

**GIGANTEA recruits deubiquitylases, UBP12 and UBP13, to regulate
accumulation of the ZTL photoreceptor complex**

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Abstract

To remain synchronous with the environment, plants constantly survey daily light conditions using an array of photoreceptors and adjust their circadian rhythms accordingly. ZEITLUPE (ZTL), a blue light photoreceptor with E3 ubiquitin ligase activity, communicates end-of-day light conditions to the circadian clock. To function properly, ZTL protein must accumulate but not destabilize target clock transcription factors before dusk, while in the dark ZTL mediates degradation of target proteins. It is not clear how ZTL can accumulate to high levels in the light while its targets remain stable. Two deubiquitylating enzymes, UBIQUITIN-SPECIFIC PROTEASE 12 and UBIQUITIN-SPECIFIC PROTEASE 13 (UBP12 and UB13), which have opposite genetic and biochemical functions to ZTL, were shown to associate with the ZTL protein complex. Here we demonstrate that the ZTL light-dependent interacting partner, GIGANTEA (GI), recruits UB12 and UB13 to the ZTL photoreceptor complex. We show that loss of *UBP12* and *UBP13* reduces ZTL and GI protein levels through a post-transcriptional mechanism. Furthermore, the ZTL target protein TOC1 is unable to accumulate to normal levels in *ubp* mutants, indicating that UB12 and UB13 are necessary to stabilize clock transcription factors during the day. Our results demonstrate that the ZTL photoreceptor complex contains both ubiquitin-conjugating and -deconjugating enzymes, and that these two opposing enzyme types are necessary for the complex to properly regulate the circadian clock. This work also shows that deubiquitylating enzymes are a core design element of circadian clocks that is conserved from plants to animals.

29 Main

30 Circadian clocks in all organisms rely on photoreceptors to sense light and entrain the central
 31 oscillator. The exact timing of the light-to-dark transition (dusk) is especially important for
 32 plants, as this indicates the length of the day and provides seasonal timing information necessary
 33 for the adjustment of plant developmental processes (Carre, 2001; Yanovsky and Kay, 2002;
 34 Imaizumi et al., 2003; Salome and McClung, 2004; Imaizumi and Kay, 2006; Nozue et al., 2007;
 35 Mizoguchi and Yoshida, 2009; Ito et al., 2012). One way that plants sense the end of the day is
 36 by using a unique photoreceptor called ZEITLUPE (ZTL) to control the stability of circadian
 37 clock transcription factors differentially in the light and the dark (Somers et al., 2000). ZTL
 38 contains an N-terminal light-oxygen-voltage sensing (LOV) domain which senses blue light.
 39 Adjacent to the LOV domain are the F-box domain, which allows ZTL to function as an E3
 40 ubiquitin ligase, and a Kelch-repeat domain. ZTL mediates degradation of transcription factors
 41 that are at the core of the plant circadian clock including TIMING OF CAB2 EXPRESSION 1,
 42 PSEUDO-RESPONSE REGULATOR 5, and CCA1 HIKING EXPEDITION (TOC1, PRR5, and
 43 CHE) (Mas et al., 2003; Han et al., 2004; Kiba et al., 2007; Fujiwara et al., 2008; Baudry et al.,
 44 2010; Lee et al., 2018). In the light, ZTL accumulates to high levels but is unable to mediate
 45 degradation of the clock transcription factors (Kim et al., 2003; Kim et al., 2007). The
 46 accumulation of ZTL protein during the day is dependent on interaction with the co-chaperone
 47 protein GIGANTEA (GI) (Kim et al., 2013; Cha et al., 2017; Cha et al., 2017). GI interacts with
 48 ZTL through the LOV domain in the light and dissociates from ZTL in the dark, allowing ZTL to
 49 mediate degradation of its target proteins and then be degraded by the ubiquitin proteasome
 50 system, likely through autocatalytic activity (Kim et al., 2003; Mas et al., 2003; Somers et al.,
 51 2004; Kiba et al., 2007; Kim et al., 2007; Kim et al., 2011; Kim et al., 2013). One of the roles of

GI is to recruit HSP70/HSP90 for maturation of the ZTL protein in the light, but ZTL is unable to mediate ubiquitylation and degradation of target proteins until dark (Mas et al., 2003; Kiba et al., 2007; Fujiwara et al., 2008; Cha et al., 2017; Pudasaini et al., 2017). It was proposed that GI can promote maturation of ZTL and block or counteract ZTL activity; however, this second role for GI has not been investigated in depth (Fujiwara et al., 2008; Pudasaini et al., 2017).

We previously identified ZTL-interacting proteins using immunoprecipitation followed by mass spectrometry (IP-MS) with a “decoy” ZTL that lacks E3 ubiquitin ligase activity and stably binds interacting proteins (Lee et al., 2018). We identified UBIQUITIN-SPECIFIC PROTEASE 12 and 13 (UBP12 and UB13) as high confidence ZTL-interacting proteins which were shown previously to have an unspecified role in clock function (Cui et al., 2013; Lee et al., 2018). UB12 and UB13 also interact with GI in IP-MS experiments (Krahmer et al., 2019), suggesting that either the UBPs interact with ZTL and GI independently or that ZTL, GI, and the UBPs exist together in a complex. UB12 and UB13 are closely related deubiquitylating enzymes that can cleave lysine 48-linked mono- or poly-ubiquitin from substrates (Ewan et al., 2011; Cui et al., 2013), interestingly, a biochemical role opposite to that of ZTL. In addition to regulating the circadian clock, they are also involved in flowering time, pathogen defense, root differentiation, and hormone signaling (Ewan et al., 2011; Derkacheva et al., 2016; Jeong et al., 2017; Zhou et al., 2017; An et al., 2018). We performed yeast two-hybrid assays and found that UB12 and UB13 interacted with GI but not with ZTL or the ZTL target proteins TOC1, PRR5, or CHE (Fig.1a). We next tested the interaction between GI and UB12 and UB13 *in planta* via bimolecular fluorescence complementation (BiFC) in *Arabidopsis* protoplasts (Fig.1b). GI, UB12, and UB13 are localized in the cytoplasm and nucleus (Cui et al., 2013;

Kim et al., 2013), and our BiFC results show that UBP12 and UBP13 interact with GI in both compartments with strong signal in the nucleus and weaker but detectable signal in the cytoplasm. The interacting complexes of UBP12 and GI formed nuclear foci, similar to the localization of GI alone (Kim et al., 2013). UBP12 and UBP13 contain a MATH-type (meprin and TRAF homology) protein interaction domain and a ubiquitin-specific protease (USP) domain (Fig. S1). The MATH domains of UBP12 and UBP13 were necessary for interaction with GI while the protease domain and the C-terminal portions did not mediate GI-interaction (Fig. 1c). This suggests that the interaction between GI and UBP12 or UBP13 is not dependent on the UBP USP domains binding to poly-ubiquitylated GI protein.

We next determined whether GI was necessary to bridge the interaction between UBP12 or UBP13 and ZTL *in vivo* by performing IP-MS on wild type (Col-0) and *gi-2* mutant transgenic lines expressing the decoy ZTL protein (Fig. S2). We collected samples at 9 hours after dawn from plants grown in 12h light/12h dark cycles to capture the time when ZTL and GI are normally interacting. We found that UBP12 and UBP13 were enriched in the Col-0 samples (p -value= 3.58E-5 and 0.0113 for UBP12 and UBP13 respectively), but not in the *gi-2* mutant (p -value= 1 for both) (Fig.1d and Table S1). These results indicate that GI is required for UBP12/UBP13 to form a complex with ZTL, substantiating our interaction studies in heterologous systems. Notably, LKP2, a known ZTL interacting partner, associated with ZTL in the presence or absence of GI and suggests that the decoy ZTL is able to form biologically relevant protein complexes even in the *gi-2* mutant (Takase et al., 2011). Together these results suggest that the GI protein physically bridges the interaction between UBP12 or UBP13 and ZTL *in vivo*.

