Twist-dependent ratchet functioning downstream from Dorsal revealed using a light-inducible degron

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Graded transcription factors are pivotal regulators of embryonic patterning, but whether their role changes over time is unclear. A light-regulated protein degradation system was used to assay temporal dependence of the transcription factor Dorsal in dorsal–ventral axis patterning of Drosophila embryos. Surprisingly, the high-threshold target gene snail only requires Dorsal input early but not late when Dorsal levels peak. Instead, late snail expression can be supported by action of the Twist transcription factor, specifically, through one enhancer, sna.distal. This study demonstrates that continuous input is not required for some Dorsal targets and downstream responses, such as twist, function as molecular ratchets.

[Keywords: Drosophila melanogaster; BLID; optogenetics; Dorsal Rel transcription factor; Snail; Twist; morphogen; cis-regulatory module; light-inducible degron]

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Results

An optogenetic approach was used to examine the temporal action of Dl in supporting target gene expression, initially focusing analysis on the target gene snail (sna). sna is expressed in ventral regions of the embryo in cells that ultimately contain the highest levels of nuclear Dl [Kosman et al. 1991] and is therefore considered a high-threshold target. However, while Dl levels peak in ventral regions of the embryo during nuclear cycle [nc] 14, studies have shown that sna is expressed within ventral regions at nc13, suggesting that lower levels of Dl are in fact sufficient for this high-threshold target [Reeves et al. 2012].

To assay the temporal dependence on Dl for expression of target genes, including the high-threshold response gene sna as well as low-threshold responses including genes short gastrulation [sog] and decapentaplegic [dpp] [for review, see Reeves and Stathopoulos 2009], an optogenetic Blue Light-Inducible Degron (BLID) sequence was fused to Dl in-frame at the C terminus through modification of the endogenous gene locus using CRISPR/Cas9 technology [Fig. 1A]. BLID consists of a LOV2 domain and a degron sequence, such that in the dark, when the α-helix of the LOV2 domain interacts with the LOV core.

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domain, the degron is inaccessible, but upon illumination with blue light (~400–500 nm), the helix dissociates from the LOV core domain, the degron is exposed, and the entire fusion protein, Dl-BLID in this case, is degraded (Bonger et al. 2014).

To assay the degradation efficiency of Dl-BLID, embryos laid by homozygous dl-BLID mothers were collected and illuminated with blue light for 4 h [see the Materials and Methods]. Larval cuticles were examined as [1] a proxy for changes to Dl levels that manifest as DV patterning defects [Roth et al. 1989], and [2] to assay for any phenotypes induced indirectly by blue-light treatment. The majority of dl-BLID embryos illuminated for 4 h with blue light exhibit cuticles similar in phenotype to dorsalized embryos laid by dl null (dl\(^{+/+}\)) mothers [Fig. 1, cf. I and 1K] suggesting that Dl-BLID is successfully degraded upon blue light illumination. However, while half of the dl-BLID embryos that were not subjected to blue light exhibited normal cuticles [Fig. 1B,F], the remaining half exhibited a range of subtle defects including a small number with the more severe, dorsalized cuticle phenotype [Supplemental Fig. S1B–D]. In contrast, wild-type and dl null mutant embryos appear unaltered when exposed to blue light for 4 h [Supplemental Fig. S1A,E], supporting the view that differences in dl-BLID cuticles, in the light versus dark, result from light-induced degradation and not indirect effects of blue-light exposure. These results suggest that blue light degrades Dl, but that the degradation process is likely leaky, occurring to some degree even in the dark.

To directly test whether Dl is degraded upon illumination, we stained embryos with anti-Dl antibody and imaged cross-sections to assay for changes to the nuclear concentration gradient. As expected, we found that levels of Dl in wild-type embryos containing an unmodified, native dl gene are unaltered both for embryos kept in the dark as well as those exposed to blue light for 1 h [Fig. 1C,D]. In the dark, the Dl gradient signal associated with dl-BLID embryos appears qualitatively lower compared with wild type [Fig. 1C,G]. However, when dl-BLID embryos were exposed to blue light for 1 h, almost all of the signal, especially the nuclear gradient, is lost [Fig. 1H]. Taken together, Dl-BLID appears to support a relatively normal Dorsal nuclear gradient that is efficiently degradable with blue-light illumination but exhibits increased variability in levels/shape compared with wild type, even in the dark. We used this finding to our advantage, as lower levels of Dorsal initially are likely to be more easily manipulated by short light exposures.

