[gen3xFLAG-BiP^{T366A}]^{AttP-89E11}$  stocks were made  
304 by crossing males harboring the genomic transgene to  $BiP^{G0102}/FM7c$  female flies. Surviving  
305 males were backcrossed to  $BiP^{G0102}/FM7c$  female flies, and stable stocks were established from  
306 the resulting progeny. None of the rare escaping  $BiP^{G0102};;p[gen3xFLAG-BiP^{T518A}]^{AttP-89E11}$   
307 male flies were fertile. The  $LongGMR-Gal4, UAS^{Scer}-V5-Fic^{E247G-attP-B3}/TM6B, Hu$  and  $Da-$   
308  $Gal4, UAS^{Scer}-V5-Fic^{E247G-attP-B3}/TM6B, hu$  stocks were made using standard Drosophila  
309 recombination and crossed into  $w^{1118}$  and  $w^{1118};fic^{30C}$  backgrounds.

310

311 **List of flies strains and stocks used**

312  $w^{1118}$  (BS#3605)

313 OreR

314 ; *fic*<sup>30C</sup>  
315 *w*<sup>1118</sup> ; *fic*<sup>30C</sup>  
316 *w*<sup>1118</sup> ; p[*GMR*-dsRNA<sup>white</sup>]  
317 *w*<sup>1118</sup> ; *fic*<sup>30C</sup> , p[*GMR*-dsRNA<sup>white</sup>]  
318 *w*<sup>1118</sup> ; *fic*<sup>30C</sup> ; *LongGMR*-Gal4,UAS<sup>Scer</sup>-V5-Fic<sup>E247G</sup>-attP-B3/TM6B,hu  
319 *w*<sup>1118</sup> ; *fic*<sup>30C</sup>/CyO ; *Da*-Gal4,UAS<sup>Scer</sup>-V5-Fic<sup>E247G</sup>-attP-B3/TM6B,hu  
320 *BiP*<sup>G0102</sup>/FM7c (BS#11815)  
321 *w*<sup>1118</sup>;; p[gen3xFLAG-*BiP*<sup>WT</sup>]<sup>AttP-89E11</sup>  
322 *w*<sup>1118</sup>;; p[gen3xFLAG-*BiP*<sup>T366A</sup>]<sup>AttP-89E11</sup>  
323 *w*<sup>1118</sup>;; p[gen3xFLAG-*BiP*<sup>T518A</sup>]<sup>AttP-89E11</sup>  
324 *w*<sup>1118</sup> ; *fic*<sup>30C</sup> ; p[gen3xFLAG-*BiP*<sup>WT</sup>]<sup>AttP-89E11</sup>  
325 *w*<sup>1118</sup> ; *fic*<sup>30C</sup> ; p[gen3xFLAG-*BiP*<sup>T366A</sup>]<sup>AttP-89E11</sup>  
326 *w*<sup>1118</sup> ; *fic*<sup>30C</sup> ; p[gen3xFLAG-*BiP*<sup>T518A</sup>]<sup>AttP-89E11</sup>  
327 *BiP*<sup>G0102</sup>;; p[gen3xFLAG-*BiP*<sup>WT</sup>]<sup>AttP-89E11</sup>  
328 *BiP*<sup>G0102</sup>;; p[gen3xFLAG-*BiP*<sup>T366A</sup>]<sup>AttP-89E11</sup>  
329 *BiP*<sup>G0102</sup> ; p[*GMR*-dsRNA<sup>white</sup>]; p[gen3xFLAG-*BiP*<sup>WT</sup>]<sup>89E11</sup>  
330 *BiP*<sup>G0102</sup> ; p[*GMR*-dsRNA<sup>white</sup>]; p[Attb\_gen3xFLAG-*BiP*<sup>T366A</sup>]<sup>89E11</sup>  
331 *w*<sup>1118</sup> ; p[tub-*Atf4*<sup>5'UTR</sup>-dsRed]/p[*GMR*-dsRNA<sup>white</sup>]  
332 *w*<sup>1118</sup> ; *fic*<sup>30C</sup>,p[tub-*Atf4*<sup>5'UTR</sup>-dsRed]/*fic*<sup>30C</sup>,p[*GMR*-dsRNA<sup>white</sup>]  
333 *w*<sup>1118</sup> ; p[*GMR*-dsRNA<sup>white</sup>] ; p[*Da*-Gal4]/p[UAS<sup>Scer</sup>-Xbp1-GFP.hg]  
334 *w*<sup>1118</sup> ; *fic*<sup>30C</sup>,p[*GMR*-dsRNA<sup>white</sup>] ; p[*Da*-Gal4]/p[UAS<sup>Scer</sup>-Xbp1-GFP.hg]  
335

336 **Generation of genomic BiP transgenes**

337 BiP cDNA sequence were subcloned into a pAttB vector and a 3X-FLAG tag was  
338 inserted after the N-terminal signal sequence. To create the T366A and T518A mutations,  
339 gBlocks (IDT, Coralville, IA) for the mutant sequences were synthesized and subcloned into the  
340 pAttB\_genomic BiP vector via NEB HiFi Assembly Kit (NEB, Ipswich, MA). These constructs  
341 were sequence-verified and injected into embryos (BestGene, Chino Hills, CA) for insertion at  
342 the 89E11 landing site. Expression levels of FLAG-BiP transgenes were determined with  
343 western blotting. In brief, fly heads were homogenized in lysis buffer (10% SDS, 6M urea, and  
344 50mM Tris-HCl, pH 6.8+ 10% DTT), sonicated for 5 min, boiled for 2 min, and centrifuged for  
345 10 min at 10,000 g to remove debris. 10 $\mu$ L were separated by SDS-PAGE and transferred to  
346 nitrocellulose membranes. Blots were probed with anti-BiP (1:8000, gift from Dr. Don Ryoo,  
347 NYU, NY), anti-FLAG (1:2000 M2- F3165, Millipore Sigma, St. Louis, MO) and anti-Actin  
348 (1:4000, JLA-20, DSHB, Iowa City, IA) and detected using IRdye-labeled antibodies and an  
349 Odyssey scanner (LI-COR Biosciences, Lincoln, NE).

350

351 **Generation of GMR\_dsRNA<sup>white</sup> transgenes**

352 To make the eye-targeted dsRNA constructs against the *white* gene, the dsRNA sequence  
353 was obtained from a pAttb-UAS<sup>S.cer</sup>-dsRNA<sup>white</sup> vector (a gift from Dr. Dean Smith, UT  
354 Southwestern Medical Center, TX) and the UAS<sup>S.cer</sup>-Hsp40 promotor sequenced was replaced  
355 with a 5X-*GMR* promotor sequence, synthesized as a gBlock (IDT) and cloned with NEB HiFi  
356 Assembly Kit (NEB).

357

358

### Fly rearing conditions

359

All flies were reared on standard molasses fly food, under room temperature conditions.

360

For light treatments, flies were collected within one to two days of enclosing, and placed in 5cm diameter vials containing normal food, with no more than 25 flies, and placed at either LD (lights ON 8am/lights OFF 8pm) or LL. ERGs, head dissections and behavior assays were performed between 1pm and 4pm. The same intensity white LED light source was used for both conditions and flies were kept the same distance away from the light source, which amounted to approximately 500 lux. LD and LL treatments were done at 25°C. For the UPR and Fic<sup>E247G</sup> rough-eye interaction experiments, all flies were raised at 28°C.

367

368

### Survival analysis of flies expressing genomic BiP construct

369

*BiP*<sup>G0102</sup>/FM7c female virgin flies were crossed to males with either gen3xFLAG-BiP<sup>WT</sup>, gen3xFLAG-BiP<sup>T366A</sup> or gen3xFLAG-BiP<sup>T518A</sup>. The number of surviving non-FM7c male flies were scored by presence or lack of the Bar eye marker. Percent of expected was calculated from the actual number of recovered flies of the relevant genotypes compared to the expected Mendelian number [# observed flies/ #expected flies]. Crosses were repeated three times (n=3). Total number of flies scored was at least 100 for each BiP variant.

375

376

### Survival analysis of flies expressing BiP variants in a *Da-Gal4*, UAS-Fic<sup>E247G</sup> background

377

C-terminally V5-His6-tagged UAS-Fic<sup>E247G</sup> (Casey et al., 2017) was expressed via the ubiquitous *Da-Gal4* driver in *fic*<sup>30C</sup>/CyO heterozygous flies. These flies were crossed to *w<sup>-</sup>*; *fic*<sup>30C</sup> (controls), *w<sup>-</sup>*; *fic*<sup>30C</sup>; gen3xFLAGBiP<sup>WT</sup>, *w<sup>-</sup>*; *fic*<sup>30C</sup>; gen3xFLAG<sup>T366A</sup>, *w<sup>-</sup>*; *fic*<sup>30C</sup>; gen3xFLAG-BiP<sup>T518A</sup>, or *BiP*<sup>G0102</sup>; *fic*<sup>30C</sup>; gen3xFLAG<sup>T366A</sup>. Offspring were scored and the number of adults

381 homozygous for *fic*<sup>30C</sup> with the *Da*-Gal4, UAS-*Fic*<sup>E247G</sup> allele and were compared to the number  
382 of *fic*<sup>30C</sup> heterozygous sibling controls. Percent of expected was calculated from the actual  
383 number or recovered flies of the relevant genotypes compared the expected Mendelian number  
384 [# observed flies/ #expected flies]. Crosses were repeated three times (n=3). Total number of  
385 flies scored was at least 100 for each BiP variant each repeat.

