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Investigations into the mechanisms that regulate *C. elegans* developmental timekeeping

A dissertation submitted in partial satisfaction

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

MOLECULAR, CELLULAR, DEVELOPMENTAL BIOLOGY

by

Guinevere E Ashley

September 2022

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Peter Biehl, Provost and Dean of Graduate Studies

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Abstract

Investigations into the mechanisms that regulate *C. elegans* developmental timekeeping

By Guinevere Ashley

All living organisms have biological clocks that drive and coordinate repetitive developmental processes. These intrinsic timing devices are essential for animal development and survival but, in many cases, the underlying mechanisms are poorly understood. *C. elegans* larval development is an ideal system in which to study this problem. *C. elegans* employ two distinct but interrelated biological timing mechanisms to regulate progression through larval development. The heterochronic pathway ensures that stage-specific cellular events happen at the correct time and in the correct order, while a molt timer ensures the proper timing of each molt in relation to developmental progression. Chapter one of this work describes our investigations into the mechanisms employed by *C. elegans* to regulate and coordinate developmental progression and molting. *lin-42*, the *C. elegans* homolog to the circadian clock protein PERIOD2 (PER2), is unique among heterochronic genes for its dual role in regulating both stage-specific cellular events, and the precise timing and execution of molting, and is thought to coordinate molt cycles with developmental progression. To investigate the mechanisms through which LIN-42 regulates and coordinates developmental timing in *C. elegans*, we looked to the well-characterized mechanisms employed by its mammalian homolog PER2. The circadian period in mammals is determined by the stability of PER2 and the stability is largely determined through its interaction with Casein Kinase I δ (CK1δ). Our work shows that CK1δ can bind and phosphorylate a LIN-42 substrate *in vitro* with similar

kinetics compared to a PER2 substrate and that these interactions depend on conserved kinase binding domains. Further, we show that deletion of the SYQ and LT domains, two highly conserved alpha helices within the conserved kinase binding domain, results in dysregulation of LIN-42::GFP expression in vivo. However, severe developmental abnormalities and molting defects are only observed once those deletions are extended to encompass the whole kinase binding domain. Together, these data suggest that PER2/LIN-42 phosphorylation by CK1δ kinases is evolutionarily conserved and that this mechanism may be employed to coordinate developmental timing in C. elegans.

When investigating essential systems and critical gene products, we often cannot employ classical genetic approaches to study gene function. Critical regulators of developmental progression and timing often function with distinct co-regulators in various developmental contexts and may have reiterative functions throughout development. In knockout or null mutants, if disruption of the first developmental event results in embryonic or larval lethality, later functions will be missed. Chapter 2 of this work describes a set of genetic tools that we produced to complement existing reagent and augment the use of the auxin inducible degron (AID) system in C. elegans. The AID system is a powerful tool to conditionally deplete proteins. We generated a set of single-copy, tissue-specific and pan-somatic TIR1-expressing *C. elegans* strains carrying a co-expressed blue fluorescent reporter to enable use of both red and green channels in experiments. We also generated a set of plasmids for constructing repair templates to generate fluorescent protein::AID fusions through CRISPR/Cas9-mediated genome editing. Together, these reagents will complement existing TIR1 strains and facilitate rapid and high-throughput fluorescent protein::AID tagging of genes.

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talk science with me, but life would be a lot more stressful and a heck of a lot lonelier

without him. Finally, I must acknowledge all the pups (Spotty, Berserker, Katelyn, Nordy, and

Kona) who provided me endless supplies of cuddles and joy. What a sad life this would be

without you.

Chapter 1:

Investigating the links between biological timing mechanisms in nematodes and mammals

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Author contributions: G.A, B.S, C.P, J.W contributed intellectually to this work; conceived and designed the experiments and collaborated to interpret results, analyzed and quantified data, and planned project direction. B.S constructed necessary reagents and performed *in vitro* biochemical assays. G.A constructed necessary reagents/*C. elegans* mutant strains and performed *in vivo* assays and phenotypic analysis, except for the luciferase assays, which were performed and analyzed by Kathrin Braun.

Abstract:

All living organisms have biological clocks that drive and coordinate repetitive developmental processes. These intrinsic timing devices are essential for animal development and survival but, in many cases, the underlying mechanisms are poorly understood. *C. elegans* larval development is an ideal system in which to study this problem. *C. elegans* employ two distinct but interrelated biological timing mechanisms to regulate progression through larval development. The heterochronic pathway ensures that stage-specific cellular events happen at the correct time and in the correct order, while a molt timer ensures the proper timing of each molt in relation to developmental progression. Animal survival depends on precise

coordination of molting with developmental progression but the mechanisms that ensure this coordination are poorly understood. LIN-42, the *C. elegans* homolog to the circadian clock protein PERIOD2 (PER2), stands out for its unusual role as a regulator of both stage-specific

cellular events, and the precise timing and execution of molting. The key domains conserved between LIN-42 and PER2 are essential for PER2 function in regulating circadian rhythms. In mammals, circadian clocks are controlled by a transcription-translation feedback loop involving PER2 expression and degradation. The stability of PER2 is largely determined through its interaction with Casein Kinase I δ (CK1δ). This kinase binds to and phosphorylates PER2 at one of two regions leading to either stabilization or degradation of PER2. Sequence analysis indicates two highly conserved kinase-binding motifs, and our *in vitro* pull-down assays show that these conserved domains in LIN-42 can bind CK1δ suggesting that a common mechanism may anchor the kinase to LIN-42. The kinase domain of CK1δ has a 79% sequence identity with the C. elegans kinase KIN-20. While we have been unable to purify recombinant KIN-20 thus far, mammalian CK1δ phosphorylates the LIN-42 C-terminus to a similar degree and within the same timeframe as CK1δ phosphorylation of a PER2 substrate. Purified LIN-42 lacking the conserved kinase binding motifs has weaker binding to CK1δ and reduced phosphorylation of the LIN-42 C-terminus compared to wild type LIN-42 protein. When we engineered the equivalent mutations into the *lin-42* endogenous locus, we observed profound developmental delay as well as extended and asynchronous molts. Together, these data suggest that PER2/LIN-42 phosphorylation by CK1δ kinases is evolutionarily conserved and that this mechanism may be employed to coordinate developmental timing.

Introduction:

Biological clocks

Every living organism possesses innate molecular mechanisms that control physiology and development. These biological clocks direct periodic expression patterns of certain genes, which in turn direct periodic changes in cellular and physiological activities that occur on a daily, seasonal, yearly, or other regular cycle. These cyclical or periodic changes are referred to as rhythms and are characterized by the period with which they occur relative to a day. Ultradian rhythms include sleep cycles and cellular respiration cycles and have a period of less than 24-hours so that the cycle may repeat multiple times within a day (Schulz and Lavie 1985; Halberg et al. 1965). Infradian rhythms, on the other hand, have a period greater than 28-hours, but less than one year and can include menstrual cycles, seasonal migration patterns, and seasonal breeding patterns (Mahesh 1985; Halberg et al. 1965) The most well-characterized biological clocks direct circadian rhythms, which exhibit a periodicity of approximately 24 hours, are found in nearly all living organisms, and coordinate a wide range of biological processes with the 24-hour night/day cycles of the
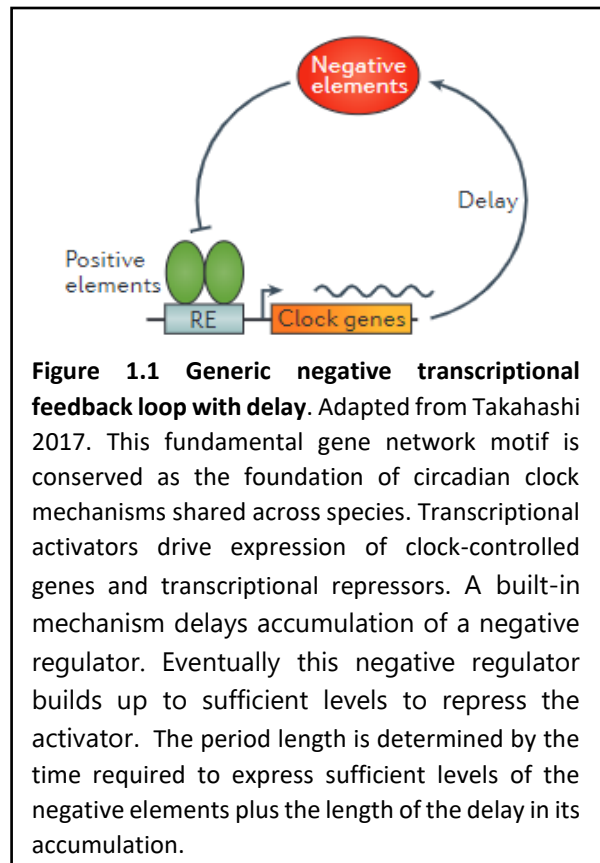
Figure 1.1 Generic negative transcriptional feedback loop with delay. Adapted from Takahashi 2017. This fundamental gene network motif is conserved as the foundation of circadian clock mechanisms shared across species. Transcriptional activators drive expression of clock-controlled genes and transcriptional repressors. A built-in mechanism delays accumulation of a negative regulator. Eventually this negative regulator builds up to sufficient levels to repress the activator. The period length is determined by the time required to express sufficient levels of the negative elements plus the length of the delay in its accumulation.

earth. Circadian clocks are thought to be a product of convergent evolution because, while specific components of circadian clocks diverge across species and kingdoms, the fundamental design is generally conserved (Doherty and Kay 2010; Bell-Pedersen et al. 2005; Young and Kay 2001; Dunlap 1999). Namely, circadian rhythms are generated by cell-autonomous negative transcriptional feedback loops with a built-in delay (Alon 2007) (Fig. 1.1). Depending on the organism, this mechanism can be more complicated and can involve additional layers of regulation at every stage of gene expression, but the underlying 'network motif' is conserved (Takahashi 2017).

In mammals, the core circadian clock is driven by a transcription-translation feedback loop that consists of two transcriptional activators: circadian locomotor output cycles protein kaput (CLOCK) and brain and muscle ARNT-like 1 (BMAL1). CLOCK and BMAL1 heterodimerize and drive expression of a large number of clock-controlled genes including transcriptional repressors PERIOD (PER1, PER2 and PER3) and cryptochrome (CRY1 and CRY2) (Takahashi 2017; Aryal et al. 2017). PER and CRY proteins form a complex together with the serine/threonine kinases casein kinases (CK1δ) and/or (CK1ε) and translocate to the nucleus where they repress the activity of CLOCK/BMAL1, thereby inhibiting their own expression (Gallego and Virshup 2007; Gekakis et al. 1998; Lee et al. 2001; Lowrey and Takahashi 2011). Once PER and CRY levels have decreased, the repression is relieved and CLOCK/BMAL1 drive expression again to start a new cycle (Gallego and Virshup 2007; Preußner and Heyd 2016) (Fig. 1.2). To maintain the 24-hour period of circadian rhythms, a delay between the activation of transcription and the repression of transcription is required. This delay is accomplished, in part, by post-translational modifications of the transcriptional

repressor PER2 that determine its stability and therefore regulate its accumulation (Crosby

and Partch 2020). Circadian rhythms and other biological clocks in mammals are

exceptionally complicated. There are many additional layers of regulation, from well-studied

accessory transcriptional feedback loops that help drive rhythmic expression of core clock

proteins, to an ever-growing litany of post-transcriptional modifications that regulate their

activity and function (Crosby and Partch 2020; Liu et al. 2008; Ikeda et al. 2019). The current

understanding of circadian clocks in mammals indicates a master clock in the hypothalamus

responsible for synchronizing peripheral clocks located in every cell of the body (Albrecht

2012; Dibner et al. 2010; Mohawk, Green, and Takahashi 2012).   The mechanisms utilized

for this coordination and integration remain unclear.

Figure 1.2 Core Mammalian Circadian clock and Auxiliary loop. Adapted from Philpott et al. 2022. Transcriptional activators CLOCK and BMAL1 drive expression of clock-controlled genes and transcriptional repressors PER/CRY. Accumulation of PER/CRY is largely regulated by post-transcriptional modifications of PER. PER/CRY eventually accumulate, form a complex with CK1 and enter the nucleus to repress CLOCK/BMAL1. This cycle drives oscillatory gene expression patterns with an approximately 24-hour circadian period. An auxiliary loop driven by transcription factors ROR and REV-ERB further regulate transcription of BMAL.

C. elegans developmental progression and the molt timer:

C. elegans is an ideal system in which to tease apart the underlying mechanisms that control

and integrate biological timekeeping. Its developmental anatomy has been described in

extraordinary detail; cell differentiation patterns and morphological events are almost

perfectly predictable throughout its development (Kimble and Hirsh 1979; Sulston et al.

1983; Sulston and Horvitz 1977). Two independent, but interconnected, biological timers

drive their progression through larval development. The heterochronic pathway regulates

temporal identities such as stage-specific cell differentiation patterns, and the molt timer

controls the periodicity of molt cycles and coordinates apical extracellular matrix

regeneration, and shedding of the old skin (cuticle) (Tennessen et al. 2006; Rougvie
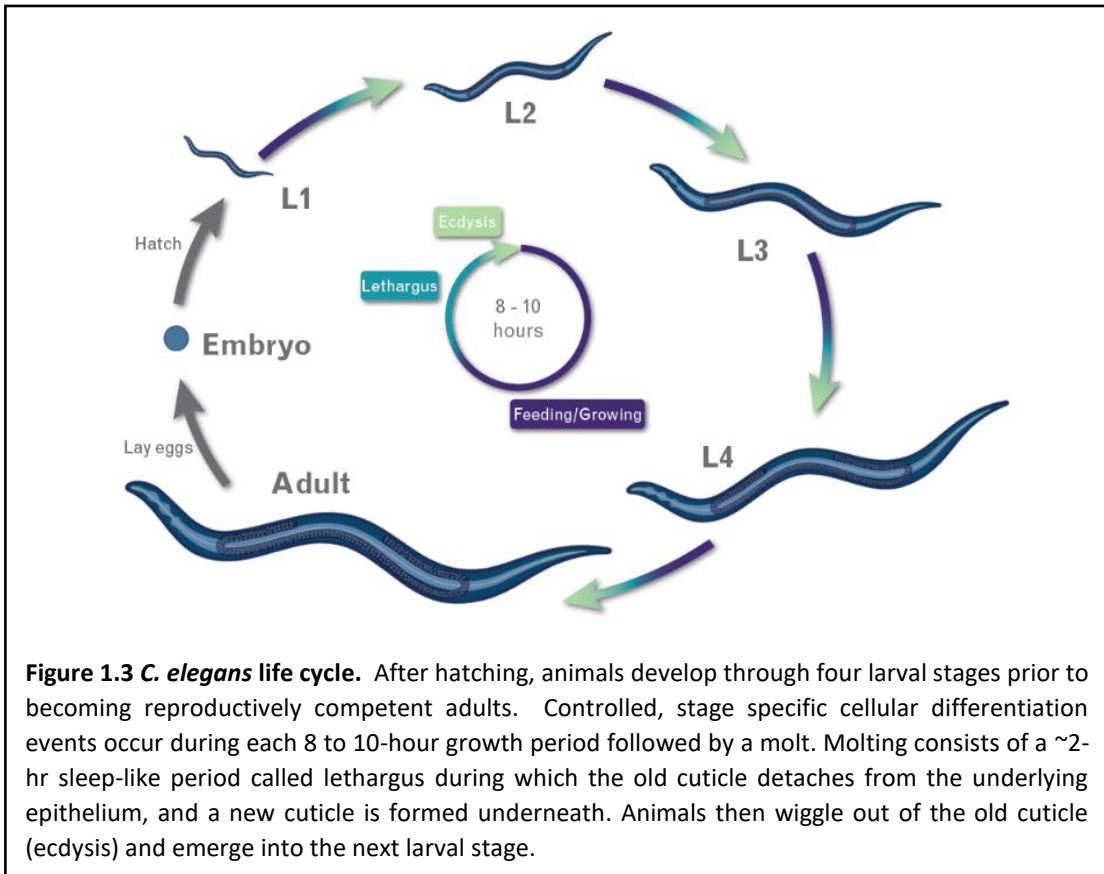
Figure 1.3 *C. elegans* life cycle. After hatching, animals develop through four larval stages prior to becoming reproductively competent adults. Controlled, stage specific cellular differentiation events occur during each 8 to 10-hour growth period followed by a molt. Molting consists of a ~2-hr sleep-like period called lethargus during which the old cuticle detaches from the underlying epithelium, and a new cuticle is formed underneath. Animals then wiggle out of the old cuticle (ecdysis) and emerge into the next larval stage.

and Moss 2013; Monsalve and Frand 2012; Moss 2007; Lažetić and Fay 2017) *C. elegans*

hatch from the embryo into the first larval stage with precisely 558 cells (Gilbert and Barresi

2000) They develop through four larval stages, each defined by a tightly regulated series of

stage-specific cellular and morphological events that are so invariable that we commonly

use cell morphology to determine the age of an animal. *C. elegans* hermaphrodites reach

adulthood with exactly 959 somatic cells, and we know precisely when each cell was

formed, where it was formed, and where it should be located (Herman 2006). The genes

responsible for ensuring that stage-specific events happen at the correct time and in the

correct sequence are referred to as heterochronic genes and are members of a well-

established biological timing mechanism called the heterochronic pathway (Rougvie and

Moss 2013; Ambros and Horvitz 1984; Ambros 1989).