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99 As a complementary approach to the IP-MS (Fig.1d) we co-expressed FLAG-UBP12 or FLAG-

100 UBP13 with HA-GI and Myc-ZTL in *N. benthamiana* leaves. We then performed

101 immunoprecipitation with anti-FLAG antibody and detected the presence of FLAG-UBP12,

102 FLAG-UBP13, HA-GI, and Myc-ZTL using western blotting (Fig.1e). In the FLAG

103 immunoprecipitation samples, HA-GI was always detected when co-expressed with FLAG-

104 UBP12 or FLAG-UBP13, showing that UBP12 and UBP13 interact with GI independently of the

105 presence of Myc-ZTL. Furthermore, Myc-ZTL was undetectable in the FLAG

106 immunoprecipitation samples unless co-expressed with HA-GI showing that the interaction

107 between UBP12 or UBP13 and ZTL is dependent on GI. These assays support our previous

108 results (Fig.1a-d) and show that a trimeric complex between full-length ZTL, GI, and UBP12 or

109 UBP13 can form *in vivo* (Fig.1f).

110

111 Our physical interaction model (Fig. 1f) led us to hypothesize that *UBP12* and *UBP13* regulate

112 the circadian clock through the same genetic pathway as *ZTL* and *GI*. We tested this via epistasis

113 analyses with loss-of-function mutants in *ZTL*, *GI*, *UBP12*, and *UBP13*. Previously, it was

114 shown that knockdown of *UBP12* and *UBP13* results in shortened clock periods (Cui et al.,

115 2013). We first determined the period of a series of mutant alleles in *UBP12* and *UBP13* by

116 crossing them to the *pCCA1::LUC* clock reporter transgenic line and measuring luciferase

117 activity (Fig. 2a-d). We found that single mutations in either *UBP12* or *UBP13* shortened the

118 clock period with period lengths that varied from 0.4 to 1 hour shorter than wild type. We next

119 generated *ubp12-1/gi-2* and *ubp13-1/gi-2* double mutants and measured the expression of the

120 core clock gene *CCA1* during a 2-day circadian time course in constant light using qRT-PCR

(Fig. 2e-f and Table S2). LS Periodogram analysis using the Biodare2 platform [biodare2.ed.ac.uk (Zielinski et al., 2014)] showed that the *ubp12-1/gi-2* double mutant had a similar phase and amplitude of *CCA1* expression to the *gi-2* mutant alone and a period more similar to *ubp12-1* (Table S3). These results show a non-additive interaction and suggest they function in the same circadian genetic pathway. The *ubp13-1/gi-2* double mutant had a similar amplitude to the *gi-2* mutant but had a more similar phase and period to the *ubp13-1* mutant (Table S3). This again shows a non-additive genetic interaction but also suggests that the roles of *UBP12* and *UBP13* have slightly diverged with respect to clock function. We also crossed the *gi-2* mutant with the *ubp12-2w* mutant which had reduced expression of both *UBP12* and *UBP13* and the shortest clock period of the tested *ubp* mutant alleles (Fig.S3, 2a-d). The pattern of *CCA1* expression in the *ubp12-2w/gi-2* double mutant was nearly identical to the *gi-2* mutant, further confirming that the effects of the *UBPs* and *GI* are not additive (Table S3). These results indicate that *UBP12* and *UBP13* work in the same pathway as *GI* to control clock function.

ZTL functions downstream of *GI* to regulate the circadian clock (Kim et al., 2007). Thus, we hypothesized that *ZTL* would function downstream of *UBP12* and *UBP13* as well. To test the genetic interaction between *UBP12* or *UBP13* and *ZTL*, we crossed *ubp12-1* and *ubp13-1* to the *ztl-4* null mutant (Fig. 2g and h). The daily expression patterns of *CCA1* in the *ubp12-1/ztl-4* and *ubp13-1/ztl-4* double mutants were nearly identical to the *ztl-4* mutant alone in phase and amplitude (Table S3). Interestingly, the period data showed that the *ubp12-1/ztl-4* was more similar to *ztl-4* than *ubp12-1*, but the *ubp13-1/ztl-4* is more similar to *ubp13-1*. This data suggests that *ZTL* is epistatic to *UBP12* and *UBP13* but that *UBP13* has diverged in function from *UBP12*. It is important to note that the qRT-PCR data is below the suggested resolution for

Biodare2 analysis which can result in inaccurate period calls (i.e. *ubp13-1* period is estimated by Biodare2 as the same period as wild type in this experiment). These results corroborate our physical interaction studies and suggest that *UBP12* and *UBP13* regulate the circadian clock upstream of *ZTL*.

UBP12 and *UBP13* are functional deubiquitylases that can cleave poly-ubiquitin from generic substrates (Ewan et al., 2011; Cui et al., 2013). We tested whether this deubiquitylation activity is necessary for their role in circadian clock function. To do this we performed complementation studies with wild-type *UBP12* and mutant *UBP12^{C208S}*. *UBP12^{C208S}* has a mutation in the cysteine-box of the USP enzymatic core (Fig. S1) that renders it non-functional as a deubiquitylase (Cui et al., 2013; Derkacheva et al., 2016; Jeong et al., 2017). We transformed *UBP12-YFP* or *UBP12^{C208S}-YFP* driven by the *UBP12* native promoter into the *ubp12-1* mutant and analyzed a population of T1 transgenic lines. In this experiment we consider a line to have rescued the *ubp12-1* mutant clock phenotype if it has a period length longer than the average period length of the *ubp12-1* plus one standard deviation. Using this criteria, 10 of 32 transgenic lines (31%) transformed with catalytically active *UBP12* rescued the short period defect of the *ubp12-1* mutant. Strikingly, only one transgenic line transformed with the inactive *UBP12^{C208S}* was able to rescue the short period phenotype of *ubp12-1* (Fig. 2i-j). As reference, approximately 13% of the *ubp12-1* plants themselves and 62% of the wild type plants fell into the rescue category. This is likely due to normal variations in population level data of this type. We further confirmed that *UBP12-YFP* and *UBP12^{C208S}-YFP* were both localized to the cytoplasm and nucleus, suggesting that differences in the clock phenotypes are not due to mislocalization of the

UBP12^{C208S} protein (Fig S4). These results indicate that the deubiquitylating functions of UBP12 are necessary for its role in regulating the circadian clock.

By cleaving poly-ubiquitin from proteins, deubiquitylase enzymes can regulate protein stability and accumulation (Komander et al., 2009; Jeong et al., 2017; Mevissen and Komander, 2017; An et al., 2018). The physical and genetic interactions shown for UBP12, UBP13, GI and ZTL prompted us to hypothesize that the UBP12 and UBP13 regulate GI or ZTL protein levels, allowing for accumulation of the proteins in the end of the day. We measured the level of HA-tagged GI under the control of the GI native promoter (*pGI::GI-HA*) in the *ubp12-1* and *ubp13-1* mutants during a 12h light/12h dark time course (Fig. 3a). GI protein levels were approximately 50% lower in the *ubp12-1* and *ubp13-1*. mRNA expression of *GI-HA* was also approximately 25% lower than wild type at the peak of *GI* mRNA expression, ZT8 (Fig. 3b). This suggests that GI protein accumulation is partially dependent on UBP12 and UBP13, but that altered transcription of *GI* could also have an effect on GI protein.