386

387 **Electroretinograms**

388 ERGs were recorded as previously described (Montell, 2012). Glass electrodes filled with  
389 2M NaCl were placed in the fly thorax and surface of the corneal lens (recording). A computer-  
390 controlled LED light source (MC1500; Schott, Mainz, Germany) was pulsed for 1s at 4s  
391 intervals. The resulting ERG traces were collected by an electrometer (IE-210; Warner  
392 Instruments, Hamden, CT), digitized with a Digidata 1440A and MiniDigi 1B system (Molecular  
393 Devices, San Jose, CA), and recorded using Clampex 10.2 (Molecular Devices) and quantified  
394 with Clampfit software (Molecular Devices). Flies were assayed in batches of eight to ten, and  
395 resulting quantifications are pooled from three independent biological repeats.

396

397 **Deep pseudopupil analysis**

398 Flies were anesthetized on CO<sub>2</sub> and aligned with one eye facing up. Using a stereoscopic  
399 dissection microscope, each fly was scored for presence or loss of the deep pseudopupil  
400 (Franceschini & Kirschfeld, 1971), and the percentage of flies with intact pseudopupils was  
401 calculated. For each genotype/treatment, over 50 flies were scored per replica and three  
402 biological replicas were performed (n=3).

403

404

### **Light-startle behavior assay**

405

Assay was adapted from a previously described method (Ni et al., 2017). After 72 hours of LD or LL treatment, 16 flies per genotype were collected at the same time each morning and placed into individual Drosophila Assay Monitoring (DAM) chambers (TriKinetics Inc, Waltham, MA). The DAM monitors were placed into a dark incubator. Two hours later, a 500-lux light was turned on by a timer for five minutes. Data was collected with DAMSystem3.0 and DAMFileScan11.0 (TriKinetics Inc). The resulting data was exported to Microsoft Excel and graphed in GraphPad Prism. Three replica experiments were averaged and plotted as Time (min) vs Average activity per 2 min. bin (n=3). The change in response to light was calculated for each light pulse as [*mean beam breaks for 10min. post-pulse*] – [*mean beam breaks for 10min. pre-pulse*].

415

416

### **Scanning electron microscopy**

417

SEMs of fly eyes were obtained as previously described (Wolff, 2011). Eyes were fixed in 2% paraformaldehyde, 2% glutaraldehyde, 0.2% Tween 20, and 0.1 M cacodylate buffer, pH 7.4, for 2 hours. Samples were washed four times with increasing ethanol (25–100%) for 12 hours each followed by a series of hexamethyldisilazane washes (25–100% in ethanol) for one hour each. Flies were air dried for 24 hours, mounted on SEM stubs, and the bodies were coated in fast-drying silver paint. Flies were sputter coated with a gold/palladium mixture for 60s and imaged at 900X magnification, with extra high tension set at 3.0 kV on a scanning electron microscope (Sigma SEM; Carl Zeiss, Germany). Ten flies per genotype were mounted and three were imaged (n=3). Blinding of the samples' identity to the user acquiring the images was performed.

427

428 **Transmission electron microscopy**

429 TEMs of retina sections were performed as previously described (Jenny, 2011; Rahman  
430 et al., 2012). Briefly, 550 nm sections were cut and stained with toluidine blue to confirm  
431 orientation and section depth. Blocks were subsequently thin-sectioned at 70 nm with a diamond  
432 knife (Diatome, Hatfield, PA) on a Leica Ultracut 6 ultramicrotome (Leica Microsystems,  
433 Wetzlar, Germany) and collected onto formvar-coated, glow-discharged copper grids, post-  
434 stained with 2% aqueous uranyl acetate and lead citrate. Images were acquired on a Tecnai G2  
435 spirit transmission electron microscope (FEI) equipped with a LaB6 source using a voltage of  
436 120 kV. Blinding of the samples to the technicians performing the processing and the user  
437 acquiring the images was performed. Two fly heads per genotype/condition and at least three  
438 thin sections per sample were examined (n=2). Samples were unmasked after the images were  
439 processed.

440

441 **Immunohistochemistry for BiP and UPR reporters**

442 Fly heads were dissected in HL3 hemolymph-like solution, fixed for four hours in ice-  
443 cold 4% para-formaldehyde in filtered PBS, washed overnight in 25% (wt/vol) sucrose in  
444 phosphate buffer (pH 7.4), embedded in Optimal Cutting Temperature compound (EMS,  
445 Hatfield, PA) frozen in dry ice and sectioned at 20- $\mu$ m thickness on a cryostat microtome (CM  
446 1950, Leica Microsystems, Wetzlar, Germany). Sections were probed overnight with primary  
447 antibodies against Drosophila BiP (1:2000, Gift from Don Ryoo(Ryoo et al.), GFP (1:1000,  
448 A10262, ThermoFisher Scientific, Waltham, MA) or RFP (1:1000, 600-401-379, Rockland,  
449 Limerick, PA). Secondary antibodies were labeled with Alexa488-conjugated Goat anti-Chicken

450 (Molecular Probes, P/N# A-11039), Alexa488-conjugated Goat anti-Guinea Pig (Molecular  
451 Probes, P/N# A-11073), or Alexa568-conjugated Goat anti-Rabbit (Molecular Probes, P/N# A-  
452 11011). Alexa 647-conjugated phalloidin was also added to label Actin for identifying structures.  
453 Images were captured with an oil-immersion 63 $\times$  NA-1.4 lens on an inverted confocal  
454 microscope (LSM710, Carl Zeiss). For each genotype and light rearing conditions,  
455 immunohistochemistry experiments were performed in two biological replicas with new sets of  
456 flies, using identical acquisition settings. Blinding of the samples to the user acquiring the  
457 images was performed when appropriate.

458

459 **Quantification of fluorescence staining**

460 Fluorescence images were quantified using ImageJ (NIH) adapting previous methods  
461 (Nandi et al., 2017). For each antibody, a threshold was determined, removing the lowest 10% of  
462 signal in LD control samples (to reduce variation from low level background signals). This same  
463 threshold was applied, and a mask was created for every image in a batch of staining. Within a 1-  
464  $\mu$ m optical slice, the retina and lamina regions were selected manually using an Actin stain and  
465 assigned as Regions of Interest. The integrated pixel intensity per unit area was measured within  
466 this selected area, redirecting to the threshold mask. In each fly, four sequential optical slices  
467 were quantified and averaged. For each genotype and treatment, four flies were quantified from  
468 two independent biological replicas for a total of eight flies. Data was normalized to the wild-  
469 type LD control for each replica. Outliers of greater than three standard deviations were omitted  
470 from the analysis.

471

472

## Yeast plasmids and strains

473

Yeast genetic techniques were performed by standard procedures described previously.

474

(Sherman et al., 1981). All strains were cultured in either rich (YPD: 1% yeast extract, 2% peptone, and 2% glucose) or complete synthetic minimal (CSM) media lacking appropriate amino acids with 2% glucose. Yeast were grown to log phase, serially diluted, and spotted onto agar plates to assay fitness and temperature sensitivity as previously described (Tran et al., 2007).

479

DNA fragments of KAR2 was generated by PCR amplification of the endogenous KAR2 gene using the primers 5'-GCATCCGCGGATACTCTCGTACCCCTGCCGC-3' and 5'-ATGCGAGCTCCGTATATACTCAGTATAATC-3'. Plasmid pKAR2:LEU2 and pKAR2:URA3 were generated by subcloning genomic DNA fragments containing promoter and coding sequence of *KAR2* into the *SacI* and *SacII* sites of pRS315 and pRS316, respectively.

484

pKAR2T386A:LEU2 was generated by site directed mutagenesis of pKAR2:LEU2 using the primers 5'-

486

GGTTGGTGGTTCTGCTAGAATTCCAAAGGTCCAACAATTGTTAGAATCATACTTGATGG-3' and 5'-ACCTTTGGAATTCTAGCAGAACCAACCAACCAAAACGATATCATCACACATCCTCTTTCC-3'.