To further confirm that Dl-BLID is being degraded, Dl protein levels in embryos were examined by Western blot using anti-Dl antibodies [Fig. 1E]. After 30 min in the blue light, Dl-BLID protein levels were indeed reduced to barely detectable levels [Fig. 1E]. For embryos that were kept in the dark, Dl-BLID proteins levels were lower compared with wild-type embryos, possibly due to leaky degradation of the degron [Fig. 1E]. This lower Dl level may contribute to the broad range of cuticle phenotypes observed in dl-BLID embryos kept in the dark [Supplemental Fig. S1B]. The results from the cuticle preparation, Dl antibody staining, and Western suggested that controlling Dl levels using blue light illumination with temporal resolution is feasible.

To directly observe Dl-BLID degradation by blue light, we created and assayed Dl-BLID fluorescent protein fusions. While we found that Dl-mCherry-BLID fusions do not retain Dl function, this fusion does permit visualization of the kinetics of blue-light-induced degradation. Embryos expressing Dl-mCherry and Dl-mCherry-BLID were imaged live using confocal microscopy. When control embryos are exposed to a high power (40%) blue laser of 488-nm wavelength for 10 min, Dl-mCherry embryos (lacking BLID sequence) exhibit little to no decrease in Dl signal [Fig. 1J, Supplemental Movie S1]. On the other hand, Dl-mCherry-BLID embryos undergo a dramatic decrease in Dl signal [Fig. 1L, Supplemental Movie S1] indicating that Dl-BLID degradation is occurring in embryos, with
appreciable degradation observable within minutes rather than hours observed in other systems [Baaske et al. 2018]. Taken together, these results warrant use of the DI-BLID system to finely assay temporal dependence of target genes on DI over time during early embryonic development.

To determine whether high levels of DI are required continuously throughout early embryonic development, we utilized confocal microscopy to illuminate individual embryos with blue light for either 20 min starting at nc14a (laser early, LE) or 20 min starting at nc14c (laser late [LL]) (Fig. 2A). In addition, a triple fluorescent protein (FP) reporter system (H2A-BFP, MCP-GFP, and PCP-mCherry) [Bothma et al. 2015] was introduced by genetic crosses into the dl-BLID background in order to monitor embryonic development and gene expression responses. The H2A-BFP fusion identifies nuclei, which is useful for monitoring all cells in the developing embryos, whereas the MCP-GFP and PCP-mCherry fusions bind to particular RNA stem–loops, which can be used to monitor nascent transcription.

To start, H2A-BFP signal was used to assay whether blue-light illumination affects developmental progression of embryos by observing gastrulation, which involves invagination of the presumptive mesoderm. dl-BLID embryos invaginate ventrally and proceed through gastrulation even when illuminated at the low power (0.8%) blue laser needed to image H2A-BFP, despite some low-level degradation of dl-BLID (“dark”) [Fig. 2C; Supplemental Movie S2]. Alternatively, when additionally subjected to high power (40%) blue laser illumination during an early time window (“LE”) [Fig. 2A], dl-BLID embryos fail to ventrally invaginate, and therefore do not gastrulate [Fig. 2D; Supplemental Movie S3]. Embryos obtained from females lacking nuclear DI also fail to undergo gastrulation [Leptin and Grunewald 1990] supporting the idea that the failure of dl-BLID embryos illuminated early to gastrulation is due to decrease in DI levels. In contrast, dl-BLID embryos illuminated during a late time window (“LL”) [Fig. 2A], surprisingly, are able to invaginate [Fig. 2E; Supplemental Movie S4]. These differences in developmental progression between embryos illuminated early or late suggest that high levels of DI achieved by late nc14 are not necessary for embryos to proceed through gastrulation.