Next, we measured ZTL protein levels in the *ubp12-1* and *ubp13-1* mutants (Fig. 3c). ZTL protein levels were substantially decreased in the *ubp12-1* and *ubp13-1* mutants throughout the entire day/night cycle. Overexposure of the immunoblot showed that a small amount of ZTL protein can still accumulate in the *ubp* mutants (Fig. 3c). The expression of *ZTL* mRNA was largely unaffected in these lines (Fig. 3d), suggesting that the decrease in ZTL protein levels was caused by a post-transcriptional mechanism. This is similar to the post-transcriptional control of ZTL reported in *gi* loss-of-function mutants (Kim et al., 2007), and indicates that UBP12 and UBP13 are necessary for robust accumulation of the ZTL protein.

189
 190 Interestingly, the *ubp12-1* and *ubp13-1* mutants caused severe reduction in the levels of the ZTL
 191 protein but had a short period phenotype, opposite to the long period phenotype of *ztl* loss-of-
 192 function mutants. Normally, loss of ZTL causes aberrantly high levels of TOC1 protein while
 193 overexpression of ZTL causes low levels of TOC1 protein (Mas et al., 2003; Kiba et al., 2007;
 194 Pudasaini and Zoltowski, 2013; Pudasaini et al., 2017). To determine if UBP12 and UBP13
 195 affect TOC1 protein levels, we crossed a transgenic line expressing TOC1 fused to YFP under
 196 the *TOC1* promoter (*TMG*) to the *ubp12-1* and *ubp13-1* mutants and measured TOC1 protein
 197 levels (Fig. 3e). TOC1 protein levels were severely reduced in the *ubp12-1* and *ubp13-1* mutants
 198 while mRNA expression of the *TOC1-YFP* transgene was similar in the wild type and mutant
 199 backgrounds, suggesting that the decrease in TOC1 protein levels was caused by a post-
 200 transcriptional mechanism (Fig. 3f). Notably, TOC1 protein was unable to accumulate to high
 201 levels in the light in the *ubp* mutants (Fig. 3e at 12 hours after dawn). This is similar to the
 202 effects of the *gi-2* mutant, where TOC1 protein levels never accumulate to full wild-type levels
 203 (Kim et al., 2007). Lowered levels of the TOC1 protein result in shortened period, suggesting
 204 this was the cause of the short period phenotype of the *ubp12* and *ubp13* mutants.

205
 206 We have shown that UBP12 and UBP13 are components of the ZTL-GI photoreceptor complex
 207 that are necessary for accumulation of the proteins in the end of the day. UBP12 and UBP13 can
 208 remove poly-ubiquitin from targets non-specifically (Ewan et al., 2011; Cui et al., 2013). Thus,
 209 we hypothesize that UBP enzymes are recruited by GI to the ZTL photoreceptor complex to
 210 prevent formation of poly-ubiquitin chains, resulting in increased stability of the protein complex
 211 (Fig. S5). Interestingly, ZTL protein levels were severely damped in the *ubp12* and *ubp13*

mutants, but counterintuitively the ZTL target, TOC1, also had reduced levels (Fig. 3c-f). This effect is similar to what was observed in a *gi* loss-of-function mutant, and suggests that GI and UBP12 and UBP13 can counterbalance the activity of ZTL during the day, allowing TOC1 to accumulate to high levels before being degraded (Kim et al., 2007). Although ZTL levels were decreased in the *ubp* mutants, there was still a small amount that could potentially decrease TOC1 levels in the light (Fig. 3c long exposure). This is different than what was seen when HSP90 activity was inhibited, resulting in lower ZTL levels but higher TOC1 levels. This suggests that HSP90 is necessary for ZTL protein maturation and to promote its activity (Kim et al., 2011). This data in combination with our results suggest that GI performs two roles in the ZTL photoreceptor complex: (1) acting as a co-chaperone that recruits HSP proteins to facilitate ZTL maturation (Cha et al., 2017; Cha et al., 2017), and (2) counterbalancing ZTL's role in ubiquitin conjugation with UBP12 and UBP13 present to deconjugate ubiquitin. The light-regulated nature of the ZTL-GI interaction also indicates that light is controlling the balance of ubiquitin conjugation and deconjugation that allows the ZTL photoreceptor complex to accurately degrade proteins at the correct time of day. It was previously shown that mammalian and insect circadian clocks utilize deubiquitylation to regulate stability and subcellular localization of clock proteins (Scoma et al., 2011; Luo et al., 2012; Yang et al., 2012). In light of this, our results further demonstrate that deubiquitylation activity is an evolutionarily conserved architectural design feature of the clocks of higher eukaryotes. Furthermore, the mammalian orthologue of UBP12 and UBP13, USP7, impacts clock function in response to environmental stress (Papp et al., 2015; Hirano et al., 2016) suggesting that these deubiquitylases are conserved clock regulators across evolution.

235

236 **Materials and Methods**

237 See supplemental information

238

239 **End notes:**

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254

255 **Author contribution**

256 JMG and CML conceived of the project. CML, MWL, AF, AMS and WL conducted the

257 experiments and analyzed the data. JMG and CML wrote the manuscript.

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264

265 **Competing interests**

266 The authors declare no competing financial interests.

267

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270

271 **Figure legends:**

272 **Figure 1. GI bridges the interactions between ZTL and UBP12 or UBP13.** (a) Yeast two-

273 hybrid showing interaction between GI and UBP12 or UBP13. The GAL4 DNA binding domain

274 (GAL4-BD) fused to UBP12 or UBP13 and either ZTL variants (ZTL and ZTL decoy), ZTL

275 targets (TOC1, PRR5 and CHE) or GI fused to GAL4 activation domain (GAL4-AD) were

276 grown on SD-LW medium for autotrophic selection and on SD-LWHA medium to test for

277 interaction. (b) Bimolecular fluorescence complementation (BiFC) assays to examine the

278 interactions of UBP12 or UBP13 and GI fused to the N- or C-terminus of Venus (YFP) were

279 performed in *Arabidopsis* protoplasts. The blue arrows indicate the interacting complex forming

280 nuclear foci. The white arrows show fluorescence signal in the cytoplasm. mCherry-VirD2NLS

was co-expressed as a nuclear marker, and the scale bar indicates 10 μ m. (c) The protein domains of UBP12 and UBP13 required to interact with GI were tested using yeast two-hybrid assays. The full-length (FL) or truncated UBP12 or UBP13 fragments as diagramed in the lower portion of the panel were fused to GAL4-BD to test for interaction with GAL4-AD-GI. (d) Scatter plot of proteins identified by IP-MS of ZTL decoys in the Col-0 and *gi-2* genotypes. The significance of the interactions were evaluated by SAINTexpress (see Supplemental Information and Table S1 for complete information) with a false discovery rate (FDR) cutoff < 0.01 and *p*-value $\leq$ 5.37E-4 to separate interacting proteins into four groups. Group I: significant interactions with ZTL decoy in the *gi-2* but not Col-0. Group II: significant interactions with ZTL decoy in both Col-0 and *gi-2*. Group III: significant interactions with ZTL decoy in the Col-0 but not *gi-2*. Group IV: Non-significant interactions with ZTL decoy in both Col-0 and *gi-2*. The interacting proteins significantly enriched in the *gi-2* mutant over Col-0 were labeled along the y-axis, and the proteins enriched in the Col-0 over the *gi-2* mutant were labeled along the x-axis. (e) Co-immunoprecipitation assays showing that UBP12 or UBP13 interact with ZTL in a GI-dependent manner. FLAG-UBP12 or FLAG-UBP13 were co-infiltrated with HA-GI and Myc-ZTL in *Nicotiana benthamiana* leaves. Anti-FLAG antibody was used to immunoprecipitate FLAG-UBP12 or FLAG-UBP13. Western blotting with anti-FLAG, anti-HA, or anti-Myc was used to detect the presence of FLAG-UBP12, FLAG-UBP13, HA-GI, or Myc-ZTL in the immunoprecipitated samples and inputs. (f) The diagram depicts the interaction between GI and the MATH domain of UBP12 or UBP13, and between GI and the LOV domain of ZTL.