488

pKAR2T538A:LEU2 was generated by site directed mutagenesis of pKAR2:LEU2 using the primers 5'-AGATAAGGGAGCTGGTAAATCCGAATCTATCACCATCACTAACG-3' and 5'-GGATTTACCAAGCTCCCTATCTGTGGCAGACACCTTCAGAATACC-3'.

491

ACY008 yeast (mat A kar2::KAN his3Δ0 leu2Δ0 LYS met15Δ0 ura3Δ0 pKAR2:URA)

492

were obtained by sporulation and dissection of KAR2 heterozygous null yeast (Mata/mat@KAR2::KAN/KAR2 his3Δ0/his3Δ0 leu2Δ0/leu2Δ0 LYS/lys MET/met15Δ0 ura3Δ0/ura3Δ0) (GE) transformed with pKAR2:URA. Standard plasmid shuffle techniques with 5-FOA(Zymo)

495 were utilized to obtain ACY016 (mat A kar2::KAN his3Δ0 leu2Δ0 LYS met15Δ0 ura3Δ0  
496 pKAR2:LEU2) ACY017(mat A kar2::KAN his3Δ0 leu2Δ0 LYS met15Δ0 ura3Δ0  
497 pKAR2T386A:LEU2), and ACY020(mat A kar2::KAN his3Δ0 leu2Δ0 LYS met15Δ0 ura3Δ0  
498 pKAR2T538A:LEU2)

499

500 **Statistics**

501 Statistics were performed using GraphPad Prism 7. Normality of data distribution was  
502 determined using D'Agostino's & Pearson's normality test. For the genetic analysis in Figure 1  
503 and the ERG measurements in Supplemental Figures 2,3,5, and 6, significance was determined  
504 using one-way ANOVA, followed by Tukey's multiple comparisons tests. Statistical significance  
505 for non-parametric data, including the ERGs with light treatment quantifications in Figure 2, was  
506 determined by Kruskal-Wallis tests followed by multiple comparisons testing with Dunn's  
507 correction. For the image quantification data in Figure 4, significance was determined by two-  
508 way ANOVA followed by multiple comparisons with Benjamini-Krieger-Yekutieli's False  
509 Discovery Rate correction. All tests were two-sided with no experimental matching. RStudio  
510 (version 1.1.442, 2018, RStudio, Inc.) was used for Fisher's Exact Tests for the eye interaction  
511 screen, with Bonferroni's multiple comparison method to determine significance. Standard R  
512 functions were used, no custom scripts were developed. Tests were two-sided. When possible,  
513 blinding of sample identities was performed for image acquisition and fluorescence intensity  
514 quantification. Sample sizes for ERG assays, EM experiments, fluorescence quantifications and  
515 fly genetic analysis were determined based from previous experience (Nandi et al., 2017;  
516 Rahman et al., 2012; Stenesen et al., 2015).

517

**Competing Interests:**

518

The authors declare no competing interests.

519

520

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695 A.T.M, A.C. K.O and H.K. conceived, designed, and analyzed experiments and wrote the  
696 manuscript. All experiments except yeast experiments were performed by A.T.M. A.C. and K.S.  
697 performed mass spec experiments.

698

699 **Data and materials availability:** Fly stocks are available upon request. All data are contained  
700 in the source files.

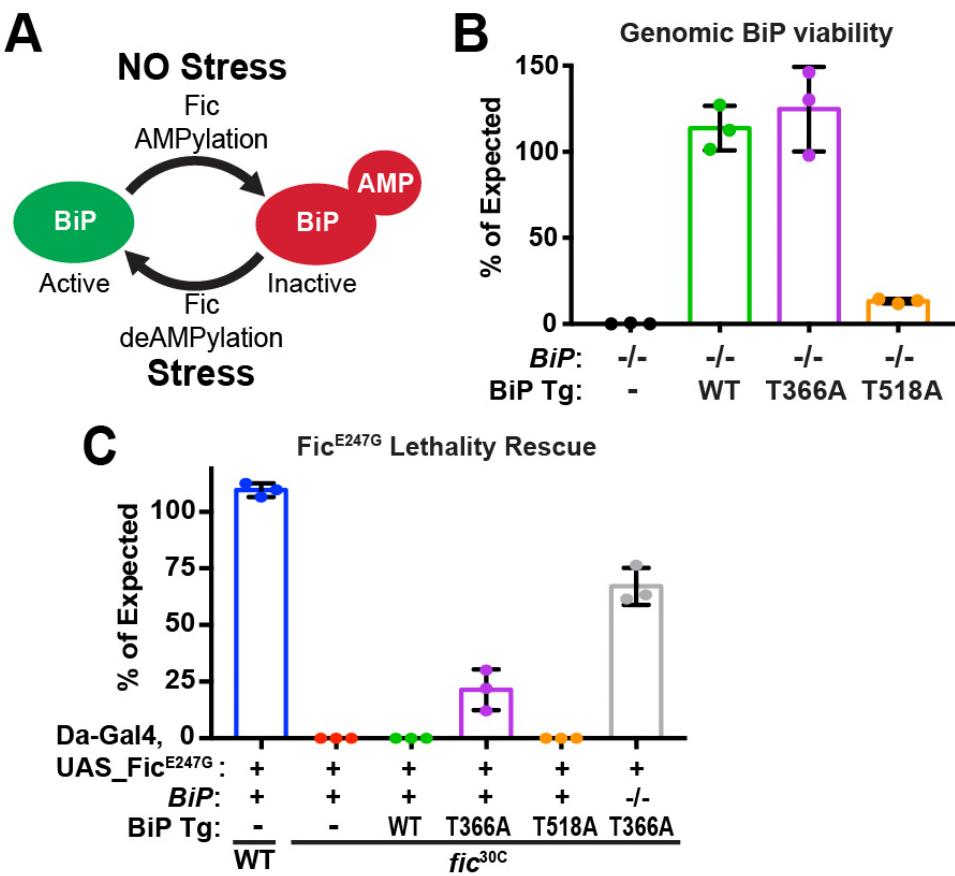
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## Figures