A molt marks the transition between each larval stage. Molting is a large-scale metamorphic

event involving the execution of a complex gene expression program that ultimately leads to

apical extracellular matrix remodeling and regeneration of a new cuticle (Singh and Sulston

1978; Iwanir et al. 2013; Lažetić and Fay 2017; Hendriks et al. 2014). Molting begins with a

period of lethargus, a sleep-like state lasting 2-3 hours (Singh and Sulston 1978). During

lethargus, animals partially detach from the old cuticle (apolysis) and a new cuticle is

synthesized underneath (Singh and Sulston 1978; Iwanir et al. 2013). Once the new cuticle is

fully synthesized, animals wiggle out of the old cuticle (ecdysis) and begin a new larval stage

(Singh and Sulston 1978; Lažetić and Fay 2017).

The periodicity of the molt cycles is also tightly regulated; molts occur at regular 8-10 hour

intervals at 25°C and must be precisely coordinated with stage-specific developmental

progression (Monsalve and Frand 2012; Edelman et al. 2016; Monsalve et al. 2011).

Uncoupling of the heterochronic pathway and the molt timer through pharmacological

activation of a nicotinic receptor during the second larval stage resulted in lethality;

developmental progression was slowed, but the molt cycle continued so that animals began

to molt before stage-specific cellular events were completed (Ruaud and Besssereau, 2006).

The mechanisms that accomplish this regularity and coordination remain unclear, but

several key players have been identified. Most notably, *lin-42*, the *C. elegans* homolog to

mammalian and *Drosophila* PERIOD (PER) proteins, is an established member of the

heterochronic pathway where it temporally regulates seam cell dynamics during larval

development by negatively regulating a number of micro-RNAs (Perales et al. 2014;

Tennessen et al. 2006; McCulloch and Rougvie 2014; Van Wynsberghe et al. 2014). *lin-42* is

unique among heterochronic genes for the molting phenotypes produced when it is

mutated. Generally, molting phenotypes caused by mutations to heterochronic genes result

from inappropriate cessation or continuation of the molting program leading to too few

molts or too many molts (Ambros and Horvitz 1984; Ambros 1989; Ambros and Ruvkun

2018). Mutations in *lin-42*, however, affect not just the number of molts, but also the

execution and timing of each molt (Monsalve et al. 2011; Edelman et al. 2016). As a result,

lin-42 is thought to coordinate molting cycles with developmental progression, although the

mechanisms involved in this regulation are unclear (Tennessen et al. 2006; Monsalve and

Frand 2012).

Lin-42 is the _C. elegans_ homolog to mammalian circadian clock protein PER2

The structural and functional homology shared between LIN-42, and its mammalian homolog PER2 is striking. PER2 and LIN-42 both function to regulate the timing of biological processes. Mutations in PER2 alter the timing of circadian rhythms so that the circadian period is either shortened or extended, and mutations in LIN-42 alter the period between molts and uncouple molting from developmental progression (Steinlechner et al. 2002; Masuda et al. 2020; Monsalve et al. 2011; Edelman et al. 2016). Expression of PER2 and LIN-42 oscillate in time with their respective cycles. PER2 mRNA and protein levels peak one time in each 24-hour period (Shearman et al. 1997; Lee et al. 2011; Chen et al. 2009; Tei et al. 1997). Similarly, _lin-42_ mRNA expression oscillates in time with _C. elegans_ 8 to 10- hour molt cycles so that mRNA levels peak once during each intermolt period (Jeon et al. 1999; Tennessen et al. 2006). Additionally, key structural domains are highly conserved between LIN-42 and PER2, namely, the PAS domains and the CKBD, SYQ/ LT domains, which are essential for the circadian function of PER2 (Militi et al. 2016; Zheng et al. 1999; Hennig et al. 2009; Beesley et al. 2020; Lee et al. 2004; Eide et al. 2005) (Fig. 1.4A; Sup. Fig 2). Some Structural differences are also apparent between LIN-42 and PER2, which makes sense given the differences in the functions of the two systems. For example, circadian rhythms are temperature compensated, while _C. elegans_ development is expressly not, and there is evidence suggesting that regulation PER2 by CK1δ phosphorylation is a key component in of the temperature compensation mechanism (Shinohara et al. 2017; Isojima et al. 2009).

Fig. 1.4 Conservation of key domains between LIN-42 and PER and high sequence similarity between KIN-20 and human CK1. A) *C. elegans* LIN-42 isoform B and mouse PER2 protein structures. PAS-A/B, SYQ, and LT domains are highly conserved. CBD is Cry-binding domain. LIN-42 construct used in this work are indicated in teal blue and pink. ShortC includes residues 402 - 475. LongC includes residues 402 – 598. **B)** Protein structure of LIN-42A, LIN-42B, and LIN-42C isoforms **C)** Sequence alignment of *C. elegans* KIN-20 and two isoforms of human CK1. In the kinase domain, the two proteins have 79% sequence identity is highlighted in dark blue, and 89% sequence similarity is highlighted in light blue.

In *C. elegans*, the *lin-42* locus expresses three distinct protein isoforms that separate these key domains (Edelman 2016) (Fig. 1.5). Edelman et al. (2016) reported strong evidence for *lin-42a* and *lin-42b* based on RNA-seq data. They also noted that support for *lin-42c* comes from 3'RACE (Rapid amplification of cDNA ends) sequence tags (WormBase release WS252).

Figure 1.5 *lin-42* genomic locus and described mutant alleles. Adapted from Edelman et al. 2016 **A)** *lin-42* genomic locus and exons transcribed in *lin-42a*, *lin-42b*, and *lin-42c*. **B**) *lin-42(n1089)* allele deletes most of PAS-A/B domains. *lin-42(ok2385)* allele deletes the entire *lin-42a* coding region, including the SYQ and LT domains. *lin-42(ox461)* is a null allele, with the entire *lin-42* coding region deleted.

lin-42b, the longest isoform with the most homology to PER2, contains both the PAS domains and the SYQ/LT domains. *lin-42a* and *lin-42c* are smaller, non-overlapping isoforms expressed from distinct promoters. *lin-42a* contains the SYQ/LT domains but not the PAS domains, while *lin-42c* contains the PAS domains but not the SYQ/LT domains. (Jeon et al. 1999; Tennessen et al. 2006). Studies characterizing and comparing two hypomorphic mutations and a null mutation suggest that isoforms containing the SYQ/LT domains are key to LIN-42 function in regulating molting and molt timing (Tennessen et al. 2006; Monsalve et al. 2011; Edelman et al. 2016). *lin-42(ok2385)* an allele that deletes *lin-42a* and the corresponding portion of *lin-42b,* results in developmental delay, egg laying defects, and severe molting defects, including asynchronous molts, fewer molts, and larval arrest caused by failure to detach from and shed the previous cuticle. (Monsalve et al. 2011). *lin-42(n1089)*, an allele deleting
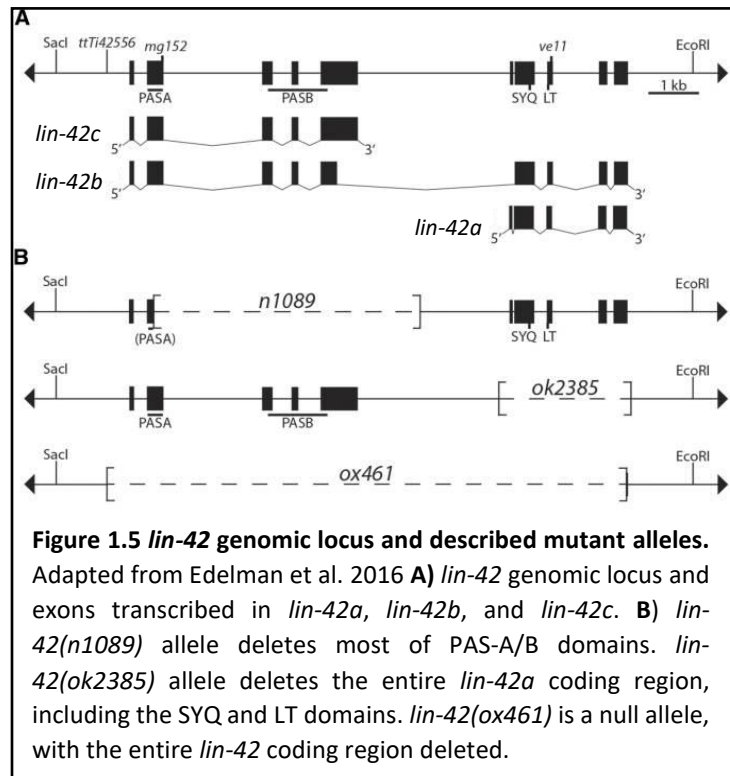
lin-*42c*, the PAS-containing region, and the corresponding portion of *lin-42b*, causes

comparably subtle heterochronic defects but is not required for proper timing or execution

of the molts (Tennessen et al. 2006). Additionally, deletion of the entire *lin-42* coding region

results in similar but more severe phenotypes compared to the partial deletions, suggesting

that the PAS-containing region does have some role in molting and molt timing (Edelman et

al. 2016). In mammals, the region encompassing the SYQ and LT domains, referred to as the

Casein Kinase-Binding Domain (CKBD), is bound by CK1δ and CK1ε and is essential for

regulating the period of circadian rhythms (Eide et al. 2005; Lee et al. 2004; Toh et al. 2001;

Xu et al. 2007). Mutations to either CK1δ or the CKBD of PER2 that disrupt CK1 binding

result in longer or shorter circadian periods and can correspond to human disease (Parico

and Partch 2020; Hirano et al. 2016; Patke et al. 2017; Xu et al. 2005; Xu et al. 2007).

Binding and phosphorylation of PER2 by CK1δ regulates circadian periods: phospho-switch

mechanism. The ~24-hour periodicity of circadian rhythms depends, in part, on the

accumulation and degradation of PER2 in the cytoplasm. Transcriptional activation by CLOCK

and BMAL1 occurs in the daytime, resulting in the accumulation of PER and CRY proteins in

the cytoplasm later in the day (Lee et al. 2001). At night, when PER and CRY proteins

accumulate to a sufficient level, they form a complex with CK1δ, migrate to the nucleus and

repress the activity of CLOCK/BMAL1 until levels of PER and CRY are reduced and the cycle

repeats (Aryal et al. 2017; Philpott et al. 2022) (Fig. 1.2). After transcription, the stability and

accumulation of PER2 are largely determined by the balance of phosphorylation by CK1

between two regions. Phosphorylation of a region in between the SYQ and LT domains

referred to as the familial advanced sleep phase (FASP) region results in stabilization of PER2, allowing its accumulation in the cytoplasm (Hirano et al. 2016; Crosby and Partch 2020; Aryal et al. 2017; Lee et al. 2001; Toh et al. 2001). Phosphorylation of an upstream phospho-degron region results in PER2 ubiquitination and degradation of PER2, delaying its accumulation (Hirano et al. 2016; Crosby and Partch 2020) (Fig. 1.6A). Mutations in CK1δ and PER2 result in longer or shorter circadian periods (Parico and Partch 2020; Hirano et al. 2016; Toh et al. 2005; Xu et al. 2005). Thus, CK1

Figure 1.6 CK1δ/PER2 phospho-switch mechanism regulates 24-hour circadian periodicity. 1.6B was adapted from Philpott et al. 2022 **A)** PER2 stability is determined by a CK1δ mediated phospho-switch. Phosphorylation of the FASP region results in stabilization of PER2 while phosphorylation of an upstream phosphodegron site results in degradation of PER2. **B)** Mutations in PER2 and CK1δ result in longer or shorter circadian periods.

plays an integral role in dictating circadian timing through regulating PER2 stability (Fig 1.6B).

Similarly, KIN-20, the *C. elegans* homolog to CK1δ, appears to have an important function in the temporal regulation of *C. elegans* development and molting cycles, although the exact mechanisms underlying this role are largely unexplored (Banerjee et al. 2005; Rhodehouse

13

et al. 2018). In this work, we investigate whether elements of the interactions between PER2 and CK1δ are conserved in *C. elegans* LIN-42 and KIN-20. We show that CK1δ can bind and phosphorylate a LIN-42 substrate *in vitro* and that the conserved CKBD domains are required for a robust interaction. Further, we show that deletion of the SYQ and LT domains, two highly conserved alpha helices within the CKBD, results in upregulation of LIN-42::GFP, but severe developmental abnormalities and molting defects are only observed once those deletions are extended to encompass the whole CKBD.

Results:

LIN-42 SYQ and LT domains are conserved enough to mediate an interaction with CK1δ

PER2 and CK1δ have a very tight and stable binding interaction that allows for phosphorylation of PER2 by CK1δ which is critical for regulating the mammalian circadian clock (Hirano et al. 2016; Crosby and Partch 2020; Aryal et al. 2017; Lee et al. 2001; Liu et al. 2019).   Given that the kinase binding motifs of PER2 are highly conserved in LIN-42 and the extensive sequence identity shared between CK1δ and KIN-20 in their kinase domains, we first set out to determine if KIN-20 binds to and phosphorylates LIN-42 using a biochemical approach. Unfortunately, we have not been able to purify recombinant protein from bacteria or insect cells as KIN-20 has been exclusively insoluble or poorly behaved. However, there is precedent for using mammalian CK1δ on *C. elegans* substrates to provide functional insight into KIN-20 (LaBella et al. 2020). We purified two distinct C-terminal LIN-42

fragments. A 196 amino acid fragment (longC) includes the majority of *lin-42a*, while a smaller, 73 amino acid fragment (shortC) contains only the conserved CKBD (Fig.1.4A, Sup. 1). We performed pull-down assays with biotinylated LIN-42 fragments to test for interaction with KIN-20. LIN-42 longC showed a near stoichiometric interaction between CK1δ and the C-terminus of LIN-42 (Fig. 1.7A). To confirm that this interaction is mediated by the conserved CKBD, which encompasses the SYQ and LT domains of LIN-42, we performed similar pull-down assays using LIN-42 longC fragments lacking either the SYQ domain or the LT domain. Removing either domain resulted in decreased interaction with mammalian CK1δ, although deletion of the LT domain resulted in a slightly larger decrease (Fig. 1.7A). These data suggest that the kinase binding domain of LIN-42 is conserved enough to mediate an interaction with mammalian CK1δ and that both the SYQ and LT domains are necessary for the robustness of this interaction.

CK1δ phosphorylates the C-terminus of LIN-42 *in vitro* and deletion of the SYQ and LT domains (CKBD) results in decreased kinase activity.