Figure 2. UBP12 and UBP13 regulate the circadian clock through the same pathway as GI and ZTL. (a-d) The *ubp12* and *ubp13* mutants have short period phenotypes. (a, c) The periods

of circadian marker *pCCA1:Luciferase* (*pCCA1::LUC*) in the wildtype (Col-0) (n=20 for a and n=19 for c), *ubp12-1* (n= 16), *ubp12-2w* (n= 20), *ubp13-1* (n= 15), *ubp13-2* (n= 20) and *ubp13-3* (n= 14) were measured with bioluminescent assays. Each symbol represents the period from one seedling, and the average period and standard deviation (SD) are labeled with gray bars. The significance of period changes between wildtype and mutants were analyzed with a Welch's *t*-test (** for *p*-value< 0.001; **** for *p*-value< 0.0001). Three biological replicates were performed with similar results, and one dataset is presented. (b, d) The average bioluminescence of the lines displayed in a and c were plotted against time after transfer from 12h light/12h dark entrainment conditions to constant light (LL). (e-f) Circadian expression of *CCA1* in Col-0, *ubp12-1*, *ubp13-1*, *gi-2*, *gi-2/ubp12-1* and *gi-2/ubp13-1* after transferring to LL for 48h from the entrainment conditions was measured using qRT-PCR. Subjective dark is colored with light grey. The data represents the average relative expression from three biological replicates, and the error bars are SD. The same Col-0 and *gi-2* data was plotted twice (in e and f) for clarity in the data presentation and for comparison with the other mutant lines. (g-h) The circadian expression of *CCA1* in Col-0, *ubp12-1*, *ubp13-1*, *ztl-4*, *ztl-4/ubp12-1* and *ztl-4/ubp13-1* after transferring to LL for 48h from the entrainment conditions was measured using qRT-PCR. The data analyses and presentation are the same as e-f. The same Col-0 and *ztl-4* data was plotted twice (in g and h). (i) The circadian period of *pCCA1::LUC* in the wild type (n= 76), *ubp12-1* (n= 54), *ubp12-1* mutant complemented with *pUBP12::UBP12-YFP* (n= 32) or deubiquitylating activity-dead *pUBP12::UBP12CS-YFP* (n= 20). Each symbol represents the period from one seedling, and the black bars indicate the average period and SD. The wild type and *ubp12-1* mutants are homogenous populations, and the complementation lines are individual T1 transgenic lines. The presented data is from three independent biological replicates. (j) Quantitation of the number of

lines, from panel i, with periods greater than the average of the *ubp12-1* mutant plus one standard deviation.

Figure 3. ZTL, GI and TOC1 protein levels are regulated by UBP12 and UBP13. (a, c, e)

The protein levels of HA-tagged GI driven by native promoter (*pGI::GI-HA*), ZTL and YFP-tagged TOC1 driven by the TOC1 promoter (TOC1 minigene or TMG) in the wild type (Col-0), *ubp12-1* or *ubp13-1* mutants under diurnal conditions (12h light/12h dark) were detected by immunoblotting. The samples from 0h to 12h after dawn were harvested in light, and the samples from 16h and 20h after dawn were harvested in the dark (indicated by grey shading). The relative protein levels were quantified by normalization to actin. The Col-0 or *ztl-4* samples were used as negative controls for the antibodies. Plots represent the average protein levels from three biological replicates, and the error bars represent standard deviation. Compared to wild type, the levels of ZTL proteins in the *ubp12-1* and *ubp13-1* were below the linear range for quantification. In the *ztl-4* sample, the anti-ZTL antibody recognizes a non-specific band close to the size of endogenous ZTL in the long-exposure blots. (b, d, f) The relative mRNA levels of *GI-HA*, *ZTL* or *TOC1-YFP* from the same time course samples were measured by qRT-PCR.

Figure S1. Protein structures of UBP12 and UBP13. UBP12 and UBP13 homologous proteins

contain a conserved Meprin And TRAF Homology (MATH) domain (blue) and a Ubiquitin-Specific Protease (USP) domain (green) in the N-terminus. The USP has a conserved cysteine protease enzymatic core (red): Cysteine Box (Cys box) and Histidine Box (His box). Mutations of the conserved cysteine residue to serine in the Cys box have been shown to disrupt the

deubiquitylating activities of UBP12 and UBP13 (Cui et al., 2013). The numbers represent the position of the amino acid sequences.

Figure S2. Immunoprecipitation of FLAG-His-ZTL decoy in the Col-0 or *gi-2* genotypes.

Immunoblots detected by anti-FLAG antibody showed that the FLAG-His-ZTL decoy and FLAG-His-GFP in the Col-0 (top panel) or *gi-2* (bottom panel) can be immunoprecipitated (IP) from the total protein extract (IN). The Col-0 (top) and *gi-2* (bottom) parental lines were negative controls. FT: flow-through.

Figure S3. Circadian expression of *CCA1* in the *gi-2/ubp12-2w* double mutant. Col-0, *ubp12-2w*, *gi-2* and *gi-2/ubp12-2w* were entrained under 12h light/ 12h dark for 10d and then

transferred to continuous light (LL) for 48h before harvest. The expression of *CCA1* was measured using qRT-PCR. The data represents the mean of the relative expression from three biological replicates with the error bars showing standard deviation.

Figure S4. The subcellular localization of UBP12 variants transiently expressed in

Nicotiana benthamiana leaves. Leaves from 5-week-old *Nicotiana benthamiana* grown under 12h light/ 12h dark at 22°C were infiltrated with *Agrobacterium* expressing *pABindGFP-UBP12* or *pABindGFP-UBP12C208S* with nuclear marker *pABindcherry-AS2*. The scale bar indicates 50µm.

Figure S5. The proposed model for UBP12/UBP13 regulation of ZTL. (a) In the light, GI

interacts with ZTL and acts as a co-chaperone, recruiting HSP90 to facilitate folding and

maturation of the ZTL protein. Additionally, GI physically bridges an interaction between ZTL and UBP12 or UBP13. UBP12 or UBP13 stabilize the GI-ZTL protein complex before dusk. At night, GI dissociates from ZTL, and ZTL mediates ubiquitylation and degradation of the TOC1 protein. (b) Loss of *UBP12* or *UBP13* causes instability of ZTL and GI. Interestingly, the TOC1 protein levels are also reduced by loss of *UBP12* or *UBP13*, mimicking the *gi* loss-of-function mutant.

Tables:

Table S1. The list of proteins identified by immunoprecipitation followed by mass spectrometry using 35S::FLAG-His-ZTL decoy in the Col-0 or *gi-2* background.

Table S2. List of primers for cloning and qRT-PCR.

Table S3. Results of LS Periodogram analysis of the qRT-PCR data from figure 2 e-h and figure S3.