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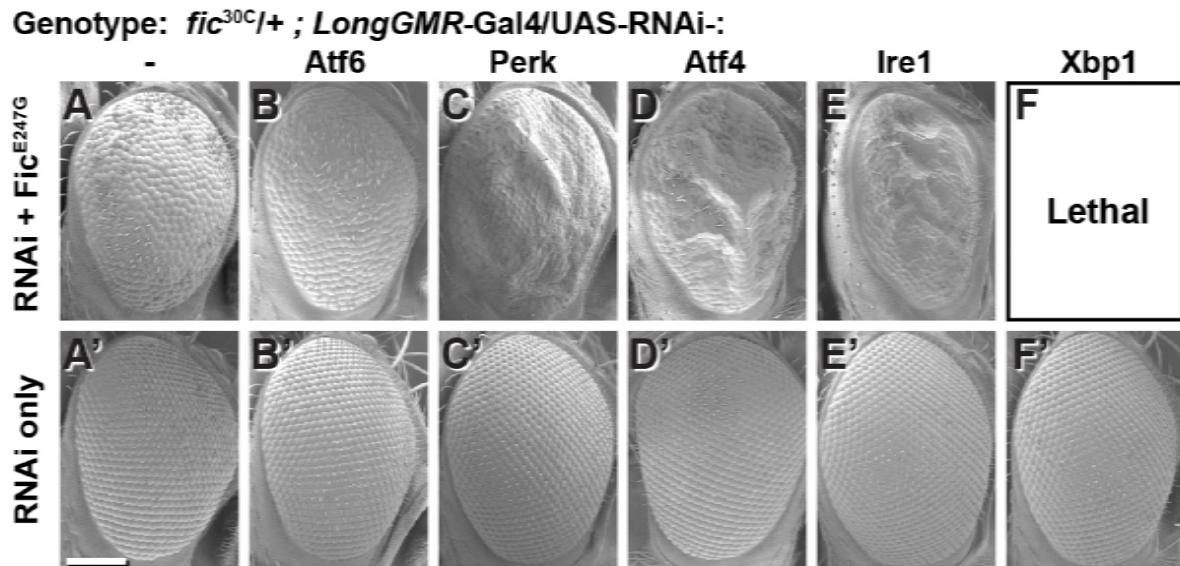
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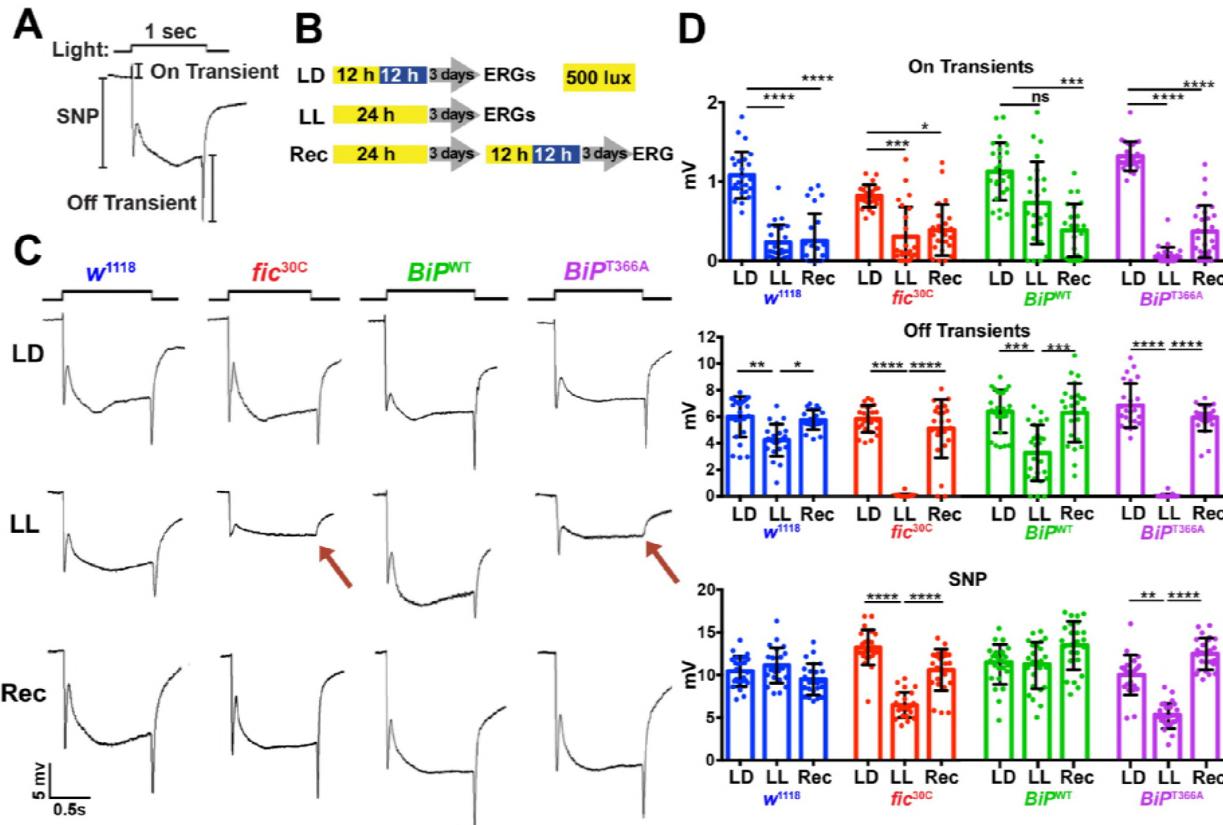
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**Figure 1. BiP is a target of Fic AMPylation and deAMPylation *in vivo*.** (A) BiP AMPylation during times of low ER stress reserves a portion of the chaperone to allow for a rapid, deAMPylation-driven, response to high ER stress (Casey et al., 2017; Preissler, Rato, et al., 2017). (B) Bar graphs show the percentage of null mutant  $BiP^{G0102}/y$  males rescued by the indicated genomic  $BiP^{WT}$ ,  $BiP^{T366A}$  or  $BiP^{T518A}$  genomic transgene (Tg) relative to sibling controls. N=3 biological replicas. At least 50 flies scored for each replica. Bar graphs show means +/- Standard Deviation (SD). (C) Bar graphs show the percentage of viable flies of the indicated wild type or  $fic^{30C}$  genotypes expressing the overactive  $Fic^{E247G}$  under the ubiquitous Da-Gal4 driver relative to sibling controls. Among the indicated genomic BiP transgenes, only  $BiP^{T366A}$  provides partial rescue of lethality in the  $BiP^{+/+}$  background and near complete rescue in a  $BiP^{G0102}$  null background. N=3 biological replicas. At least 100 total flies scored for each replica. Bar graphs show means +/- SD.



719

720 **Figure 2. Genetic interactions between Fic and UPR genes.** Representative SEM images of  
721 heterozygote mutant *fic*<sup>30C/+</sup> eyes expressing the indicated UAS-RNAi transgenes with (A-F) or  
722 without (A'-F') UAS-*Fic*<sup>E247G</sup> under *longGMR-Gal4* control. See Supplemental Table 1 for  
723 quantification. Scale bar: 100 $\mu$ M.



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725

**Figure 3. Fic-mediated AMPylation of BiP is required for photoreceptor maintenance. (A)**

726

A representative ERG trace in response to a 1-sec light pulse displaying the sustained negative potential (SNP), representing the depolarization within photoreceptor neurons, and the ON & OFF transients, reflecting post-synaptic activity of lamina neurons. **(B)** Representation of the different light treatments of flies before ERG recordings: 3 days of 12hr light (500 lux) and 12hr dark (LD), 3 days of continuous light (LL) or 3 days of continuous light followed by 3 days of LD (Rec). 1-sec light pulses were performed at 4 sec intervals. **(C)** Representative traces from *w*<sup>1118</sup>, *fic*<sup>30C</sup>, *BiP*<sup>WT</sup> and *BiP*<sup>T366A</sup> flies. Under LL, *fic*<sup>30C</sup> and *BiP*<sup>T366A</sup> mutants lose ON and OFF transients (red arrows) and have reduced SNPs. The changes are reversed after 3-days of recovery (Rec). **(D)** Quantification of key components of ERGs shown in panel C. Bar graphs show means +/- SD. \*\*\*\*, p < 0.0001; \*\*\*, p < 0.001; \*\*, p < 0.01; \*, p < 0.05; n= 24 flies for each genotype/condition, pooled from three independent biological replicas.

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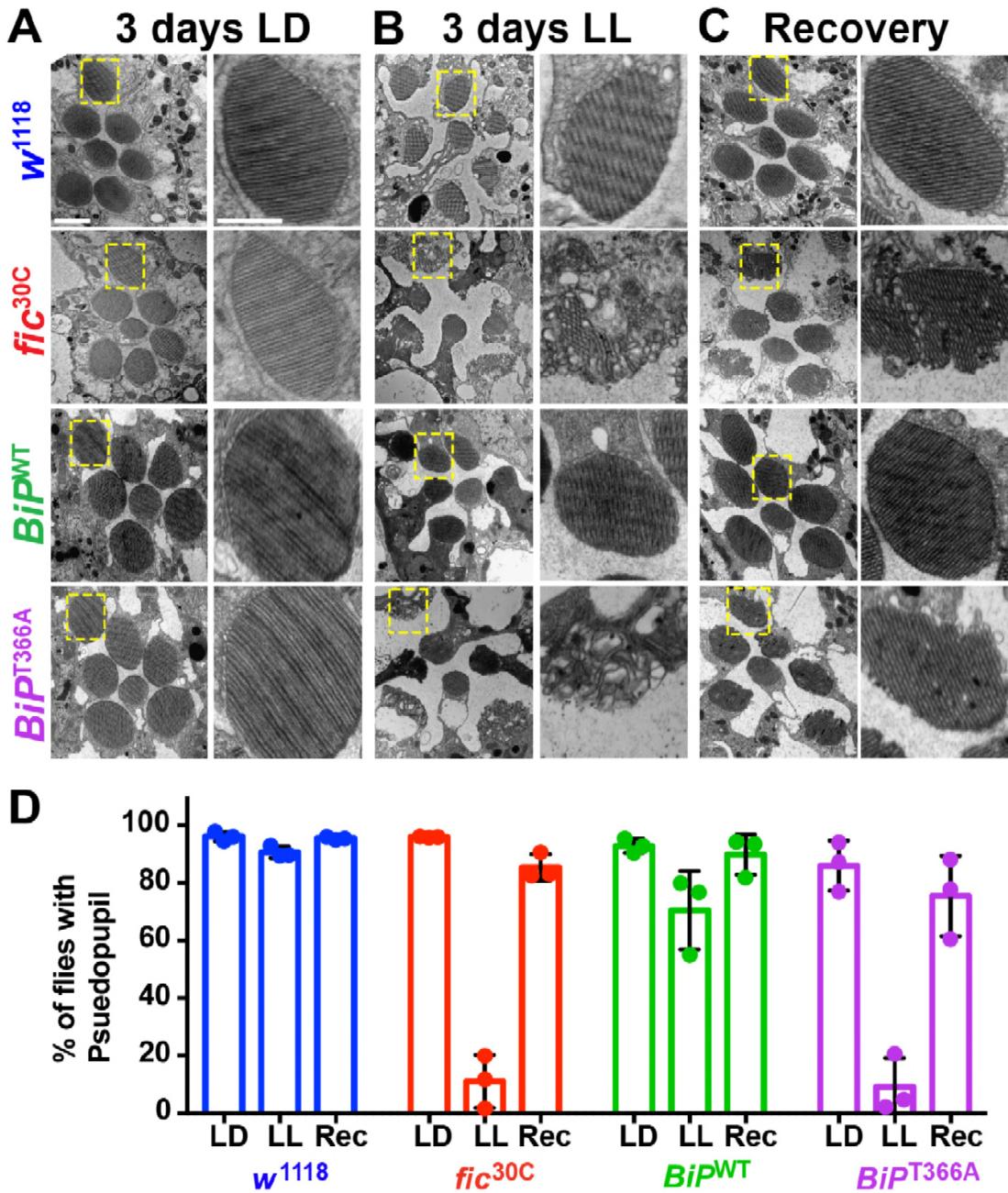
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**Figure 4. AMPylation of BiP is required for maintaining structural plasticity in the retina.**

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(A-C) Representative TEM images of retina thin sections from the indicated genotypes with either standard LD (A), the stress-inducing LL (B) or recovery treatment (C). Scale bars: 1  $\mu$ M. Yellow boxes indicate rhabdomeres shown in high magnification images. High magnification scale bars: 0.5  $\mu$ M. (D) Percentages of flies with intact deep pseudopupil following LD, LL and Rec. N=3 independent biological replicas with approximately 50 flies scored per genotype per replica. Bar graphs show means +/- SD.