To test how DI target gene expression is altered by lower DI levels, we performed fluorescent in situ hybridization (FISH) using riboprobes to monitor expressions of the genes dpp, sog, and sna, which span the DV axis [Reeves et al. 2012] comparing expression in the dark to that after illumination. In order to collect enough embryos to carry out FISH experiments, we illuminated embryos en masse on plates as opposed to using confocal microscope laser illumination (Fig. 2B; see the Materials and Methods). dl-BLID embryos kept in the dark were analyzed by FISH and show dorsal dpp expression at nc14a-b [Fig. 2G] but a narrower sna expression domain with increased variability at the anterior [Fig. 2H; Supplemental Fig. S2]. In addition, sog expression is repressed in this more narrow domain encompassed by its repressor, Sna [Fig. 2F,H;J;Cowden and Levine 2002]. Narrowing of the sna domain is likely due to lower levels of total DI present in the dl-BLID background, even in the dark (Fig. 1E,G).

Embryos illuminated for 30 min before being fixed at nc14a [likely illuminated between nc13-nc14a] exhibit ventrally expanded dpp [Fig. 2K] but retracted sog [Fig. 2J]. As dpp and sog expression share a boundary, where dpp is repressed by DI and sog expression is supported by DI, these genes likely share the same threshold response but with opposite effect [for review, see Reeves and Stathopoulos 2009]. Furthermore, sna expression is lost in embryos fixed at nc14b [likely illuminated between nc14a-b], but sog expression appears unaltered [Fig. 2L; see Discussion]. sog transcription is absent from ventral-most regions, presumably due to the presence and action of Sna protein despite the lack of sna transcripts. As sna transcripts have a half-life of ~15 min [Boettiger and Levine 2013], Sna protein made before blue-light illumination may perdure and continue to repress sog at least partially in ventral regions [Bothma et al. 2011].

In contrast, embryos illuminated for 30 min before being fixed at nc14d [likely illuminated later between nc14c-d], express both sna and sog similar to embryos
kept in the dark [Fig. 2I,M]. These results support the view that the decrease in Dl levels upon illumination affects multiple target genes, but in a temporally dependent manner. Collectively, these results suggest that embryos exposed to light late [i.e., nc14c to nc14d] can still gastrulate because of maintained expression of target genes including sna, a critical regulator of gastrulation [Leptin and Grunewald 1990], whereas embryos exposed to light early [i.e., nc14a to nc14b] fail to gastrulate due to loss of sna.

To distinguish whether maintenance of sna expression at the late time point relates to retention of transcripts made earlier or to an ability to produce new transcripts late, even when Dl is degraded, we turned to live imaging. The sna transcripts identified by in situ hybridization within fixed embryos comprise both mature and nascent transcripts; it is difficult to distinguish nascent sna transcripts in part because this gene is expressed at high levels and transcripts accumulate. Instead, the MS2-MCP system was used to monitor nascent transcription in vivo. Combining the MS2-MCP system with dl-BLID allows nascent transcription to be assayed under different illumination schemes. Specifically, transgenic lines containing a previously defined sna MS2-based reporter were used to assay sna transcriptional activity [Bothma et al. 2015]. In these constructs, ~20 kb spanning the sna locus is used as a reporter in which sna is replaced with the yellow gene sequence including intronic MS2 RNA stem loop sequences [Fig. 3A; Bothma et al. 2015]. When this reporter is actively transcribed, MCP-GFP fusion proteins bind to the stem loops and produce visible nuclear puncta, allowing live monitoring of sna expression.

An intermediate power laser setting (5%) was used to image the MS2-MCP signal, while the high-power setting was used to degrade DI-BLID [Supplemental Fig. S3A; see the Materials and Methods]. Again, under these imaging conditions, illumination of dl-BLID embryos with high power at the early time point [i.e., nc14a-nc14b, “mLE”] leads to gastrulation failure, whereas illumination of embryos later [i.e., nc14c, “mLL”] has no effect on gastrulation despite the use of intermediate laser power to image the MS2-MCP signal for an extended period of time [Supplemental Fig S3B]. We used this scheme, in which MS2-MCP imaging and DI-BLID degradation are compatible, to determine how sna transcription is affected by temporal changes in Dl levels.