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Supplemental Information

Materials and Methods

Plant materials and growth conditions

The *Arabidopsis* seeds of Col-0, *ubp12-1* (CS423387), *ubp12-2w* (CS2103163), *ubp13-1* (SALK_128312), *ubp13-2* (SALK_024054), *ubp13-3* (SALK_132368)¹, *gi-2* (cs3370)^{2,3}, *ztl-4* (SALK_012440)⁴, *pGI::GI-HA* (CS66130)⁵ and TMG (CS31390)⁶ were described previously and obtained from ABRC. The *ubp12-1/gi-2*, *ubp12-2w/gi-2*, *ubp13-1/gi-2*, *ubp12-1/ztl-4* and *ubp13-1/ztl-4* double mutants were generated by crossing and genotyped by PCR. The *pGI::GI-HA* and TMG lines were crossed to *ubp12-1* and *ubp13-1*, and the homozygous lines were selected by genotyping and gentamycin resistance.

For IP-MS, the *35S::FLAG-His-ZTL-decoy* transgenic lines and *35S::FLAG-His-GFP* control were described previously⁷, and the same constructs were transformed into the *gi-2* background by floral-dip method⁸.

For the bioluminescent assays, the circadian reporter line *pCCA1::Luciferase* (*pCCA1::LUC*)⁹ was crossed to the *ubp12* and *ubp13* mutants. The *pUBP12::UBP12-YFP* variants (see Cloning section) were transformed into *pCCA1::LUC/ubp12-1* by floral-dip⁸ for complementation experiments.

For growth conditions of *Arabidopsis* seedlings, the seeds were surface sterilized with ethanol, cold stratified, plated on ½ strength MS (Murashige and Skoog medium, Caisson Laboratories, MSP01) medium with 0.8% Agar (AmericanBio, AB01185), and grown at 22°C under 12h light/12h dark as described previously⁷ unless specified otherwise. For soil-grown conditions, plants were grown in Fafard-2 mix under 16h light/8h dark at 22 °C.

For circadian experiments, seedlings were grown on ½ strength MS medium under 12h light/12h dark at 22 °C for 10d, transferred to continuous light (LL) at 22 °C for 48h before starting harvest. For the 12h light/12 dark (LD) experiments, 12-day-old seedlings grown on ½ strength MS medium were used.

Cloning

The GATEWAY pENTR™/D-TOPO entry vectors (Thermo Fisher Scientific, K240020) of ZTL full-length, ZTL decoy, CHE, TOC1 and PRR5 were obtained from previous reports^{7,9,10}. For GI, UB12 and UB13, the full-length coding regions were amplified from cDNA by PCR and cloned into pENTR™/D-TOPO vectors. These entry clones were then sub-cloned into GATEWAY compatible yeast two-hybrid vectors (pGADT7-GW and pGBKT7-GW)¹¹ or BiFC vectors (pUC-DEST-VYCE®GW and pUC-DEST-VYNE®GW)¹² with GATEWAY recombination cloning (Thermo Fisher Scientific).

To construct the fragments of UB12 and UB13 into yeast two-hybrid pGADT7-GW vectors, the desired fragments were first amplified from the full-length UB12 or UB13 entry vectors by PCR and cloned into pENTR™/D-TOPO vectors before being sub-cloned into pGADT7-GW with GATEWAY cloning.

For the UBP12 complementation plasmids, the pENTRTM/D-TOPO-UBP12-NS vector served as template for site-directed mutagenesis to introduce a Cys to Ser mutation at a.a. 208 position using Q5® Site-Directed Mutagenesis Kit (NEB, E0554). Subsequently, UBP12-NS and UBP12C208S-NS in the pENTRTM/D-TOPO entry vectors were sub-cloned into a modified GATEWAY compatible pGreenBarT vector¹² with 1.7k bp upstream of ATG of *UBP12* promoter region in the KpnI/XhoI sites. The primers used for cloning were listed in Table S2.

Yeast two-hybrid

ZTL, ZTL decoy, GI, TOC1, PRR5 and CHE were fused to the GAL4-BD in pGBKT7-GW vectors, and the full-length or fragments of UBP12 and UBP13 were fused to the GAL4-AD in pGADT7-GW vectors by GATEWAY cloning. The interactions were tested on synthetic dropout medium as described previously⁷.

Bimolecular fluorescence complementation (BiFC) and confocal microscopy

The coding region of GI, UBP12 or UBP13 in the GATEWAY entry vectors were cloned into protoplast GATEWAY destination vectors pUC-DEST-VYCE®GW and pUC-DEST-VYNE(R)GW¹² respectively for transient transfections into protoplasts. *pSAT6-mCherry-VirD2NLS* was used as a nuclear marker. The protoplasts were isolated from 3- to 4-week-old *Arabidopsis* (Col-0) grown at 22°C under 8h light/16h dark and transfected following the protocol of tape-*Arabidopsis* sandwich method¹³. After 14-18 h incubation in low-light conditions, protoplasts were imaged on a Nikon Ti microscope with using a 60X 1.4 NA plan Apo objective lens as described previously¹⁴. The images were analyzed with FIJI¹⁵.

Immunoprecipitation and mass spectrometry (IP-MS)

For the ZTL decoys in Col-0 background, homozygous *35S::FLAG-His-ZTL-decoy* transgenic lines along with Col-0 and *35S::FLAG-His-GFP* controls were used. For the ZTL decoys in the *gi-2* background, three independent T2 transgenic lines of *35S::FLAG-His-ZTL-decoy/gi-2* and *35S::FLAG-His-GFP/gi-2* were selected on ½ strength MS plates with 15 µg/ml ammonium glufosinate before being transferred to soil. Twenty-one-day-old soil-grown plants were entrained in 12 h light/12 h dark at 22°C for 7 days prior to harvest. Leaf tissues were collected at 9 h after dawn for subsequent IP-MS. One-step IP-MS and MS spectral analyses were carried out as documented⁷ with minor changes. The MS/MS spectral were searched against the SwissProt_2017 tax: *Arabidopsis thaliana* (thale cress) database (February 2017) using MASCOT MS/MS ion search engine version 2.6.0¹⁶ with the following parameters: up to 2 missed cleavages; variable modifications included Acetyl (K), GlyGly(K), Oxidation (M), Phospho (ST), Phospho (Y); peptide tolerance ± 10 ppm; MS/MS tolerance ± 5 Da; peptide charge 2+ and 3+. The protein lists identified by MASCOT were first filtered out non-specific interactions by removing proteins only present in the controls (Col-0, *gi-2*, *35S::FLAG-His-GFP/Col-0* and *35S::FLAG-His-GFP/gi-2*). The SAINTexpress algorithm^{17,18} were further performed to determine the significance of protein-protein interactions.

Bioluminescent assays

The *Arabidopsis* seedlings bearing *pCCA1::LUC* in the wild type (Col-0), *ubp12* or *ubp13* mutants were grown in ½ strength MS medium and entrained in 12h light/12h dark for 7 days prior to being transferred to new ½ strength MS plates and constant light (LL) for circadian free-run experiments. For the various *pUBP12::UBP12-YFP* complementation T1 lines in the *pCCA1::LUC/ubp12-1* background, seedlings were first screened and entrained on the ½ strength MS plates containing 7.5 µg/ml ammonium glufosinate prior to being transferred to ½ strength MS medium and LL. The measurement of luciferase activities and analyses were described as previously⁷.

Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

RNA extraction, reverse-transcription and constitution of qPCR reactions were followed as described previously⁷, except for minor modifications. Four hundred ng total RNA were used for reverse transcription reactions. For semi-quantification of gene expression, *IPP2* (AT3G02780) was used as an internal control. The relative expression represents means of $2^{-(\Delta CT)}$ from three biological replicates, in which $\Delta CT = (CT \text{ of Gene of Interest} - CT \text{ of } IPP2)$. The primers were listed in Table S2.

Immunoblotting

The procedure of protein extraction from *Arabidopsis* seedlings, separation, detection with antibodies and quantification are described as previously⁷, except 60µg total protein were used for immunoblotting. The primary antibodies used for detection are: for GI-HA, anti-HA-Biotin antibody (1:1000, 12158167001, Millipore-Sigma); for ZTL, anti-ZTL antibody¹⁹ (1:200); for TMG, anti-GFP (1:10000, ab-290, Abcam); for FLAG-ZTL decoy, anti-FLAG antibody (1:1000, F7425, Millipore-Sigma). To quantify expression levels, the levels of target proteins were normalized to actin (anti-Actin antibody, 1:2000, SAB4301137, Millipore-Sigma).

Transient expression in *Nicotiana benthamiana* and confocal microscopy

UBP12-NS and, UBP12C208S-NS in the pENTR™/D-TOPO vectors were subcloned into inducible GATEWAY destination pABindGFP vectors²⁰ and transformed into the *Agrobacterium tumefaciens* strain *GV3101* for transient expression in *Nicotiana benthamiana*. The *Agrobacterium* culture of pABindGFP-UBP12 or pABindGFP-UBP12C208S and the nuclear marker pABindcherry-AS2²¹ were pelleted and resuspended in the infiltration solution (5% (w/v) Sucrose, 450 µM acetosyringone and 0.01% (v/v) Silwet). The bacterial infiltration solution was incubated at 4°C for 2h before infiltrated into 5-week-old *Nicotiana benthamiana* leaves. After 20h of infiltration, the protein expression was induced by spraying leaves with 20 µM β-estradiol in 0.1% Tween 20. The leaves were imaged after 18h of induction.

The leaf samples were imaged on a Zeiss LSM510 confocal microscope with a Plan-Apochromat 40x/1.3 Oil objective. GFP was excited using 488 nm Argon laser and observed through a

505/530 nm bandpass filter. mCherry was excited using 543 nm HeNe laser and observed through a 585/615 nm bandpass filter. The images were processed with FIJI¹⁵.

Co-immunoprecipitation (co-IP) using *Nicotiana benthamiana* transient expression system

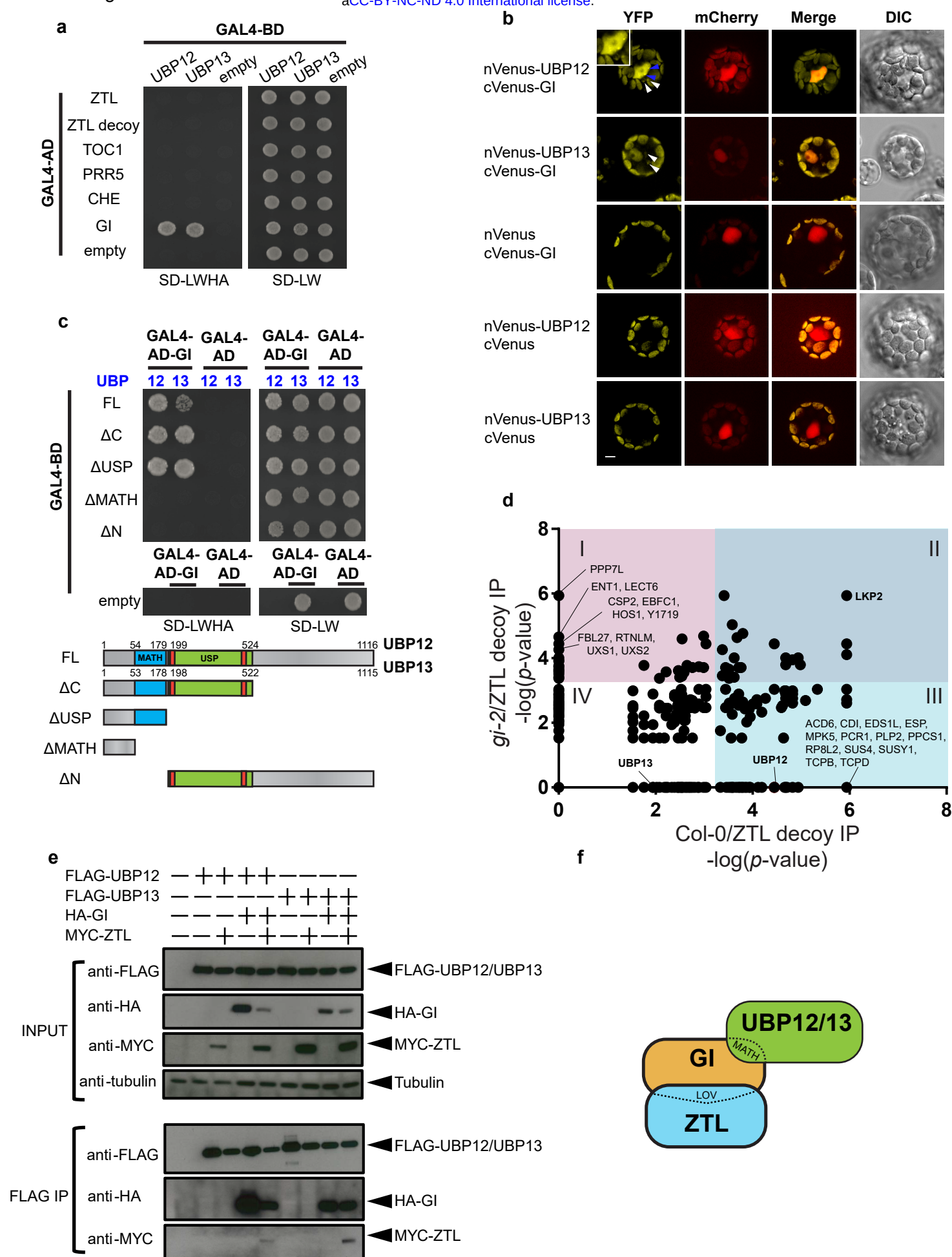
The full-length coding sequences of ZTL, GI, UBP12 and UBP13 in the pENTR™/D-TOPO vectors were subcloned into pEarlygate203, pEarlygate201 and pEarlygate202 plant binary vectors²² respectively and transformed into *Agrobacterium tumefaciens* strain *GV3101*. Agro-infiltration into *Nicotiana benthamiana* leaves was described in the previous section. In this co-immunoprecipitation experiment co-infiltration with P19 in the EHA105 *Agrobacterium* strain was used to increase expression of the transgenes. The leaf samples were harvested after 48h of infiltration and snap frozen with liquid nitrogen. Protein extraction and co-immunoprecipitation with Anti-FLAG® M2 Magnetic Beads (M8823, Millipore-Sigma), and a one-step IP protocol was used as described previously^{7,23}. The inputs and IP samples were resolved on NuPAGE 4-12% Bis-Tris Protein Gels (NP0321, Thermo Fisher Scientific) for immunoblotting. The primary antibodies used for detection are: for MYC-ZTL, anti-MYC antibody (1:10000, C3956, Millipore-Sigma); for HA-GI, anti-HA antibody (1:5000, H3663, Millipore-Sigma); for FLAG-UBP12 and FLAG-UBP13, anti-FLAG antibody (1:5000, F1804, Millipore-Sigma); for loading control, anti-tubulin antibody (1:5000, T5168, Millipore-Sigma).