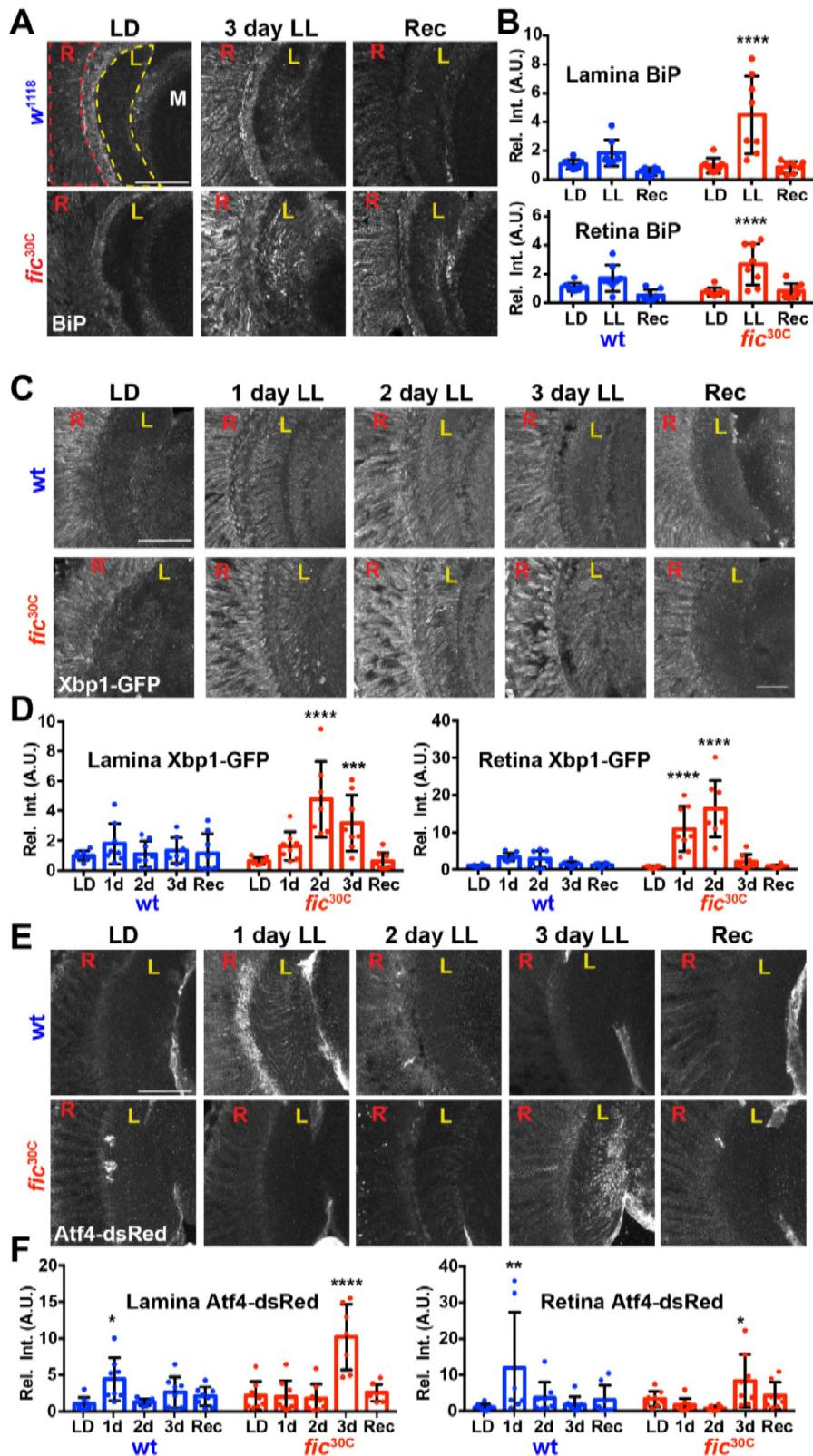
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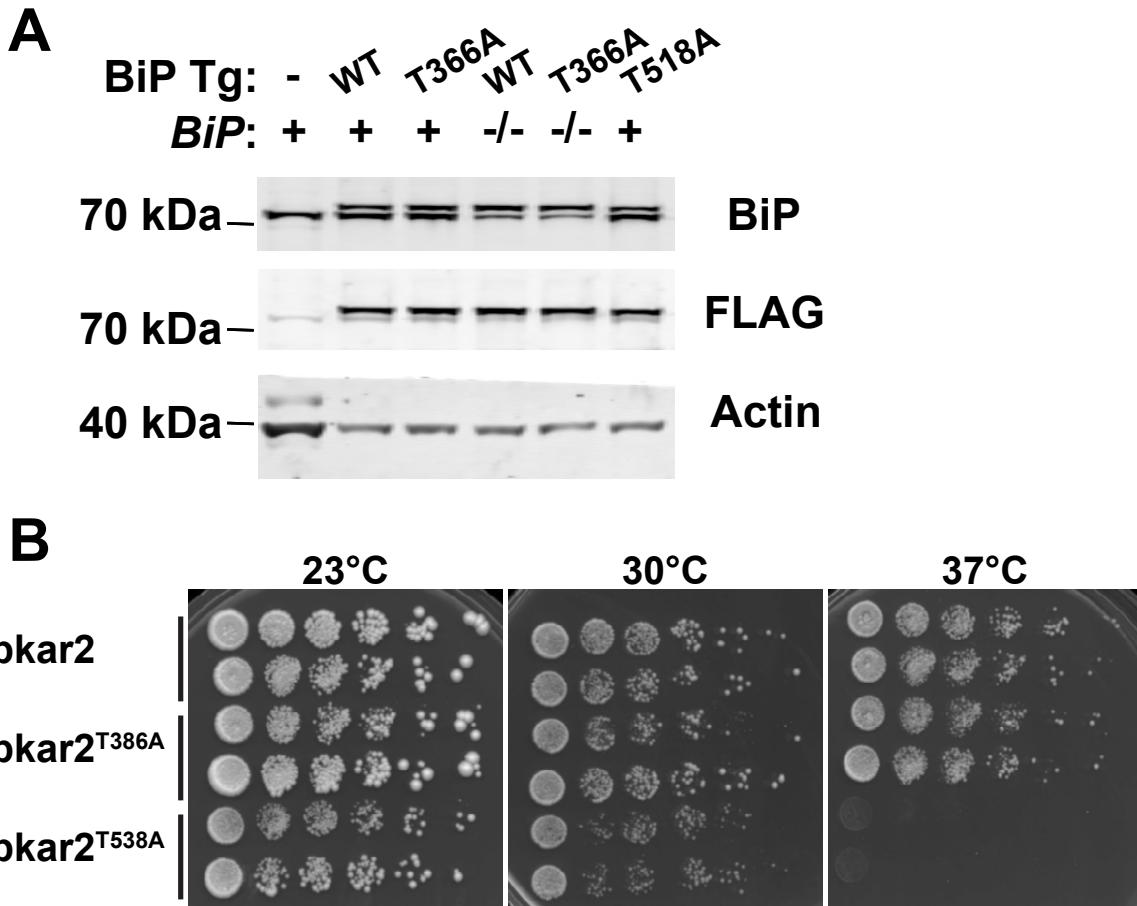
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**Figure 5. BiP AMPylation regulates ER homeostasis during prolonged light stimulation.**

747 (A) Representative images of BiP immunohistochemistry in sections of *w<sup>1118</sup>* and *fic<sup>30C</sup>* flies