We found that wild-type sna MS2-MCP signal [sna.wt] was retained when embryos were illuminated with high-power laser late [mLL] [Fig. 3E,I; Supplemental Movie S7], but was diminished when embryos were illuminated early [mLE] [Fig. 3B,H; Supplemental Movie S5]. Two enhancers are known to support early sna expression during embryogenesis, one proximal [sna.prox] and one distal [sna.dis] [Ip et al. 1992; Perry et al. 2010; Dunipace et al. 2011; Ozdemir et al. 2011]. In order to understand which cis-regulatory sequences drive sna gene expression even when DI is degraded at the late time point, we also assayed two reporter variants in which portions of these two early embryonic enhancers had been deleted, constructed in a previous study [Fig. 3A; Bothma et al. 2015]. The snaΔ-

![Figure 3](https://example.com/figure3.png)

**Figure 3.** High levels of Dl are required for sna activatation only at early stages, but not at late stages, in which sna expression is predominantly supported by the sna distal enhancer. (A) Scheme of large reporter constructs used to assay sna transcriptional activities by MS2-MCP system [Bothma et al. 2015]. (B–G) MCP.GFP signals associated with the sna MS2 reporter were imaged (false-colored red dots) in dl-BLID with early (B–D) or late (E–G) blue laser illumination that is MS2-MCP imaging compatible (“mLE” and “mLL,” respectively) [see also Supplemental Fig. S3] in various sna regulatory conditions including wildtype (sna.wt), proximal enhancer deletion (sna.Aprox; C,F), and distal enhancer deletion (sna.Adis, D,G). Images are snapshots from movies, before illumination (top) and after illumination (bottom) of each panel. Three movies were taken for each condition. Ventral views of embryos are shown with anterior oriented to the left. (H,I) Quantitative analysis of the number of MCP.GFP dots associated with the sna MS2 reporter in dl-BLID embryos with sna.wt, sna.Aprox, or sna.Adis sna regulatory condition. Number of MS2-MCP.GFP spots are counted in each time frame, and the values are normalized to the initial value detected in the first frame (before 5 min blue-laser illumination with 15% laser power) with early laser (H) or late laser (I) illumination. Blue shade indicates a time frame of 5-min 15% blue-laser illumination. Error bars represent standard error of the mean. For individual traces, see Supplemental Figure S5. For details for detection of sna-MS2-MCP.GFP and blue-laser illumination, see Supplemental Figure S3.

prox reporter behaves as the sna.wt reporter: Embryos illuminated early lose signal, whereas those illuminated late retain it [Fig. 3C,F,H,I; Supplemental Movies S6, S8]. In contrast, the sna.Adis reporter loses expression when illuminated at either time point [Fig. 3D,G,I; Supplemental Movies S6, S8]. Thus, the distal enhancer is necessary for late sna expression when no or very little DI is present, while the proximal enhancer cannot support
late \textit{sna} expression in the absence of DI. This suggests that the proximal \textit{sna} enhancer likely requires high DI levels for activity. Taken together, these results suggest a model in which DI acts through either enhancer (directly or indirectly) early, but that an additional input is required to sustain late \textit{sna} expression through the \textit{sna.dis} enhancer, specifically.

Another DI target gene encoding a bHLH transcription factor, \textit{twist} ([\textit{twi}]), is expressed in ventral regions, and also provides input to \textit{sna} [for review, see Reeves and Stathopoulos 2009]. \textit{sna} expression is either lost or greatly diminished in \textit{dl} and \textit{twi} mutants, respectively [Ip et al. 1992]. \textit{twi} transcript levels increase rapidly at the onset of nc14 and activation of mesodermal genes follows (Sandaler and Stathopoulos 2016a), suggesting that Twi may be an important input into these target genes. Furthermore, peak DI levels are not required to support \textit{sna} expression as ectopic Twi gradients can support its expression even in conditions of low, but not completely absent, DI [Stathopoulos and Levine 2002]. These previous studies had suggested that Twi may suffice to support \textit{sna} activation at the late time point, even in the absence of DI. However, it was previously not possible to remove DI but retain Twi as \textit{twi} gene expression is DI-dependent.