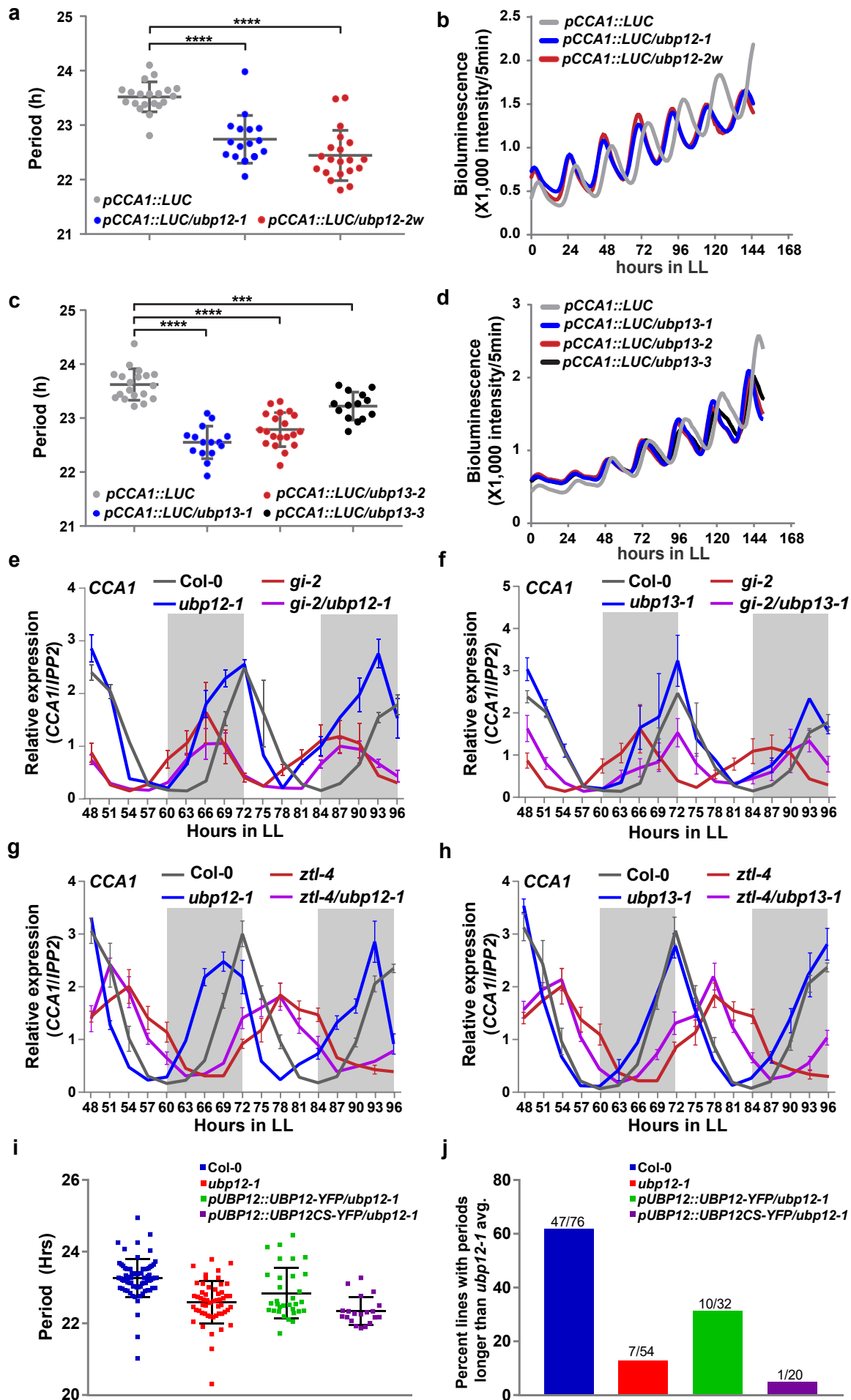
Data Availability

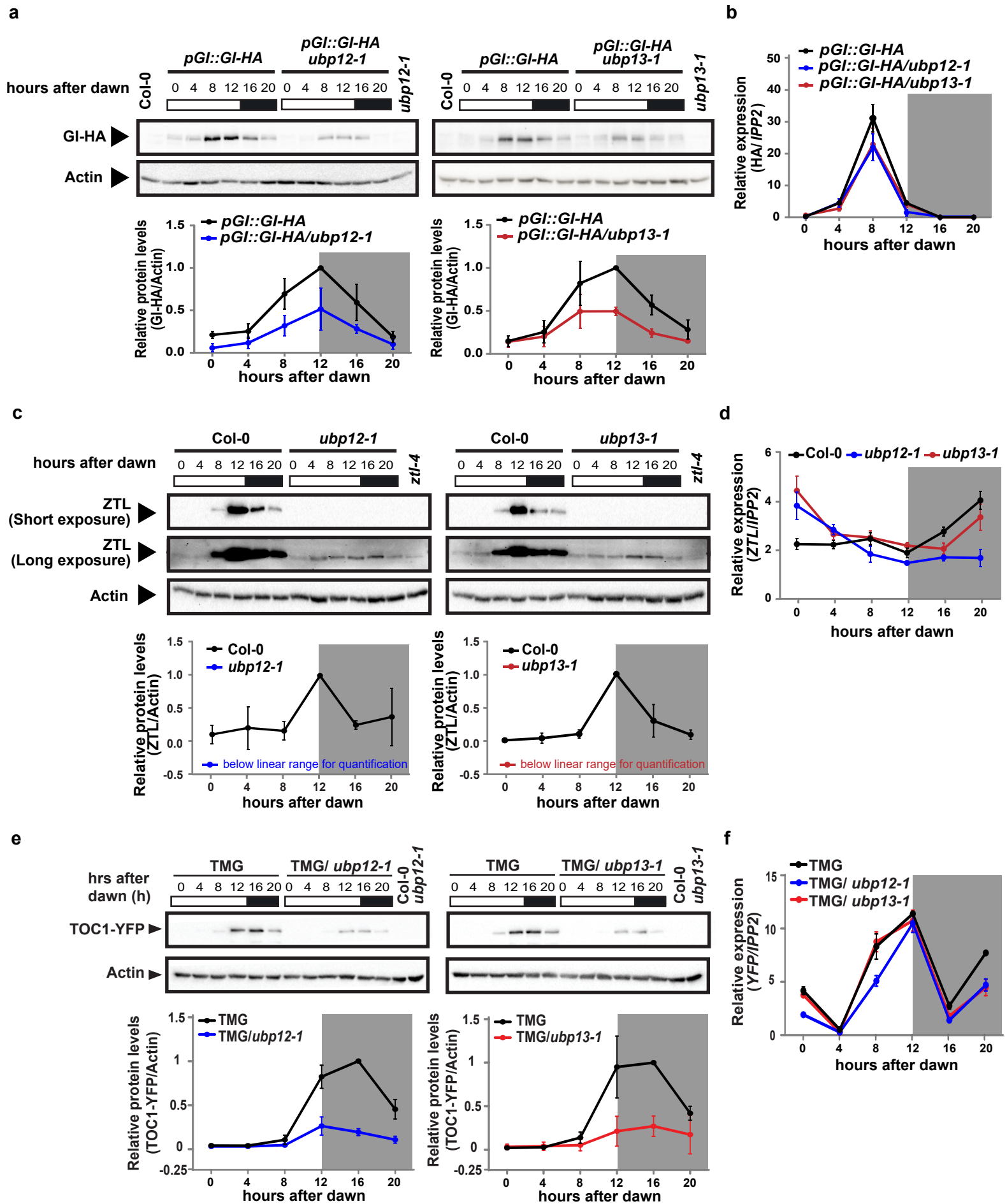
The raw data of mass spectrometry experiments will be deposited to PRIDE (<https://www.ebi.ac.uk/pride/archive/>).