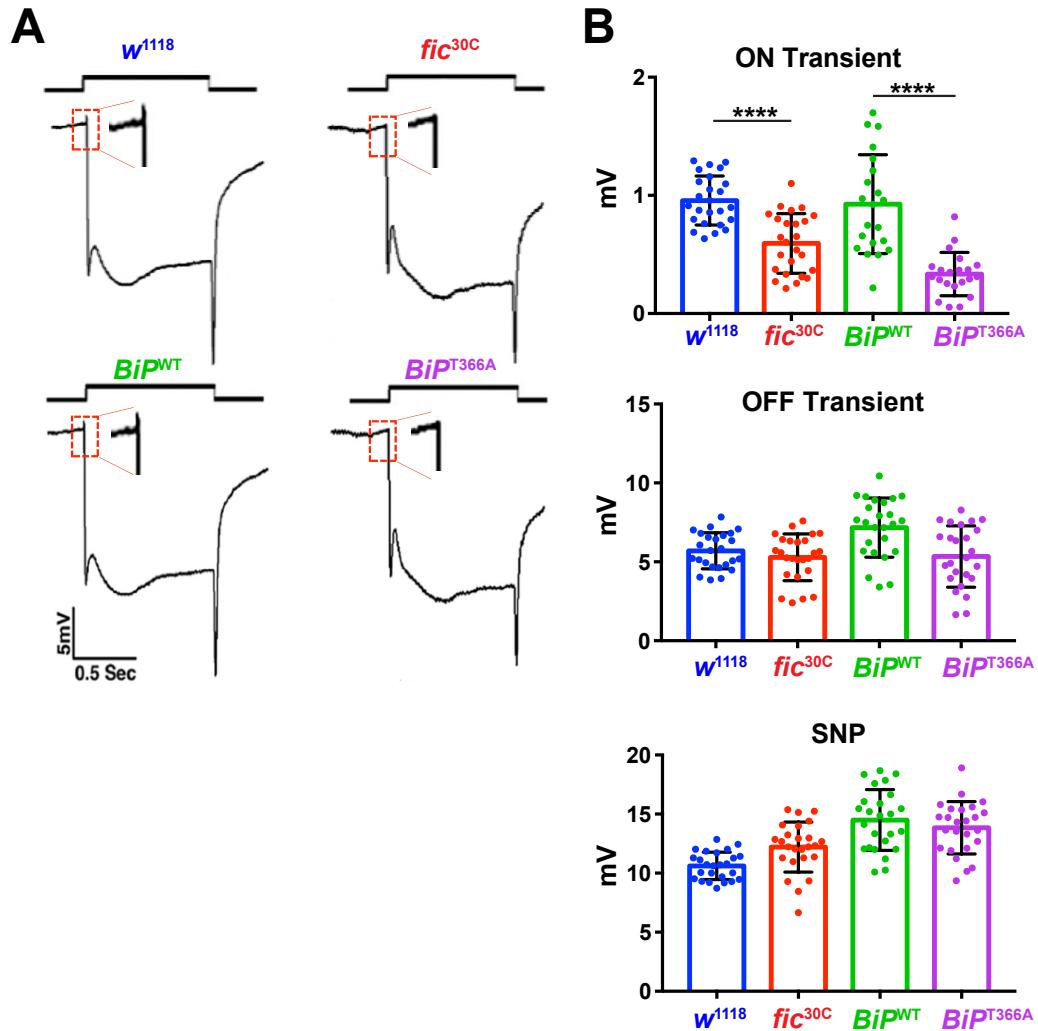
748 following 3 days LD, LL or Recovery treatments. **(B)** Quantification of BiP fluorescence  
749 intensity, normalized to wild-type LD controls, in the lamina neuropil and retina from 2  
750 independent experiments. **(C)** Representative images of a *Xbp1*-GFP splicing reporter in either a  
751 Fic wild-type or the null *fic*<sup>30C</sup> background following LD, 1-day LL, 2-day LL, 3-day LL, and  
752 Recovery conditions. **(D)** Quantification of GFP fluorescence intensity, normalized to wild-type  
753 LD controls, in the lamina neuropil and retina from 2 independent experiments. **(E)**  
754 Representative images of an *Atf4*-dsRed reporter in either a wild-type or *fic*<sup>30C</sup> background  
755 following LD, 1-day LL, 2-day LL, 3-day LL, and Recovery conditions. **(F)** Quantification of  
756 *Atf4*-dsRed intensity, normalized to wild-type LD controls, in the lamina neuropil and retina  
757 from 2 independent experiments. For all experiments, n= 8 flies per genotype/condition, with  
758 exceptions of outliers falling 3 standard deviations outside the mean. Bar graphs show means +/-  
759 SD. For all experiments, significance is indicated for treatment compared to the LD condition for  
760 the corresponding genotype. \*\*\*\*, p < 0.0001; \*\*\*, p < 0.001; \*\*, p < 0.01; \*, p < 0.05. All scale  
761 bars: 50  $\mu$ M.



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763 **Figure 1- figure supplement 1. Expression of genomic BiP transgenes**

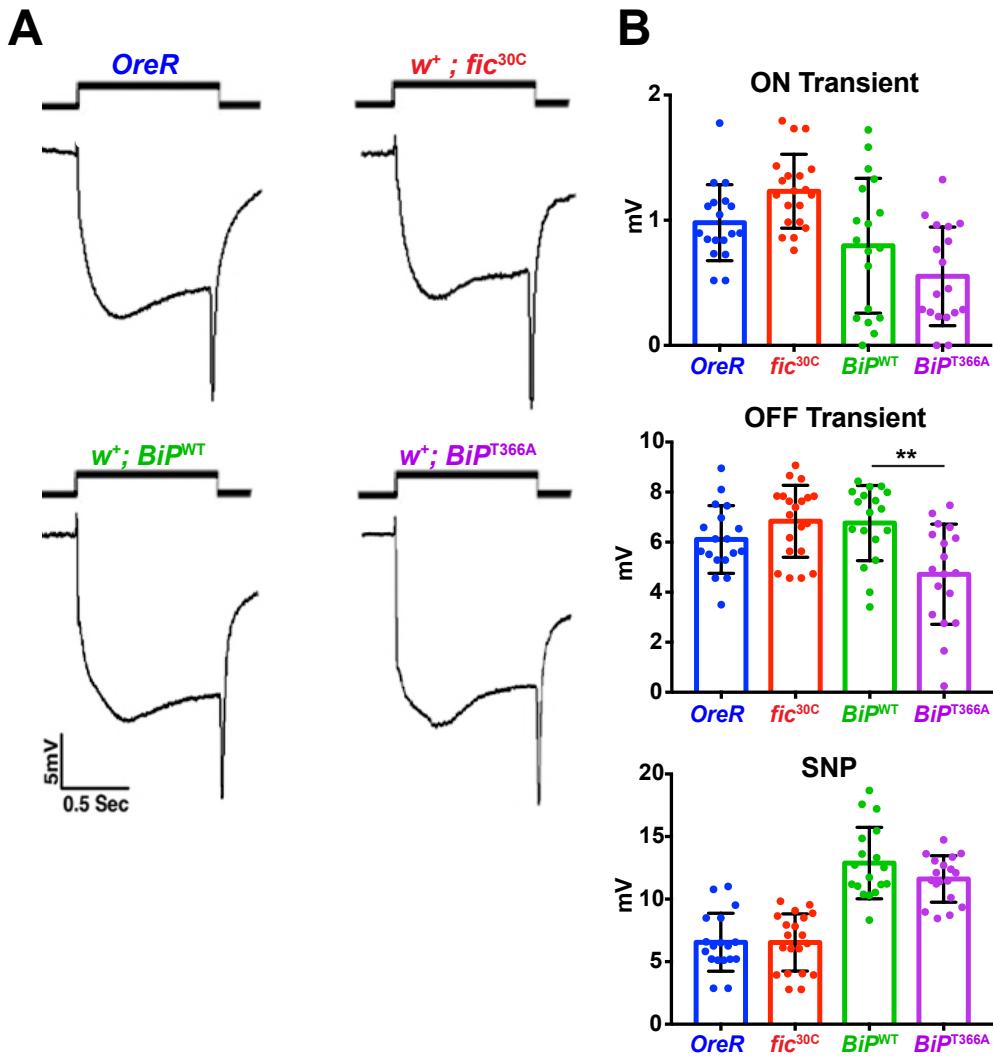
764 (A) Western blots for FLAG-tagged BiP transgenes and total BiP in whole head lysates in *BiP*  
765 wild type or homozygous mutant background as indicated. Actin (JLA-20) served as a loading  
766 control. (B) *Kar2*<sup>T538A</sup> mutants have temperature-sensitive growth defects. Yeast strains *kar2Δ*  
767 +*pKar2*, *kar2Δ* +*pkar2*<sup>T386A</sup>, and *kar2Δ* +*pkar2*<sup>T538A</sup> were grown at 25°C and five-fold serially  
768 diluted onto plates of rich media incubated at the indicated temperatures.