To determine if CK1δ binding leads to phosphorylation of the LIN-42 C-terminus, we performed kinase assays using ^{32}P-labeled ATP. CK1δ was able to phosphorylate both LIN-42 constructs; however, significantly higher phosphorylation levels were observed in longC compared to shortC (Fig. 1.7B). These data suggest that the unstructured C terminal tail could help facilitate binding or be a target of CK1δ phosphorylation. ^{32}P-ATP kinase assays were repeated over a 120-minute time course along with substrate titrations to compare CK1δ phosphorylation of longC and mammalian PER2 FASP substrate. CK1δ phosphorylated LIN-42 longC to a similar degree and within the same timeframe as CK1δ phosphorylation of

a PER2 substrate (Fig. 1.6C). Deleting the SYQ domain, LT domain, or both resulted in

decreased phosphorylation of both the longC and shortC LIN-42 constructs in a stepwise

fashion, with the SYQ deletion having the least impact and the double deletion having the

most (Fig. 1.7B,D). Taken together, these data indicate that the CKBD in LIN-42 is conserved

enough to facilitate both binding and phosphorylation by CK1δ and that the SYQ and LT

domains play an important role in mediating this interaction.
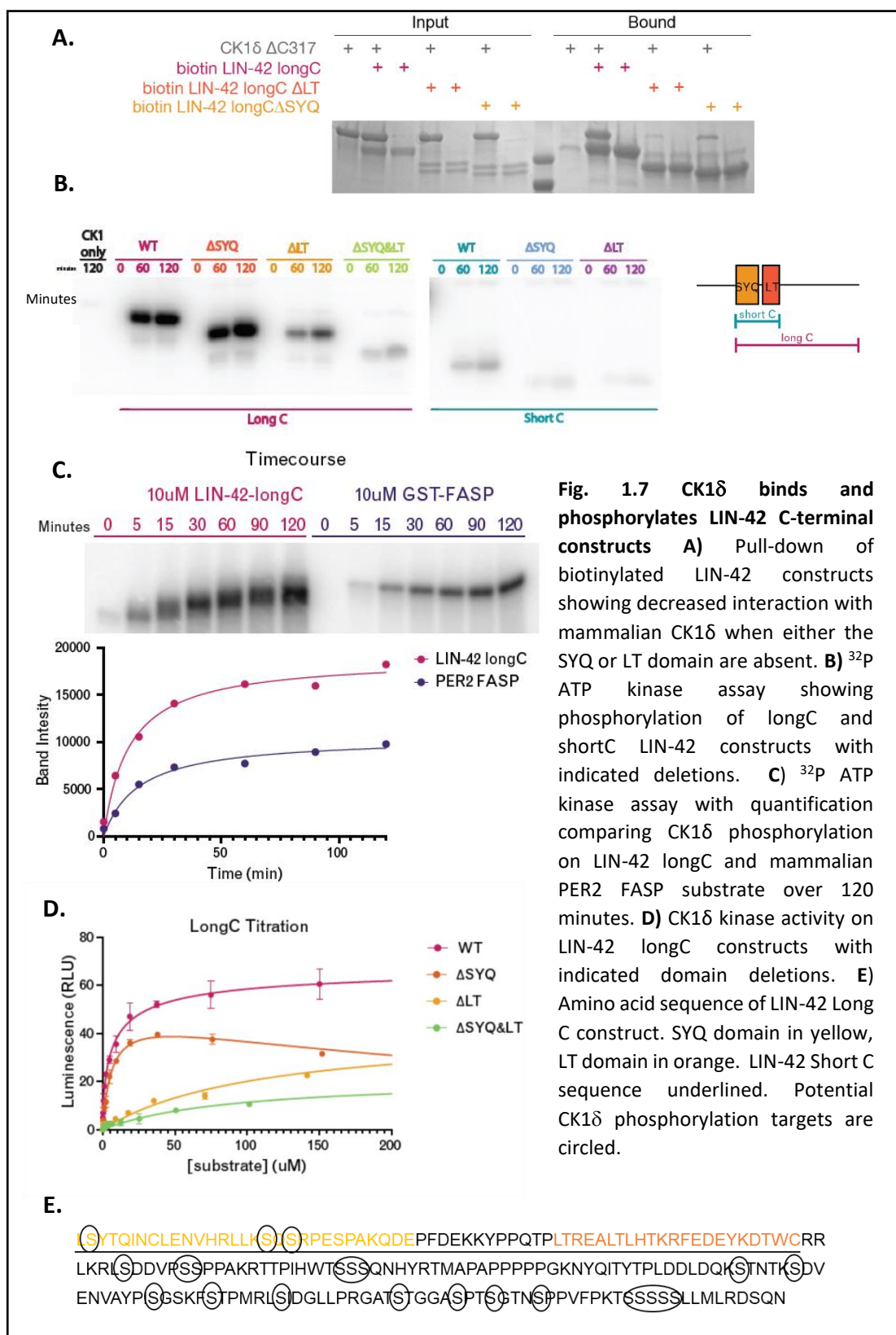
Fig. 1.7 CK1δ binds and phosphorylates LIN-42 C-terminal constructs A) Pull-down of biotinylated LIN-42 constructs showing decreased interaction with mammalian CK1δ when either the SYQ or LT domain are absent. **B)** [32]P ATP kinase assay showing phosphorylation of longC and shortC LIN-42 constructs with indicated deletions. **C)** [32]P ATP kinase assay with quantification comparing CK1δ phosphorylation on LIN-42 longC and mammalian PER2 FASP substrate over 120 minutes. **D)** CK1δ kinase activity on LIN-42 longC constructs with indicated domain deletions. **E)** Amino acid sequence of LIN-42 Long C construct. SYQ domain in yellow, LT domain in orange. LIN-42 Short C sequence underlined. Potential CK1δ phosphorylation targets are circled.

CKBD deletion mutations cause drastic upregulation of LIN-42::GFP expression in *C. elegans* but do not cause developmental or molting defects

To gauge the physiological importance of the conserved CKBD in *C. elegans* we used CRISPR/Cas9-mediated genome editing to introduce deletion mutations into a *C. elegans* strain containing a GFP::AID::3XFLAG tag at the 3' end of the endogenous *lin-42* locus. We refer to this parent strain as **lin-42::GFP** for the remainder of this document. We deleted a nine amino acid portion of the SYQ domain (*lin-42(SYQΔ403-411)*), and a 21 amino acid portion of the LT domain (*lin-42(LTΔ444-464)*). We also made a strain containing both individual deletions (*lin-42(SYQΔ403-411)*, *(LTΔ444-464)*) (Fig. 1.8A, Sup. Fig. 1). Note that the portion of the SYQ domain removed from our mutant is smaller than the portion removed from the constructs described above. We initially made this smaller deletion as we tried to make the most efficient edit based on PAM orientation and predicted crRNA efficiency (Farboud and Meyer 2015; Farboud et al. 2019; Concordet and Haeussler 2018). Given the lack of impact this deletion had *in vitro*, it has not been a priority to generate a new ΔSYQ mutant that is more closely equivalent. We observed a small but statistically relevant extension in the length of time these mutants spent in larval stages and a small but statistically relevant extension in the length of each molt compared to *lin-42::GFP* (Fig. 1.9 C,D, Sup. Fig. 2). The double mutant exhibited a relatively more severe delay that persisted through all four larval stages and all 4 molts while *lin-42(SYQΔ403-411)* and *lin-42(LTΔ444-464)* mutants exhibited relatively shorter extensions observed only in the first and fourth molts and larval stages. We observed no other significant developmental abnormalities in our *lin-42(SYQΔ403-411)*, *lin-42(LTΔ444-464)*, or *lin-42(SYQΔ403-411)*, *(LTΔ444-464)*

mutants. All reached adulthood within three days of hatching when cultured at 20°C and exhibited no significant molting defects, larval arrest, or egg laying defects (Fig. 1.8, 1.9, Sup. Fig 2).

Interestingly, we did observe an increase in LIN-42::GFP expression throughout larval development (Fig. 1.8E). Deleting the SYQ domain resulted in a slight but noticeable increase in LIN-42::GFP expression compared to wild type. By contrast, the LT deletion and the double deletion caused a significant upregulation of LIN-42::GFP, indicating that these domains play some role in regulating LIN-42 levels *in vivo.* Surprisingly, this upregulation of LIN-42 did not correspond to developmental phenotypes associated with previously described *lin-42* mutants. It is worth noting that we did see a slight developmental delay and a small percentage of dumpy adults in *lin-42::*GFP, presumably because the large GFP tag interferes with *lin*-42 activity. As expected, equivalent mild phenotypes were observed in mutants made in this background. For this reason, all mutant strains described in the remainder of this document were made in both wild type and *lin-42*::GFP backgrounds, and phenotypic analyses were performed on mutants in a wild-type background. The *lin-42*::GFP background was used primarily to test the impact of a given mutation on LIN-42::GFP expression.

Deletion of the entire CKBD results in developmental delay and asynchronous molts but does not noticeably affect LIN-42::GFP expression

We used a serial truncation approach to identify regions of the *lin-42* locus necessary for its function in regulating and coordinating molting and developmental timing. We first deleted 194 amino acids from the C-terminal end of LIN-42 relative to the sequence of isoform B

(*lin-42(C-termΔ398-591)*)) in a wild-type background, essentially removing all of *lin-42a* (Fig. 1.8A, Sup. Fig. 1). As expected, severe developmental abnormalities were observed, similar to those previously described in *lin-42a* mutants (Monsalve et al. 2011). Over 40% of *lin-42(C-termΔ398-591)* mutants failed to complete the fourth molt or reach adulthood by day three after hatching when cultured at 20°C, compared to 100% of wild-type animals (Fig. 1.8B, 1.9A,B). Interestingly, the first three molts, while delayed and extended, occurred synchronously. The fourth larval stage and the fourth molt were severely extended in of *lin-42(C-termΔ398-591)* mutants, and just over 40% of animals did not complete a fourth molt after 84 hours (Fig. 1.9B-D, Sup Fig. 2). Of those that did reach adulthood, nearly 40% were egg laying defective, exhibiting a bag-of-worms phenotype and the remaining 60% that became reproductively competent adults, had significantly smaller average brood sizes compared to wild-type animals (Fig. 1.8C,D).

We then focused our truncation approach on the conserved CKBD region to determine whether disruption of the sequence necessary for kinase binding and phosphorylation of LIN-42 resulted in the developmental delay and molting phenotypes we observed. Since deletion of individual SYQ and LT domains resulted in only mild developmental delay, we expanded that deletion to encompass the entire conserved CKBD region, including the short unstructured region between the SYQ and LT domains (*lin-42(CKBDΔ398-462)*)) (Fig. 1.8A, Sup. Fig. 1). Expanding the CKBD deletion resulted in developmental defects similar to our *lin-42(C-termΔ398-591)* mutants but with some key differences. We observed a similar developmental delay, with just under 45% of animals failing to reach adulthood by day three at 20°C after hatching, and a similar, but slightly less severe extensions in the length of each

molt. *lin-42(CKBDΔ398-462)* mutants exhibited asynchronous molt patterns beginning at the L2 molt and persisting until adulthood. We observed significantly less penetrant egg-laying defects, with only 4% of animals presenting with a bag of worms phenotype. *lin-42(CKBDΔ398-462)* animals that reached adulthood had significantly lower average brood sizes compared to wild type, similar to *lin-42(C-termΔ398-591)* mutants.

Figure 1.8: Deletions of the conserved CKBD region results in developmental abnormalities.
A) Schematic of deletions made in *lin-42.* Residue numbers correspond to LIN-42B isoform. Individual SYQ and LT deletions (*lin-42(SYQΔ403-411), lin-42(LTΔ444-464)*, and *lin-42(SYQΔ403-411), (LTΔ444-464)*)) were generated only in a *lin-42*::GFP background. *lin-42(CKBDΔ398-462)* and *lin-42(C-termΔ398-591)* alleles were generated in both wild-type and *lin-42*::GFP backgrounds. In subsequent images allele names are color coded and abbreviated. Purple *ΔSYQ, ΔLT*, and *ΔSYQ/LT* correspond to *lin-42(SYQΔ403-411), lin-42(LTΔ444-464)*, and *lin-42(SYQΔ403-411), (LTΔ444-464)*. Blue *ΔCKBD* is *lin-42(CKBDΔ398-462)*, and red *ΔC-term is lin-42(C-termΔ398-591).***B)** Percentage of animals that were adults by day 3 after hatching. n>24 for all strains. **C)** Percentage of animals with bag of worms phenotype. n>21 for all strains. C. Average number of live progeny. n>12 for all strains. Statistical significance was determined using a two-tailed unpaired Student's t-test. P < 0.05 was considered statistically significant. **** indicates P < 0.0001 **D)** Representative images of late L3 or early L4 *lin-42::GFP* animals with indicated domain deletions. Animals staged based on germline morphology. Images acquired at 40X magnification, 1000ms exposure, and brightness was increased by 40% post acquisition uniformly for all images.

A.

LIN-42
598 aa
PAS-A | PAS-B | SYQ | LT

LIN-42::GFP
PAS-A | PAS-B | SYQ | LT | GFP | 3X FLAG

SYQΔ403-411
PAS-A | PAS-B | ΔSYQ | LT

LTΔ444-446
PAS-A | PAS-B | SYQ | ΔLT

SYQΔ403-411;
LTΔ444-446
PAS-A | PAS-B | ΔSYQ | ΔLT

CKBDΔ389-462
PAS-A | PAS-B | Δwhole SYQ-LT

C-termΔ398-591
PAS-A | PAS-B | Δwhole C-term

B. Developmental Delay

C. Egg-Laying Defective

D. Brood Size

E.

lin-42::GFP | *lin-42ΔSYQ ::GFP* | *lin-42ΔLT::GFP* | *lin-42 ΔSYQ/LT::GFP* | *lin-42 ΔCKBD::GFP*

40X, 1100ms exposure, Brightness +40%

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Fig. 1.9 Deletions of the conserved *lin-42* CKBD region results asynchronous molts and developmental delay. Indicated mutant alleles were crossed to a strain that expressed luciferase from a single copy integrated transgene (details in table 1). Luciferase is expressed while animals feed and signal drops during the molt when animals stop feeding. (n = 24) for all strains. in all images allele names are color coded and abbreviated. Purple *ΔSYQ, ΔLT*, and *ΔSYQ/LT* correspond to *lin-42(SYQΔ403-411), lin-42(LTΔ444-464)*, and *lin-42(SYQΔ403-411), (LTΔ444-464)*. Blue *ΔCKBD* is *lin-42(CKBDΔ398-462)*, and red *ΔC-term is lin-42(C-termΔ398-591)***A)** Bar plot showing number of molts observed in animals with specified allele over 84hrs. Empty wells and unreadable traces labeled as undefined. **B)** Heat maps representing luciferase activity in specified alleles. Grey represents feeding animals, Black represents lethargus. Each line represents an individual animal **C/D/E)** Boxplots of molt, intermolt, and larval stage durations in specified strains. Period is represented in hours.

C. Molt Duration

D. Intermolt Duration

E. Larval Stage Duration

Legend:
- *lin-42*::GFP
- *lin-42ΔSYQ::GFP*
- *lin-42ΔLT::GFP*
- *lin-42ΔSYQ/LT::GFP*
- *wild type*
- *lin-42ΔCKBD*
- *lin-42ΔC-term*
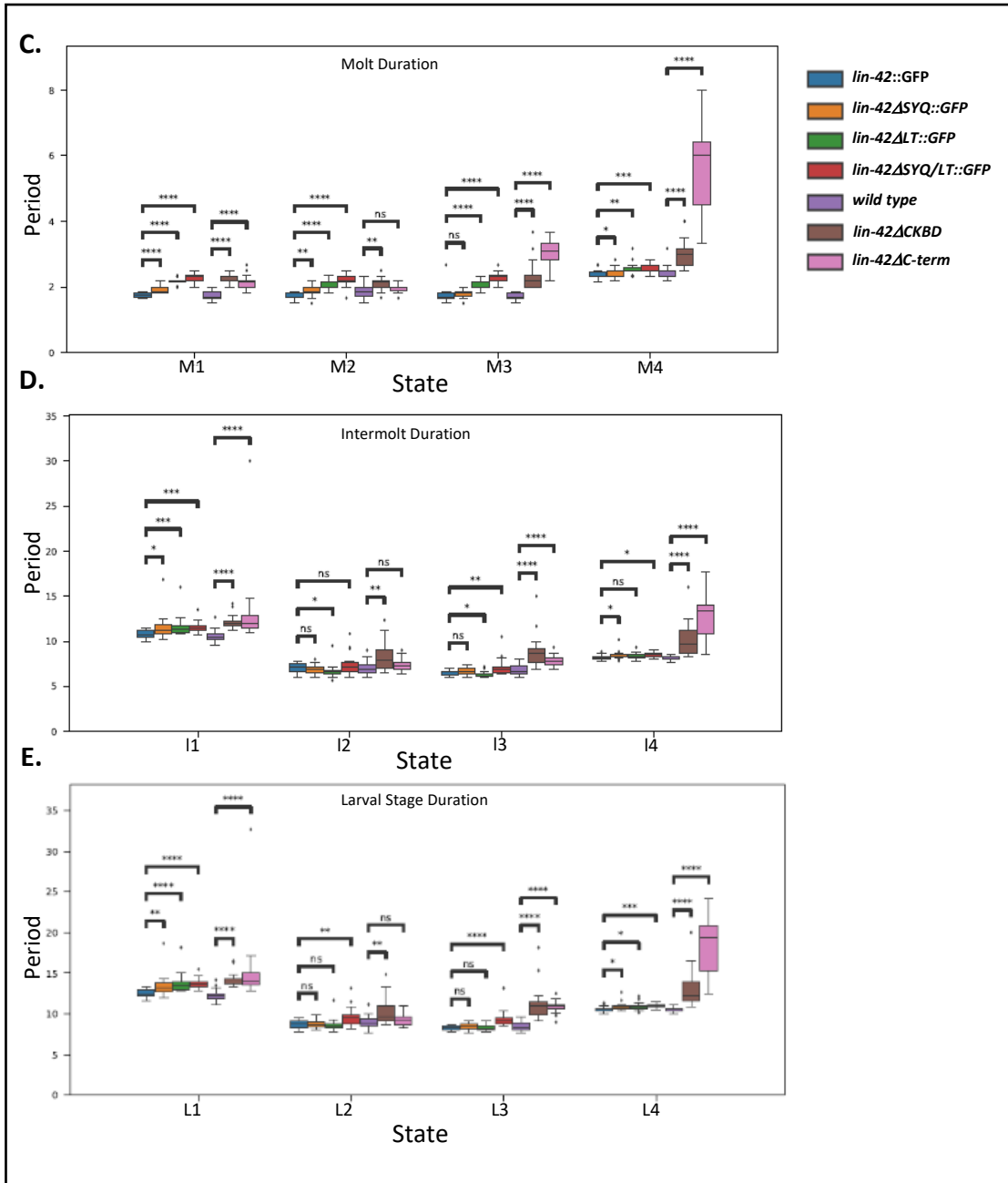
Discussion

To determine the mechanisms through which LIN-42 regulates and coordinates developmental timing in *C. elegans*, we looked to the well-studied mechanisms employed by its mammalian homolog PER2. The striking similarities between LIN-42 and PER2 and the extensive sequence identity shared between their cognate kinases lead us to consider the possibility that some aspects of these timekeeping mechanisms may also be conserved. The ~24-hour period of the circadian clock depends on a tight and stable binding interaction between PER2 and CK1δ that allows for CK1δ kinase activity on PER2. Here we show that CK1δ binds and phosphorylates a LIN-42 substrate *in vitro* with similar kinetics compared to a PER2 substrate and that the SYQ and LT domains are required for a robust interaction. Further, we show that deletion of the SYQ and LT domains, two highly conserved sequences that are predicted to take on alpha helical structure within the CKBD, results in dysregulation of LIN-42::GFP expression *in vivo*. However, severe developmental abnormalities and molting defects are only observed once those deletions are extended to encompass the whole CKBD.