We hypothesized that Twi is responsible for the late expression of \textit{sna}, essentially taking over for DI. To test this idea, embryos were fixed after 30-min blue LED illumination [Fig. 2B] and assayed for DI and Twi proteins using antibodies, and for \textit{sna} transcripts by FISH. Embryos exposed to light early or late exhibited low levels or no DI as expected but, surprisingly, retained Twi expression [Supplemental Fig. S4] demonstrating that even low levels of Twi in nc14 are sufficient to support low levels of Twi expression. \textit{sna} expression is also retained when embryos are illuminated late [Fig. 4A–B′′], but not early [Supplemental Fig. 4A–B′] suggesting early nc14 \textit{sna} expression is DI-dependent. However, when the \textit{twi} mutant is recombined with \textit{dl-BLID}, even when embryos are kept in the dark and high levels of DI are present, \textit{sna} expression is lost if Twi is absent [Fig. 4C–C′′]. Taken together, these results suggested that Twi is a pivotal input for \textit{sna} activation, particularly at late stages when \textit{sna} expression is independent of high DI levels.

In order to understand the temporal relationship between DI and Twi transcription factor dynamics, we assayed Twi dynamics with fine time resolution in combination with temporally controlled DI-BLID levels. Twi levels were detected in \textit{dl-BLID} embryos using a previously described Twi-mCherryLlamaTag fusion protein, which allows early zygotic proteins to be visualized without having to wait for fluorescence maturation [Bothma et al. 2018]. When embryos are kept in the dark, mCherry signal intensities throughout nc14, suggesting exponential production of Twi protein [Fig. 4D,G; Supplemental Movie S9]. However, in embryos exposed to high-power blue laser illumination at nc14a, no increase in Twi levels is observed (“LE”)[Fig. 4E,G; Supplemental Movie S10]. In contrast, for embryos illuminated at nc14c, Twi levels increase (“LL”)[Fig. 4F,G; Supplemental Movie S10] similarly to embryos without illumination (“dark”)[Fig. 4D,G;
levels of Dl in early nc14 determine above which Twi can activate its own expression independently. Our results suggest that Twi levels are reflective of the underlying DI levels early, and that levels of DI early impact levels of Twi present later.

Interestingly, intermediate exposure of DI-BLID to blue laser (e.g., 5–10 min) results in loss of the late nc14 exponential increase in Twi levels that is normally observed in control embryos (e.g., dark) [Fig. 4G] as the rate of change in levels decreases, but it initially remained unchanged why [Fig. 4H]. In order to explain why Twi levels do not grow exponentially after intermediate duration DI degradation, we hypothesized that low levels of DI are retained that continue to support low levels of Twi. In this scenario, a second blue-light illumination to knock down the remaining DI would be expected to further decrease Twi levels. However, we found that exposure to a second illumination (e.g., 15 min at nc14c) has no effect; Twi is maintained at levels similar to embryos exposed to a single 5-min illumination at nc14b [Fig. 4I]. This observation suggests that in late nc14 activation of Twi shifts to a gene regulatory state that is independent of DI levels. Collectively, these data support the view that a Twi-dependent threshold exists above which Twi can activate its own expression independently of DI at this late stage and supports a model where levels of DI in early nc14 determine twi expression, but during late nc14, twi expression is DI independent.