769

770 **Figure 1- figure supplement 2. AMPylation-resistant *BiP<sup>T366A</sup>* phenocopies *fic*.**

771 (A) ERGs of *fic<sup>30C</sup>* flies show reduced ON transients (arrows). Flies homozygous for a lethal  
772 *BiP<sup>G0102</sup>* allele rescued by *BiP<sup>WT</sup>* transgene have normal vision but flies rescued with the mutant  
773 *BiP<sup>T366A</sup>* transgene display reduced ON transients. (B) Quantification of ERG traces. Bar graphs  
774 show means +/- SD. \*\*\*\*, p < 0.0001; \*\*, p < 0.001; \*, p < 0.05; n= 24 flies per genotype and  
775 condition.



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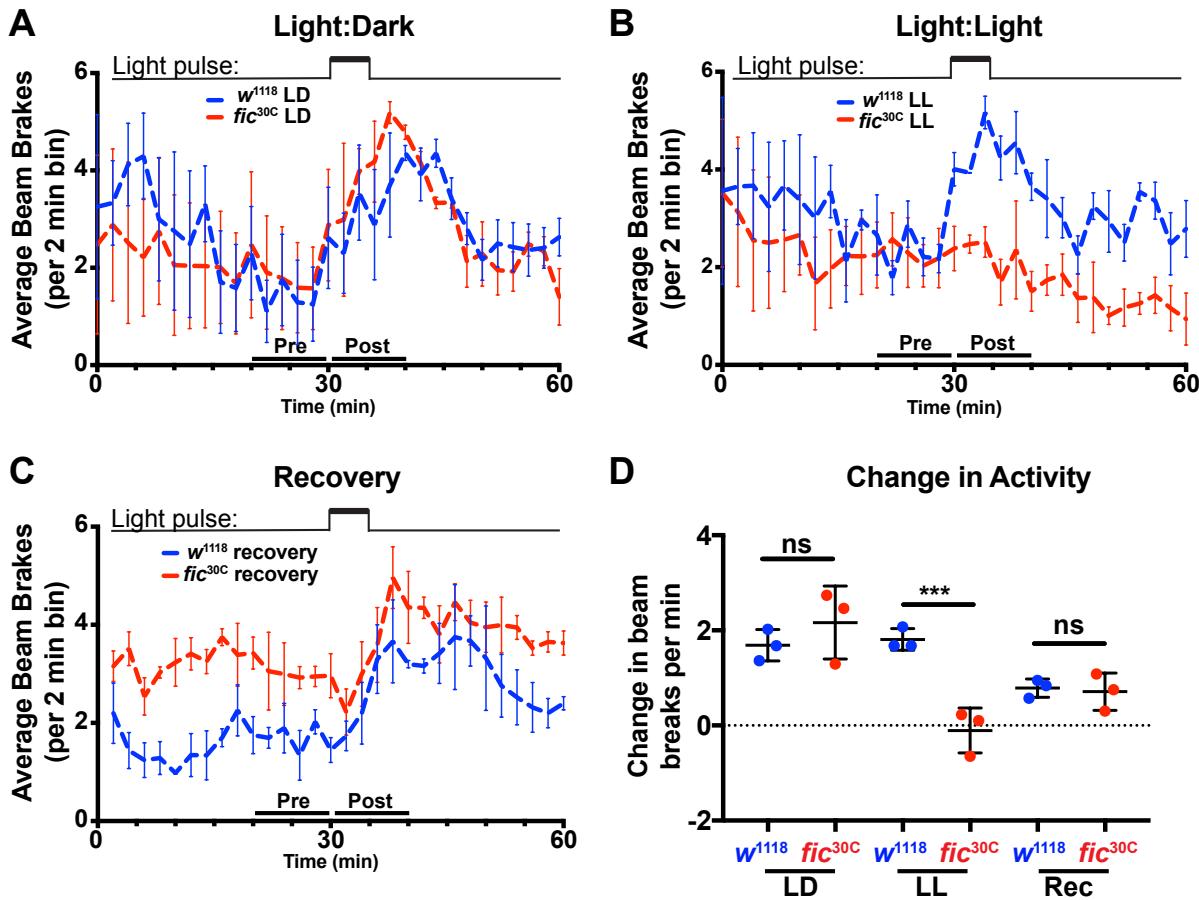
777 **Figure 1- figure supplement 3. Red eye pigment suppresses ERG phenotypes of *fic<sup>30C</sup>* and**

778 *BiP<sup>T366A</sup>* mutants.

779 **(A)** ERGs of OreR and red-eyed *fic<sup>30C</sup>* flies as well as *BiP<sup>WT</sup>* and *BiP<sup>T366A</sup>* animals. **(B)**

780 Quantification of ERG data. Bar graphs show means +/- SD. \*\*, p < 0.01, n= 18 flies per

781 genotype and condition.



782

783 **Figure 3- figure supplement 1. Light-induced defects in light-startle activity in *fic*<sup>30C</sup> mutants.**

784

785 (A, B, & C) Actogram of *w<sup>1118</sup>* or *fic<sup>30C</sup>* flies reared in LD for three days (A), LL for three days (B), or recovery condition (three days in LL then three days in LD) (C). Light pulse is indicated by upper bars. Data is averaged from three biological replicas, each containing 16 flies per genotype. Data were collected in two-minute bins. Error bars represent Standard Error. (D) Quantification of change in beam breaks per 2-min bin for the 10 min intervals before and after the onset of the light pulse in each experiment. Bar graphs show means +/- SD. \*\*\*, p < 0.01, n= 3 experimental repeats with 16 flies per genotype and condition. Dead flies and those with a change in activity greater than 3 deviations from the median were excluded.