Our *in vitro* work indicated that removing the SYQ and LT domains disrupts the interaction between CK1δ and LIN-42 and decreases CK1δ kinase activity on LIN-42 (Fig. 1.7A,B). These data are in agreement with recently published work demonstrating that deletion of these domains in PER2, decreases CK1δ activity in that system and alters circadian activity. (Liu et al. 2019; Marzoll et al. 2022).

When these domains were deleted *in vivo*, presumably disrupting kinase binding, we observed a striking upregulation of LIN-42::GFP expression (Fig 1.8E). These data could suggest that LIN-42 levels depend on CK1δ/KIN-20 kinase activity, similar to PER2 in mammals. Additional work will be necessary to determine whether the increased levels of LIN-42::GFP we observed resulted from an increase in protein stability or an overexpression at the level of transcription or translation. Upregulation of *lin-42* mRNA was not observed in a *kin-20* null mutant, however, suggesting that the upregulation we observed in LIN-42::GFP protein levels may be posttranscriptional (Rhodehouse et al. 2018).

Still perplexing is the question of why the upregulation we saw in our *lin-42(SYQ∆403-411)* and *lin-42(LT∆444-464)* mutants did not obviously impact developmental timing or larval progression. This result is surprising considering previous reports that forced expression of *lin-42a* from an integrated array driven by a heat shock promoter resulted in anachronistic molts and lethargy (Monsalve 2011). It is possible that LIN-42 levels in our mutants, while increased in comparison to control animals, may not reach a particular threshold of overexpression required to disrupt developmental timing. Another possibility is that there is some sort of mechanism to maintain LIN-42 activity at certain levels and mutations that reduce LIN-42 activity may trigger upregulation to compensate. We attempted to attenuate LIN-42 levels using RNAi and auxin systems to see if reducing LIN-42 levels in these mutants would result in developmental phenotypes, but our results were unclear (data not shown). Overexpression of LIN-42 in wildtype and mutant backgrounds is another avenue we could pursue to answer these questions. Further work will be necessary to fully understand this discrepancy. Interestingly, when we expanded our *in vivo* deletions to encompass the whole

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CKBD, LIN-42::GFP expression appeared to decrease slightly throughout larval development compared to controls (Fig. 1.8E). Further, we observed developmental abnormalities and asynchronous molt patterns similar to those observed when the entire *lin-42a* isoform was deleted (Fig 1.8B-D, Fig. 1.9A-E). These data indicate that an intact CKBD is essential for *lin-42* function in coordinating developmental timing, further indicating a role for kinase binding and the phosphorylation state of LIN-42.

The next steps in this work will require linking the phosphorylation state of LIN-42 to the phenotypes we observe and to KIN-20 specifically. We plan to map potential phosphorylation sites on the C-terminal end of LIN-42 through mass spectrometry and then test the impact of mutations in these sites. PER2 stability is maintained through a phospho-switch mechanism. CK1 phosphorylation of a phospho-degron region results in ubiquitination and degradation of PER2 (Hirano et al. 2016; Crosby and Partch 2020; Zhou et al. 2015), while phosphorylation of the FASP region results in stabilization of PER2 (Toh et al. 2001; Philpott et al. 2020). The FASP region includes a series of five serine residues that are consecutively phosphorylated (Narasimamurthy et al. 2018). CK1δ phosphorylation of the first 'priming' residue in this series results in the obligately sequential phosphorylation of downstream residues. We observe a cluster of serine residues between the SYQ and LT domains of LIN-42 that may be especially important given that they are located in an area left intact in our individual SYQ and LT domain deletions and that there is evidence suggesting these sites regulate CK1 activity in mammalian systems. (Philpott et al. 2022).

Some unanswered questions remain that could be interesting to explore further. In our serial truncation approach, we began by deleting the entire C-terminus and the entire PAS-

containing region (data not shown). In the circadian clock, the tandem PAS domains allow

dimerization of PER proteins and are therefore critical for circadian rhythms (Yagita et al.

2000; Kucera et al. 2012; Hennig et al. 2009). Preliminary data suggested that LIN-42 PAS-B

folds similarly to PER2 and that LIN-42 dimerizes *in vitro* (Data not shown). However,

deletion of PAS-A and B from LIN-42 did not have any significant impact on developmental

progression that we observed. We did not investigate whether they exhibit the subtle but

expected heterochronic phenotypes associated with disruptions in *lin-42c,* but the lack of

severe developmental phenotypes associated with these mutations suggests that

dimerization is not as important for LIN-42 as it is for PER2, or that other LIN-42 domains

promote dimerization. We did notice and note a significant upregulation of LIN-42::GFP

levels similar to our individual *lin-42(SYQ403-411)/ (LT444-464)* mutants. I am currently

working to generate a *lin-42(C-termΔ398-591)* mutant in a *lin-42::GFP* background to

compare levels of LIN-42::GFP and will continue to generate all future strains in both to

continue this structure/function approach.

While it is likely we will uncover similarities in these timing mechanisms, there are sure to

be differences as well. A key characteristic of the core circadian clock in mammals is that the

rhythmic gene expression patterns are achieved by a feedback mechanism that is strongly

influenced by PER2 levels (Chen et al. 2009). This doesn't appear to be the case in *C.

elegans*. *lin-42* mRNA continued to oscillate in mutants with non-functional LIN-42 (Jeon et

al., 1999), and upregulation of LIN-42::GFP did not, in itself, result in severe perturbations in

developmental timing or molting. It is clear, however, that the kinase binding domain, and

therefore, the phosphorylation state of LIN-42, is essential for LIN-42 function, but it does

not appear that LIN-42 levels largely impact developmental timing. These differences make

sense given that the circadian clock controls cyclical changes in behavior and physiology

with an invariant ~24-hour period that is not affected by temperature (Bass and Takahashi,

2010; Buhr et al. 2010; Pittendrigh, 1954; Sweeney and Hastings, 1960). The circadian clock

requires such a feedback mechanism to ensure the fidelity of these ~24-hour periods every

single day for the life of an organism. *C. elegans* developmental timing, on the other hand,

controls a linear progression of cell fate patterns over a set time, coordinated with a finite

number of molts. The molt cycles are similar to circadian cycles, in that they are controlled

by rhythmic gene expression programs, but they are not temperature compensated and the

intermolt periods can be variable. A wild-type population will molt synchronously, but the

intermolt periods change in response to environmental cues such as temperature and

nutrient availability, to match the speed at which animals are progressing through larval

stages. Interestingly, there are reports of circadian rhythms in adult *C. elegans* (Simonetta et

al. 2009; Hasegawa et al. 2005; Edgar et al. 2012; De Temmerman et al. 2011), with one

report linking *lin-42* (Simonetta et al. 2009). It would be interesting to further investigate

the role of *lin-42* and potentially *kin-20* within that mechanism.

Supplemental Figures:

SYQ domain = DPPLSYTQINCLENVHRLLKSQSRPESPAKQDE   in orange
LT domain = QTPLTREALTLHTKRFEDEYKDTWC   in blue
Constructs (aka purified proteins sequences) are in yellow

>LIN-42 isoform b protein sequence with shortC construct highlighted (402-475)

LIN-42
598 aa

MEPAGHSSATHNIVVPNANPTQPQPLAPAMREEGATLSPPNTWSSSSVEFLDDADDNRLLFTCTFTLP
HGTVLSSATYADGFHEQYLTIGDNFLARLEPKGQSFILSAAAASVKQRIFARVTMPDGALRACELLCEF
ETDRAKITVLALRSAFSLQASHVSSNFHVFTFITKHSSTCALTHIDYASIPYLGLLPTDLIGKSLLAFVYSP
DVHVVRQAHIDLHNSRGKIVKSIADLRLVAHNGSILRCQTEWSAYVNPWTRKMELVVARHRICSLPIG
DSDVISSPPPGIQSNTLPPVMAKTFEDELRTIMNKPVPSTSRHSHHHHHSSLKDQNQGFPANIDLGAYI
DKIVEQLVVNSTAQQQQKVAVAAAAAAQAAQAAVVATAQIRKVASAPPTTSTDPPLSYTQINCLENV
HRLLKSQSRPESPAKQDEPFDEKKYPPQTPLTREALTLHTKRFEDEYKDTWCRRLKRLSDDVPSSPP
AKRTTPIHWTSSSQNHYRTMAPAPPPPPGKNYQITYTPLDDLTDQKSTNTKSDVENVAYPISGSKFST
PMRLSIDGLLPRGATSTGGASPTSGTNSPPVFPKTSSSSSLLMLRDSQN

>LIN-42 isoform b protein sequence with longC construct highlighted (402-598)

LIN-42
598 aa

MEPAGHSSATHNIVVPNANPTQPQPLAPAMREEGATLSPPNTWSSSSVEFLDDADDNRLLFTCTFTLP
HGTVLSSATYADGFHEQYLTIGDNFLARLEPKGQSFILSAAAASVKQRIFARVTMPDGALRACELLCEF
ETDRAKITVLALRSAFSLQASHVSSNFHVFTFITKHSSTCALTHIDYASIPYLGLLPTDLIGKSLLAFVYSP
DVHVVRQAHIDLHNSRGKIVKSIADLRLVAHNGSILRCQTEWSAYVNPWTRKMELVVARHRICSLPIG
DSDVISSPPPGIQSNTLPPVMAKTFEDELRTIMNKPVPSTSRHSHHHHHSSLKDQNQGFPANIDLGAYI
DKIVEQLVVNSTAQQQQKVAVAAAAAAQAAQAAVVATAQIRKVASAPPTTSTDPPLSYTQINCLENV
HRLLKSQSRPESPAKQDEPFDEKKYPPQTPLTREALTLHTKRFEDEYKDTWCRRLKRLSDDVPSSPP
AKRTTPIHWTSSSQNHYRTMAPAPPPPPGKNYQITYTPLDDLTDQKSTNTKSDVENVAYPISGSKFST
PMRLSIDGLLPRGATSTGGASPTSGTNSPPVFPKTSSSSSLLMLRDSQN

Supplemental Figure 1. Protein sequence of LIN-42B used to make constructs and mutants strains. A/B) Protein coding sequence of LIN-42B. SYQ domain is colored orange and LT domain is colored blue. Whole conserved kinase binding domain is underlined. Highlighted in yellow are the residues included in LIN-42 constructs used for biochemical assay. **C/D/E)** Protein coding sequence of LIN-42B. SYQ domain is colored orange and LT domain is colored blue. Whole conserved kinase binding domain is underlined. Highlighted in yellow are the residues that were deleted in mutant strains.

```
SYQ domain = DPPLSYTQINCLENVHRLLKSQSRPESPAKQDE        in orange
LT domain = QTPLTREALTLHTKRFEDEYKDTWC                 in blue
Deletions made highlighted in yellow
```

>LIN-42 isoform b protein sequence with individual SYQ and LT deletions highlighted

Lin-42ΔSYQ/LT — PAS-A? | PAS-B | ΔSYQ ΔLT — **SYQΔ(403-411), (ΔLT(444-446)**

MEPAGHSSATHNIVVPNANPTQPQPLAPAMREEGATLSPPNTWSSSSVEFLDDADDNRLLFTCT
FTLPHGTVLSSATYADGFHEQYLTIGDNFLARLEPKGQSFILSAAAASVKQRIFARVTMPDGALR
ACELLCEFETDRAKITVLALRSAFSLQASHVSSNFHVFTFITKHSSTCALTHIDYASIPYLGLLPTD
LIGKSLLAFVYSPDVHVVRQAHIDLHNSRGKIVKSIADLRLVAHNGSILRCQTEWSAYVNPWTRK
MELVVARHRICSLPIGDSDVISSPPPGIQSNTLPPVMAKTFEDELRTIMNKPVPSTSRHSHHHHH
SSLKDQNQGFPANIDLGAYIDKIVEQLVVNSTAQQQQKVAVAAAAAQAAQAAVVATAQIRKVA
SAPPTTSTDPPLSYTQINCLENVHRLLKSQSRPESPAKQDEPFDEKKYPPQTPLTREALTLHTKR
FEDEYKDTWCRRLKRLSDDVPSSPPAKRTTPIHWTSSSQNHYRTMAPAPPPPPGKNYQITYTPL
DDLTDQKSTNTKSDVENVAYPISGSKFSTPMRLSIDGLLPRGATSTGGASPTSGTNSPPVFPKTS
SSSSLLMLRDSQN

**>LIN-42 isoform b protein sequence with whole SYQ-LT region deletion highlighted**

Δ Lin-42ΔCKBD — PAS-A? | PAS-B | Δwhole SYQ-LT — **CKBDΔ398-462**

MEPAGHSSATHNIVVPNANPTQPQPLAPAMREEGATLSPPNTWSSSSVEFLDDADDNRLLFTCT
FTLPHGTVLSSATYADGFHEQYLTIGDNFLARLEPKGQSFILSAAAASVKQRIFARVTMPDGALR
ACELLCEFETDRAKITVLALRSAFSLQASHVSSNFHVFTFITKHSSTCALTHIDYASIPYLGLLPTD
LIGKSLLAFVYSPDVHVVRQAHIDLHNSRGKIVKSIADLRLVAHNGSILRCQTEWSAYVNPWTRK
MELVVARHRICSLPIGDSDVISSPPPGIQSNTLPPVMAKTFEDELRTIMNKPVPSTSRHSHHHHH
SSLKDQNQGFPANIDLGAYIDKIVEQLVVNSTAQQQQKVAVAAAAAQAAQAAVVATAQIRKVA
SAPPTTSTDPPLSYTQINCLENVHRLLKSQSRPESPAKQDEPFDEKKYPPQTPLTREALTLHTKR
FEDEYKDTWCRRLKRLSDDVPSSPPAKRTTPIHWTSSSQNHYRTMAPAPPPPPGKNYQITYTPL
DDLTDQKSTNTKSDVENVAYPISGSKFSTPMRLSIDGLLPRGATSTGGASPTSGTNSPPVFPKTS
SSSSLLMLRDSQN

>LIN-42 isoform b protein sequence Whole C-term deletion highlighted (398-591)

Lin-42ΔC-term — PAS-A? | PAS-B | Δwhole C-term — **C-termΔ398-591**

MEPAGHSSATHNIVVPNANPTQPQPLAPAMREEGATLSPPNTWSSSSVEFLDDADDNRLLFTCT
FTLPHGTVLSSATYADGFHEQYLTIGDNFLARLEPKGQSFILSAAAASVKQRIFARVTMPDGALR
ACELLCEFETDRAKITVLALRSAFSLQASHVSSNFHVFTFITKHSSTCALTHIDYASIPYLGLLPTD
LIGKSLLAFVYSPDVHVVRQAHIDLHNSRGKIVKSIADLRLVAHNGSILRCQTEWSAYVNPWTRK
MELVVARHRICSLPIGDSDVISSPPPGIQSNTLPPVMAKTFEDELRTIMNKPVPSTSRHSHHHHH
SSLKDQNQGFPANIDLGAYIDKIVEQLVVNSTAQQQQKVAVAAAAAQAAQAAVVATAQIRKVA
SAPPTTSTDPPLSYTQINCLENVHRLLKSQSRPESPAKQDEPFDEKKYPPQTPLTREALTLHTKR
FEDEYKDTWCRRLKRLSDDVPSSPPAKRTTPIHWTSSSQNHYRTMAPAPPPPPGKNYQITYTPL
DDLTDQKSTNTKSDVENVAYPISGSKFSTPMRLSIDGLLPRGATSTGGASPTSGTNSPPVFPKTS
SSSSLLMLRDSQN