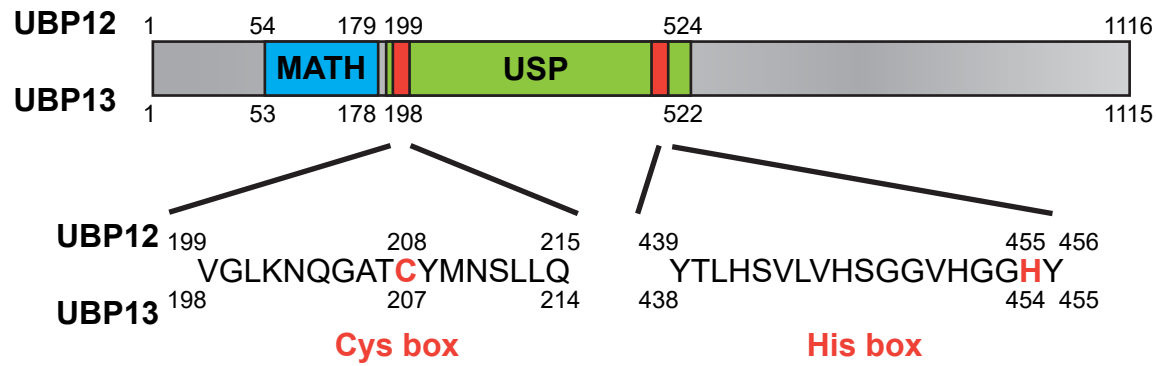
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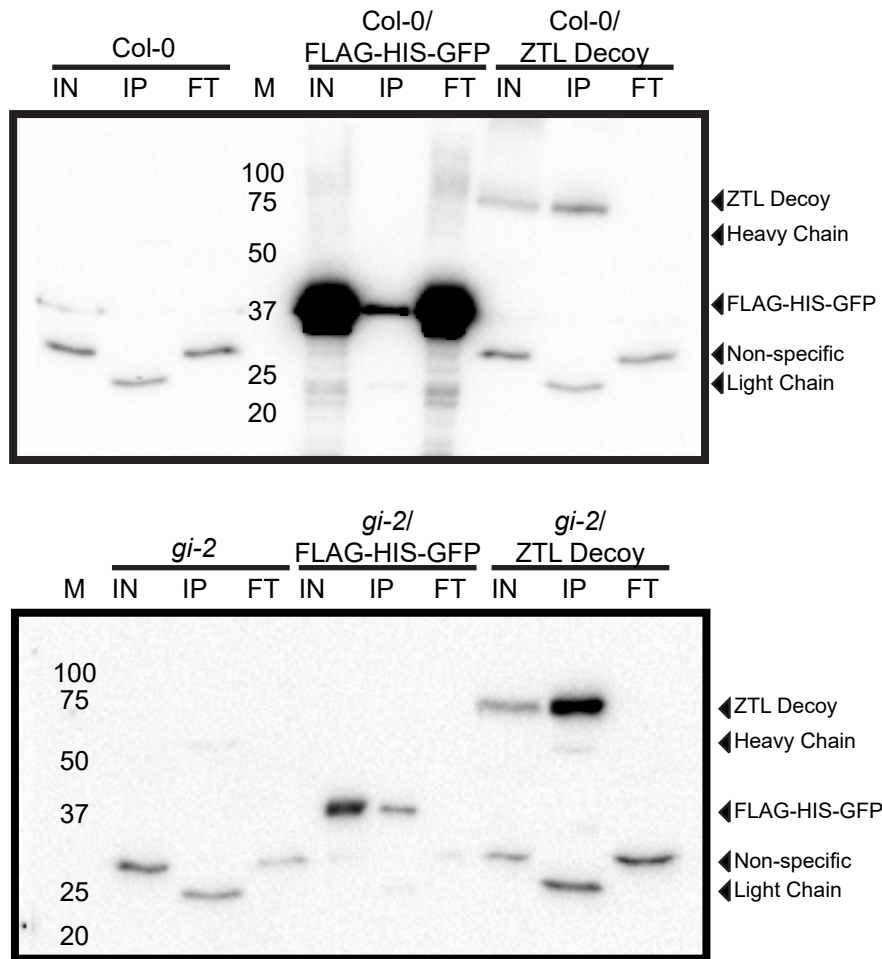




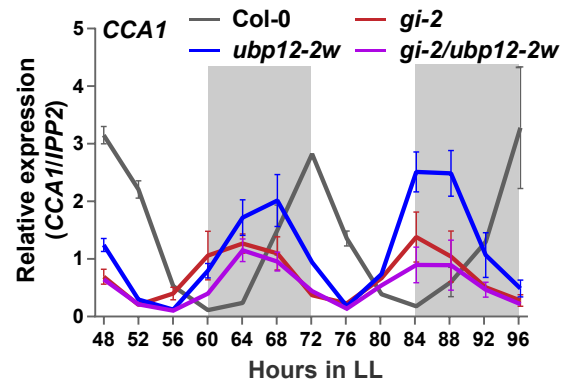




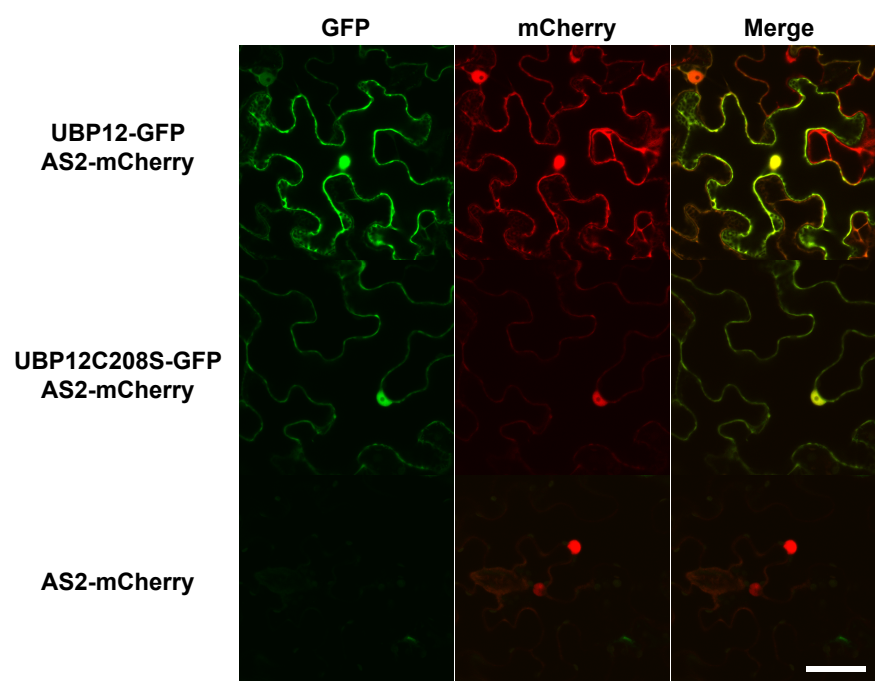
Lee *et al.* Figure S2



Lee *et al.* Figure S3



Lee *et al.* Figure S4



Lee *et al.* Figure S5