Discussion

In this study, we examined whether DI is continuously required to activate target genes in the early embryo by utilizing a DI-BLID fusion. DI is required early for the initiation of expression of the snail target gene in ventral regions, but surprisingly is not needed late to maintain its expression. Like snail, expression of htl, mes3 and netA are sustained in DI-BLID embryos illuminated with blue LED light late [Supplemental Fig. 5F] suggesting that other target genes are similarly regulated. In contrast, we found that the lateral gene sog is still expressed no matter when DI degradation occurs during nc14. This unexpected result appears to contradict the model where the sog dorsal boundary is formed by limiting levels of nuclear Dorsal. One possible explanation that is consistent with this model is that low levels of Dorsal remain after illumination and are enough to activate sog. However, this should result in either a narrow sog expression domain or requires asymmetrical degradation of Dorsal, neither of which was observed [Fig. 1H; Supplemental Fig. S2; Supplemental Movie S1]. Another explanation is that once the sog domain is established by lower levels of DI, sog does not require DI to remain active because another factor acts to retain its expression. A simpler explanation is that sog transcripts are long and the detected signal could be from sog transcripts that were initiated at an earlier time point, when Dorsal was present. These possible explanations for how sog transcription fails to respond to Dorsal degradation upon illumination are not mutually exclusive. Addressing how sog transcription becomes DI independent in future studies will be an important step forward in our understanding of how the sog dorsal boundary is set.

Our results also provide insight into how a transcriptional network may buffer changes in levels of a maternal patterning morphogen. In the case of snail, high levels of DI are required early to activate snail gene expression. DI acts both directly, and indirectly by controlling twi expression, as Twi is also an input to snail [Fig. 4J, left]. In contrast, DI is dispensable for snail activation at later time points. When DI levels are reduced, snail expression remains [Fig. 4J, right], likely maintained by Twi once Twi is expressed. The ability to retain expression of a morphogen target gene despite a decrease in morphogen levels has been termed a “ratchet response,” and was demonstrated for targets of the activin morphogen in Xenopus (Gerhart et al. 1995). Twi can maintain its own expression through autoregulation [Kosman et al. 1991; Crews and Pearson 2009], and we propose this autoregulatory feedback serves to support this ratchet response that is able to buffer against decreases in DI levels. However, simple Twi autoregulatory feedback would predict a single steady-state concentration for Twi. Instead, we observed that Twi levels increase exponentially or reach intermediate levels of Twi when varying the length of illumination. While this result would not support simple autoregulatory feedback as a mechanism for maintaining Twi expression in the absence of high DI, it requires Twi levels to be at steady state. It is possible that the observed responses have not reached steady-state, and if given enough time they might all converge to the same steady-state concentration [i.e., a single response supported by autoregulation]. It is likely that other factors contribute to twi regulation; however, these results support the model that DI activates twi, and Twi is able to maintain its own expression through autoregulation.

Taken together, we propose that once Twi reaches sufficient levels to support its own auto-activation, DI is no longer required to support snail expression [Fig. 4J, right]. This is in sharp contrast to the Bcd morphogen, which patterns the anterior–posterior [AP] axis and to the early maternal pioneer factor Zelda [Huang et al. 2017; McDaniel et al. 2019]. Both Bcd and Zelda have been found to be required continuously; perturbations at any stage cause loss...
of gene expression. Alternatively, the DV gene regulatory network shifts from a state of high DI dependence to a state of DI independence for several target genes expressed in the presumptive mesoderm. It is possible that this ratchet response relates to the ability of *twi* gene expression to buffer changes in DI concentration, and allows the DV patterning network to respond only to increasing DI levels. Taken together, ratchet-like responses are crucial steps during animal development not only because they support morphogen-dependent patterning, but also they may serve to buffer expression of target genes against fluctuations in morphogen levels due to genetic and environmental changes.

Materials and methods

Fly stocks/husbandry and plasmids

All flies were kept at 18°C, unless otherwise noted. *yw* was used as wild type. Fly stocks used were as follows: *dp* /CyO (Bloomington Drosophila Stock Center [BDSC] 7096), *dl* /CyO (#3236, BDSC), *twi* /CyO (BDSC 2381), nos > MCP-GFP, nos > mCherry-PCP, Hs2Av-eBFP2 [from Michael Levine, Princeton University] (Lim et al. 2018), snailBAC > MS2 with both proximal and distal enhancers [WT, snw, wt], proximal deletion [NoPrimary, snw A-prox], or distal deletion [NoShadow, snw Aprox] [from Michael Levine, Princeton University] (Bothma et al. 2015), *vasa-mCherry* and *Twi-mCherryLlamaTag* [from Hernan Garcia, University of California at Berkeley] (Bothma et al. 2018). For details regarding fly crosses, see the Supplemental Material.