Supplemental Figure 2
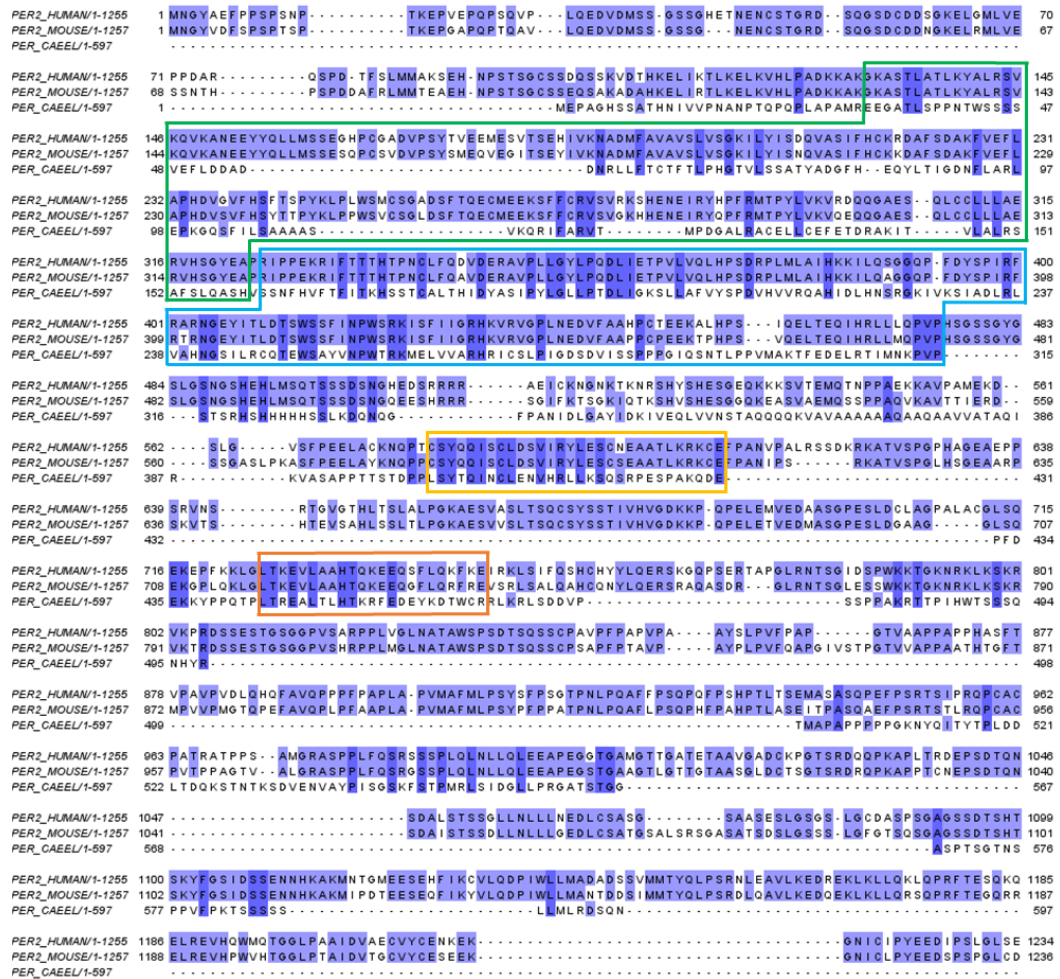
Supplemental Figure 2. Sequence alignment of *C. elegans* LIN-42, mouse PER2 and human PER2. PASA domain is boxed in green. PASB domain is boxed in Blue. SYQ domain is boxed in yellow, and LT domain is boxed in orange. Homologous sequence is highlighted in blue.

Materials and methods:

Strains and Nematode Maintenance

C. elegans were grown and maintained at 20°C and cultures as described (BRENNER 1974), except worms were grown on MYOB agar instead of NGM as described (Church, 1995). Full genotypes of strains used in this work are listed in Table 1. All mutant strains were backcrossed to N2 at least 2X. The Bristol N2 strain was used as wild type.

lin-42::GFP::AID::3XFLAG { lin-42(wrd35[lin-42::GFP^degron:3xFLAG])} parent strain used to observe *lin-42::GFP* expression was generated through self-excising cassette (SEC) selection-based editing as previously described (Dickinson et al. 2015). A single copy transgene was inserted into the 3' end of the endogenous *lin-42* locus. Repair template was injected into EG9615 at 40ng/μl along with 40 ng/μl sgRNA plasmid (pJW1858) + co-injection marker (pCFJ90).

lin-42 mutant strains used in this work were generated by injection of Cas9 or Cas12 ribonucleoprotein complexes as described (Ragle et al. 2022). Specified deletions were made in the endogenous *lin-42* locus, in N2, *lin-42*::GFP::AID::3XFLAG, or both. Repair templates were used at 100ng/μl along with Cas9 or Cas12 at 250ng/μl, crRNA oligos at 60ng/μl, and 10ng/μl co-injection marker (pCFJ90).

In Table 1, we detail the genetic background in which injections were performed and the reagents used to generate each strain.

Strains used in luciferase assays were crosses of mutant strains listed above and a luciferase reporter strain previously described (Milou et al. 2020) HW1993: EG8081; *xeSi312[eft-3p::luc::gfp::unc-54 3'UTR, unc-119(+)] IV*. Crosses and genotypes are detailed in Table 1.

Phenotypic analysis

For all phenotypic analyses, gravid adults were bleached, and embryos were immediately released or singled onto plates seeded with OP50, or gravid adults were picked onto seeded plates and removed after 1-2 hours. Animals were maintained at 20°C and observed daily. Bag-of-worms phenotype was determined when live progeny were observed moving inside adult animals. For brood counts, animals were individually transferred to fresh wells daily for five days after L4 and progeny were counted and averaged. Developmental delay was determined if animals failed to reach adulthood by day 3 after hatching or as determined by length of development in luciferase assays. Statistical significance was determined using a two-tailed unpaired Student's t-test. $P < 0.05$ was considered statistically significant. **** indicates $P < 0.0001$.

Imaging

Synchronized animals were collected from MYOB plates by washing off plates. 1000 µl of M9 + 2% gelatin was added to the plate or well, agitated to suspend animals in M9+gelatin, and then transferred to a 1.5 ml tube. Animals were spun at 700xg for 1 min. The media was then aspirated off and animals were resuspended in 500µl M9 + 2% gelatin with 5 mM levamisole. 12 µl of animals in M9 +gel with levamisole solution were placed on slides with a 2% agarose pad and secured with a coverslip. Images were acquired using a Plan-Apochromat 40x/1.3 Oil DIC lens or a Plan-Apochromat 63x/1.4 Oil DIC lens on an

AxioImager M2 microscope (Carl Zeiss Microscopy, LLC) equipped with a Colibri 7 LED light source and an Axiocam 506 mono camera. Acquired images were processed through Affinity photo software (version: 1.9.2.1035). For direct comparisons of LIN-42::GFP, we set exposure to we set the exposure conditions to avoid pixel saturation of the brightest sample and kept equivalent exposure for imaging of the other samples.

Luciferase assays

Assays were performed and analyzed as described (Milou et al. 2020). Briefly, Gravid adults were bleached, and eggs were immediately singled into wells containing 90 µl OP50/S-Basal/D-Luciferin solution per well and left in Luminometer machine and measured every 10 minutes for 0.05 seconds. In this work, the following differences are notes. The assays were performed for 84 hrs and for statistical analysis, we performed a Wilcoxon-Mann-Whitney test (implemented in the python package SciPy version 1.4.1 as the function mannwhitneyu).

Expression and purification of recombinant proteins

All proteins were expressed from a pET22-based vector in *Escherichia coli* Rosetta (DE3) cells based on the Parallel vector series (Sheffield et al., 1999). All LIN-42 constructs (Long C, residues 402-598; short C, residues 402-475, all ΔSYQ, ΔLT, and ΔBoth mutants) and human FASP peptide were expressed downstream of an N-terminal TEV-cleavable His-NusA tag. Human CK1δ catalytic domains (CK1δ ΔC, residues 1–317) were all expressed with a TEV-cleavable His-GST tag. All proteins expressed from Parallel vectors have an additional N-

terminal vector artifact of 'GAMDPEF' remaining after TEV cleavage. Cells were grown in LB media at 37°C until the O.D.600 reached ~0.8; expression was induced with 0.5 mM IPTG, and cultures were grown for approximately 16–20 hr more at 18°C. Cells were centrifuged at 3,200 x g, resuspended in 50 mM Tris, pH 7.5, 300 mM NaCl, 20 mM imidazole, 5% (vol/vol) glycerol, 1 mM tris(2-carboxyethyl)phosphine (TCEP), and 0.05% Tween-20. For purification of recombinant protein, cells were lysed with a microfluidizer followed by sonication and then the lysate was clarified via centrifugation at 140,500 x g for 1 hour at 4ºC. Ni-NTA affinity chromatography was used to extract his-tagged proteins from the lysate and then the affinity/solubility tags were cleaved using His6-TEV protease overnight at 4ºC. The cleaved protein was then separated from tag and TEV by a second Ni-NTA affinity column and further purified using size exclusion chromatography (SEC) in 50 mM Tris, pH 7.5, 200 mM NaCl, 1 mM EDTA, 5% (vol/vol) glycerol, 1 mM TCEP, and 0.05% Tween-20. Small aliquots of protein were frozen in liquid nitrogen and stored at -70ºC for long-term storage.

***In vitro* biotinylation and pull-down assays**

LIN-42 Long C constructs were biotinylated via Sortase A-mediated reactions between a Sortase A recognition motif peptide (biotin-LPETGG) and our LIN-42 long C (N-terminal G from 'GAMDPEF' artifact). Reactions were carried out in 50 mM Tris pH 7.5 and 150 mM NaCl using 5 µM His6-Sortase A, 300 µM biotin-LPETGG, and 50 µM LIN-42 protein. Ni-NTA affinity chromatography followed by SEC was used to purify labeled protein from His6-Sortase A and excess biotin, respectively. Magnetic Streptavidin beads were used to bind biotinylated LIN-42 long C WT and mutants (final concentration 5uM) in the presence and absence of CK1δ ΔC (final concentration 5uM).

32P-ATP kinase assay

1 μM CK1δ ΔC was incubated with 10 μM LIN-42 (Long C or short C WT/mutants) or 10 μM human FASP peptide in 25 mM Tris pH 7.5, 100 mM NaCl, 10 mM MgCl2, 2 mM TCEP. Reactions were started by the addition of ^{32}P-ATP (final concentration 2 mM) and samples were collected at indicated timepoints and quenched in an equivalent volume of 2x SDS-PAGE loading buffer. Protein labeled with ^{32}P were separated on an a KDTM pre-cast gel (BioRad) and gels were dried for overnight exposure to phosphoscreen. A Typhoon phophoimager was used to visualize exposed gels and ^{32}P-labeled proteins were quantified via densitometry using ImageJ.

ADP-Glo kinase assay

Substrate titrations kinase reactions were performed as done previously (Philpott et al., 2020) using the Promega ADP-Glo kinase assay kit. Reactions were incubated in 25 mM Tris pH 7.5, 100 mM NaCl, 10 mM MgCl$_2$, 20 mM TCEP, and 100 μM ATP for 1 hour at room temperature.

Table 1. List of strains used in this work

Table 1 Strains used in this work

Strain name	*lin-42* mutations	*Genotype*	*Background*	crRNA oligo(s)	repair template	Injected into	outcross	Origin
JDW136	None	*lin-42(wrd35[lin-42::GFP^degron:3xFLAG])*	wt			EG6915	X3	This work
JDW251	*SYQ Δ403-411*	*lin-42(wrd49[SYQ Δ403-411]::GFP::AID::3xFLAG) II*	JDW136	#69_GTCAGTGCTTCTCGGGTCAG TGG	5042	JDW136	X3	This work
JDW248	*LT Δ444-446*	*lin-42(wrd47[LT Δ444-446]::GFP::AID::3xFLAG) II*	JDW136	#16_ TTTG AAGACGAGTACAAGGACACTTGG	5190	JDW136	X3	This work
JDW270	*SYQ Δ403-411; LT Δ444-446*	*lin-42(wrd50[SYQ Δ403-411]::[LT Δ444-446]::GFP::AID::3xFLAG) II*	JDW136	#69_GTCAGTGCTTCTCGGGTCAG TGG	5042	JDW248	X3	This work
JDW335	*CKBD Δ398-462*	*lin-42 (wrd63[Δ398-462]) II*	wt	#143_GAGTGGTGGGTCCGTTGAGG TGG #145_AAGACGAGTACAAGGACACT TGG	6158	N2	X3	This work
JDW336	*CKBD Δ398-462*	*lin-42 (wrd64[Δ398-462]::GFP::AID*::3xFLAG) II*	JDW136	#143_GAGTGGTGGGTCCGTTGAGG TGG #145_AAGACGAGTACAAGGACACT TGG	6158	JDW136	X2	This work
DW130	*C-term Δ398-592*	*lin-42(wrd89[Δ398-592]) II*	wt	#143_GAGTGGTGGGTCCGTTGAGG TGG #144_CCTCCTCTCTCCTAATGCTA CGG	6157	N2	X2	This work

	Strains used in luciferase assays			
Strain name	Deletion	*Genotype*	HW1993 crossed to	Origin
HW1993	'wild type' control	*EG8081, xeSi312 [Peft-3::luc::gfp::unc-54 3' UTR, unc-119(+)] IV*		Milou et al. 2020
JDW408	*lin-42::GFP control*	*lin-42(wrd35[lin-42::GFP^degron:3xFLAG])*	JDW136	This work
JDW416	*SYQ Δ403-411*	*lin-42(wrd49[SYQ Δ403-411]::GFP::AID::3xFLAG); EG8081, xeSi312 [Peft-3::luc::gfp::unc-54 3' UTR, unc-119(+)] IV*	JDW251	This work
JDW415	*LT Δ444-446*	*lin-42(wrd47[LT Δ444-446]::GFP::AID::3xFLAG); EG8081, xeSi312 [Peft-3::luc::gfp::unc-54 3' UTR, unc-119(+)] IV*	JDW248	This work
JDW414	*SYQ Δ403-411; LT Δ444-446*	*lin-42(wrd50[SYQ Δ403-411]::[LT Δ444-446]::GFP::AID*::3xFLAG); EG8081, xeSi312 [Peft-3::luc::gfp::unc-54 3' UTR, unc-119(+)] IV*	JDW270	This work
JDW417	*CKBD Δ398-462*	*lin-42 (wrd63[Δ398-462]); EG8081, xeSi312 [Peft-3::luc::gfp::unc-54 3' UTR, unc-119(+)] IV*	JDW335	This work
JDW437	*C-term Δ398-592*	*lin-42(wrd89[Δ398-592]); EG8081, xeSi312 [Peft-3::luc::gfp::unc-54 3' UTR, unc-119(+)] IV*	JDW130	This work

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Chapter 2

An expanded auxin-inducible degron toolkit for *Caenorhabditis elegans*

[This chapter has been adapted from publication, **An expanded auxin-inducible degron**

toolkit for *Caenorhabditis elegans*, (Ashley et al. 2020) Genetics]

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Abstract

The auxin-inducible degron (AID) system has emerged as a powerful tool to conditionally deplete proteins in a range of organisms and cell types. Here, we describe a toolkit to augment the use of the AID system in *Caenorhabditis elegans*. We have generated a set of single-copy, tissue-specific (germline, intestine, neuron, muscle, pharynx, hypodermis, seam cell, anchor cell) and pan-somatic TIR1-expressing strains carrying a co-expressed blue fluorescent reporter to enable use of both red and green channels in experiments. These transgenes are inserted into commonly used, well-characterized genetic loci. We confirmed that our TIR1-expressing strains produce the expected depletion phenotype for several nuclear and cytoplasmic AID-tagged endogenous substrates. We have also constructed a set of plasmids for constructing repair templates to generate fluorescent protein::AID fusions through CRISPR/Cas9-mediated genome editing. These plasmids are compatible with commonly used genome editing approaches in the *C. elegans* community (Gibson or SapTrap assembly of plasmid repair templates or PCR-derived linear repair templates). Together these reagents will complement existing TIR1 strains and facilitate rapid and high-throughput fluorescent protein::AID tagging of genes. This battery of new TIR1-expressing strains and modular, efficient cloning vectors serves as a platform for straightforward assembly of CRISPR/Cas9 repair templates for conditional protein depletion.