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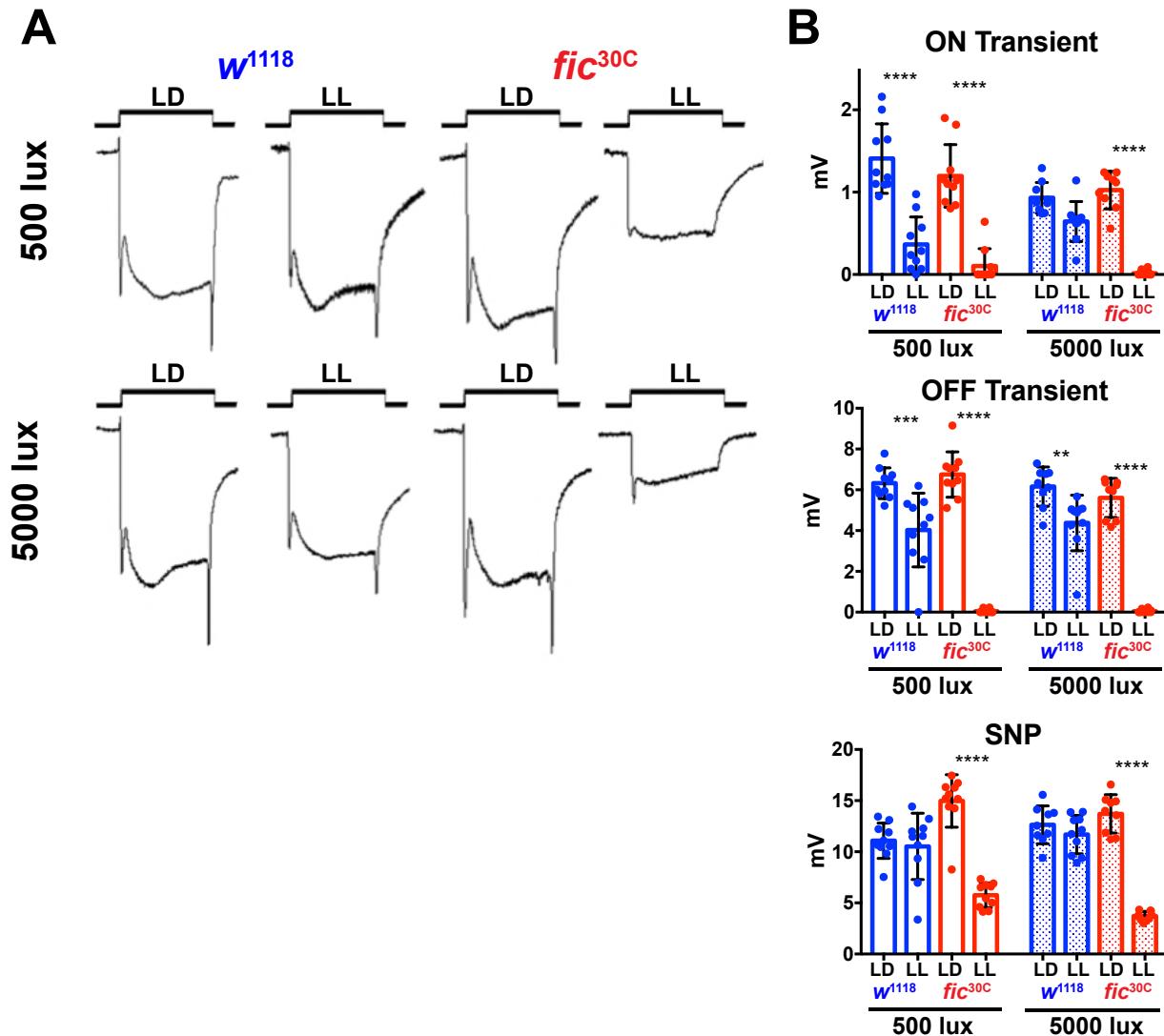
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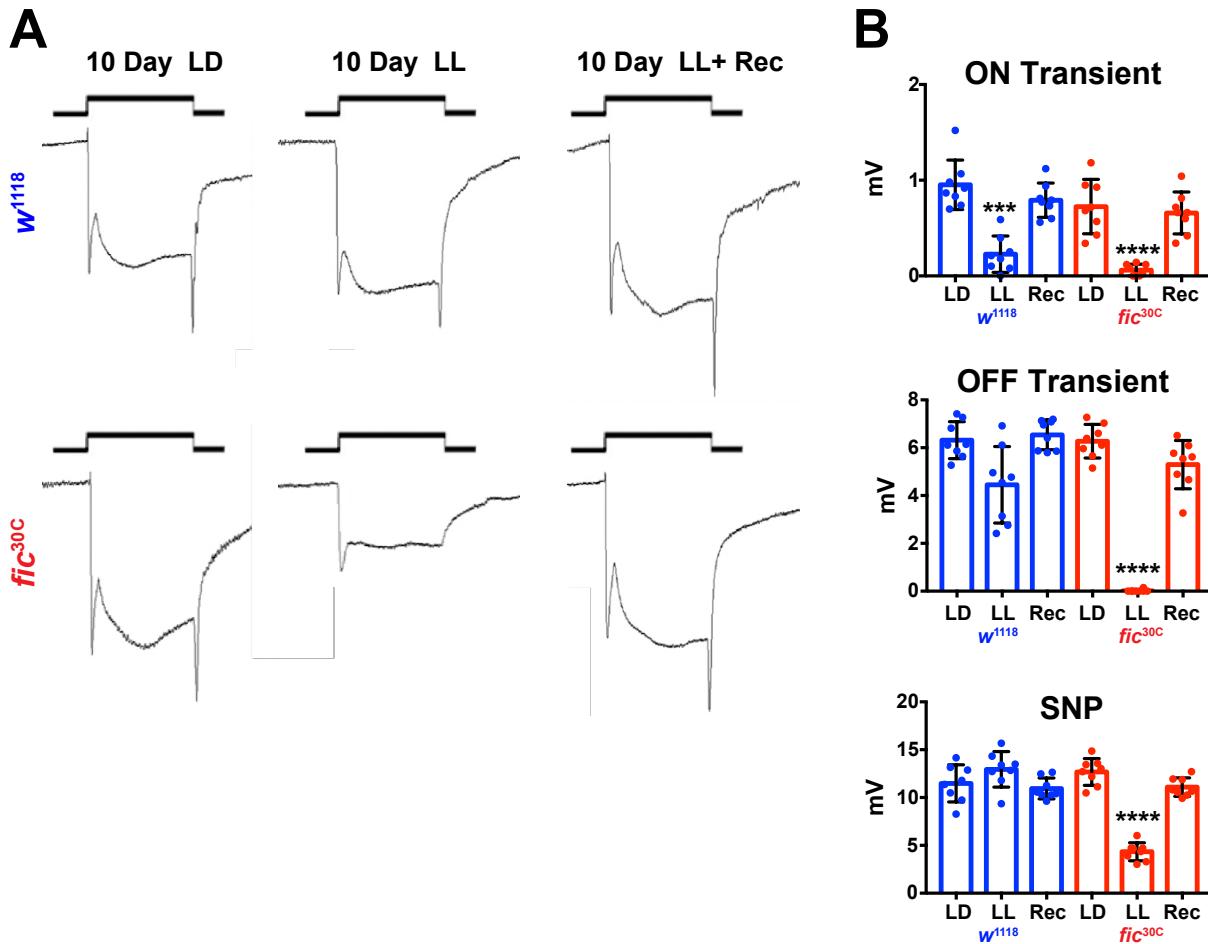
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794 **Figure 3- figure supplement 2. *fic*<sup>30C</sup> mutants are sensitive to constant light, regardless of**  
795 **total intensity.**

796 **(A)** Representative ERGs of flies following 3 days of LL or LD with either 500 lux or 5000 lux  
797 light, showing *fic*<sup>30C</sup> null animals lose ON/OFF transients and have reduced SNPs with constant  
798 light, regardless of intensity, but under LD conditions, even at 5000 lux, have healthy ERG  
799 responses. **(B)** Quantification of ERG data. Bar graphs show means +/- SD. \*\*\*\*, p < 0.0001;  
800 \*\*\*, p < 0.001; \*, p < 0.05; n= 10 flies per genotype and condition.



801

802 **Figure 3- figure supplement 3. *Fic<sup>30C</sup>* mutants recover ERG properties in 72 hours after 10  
803 days of LL.**

804 (A) Representative ERGs of flies following 10 days of LL, 10 days LD (500 lux), and 3 days  
805 Recovery following 10 days. (B) Quantification of ERG data. Bar graphs show means +/- SD.  
806 \*\*\* p < 0.0001; \*\* p < 0.001; \*, p < 0.05; n= 8 flies per genotype and condition.

Line	RNAi Target Gene	Eye Roughness Score					Weighted Average	Number of Flies	P-Value (Fisher's)
		0	1	2	3	4			
Control	<b>None</b>	0	1	68	3	0	2.03	72	n/a
BS36815	4EBP	0	0	96	0	0	2.00	96	0.032
v2935	ATF4	Lethal	Lethal	Lethal	Lethal	Lethal	Lethal	Lethal	
BS25985	ATF4	0	0	51	98	0	2.66	149	2.20E-16
BS26211	ATF6	0	2	68	2	0	2.00	72	1.000
BS64873	CaBP1	0	3	42	0	0	1.93	45	0.137
BS58172	Calnexin	0	2	41	1	0	1.98	44	0.688
v7799	eIF2a	0	2	54	0	0	1.96	56	0.302
v104562	eIF2a	0	0	48	0	0	2.00	48	0.388
BS55657	eric53	0	5	35	7	0	2.04	47	0.007
BS35023	Gadd45	0	0	45	3	0	2.06	48	0.811
BS34346	GP93	0	0	33	5	0	2.13	38	0.122
v39561	Ire1	0	0	33	40	0	2.55	73	4.14E-12
v39562	Ire1	0	0	15	47	0	2.76	62	2.20E-16
BS62156	Ire1	0	0	0	57	2	3.03	59	2.20E-16
BS36743	Ire1	0	0	0	14	13	3.48	27	2.20E-16
BS28039	PDI	0	0	20	2	0	2.09	22	0.685
v110278	PERK	0	0	3	45	4	3.02	52	2.20E-16
v16427	PERK	0	0	0	13	30	3.70	43	2.20E-16
BS35162	PERK	0	0	28	43	0	2.61	71	6.84E-14
BS42499	PERK	0	0	26	65	50	3.17	141	2.20E-16
BS36755	Xbp1	Lethal	Lethal	Lethal	Lethal	Lethal	Lethal	Lethal	Lethal
BS25990	Xbp1	Lethal	Lethal	Lethal	Lethal	Lethal	Lethal	Lethal	Lethal

807

808

**Table 1. Genetic interactions between Fic and UPR genes.**

809 UAS<sup>Scer</sup>-driven RNAi transgenes (either TRIP or VDRC lines) were used to silence candidate  
 810 UPR and ER stress-related genes in a *fic*<sup>30C/+</sup> heterozygous background, with either *LongGMR*-  
 811 Gal4, UAS-Fic<sup>E247G</sup> or *LongGMR*-Gal4 only. Eye roughness was scored for individual flies and  
 812 averaged for each cross. Table reports number of flies scored in each group (0=no roughness,  
 813 2=mildly rough (control flies), 4= severely rough, 1 and 3 are intermediate phenotypes) and the  
 814 weighted average of the eye roughness. Significance differences are highlighted in yellow, and  
 815 p-value's were determined using Fisher's Exact Test for categorical data, comparing the effects  
 816 of each gene knockdown with the control group (**top line**, *fic*<sup>30C/+</sup>; *LongGMR*-Gal4, UAS-  
 817 Fic<sup>E247G</sup>). Interactions were considered significant for any individual test if  $p < 0.003$  as  
 818 determined using Bonferroni's multiple comparison adjustment.