Genome editing

CRISPR was performed as described previously (Gratz et al. 2014; Port et al. 2014). Briefly, the gRNA fly line [targeting before the C-term and after the 3 UTR of *Dorsal*] [see the Supplemental Materials, Supplemental Table S1] and the Cas9 line *Sp/CyO, P* [nos-Cas9] [A, [NIG-FLY, CAS-0004] were mated. Embryos were collected and the homology-directed repair (HDR) template containing the C-term of *dl* fused to BLID [see the Supplemental Material] was injected into these embryos. Flies were screened for DsRed. The integration was confirmed by PCR and sequencing.

Blue-light illumination

Embryos were collected for 1 h at 18°C followed by 4-h incubation for aging and illuminated with blue light using either a set of LEDs (2501BU blue 225 LED 13.8-W square grow light panel 110) or the 488-nm laser on a Zeiss LSM 800 confocal microscope. For blue LED light illumination, embryos on agar plates were placed 6.5 cm below the LED light panel and illuminated for appropriate time lengths. After blue light exposure, the embryos were fixed. For 488-nm blue laser illumination, the embryos were dechorionated and mounted on a heptane glued slide. The embryos were immersed in water, and a blue laser was applied using a 25× water immersion objective. All the embryos were prepared under red filtered light to avoid possible DI-BLID degradation by light coming from microscopes or other ambient sources.

Cuticle preparations

Embryos were collected for 2 h at 18°C, aged 1.5 h in the dark, and illuminated with blue LEDs for 4 h. Subsequently, embryos were aged for an additional 36–40 h in the dark and then processed by standard cuticle preparation using lactic acid.

Western blot analysis

Aged embryos were dechorionated and mounted in Halocarbon 27 oil (Sigma-Aldrich). Embryos at nc14b were manually selected and illuminated for 30 min with LED blue light. After light exposure, embryos at nc14c were prepared for standard Western blot.

Immunostaining and fluorescent in situ hybridization (FISH)

Immunostaining and FISH protocols were followed as previously described (Kosman et al. 2004). Sheep antidigoxigenin [Life Technology PA185378], or rabbit anti-FITC [Invitrogen A889], mouse anti-DI [1:10, Developmental Studies Hybridoma Bank 7A4] or guinea pig anti-Twi [1:200] [Trisnadi and Stathopoulos 2015] were used together with Alexa conjugate secondaries [1:400; Thermo Fisher], DAPI staining [1:10,000; Molecular Probes] was used to mark nuclei.

Live imaging and quantification

To test efficiency of DI-BLID degradation upon blue laser illumination, 488 nm of blue laser with 40% laser power [high power] was applied to the embryos heterozygous for either *dl-mCherry* or *dl-mCherry-BLID*, while also applying 555-nm laser to monitor mCherry signal. To examine the continuous requirement of high level of DI at blastoderm stage, *dl-BLID* embryos were illuminated by 488 nm blue laser with 40% laser power [high power] for 20 min, starting at the appropriate developmental stage. Early embryonic development was examined by live imaging of H2A-BFP [i.e., Hs2Av.eBFP2] using 405 nm blue laser with 0.8% laser power. Twi protein dynamics, Twi-mCherryLlamaTag system, which recognizes maternally deposited mature mCherry fluorescent protein, was utilized [Bothma et al. 2018]. mCherry was imaged live from the onset of nc14a to gastrulation while DI-BLID was degraded by 488 nm blue laser at 40% laser power [high power] with varying lengths of time at appropriate developmental stages. All images were taken using a 25× water immersion objective for additional details regarding imaging and quantification, see the Supplemental Material.

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