Introduction

The AID system has allowed rapid, conditional, and tissue-specific depletion of tagged proteins in a wide range of organisms and cell types (Nishimura *et al.* 2009; Holland *et al.*

2012; Zhang *et al.* 2015; Natsume *et al.* 2016; Trost *et al.* 2016; Brown *et al.* 2017; Daniel *et al.* 2018; Chen *et al.* 2018; Camlin and Evans 2019). Since its introduction to *C. elegans* (Zhang *et al.* 2015), it has been promptly adopted by the community. This system has allowed for rapid depletion of proteins in tissues that are refractory to RNA interference approaches, such as the germline (Pelisch *et al.* 2017; Shen *et al.* 2018; Zhang *et al.* 2018b), vulval precursor cells (Matus *et al.* 2014), and neurons (Liu *et al.* 2017; Patel and Hobert 2017; Serrano-Saiz *et al.* 2018). The system is also powerful for studying rapid developmental events such as molting (Zhang *et al.* 2015; Joseph *et al.* 2020), organogenesis (Martinez *et al.* 2020), developmental timing (Azzi *et al.* 2020), meiosis (Zhang *et al.* 2015), and spermatogenesis (Ragle *et al.* 2020). Improvements to the auxin ligand have enhanced protein degradation in the embryo (Negishi *et al.* 2019) and removed the need for ethanol solubilization, allowing the auxin derivative to be dissolved in any aqueous buffer (Martinez *et al.* 2020). This water-soluble auxin was shown to be compatible with microfluidic devices, allowing the coupling of long-term imaging and targeted protein depletion (Martinez *et al.* 2020). Auxin-mediated depletion of a spermatogenesis regulator has been developed to conditionally sterilize animals, a valuable approach for the *C. elegans* aging field (Kasimatis *et al.* 2018).

The system is comprised of two components. First, the plant F-box protein Transport Inhibitor Response 1 (TIR1) is expressed under the control of a promoter with a defined expression pattern (Figure 1). TIR1 can then interact with endogenous Skp1 and Cul1 proteins to form a functional SCF E3 ubiquitin ligase complex (Figure 1). Second, an auxin-

inducible degron (AID) sequence from the IAA17 protein is fused to a protein of interest (Figure 1) (Nishimura *et al.* 2009; Natsume and Kanemaki 2017). Addition of the plant hormone, auxin, promotes TIR1 binding to the degron, leading to the ubiquitination and subsequent proteasome-mediated degradation of the degron-tagged protein (Figure 1). While the full length IAA17 sequence is 229 amino acids, minimal AID tags of 44 amino acids (AID*) and 68 amino acids (mAID) have been developed (Morawska and Ulrich 2013; Li *et al.* 2019). In *C. elegans,* the *Arabidopsis thaliana* TIR1, AID*, and mAID sequences are used (Zhang *et al.* 2015; Negishi *et al.* 2019), as this plant grows at a temperature range more similar to *C. elegans*, whereas rice (*Oryza sativa*)-derived sequences are used in other systems (Nishimura *et al.* 2009; Natsume *et al.* 2016; Natsume and Kanemaki 2017). The SCF ligase in *C. elegans* with which TIR1 interacts is thought to be comprised of SKR-1/2, CUL-1, and RBX-1 (Martinez *et al.* 2020).

Here, we describe a new set of strains and reagents for *C. elegans* that complement the tools originally described by Zhang *et al.* (2015). To enable both targeted protein degradation and imaging-based measurement of depletion, we have generated a set of strains that express single-copy, tissue-specific TIR1 marked with a reporter of activity (*TIR1::F2A::mTagBFP2::AID*::NLS*). Nuclear-localized mTagBFP2::AID* (hereafter referred to simply as "BFP") serves as a reporter for TIR1 expression. TIR1 should degrade BFP in the presence of auxin, providing a built-in read-out of TIR1 activity. For many of the tissue-specific promoters driving this construct, we have created strains with insertions into well-characterized, neutral target sites on both chromosomes I and II (Frøkjaer-Jensen *et al.* 2008; Frøkjær-Jensen *et al.* 2012), to facilitate crossing schemes. These strains expand

experimental possibilities with green and red fluorescent proteins (FPs) of interest. We have

also generated constructs to introduce FP::AID* tags into genes of interest using

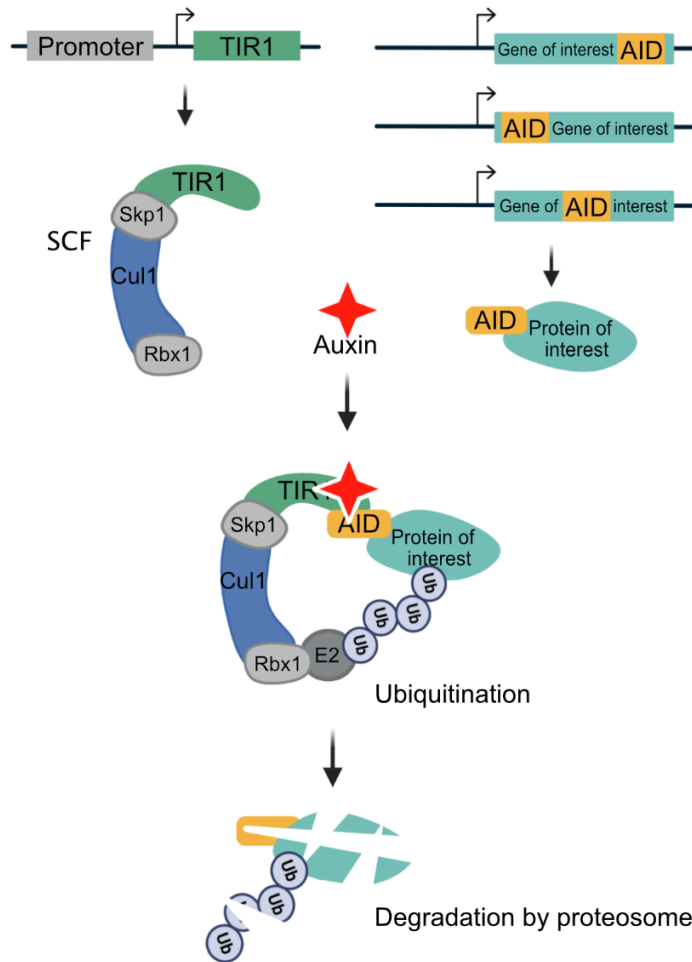conventional genome editing approaches in *C. elegans*.

Figure 1. Schematic of the auxin-inducible degron (AID) system. The plant F-box protein TIR1 is expressed using a promoter of interest with a desired spatiotemporal expression pattern. TIR1 interacts with endogenous Skp1 and Cul1 proteins to form an SCF E3 ubiquitin ligase complex. An auxin-inducible degron sequence (AID) is fused to a protein of interest. In the presence of the plant hormone auxin, TIR1 recognizes and binds the AID sequence, leading to ubiquitination and subsequent degradation of the AID-tagged protein. We use a minimal, 44 amino acid degron sequence (AID*), but a full-length 229 amino acid AID tag or a 68 amino acid mini-AID (mAID) are used in other systems. In *C. elegans*, the system is frequently used with single-copy TIR1 transgenes inserted into neutral loci, and AID* knock-ins into genes of interest, though extrachromosomal arrays can also be used.

Results:

A new TIR1 transgene with a built-in BFP::AID*::NLS reporter

The initial description of the AID system in *C. elegans* used an mRuby2 fusion to monitor the

expression of TIR1 (Zhang *et al.* 2015). This feature was useful to monitor TIR1 localization

and expression level in comparison to depletion of GFP::AID* tagged proteins. One

limitation is that the mRuby2 fusion interferes with the imaging of factors tagged with red

FPs. Blue fluorescent proteins offer an appealing alternative as reporters for TIR1

expression, since their emission spectra do not significantly overlap with commonly used

green and red FPs (Lambert 2019). To report TIR1 expression and activity, we placed an

F2A::BFP::AID::NLS* reporter downstream of the TIR1 transgene and put the transgene

under the control of a *sun-1* promoter, which drives germline and embryo expression

(Figure 2A). We used an F2A ribosome skip sequence to allow separate TIR1 and

BFP::AID*::NLS proteins to be produced from the mRNA transcript (Ryan and Drew 1994;

Ryan *et al.* 1999; Donnelly *et al.* 2001; de Felipe *et al.* 2003; Ahier and Jarriault 2014).

Our *sun-1p::TIR1::F2A::BFP::AID*::NLS* transgene was introduced in single copy through

CRISPR/Cas9 editing and self-excising cassette (SEC) selection into the same neutral locus

where MosI transposon was inserted in ttTi4348 (Dickinson *et al.* 2015). The SEC strategy

(Dickinson *et al.* 2015) first produces hygromycin resistant, rolling animals, which is useful

for tracking the allele phenotypically in crosses. The loxP-flanked SEC is then excised by heat

shock, producing the final strain with wild type locomotion. As expected, this *sun-1p*

construct drives nuclear-localized BFP in the germline and embryos, confirming the

expression of the transgene. We confirmed TIR1 activity by placing adult animals on 1 mM

auxin and observing loss of BFP::AID*::NLS (Figure 2B). To test the performance of our new

TIR1 transgene, we compared depletion of mNeonGreen (mNG)::AID*::PAR-3 using the *sun-1p::TIR1::F2A:::BFP::AID*::NLS* transgene and the original *sun-1p::TIR1::mRuby2* transgene

(Zhang *et al.* 2015). In the absence of auxin, comparable PAR-3 fluorescence was observed

(Figure 2C,D). Similarly, exposure to 1 mM auxin resulted in a comparable loss of

fluorescence (Figure 2D). Moreover, 100% of auxin-treated embryos exhibited symmetric

cell division which is the expected *par-3* loss-of-function phenotype (Figure 2C). Together,

these results indicate that our *TIR1::F2A::BFP::AID*::NLS* transgene can effectively deplete

AID*-tagged PAR-3, a cytoplasmic cell polarity determinant.

We planned to insert our constructs into the sites in chromosomes I and II, respectively,

where the ttTi4348 and ttTi5605 transposons are inserted for MosSCI-based genome editing

(Frøkjaer-Jensen *et al.* 2008; Frøkjær-Jensen *et al.* 2012). To facilitate genome editing, we

created sgRNA and Cas9+sgRNA vectors targeting these loci containing the "Flipped and

extended (F+E)" sgRNA modifications that improved editing efficiency in mammalian cells

and *C. elegans* (Chen *et al.* 2013; Ward 2015) (Table 1). We generated sgRNA expression

vectors using the two commonly used U6 promoters in *C. elegans* as it is currently unclear

whether one promoter is broadly more active (Friedland *et al.* 2013; Dickinson *et al.* 2013;

Schwartz and Jorgensen 2016) (Table 1). We also generated sgRNA vectors targeting the

cxTi10882 insertion site on chromosome IV to support future knock-ins into this locus

(Frøkjaer-Jensen *et al.* 2008; Frøkjær-Jensen *et al.* 2012).

Figure 2. A new TIR1 expression system allows assessment of TIR1 expression and activity. A) The new TIR1 expression construct contains a *TIR1::F2A::BFP::AID*::NLS* transgene cassette. An F2A skip sequence results in expression of two separate protein products: 1) TIR1, which will interact with endogenous SCF proteins to produce an E3 ubiquitin ligase complex and can only bind the AID sequence in the presence of auxin; and 2) an AID*-tagged BFP protein with a c-Myc nuclear localization signal (NLS) that functions as a readout for TIR1 expression and internal control for TIR1 activity. The use of BFP as a reporter makes this construct compatible with simultaneous green and red FP imaging. B) Adult animals expressing *sun1p::TIR1::F2A::BFP::AID*::NLS*. A control animal expresses AID*-tagged BFP in the nuclei of germline and embryonic cells (white arrows). When animals are exposed to 1 mM auxin, BFP expression is undetectable. BFP channel and DIC images are provided for each condition. Note that the fluorescence signal at the lower right-hand side of each BFP image is due to intestinal autofluorescence. Scale bars represent 50 μm. C) Images of embryos harboring endogenously-tagged mNG::AID*::PAR-3 and expressing either *sun-1p::TIR1::mRuby2* (Zhang et al. 2015) or *sun-1p::TIR1::F2A::BFP::AID*::NLS* (this study). Both TIR1 transgenes were able to deplete mNG::AID*::PAR-3 to background levels in the presence of auxin and produced a symmetric first division as expected for loss of PAR-3 function. D) Quantification of whole-embryo mNG::AID*::PAR-3 fluorescence intensity for the indicated conditions. Wild-type (N2) embryos were measured to account for autofluorescent background. Data points represent the fluorescence intensity from individual embryos; at least six embryos were imaged for each condition. The horizontal red bar depicts the mean for each condition.

A

B

sun-1p::TIR1::2A::BFP

C

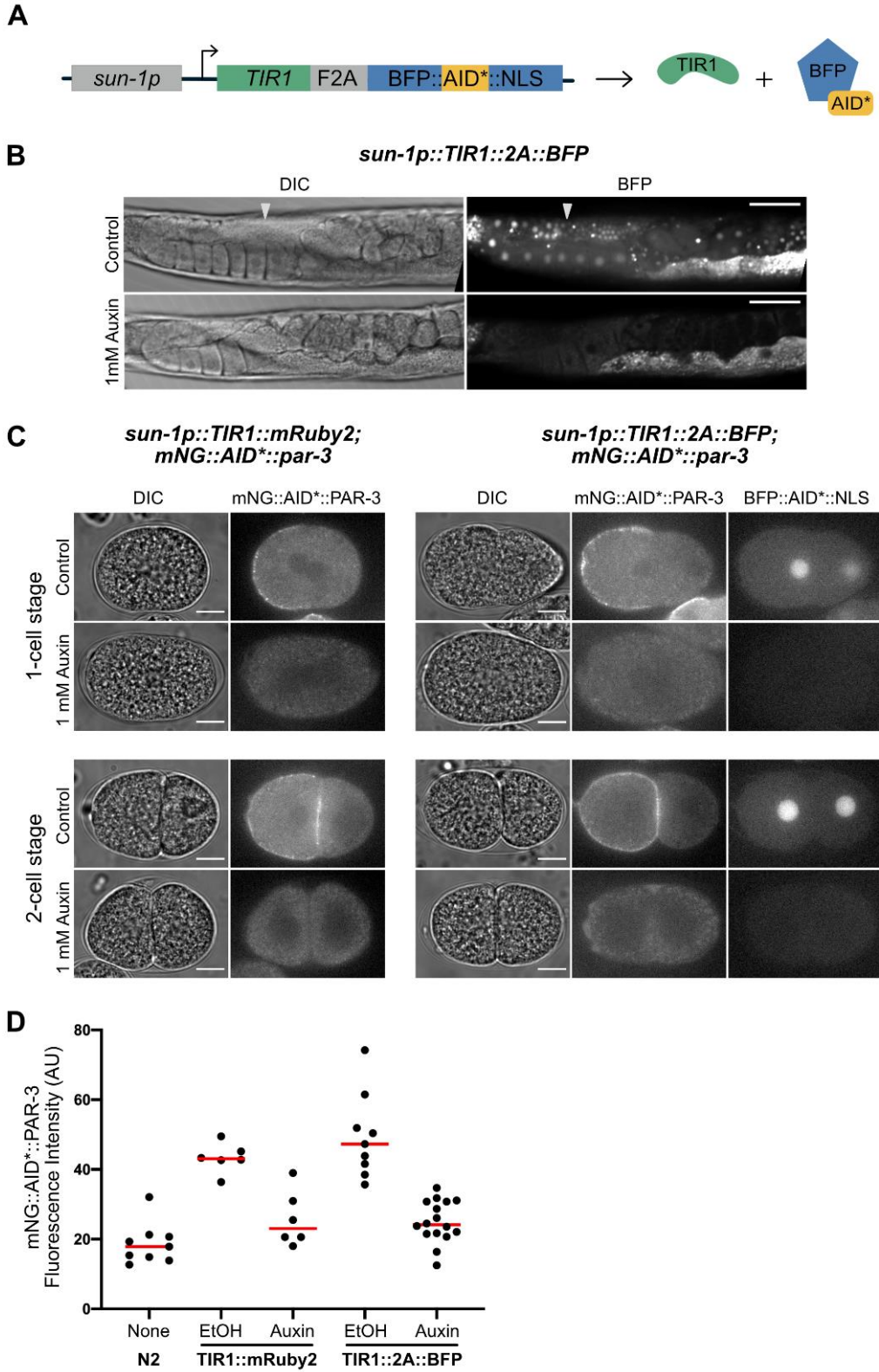| *sun-1p::TIR1::mRuby2; mNG::AID*::par-3* | *sun-1p::TIR1::2A::BFP; mNG::AID*::par-3* |

D

Table 1. Plasmids to support generation of new TIR1 alleles and other transgenes at standardized genetic loci through CRISPR/Cas-based genome editing.

Plasmid name	Description	Purpose
pJW1838	SapTrap sgRNA (F+E) vector, *K09B11.2* U6 promoter and 3'UTR	Creating new sgRNA (F+E) plasmids)
pJW1839	SapTrap sgRNA (F+E) vector, *R07E5.16* U6 promoter	Creating new sgRNA (F+E) plasmids)
pJW1836	promoterless *SV40 NLS::mScarlet-I (dpi)::PEST::tbb-2* 3'UTR vector	Promoter reporter, test new tissue-specific promoters
pJW1841	promoterless *SV40 NLS::mScarlet-I (dpi)::tbb-2* 3'UTR vector	Promoter reporter, test new tissue-specific promoters
pJW1849	ttTi4348 site targeting sgRNA (F+E) with *K09B11.2 U6* promoter and 3'UTR	CRISPR/Cas9 editing of ttTi4348 insertion site
pJW1850	ttTi5605 site targeting sgRNA (F+E) with *K09B11.2 U6* promoter and 3'UTR	CRISPR/Cas9 editing of ttTi5605 insertion site
pJW1851	cxTi10882 site targeting sgRNA (F+E) with *K09B11.2 U6* promoter and 3'UTR	CRISPR/Cas9 editing of cxTi10882 insertion site
pJW1882	ttTi4348 site targeting sgRNA (F+E) with *R07E5.16 U6* promoter and 3'UTR	CRISPR/Cas9 editing of ttTi4348 insertion site
pJW1883	ttTi5605 site targeting sgRNA (F+E) with *R07E5.16 U6* promoter and 3'UTR	CRISPR/Cas9 editing of ttTi5605 insertion site
pJW1884	cxTi10882 site targeting sgRNA (F+E) with *R07E5.16 U6* promoter and 3'UTR	CRISPR/Cas9 editing of cxTi10882 insertion site
pJW1947	*pes-10Δ minimal promoter*::*SV40 NLS::mScarlet-I (dpi)::tbb-2 3'UTR* vector	Promoter reporter, test new tissue-specific promoters
pJW1948	*pes-10Δ minimal promoter*::*SV40 NLS::mScarlet-I (dpi)::PEST::tbb-2 3'UTR* vector	Promoter reporter, test new tissue-specific promoters
pTD77	*eft-3p::Cas9, R07E5.16 U6::sgRNA* targeting LGI site where ttTi4348 Mos is inserted	CRISPR/Cas9 editing of ttTi4348 insertion site
pTD78	*eft-3p::Cas9, R07E5.16 U6::sgRNA* targeting LGII site where ttTi5605 Mos is inserted	CRISPR/Cas9 editing of ttTi5605 insertion site

The F2A ribosome skip sequence functions efficiently in an *eft-3p::TIR1::F2A::BFP::AID*::NLS* transgene

We tested the efficiency of the F2A peptide in our new TIR1 transgenes, as inefficient processing could create a TIR1::F2A::BFP::AID*::NLS fusion protein that could be nuclear localized and degraded, impairing performance of the system. The expression of BFP in our *sun-1*p strain was quite dim and we were unable to detect it via western blot. We therefore generated a new *TIR1::F2A::BFP::AID*::NLS* construct driven by a strong ubiquitous promoter (*eft-3p*) and inserted this transgene in the same site. We observed somatic BFP which was lost upon auxin exposure, indicating that our TIR1 transgene was functional (Figure 3A). We performed anti-BFP western blots on this strain (Figure 3B). We observed a doublet in the *eft-3p*::*TIR1::F2A::BFP::AID*::NLS* strain which was not present in N2 control animals. The upper band was consistent with the predicted size of the BFP reporter (34.5 kDa). The lower band is most likely a degradation product, as tRFP-derived proteins such as BFP are known to fragment in western blots (Kovacs *et al.* 2012; Breunig *et al.* 2015; Katoh *et al.* 2016). A similar doublet was also reported in the product literature for the antibody we used. We never observed a 101.5 kDa band, which is the predicted size for a TIR1::F2A::BFP::AID*::NLS fusion protein. Together, these data suggest that the F2A peptide is being effectively processed to produce a BFP::AID*::NLS reporter which marks TIR1 expression.

A *eft-3p::TIR1::2A::BFP*

Control

1 mM Auxin

DIC   BFP

B

wild type
eft-3p::TIR1::2A::BFP
wild type
eft-3p::TIR1::2A::BFP

100 kDa
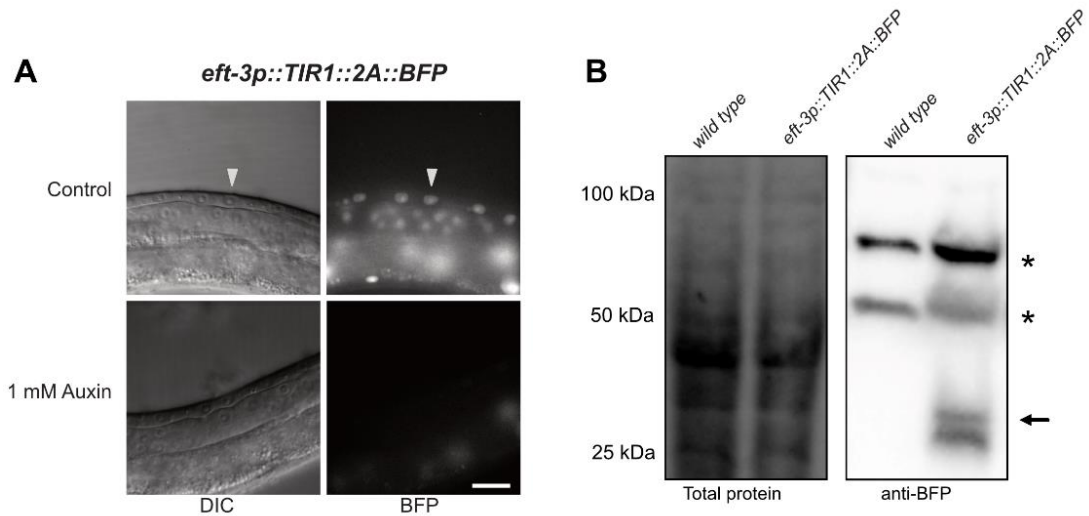
50 kDa

25 kDa

Total protein   anti-BFP

*
*
←

Figure 3. The F2A ribosome skip sequence functions efficiently in an *eft-3p::TIR1::F2A::BFP::AID*::NLS* transgene. A) L3 larvae expressing *eft-3p:: TIR1::F2A::BFP::AID*::NLS*. A control animal expresses AID*-tagged BFP in the nuclei of vulval precursor cells (VPCs; white arrows). BFP expression is undetectable in animals grown on 1 mM K-NAA—a water-soluble, synthetic auxin—for 1 hour before imaging. Scale bars represent 15 μm (*eft-3p*). B) Western blot detecting *BFP::AID::NLS*. Stain-free (Bio-Rad) analysis of total protein on the blot is provided as a loading control (left). Marker size (in kilodaltons) is provided. Anti-BFP blot showing background bands (marked with *) and a doublet consistent with the predicted size of BFP::AID*::NLS (black arrow) at approximately 34.5 kDa, and a smaller band below, likely a BFP degradation product.

eft-3p::TIR1::F2A::BFP::AID*::NLS is functionally equivalent to eft-3p::TIR1::mRuby2, but exhibits slower kinetics for degradation of substrates

To test the efficiency of our new *eft-3p::TIR1::F2A::BFP::AID*::NLS* transgene, we crossed it to an *eft-3p::AID*::GFP* reporter and compared AID*::GFP depletion level and rate between our new TIR1 strain and the original *eft-3p::TIR1::mRuby2* strain (Zhang *et al.* 2015). TIR1::mRuby2 strongly depleted the AID*::GFP reporter within 30 minutes of exposure to the natural auxin indole-3-acetic acid (IAA) used by Zhang *et al.* (2015). In contrast, TIR1::F2A::BFP::AID*::NLS took 120 minutes to completely deplete the AID*::GFP reporter after exposure to IAA. (Figure 4A). Single-cell imaging of the AID*::GFP reporter in vulval precursor cells (VPCs)(Figure 4B, Figure S2) supported the western blot data (Figure 4A). We also compared the performance of IAA to K-NAA, a water-soluble synthetic auxin recently described for use in *C. elegans* (Martinez *et al.* 2020). The reporter was strongly depleted 30 minutes after exposure to both IAA and K-NAA in the TIR1::mRuby2 strain, but took 90 minutes for equivalent depletion in the TIR1::F2A::BFP::AID*::NLS strain (Figure 4C, Figure S2). We also compared the ratio of nuclear to cytoplasmic AID*::GFP to test whether there were differences in sub-cellular depletion rates. We did not observe differences in the nuclear/cytoplasmic ratio, suggesting that both TIR1::mRuby2 and TIR1::F2A::BFP::AID*::NLS are equally effective at depleting nuclear and cytoplasmic AID*::GFP (Figure S3). IAA and K-NAA performed identically in these experiments (Figure S3).
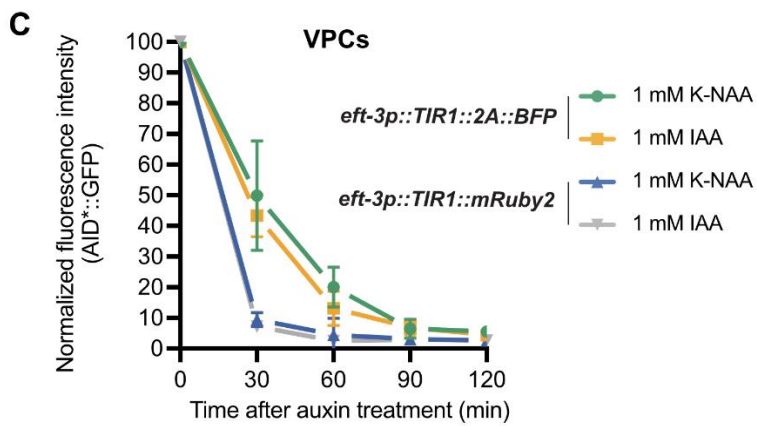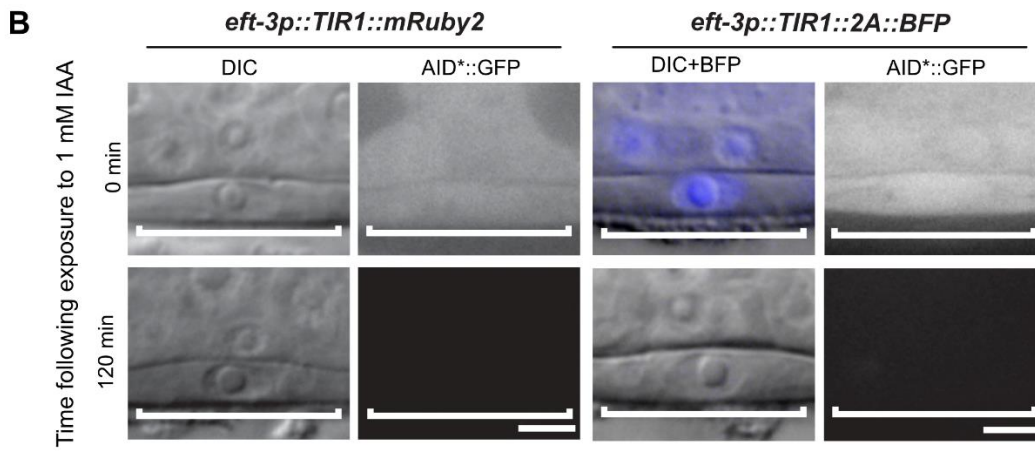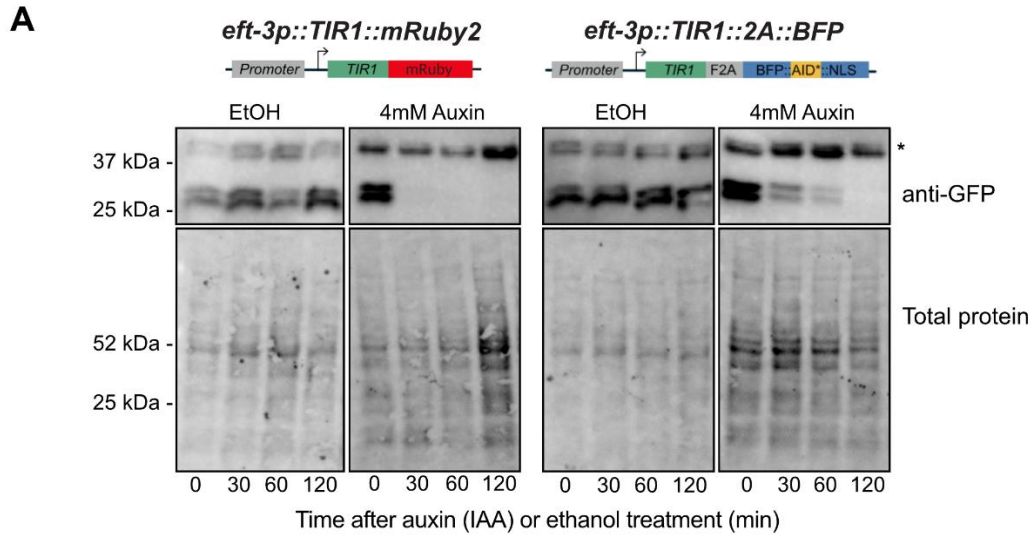
A

B

C

Figure 4. *eft-3p::TIR1::F2A::BFP::AID*::NLS* depletes AID*::GFP to the same extent as *eft-3p::TIR1::mRuby2* but exhibits a slower rate of degradation. A) Western blots detecting AID*::GFP after exposure to IAA or EtOH (control). An AID*::GFP reporter strain was crossed to either *eft-3p::TIR1::mRuby2* (left) or *eft-3p::TIR1::F2A::BFP::AID*::NLS* (right) and then exposed to 4 mM IAA or EtOH for 0 min, 30 min, 60 min, or 120 min. Anti-GFP blots (top) showing background band (marked with *) and a doublet at approximately 27 kDa, consistent with the predicted size of GFP. B) Representative images of AID*::GFP depletion in animals carrying either *eft-3p::TIR1::mRuby2* or *eft-3p::TIR1::F2A::BFP::AID*::NLS*. For animals expressing *eft-3p::TIR1::F2A::BFP::AID*::NLS*, an overlay of DIC and BFP images is provided. DIC and corresponding GFP images of VPCs (brackets) from L3 larvae at the P6.p 1-cell stage. Animals were treated with 1 mM IAA for the specified time and then imaged to visualize loss of AID*::GFP. Representative images from additional timepoints can be found in Figure S2. Scale bars represent 5 µm. C) AID*::GFP degradation kinetics. Rates of degradation were determined by quantifying AID*::GFP levels in VPCs of animals as described above. Animals were exposed to 1 mM K-NAA or IAA from 0 to 120 minutes at intervals of 30 min. The graph depicts the mean normalized fluorescent intensity from 10 or more animals from a single experimental replicate. Error bars indicate standard deviation.

To further test the performance of TIR1::F2A::BFP::AID*::NLS for depleting an endogenous AID-tagged protein, we compared depletion in the *eft-3p::TIR1::F2A::BFP::AID*::NLS*; *nhr-25::GFP::AID*::3xFLAG* strain to depletion in the previously described *eft-3p::TIR1::mRuby2; nhr-25::GFP::AID*::3xFLAG* strain (Martinez *et al.* 2020). Similar to the AID*::GFP experiments, we performed single cell quantitative imaging experiments (Figure 5). With the *eft-3p::TIR1::mRuby2* transgene we observed undetectable NHR-25::GFP::AID*::3XFLAG in both seam cells and VPCs within 30 minutes of exposure to IAA or K-NAA (Figure 5, Figure S2). In contrast, we observed differences in IAA and K-NAA treatment using our *eft-3p::TIR1::F2A::BFP::AID*::NLS; nhr-25::GFP::AID*::3xFLAG* strain (Figure 5, Figure S2). K-NAA resulted in more rapid NHR-25::GFP::AID*::3xFLAG depletion by TIR1::F2A::BFP::AID*::NLS compared to IAA (Figure 5, Figure S2). In VPCs, NHR-25::GFP::AID* was robustly depleted after 60 minutes of exposure to K-NAA and undetectable after 90 minutes (Figure 5B, Figure

S2). There appeared to be tissue-specific differences in depletion rates as NHR-

25::GFP::AID*::3xFLAG was undetectable in seam cells 60 minutes after exposure to K-NAA

(Figure 5C, Figure S2). Depletion rates were slower with IAA, taking an additional 30 minutes

for TIR1::F2A::BFP::AID*::NLS to deplete NHR-25::GFP::AID*::3xFLAG to undetectable levels

(Figure 5, Figure S2). These results suggest that: i) some element of our

TIR1::F2A::BFP::AID*::NLS transgene is limiting depletion rate; ii) we reach the same

depletion endpoint using our TIR1::F2A::BFP::AID*::NLS transgene; iii) there can be tissue-

specific differences in depletion rates; and iv) K-NAA performed equal to or better than IAA

in these depletion experiments. These results suggest that one should test IAA and K-NAA

for depletion efficiency independently for each TIR1 expression system and AID*-tagged

protein.

A

eft-3p::TIR1::mRuby2
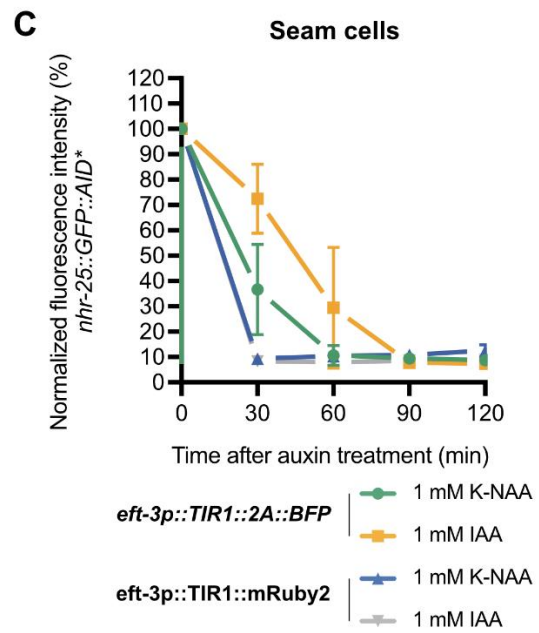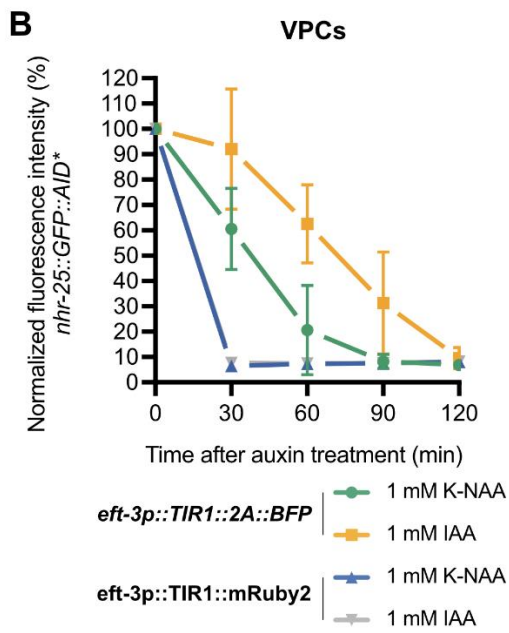
DIC | NHR-25::GFP::AID*

eft-3p::TIR1::2A::BFP

DIC+BFP | NHR-25::GFP::AID*

Time following exposure to 1 mM IAA

0 min

120 min

B VPCs

C Seam cells

Normalized fluorescence intensity (%) *nhr-25::GFP::AID**

Time after auxin treatment (min)

eft-3p::TIR1::2A::BFP — 1 mM K-NAA — 1 mM IAA

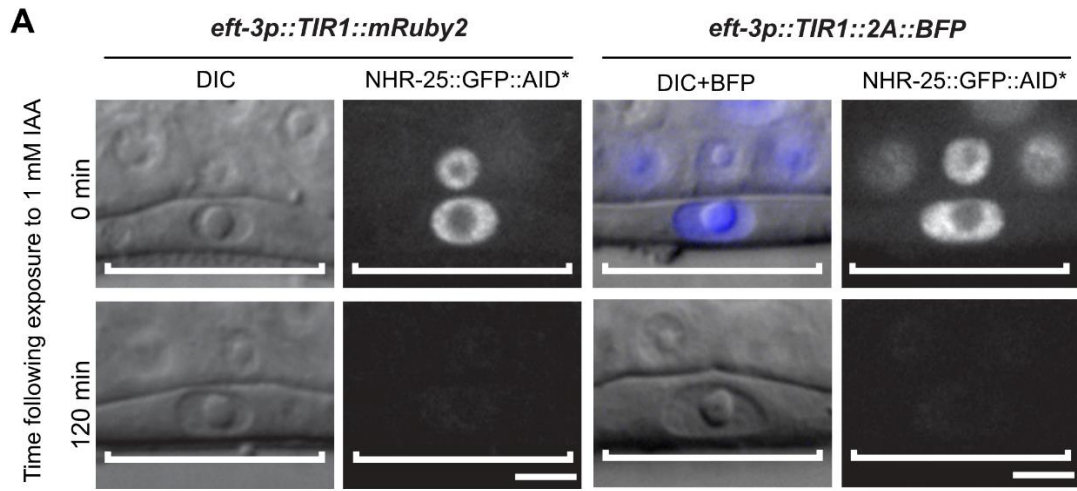eft-3p::TIR1::mRuby2 — 1 mM K-NAA — 1 mM IAA

Figure 5. ***eft-3p::TIR1::F2A::BFP::AID*::NLS* depletes NHR-25::GFP::AID* to the same extent as *eft-3p::TIR1::mRuby2* but also exhibits a slower degradation rate.** A) Representative images of NHR-25::GFP::AID* depletion in VPCs of animals expressing either *eft-3p::TIR1::mRuby2* or *eft-3p::TIR1::F2A::BFP::AID*::NLS*. For animals expressing *eft-3p::TIR1::F2A::BFP::AID*::NLS*, an overlay of DIC and BFP images were used to demonstrate BFP internal control expression in VPCs. DIC and corresponding GFP images of VPCs (brackets) from L3 larvae at the P6.p 1-cell stage. Animals were treated with 1 mM IAA for the specified time and then imaged to visualize loss of NHR-25::GFP::AID*. Additional timepoints can be found in figure S2. Scale bars represent 5 μm. NHR-25::GFP::AID* degradation kinetics in B) VPCs and C) seam cells. Kinetics were determined by measuring NHR-25::GFP::AID* levels in L3s (as described above) exposed to 1 mM K-NAA or IAA. The graph depicts the mean normalized fluorescent intensity from 10 or more animals from a single experimental replicate. Error bars indicate standard deviation.

eft-3p::TIR1::F2A::BFP::AID*::NLS produces comparable depletion phenotypes as eft-

3p::TIR1::mRuby2 for depleting both nuclear and cytoplasmic proteins

Given the slower depletion kinetics we observed in our *eft-3p::TIR1::F2A::BFP::AID*::NLS*

strain for an AID*::GFP reporter and NHR:25::GFP::AID*::3xFLAG, we wanted to test the

impact of this slowed depletion rate at the phenotypic level. We first tested the two TIR1

alleles on our *nhr-25::GFP::AID*::3xFLAG* strain (Table 2 and Table S5). With the

TIR1::mRuby2 transgene and IAA, we observed that 61% of *nhr-25::GFP::AID*::3xFLAG*

larvae arrested, and the remaining 39% of animals that made it to adulthood had gonadal

abnormalities (Table 2 and Table S5). With our *TIR1::F2A::BFP::AID*::NLS* transgene, we

observed that 85% of larvae arrested, while 93% of animals that reached adulthood had

gonadal abnormities (Table 2 and Table S5). Surprisingly, with growth on K-NAA we did not

observe any larval arrests for either strain, but all adult animals had gonadal defects (Table 2

and Table S5). We next tested the two different TIR1 strains on an *nhr-23::AID*::3xFLAG*

allele (Zhang *et al.* 2015). Similar to previous reports (Zhang *et al.* 2015), we observed a completely penetrant larval arrest with both the *nhr-23::AID*::3xFLAG; eft-3p::TIR1::mRuby2* and *nhr-23::AID*::3xFLAG, eft-3p::TIR1::F2A::BFP::AID*::NLS* strains grown on either IAA or K-NAA. Interestingly, some of the arrested larvae produced by growth on K-NAA died (Table S5), which we did not see on IAA and is consistent with a stronger phenotype. None of the strains tested presented any defects on MYOB or ethanol control plates (Table 2 and Table S5). Finally, we wished to test an additional cytoplasmic AID*-tagged protein for depletion phenotypes. We chose DAF-15/Raptor, a lysosome-localized factor which causes larval arrest when depleted (Duong *et al.* 2020). Both *eft-3p::TIR1::mRuby2* and *eft-3p::TIR1::F2A::BFP::AID*::NLS* caused a completely penetrant larval arrest in animals carrying the *daf-15::mNeonGreen::AID** (Table 3 and Table S6). Strains grown on IAA arrested at L2, while strains grown on K-NAA arrested at L3 (Table 3 and Table S6). Based on previous work (Duong *et al.* 2020), these results suggests that K-NAA is producing slower or less complete DAF-15::mNG::AID* depletion than IAA. For DAF-15::mNG::AID*, IAA appeared to perform better. For all strains tested, we observed wild-type growth on control media (Table 2, Table 3, Table S5, and Table S6). Together, these results indicated that our *eft-3p::TIR1::F2A::BFP::AID*::NLS* transgene performs comparably to the original *eft-3p::TIR1::mRuby2* with respect to phenocopying mutant phenotypes of NHR-23, NHR-25, and DAF-15.

Table 2. *eft-3p::TIR1::F2A::BFP::AID*::NLS* produces comparable depletion phenotypes to *eft-3p::TIR1::mRuby2* for depleting nuclear proteins.

eft-3p::TIR1 transgene	AID-tagged allele	Treatment	% WT developmental rate	% Larval arrest	% Gonadal abnormalities among adults
TIR1::mRuby2	n/a	Control	100	0	0
TIR1::mRuby2	n/a	IAA	100	0	0
TIR1::mRuby2	n/a	K-NAA	100	0	0
TIR1::2A::BFP	n/a	Control	100	0	0
TIR1::2A::BFP	n/a	IAA	100	0	0
TIR1::2A::BFP	n/a	K-NAA	100	0	0
TIR1::mRuby2	*nhr-23(kry61[nhr-23::AID*::TEV-3xFLAG])*	Control	100	0	0
TIR1::mRuby2	*nhr-23(kry61[nhr-23::AID*::TEV-3xFLAG])*	IAA	0	100	0
TIR1::mRuby2	*nhr-23(kry61[nhr-23::AID*::TEV-3xFLAG])*	K-NAA	0	100	0
TIR1::2A::BFP	*nhr-23(kry61[nhr-23::AID*::TEV-3xFLAG])*	Control	100	0	0
TIR1::2A::BFP	*nhr-23(kry61[nhr-23::AID*::TEV-3xFLAG])*	IAA	0	100	0
TIR1::2A::BFP	*nhr-23(kry61[nhr-23::AID*::TEV-3xFLAG])*	K-NAA	0	100	0
TIR1::mRuby2	*nhr-25(wrd18[nhr-25::GFP^AID*:3xFLAG])*	Control	100	0	0
TIR1::mRuby2	*nhr-25(wrd18[nhr-25::GFP^AID*:3xFLAG])*	IAA	39	61	100
TIR1::mRuby2	*nhr-25(wrd18[nhr-25::GFP^AID*:3xFLAG])*	K-NAA	100	0	100
TIR1::2A::BFP	*nhr-25(wrd52[nhr-25::GFP^AID*:3xFLAG])*	Control	100	0	0
TIR1::2A::BFP	*nhr-25(wrd52[nhr-25::GFP^AID*:3xFLAG])*	IAA	15	85	93
TIR1::2A::BFP	*nhr-25(wrd52[nhr-25::GFP^AID*:3xFLAG])*	K-NAA	100	0	100

Table 3. *eft-3p::TIR1::F2A::BFP::AID*::NLS* produces comparable depletion phenotypes to *eft-3p::TIR1::mRuby2* for depleting cytoplasmic proteins.

eft-3p::TIR1 transgene	AID-tagged allele	Treatment	% WT developmental rate	% L2 arrest	% L3 arrest
TIR1::mRuby2	*daf-15(re257[daf-15::mNG::AID*])*	Control	100	0	0
TIR1::mRuby2	*daf-15(re257[daf-15::mNG::AID*])*	IAA	0	100	0
TIR1::mRuby2	*daf-15(re257[daf-15::mNG::AID*])*	K-NAA	0	0	100
TIR1::2A::BFP	*daf-15(re257[daf-15::mNG::AID*])*	Control	100	0	0
TIR1::2A::BFP	*daf-15(re257[daf-15::mNG::AID*])*	IAA	0	100	0
TIR1::2A::BFP	*daf-15(re257[daf-15::mNG::AID*])*	K-NAA	0	0	100

Synchronized animals (n=120-240) of the indicated genotype were grown on the indicated plates (control, IAA, K-NAA). Animals were grown at 20°C for 4 days and scored for larval arrest. Wild-type (WT) developmental rate was determined by scoring animals that reached adulthood after 3 days at 20°C. L2 and L3 arrest were determined by animal size and gonadal development.

A new suite of TIR1 driver strains compatible with red/green FP imaging

Having confirmed our *TIR1::F2A::BFP::AID*::NLS* transgene functioned effectively in both

embryos and somatic cells, we wished to create a suite of strains for tissue-specific TIR1

expression (Figure 6A). We created chromosome I and II knock-ins expressing TIR1 in the

germline (*mex-5p* and *sun-1p*), hypodermis (*dpy-7p* and *col-10*p), muscle *(unc-54p)*, and

intestine (*ges-1p)* (Figure 6, Figure S4). We also created chromosome I knock-ins expressing

TIR1 in neurons (*rgef-1p*), pharynx (*myo-2p)*, body wall muscle (*myo-3p*), the anchor cell

(*cdh-3*), as well as the excretory cell, hypodermis, and gut (*vha-8p*) (Figure 6, Figure S4). The

vha-8 promoter also drove expression in unidentified cells in the head. Additionally, we

generated a strain expressing TIR1 in the hypodermal seam cells using a minimal SCMp

enhancer (gift from Prof. Allison Woollard) with the *pes-10* minimal promoter (Figure 6A).

While we observed robust seam cell expression in this strain, we also detected hypodermal

expression (unpublished data). We are making this strain available to the community but

encourage careful evaluation before interpretation.

A TIR1 driver strains

`- Promoter - ⌐→ TIR1 F2A BFP::AID*::NLS -`

Strain	Promoter	Tissue	Genotype	Insertion site
LP869	vha-8p	Excretory cells, Hypodermis, Gut, unidentified head cells	cpSi171[vha-8p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	I:-5.32
JDW225	eft-3p	Soma	wrdSi23[eft-3p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	I:-5.32
DV3799	col-10p	Hypodermis	reSi1[col-10p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	I:-5.32
DV3800	col-10p		reSi2[col-10p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	II:-0.77
JDW227	dpy-7p		wrdSi45[dpy-7p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	II:-0.77
JDW229	dpy-7p		wrdSi47[dpy-7p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	I:-5.32
JDW231	SCMp‡	Seam cells and	wrdSi44[SCMp*::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	II:-0.77
JDW233	SCMp‡	Hypodermis	wrdSi46[SCMp*::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	I:-5.32
JDW221	mex-5p	Germline	wrdSi18[mex-5p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	I:-5.32
JDW223	mex-5p		wrdSi35[mex-5p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	II:-0.77
JDW10	sun-1p		wrdSi3 [sun-1p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	II:-0.77
DV3801	unc-54p	Muscle	reSi3[unc-54p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	I:-5.32
DV3825	unc-54p		reSi11[unc-54p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	II:-0.77
LP871	myo-3p		cpSi174[myo-3p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	I:-5.32
DV3803	ges-1p	Intestine	reSi5[ges-1p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	I:-5.32
DV3826	ges-1p		reSi12[ges-1p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	II:-0.77
DV3805	rgef-1p	Neuron	reSi7[rgef-1p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	I:-5.32
DQM526	cdh-3p	Anchor cells, Seam cells, L4 Vulval precursor cells	bmd176[cdh-3p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	II:-0.77
LP870	myo-2p	Pharynx	cpSi172 [myo-2p::TIR1::F2A::BFP::AID*::NLS::tbb-2 3'UTR]	I:-5.32

‡ SCM promotor is a 573bp enhancer from *arf-3* intronic sequence + *pes-10Δ*

B

Hypodermis (*col-10p*)

Muscle (*unc-54p*)

Neuron (*rgef-1p*)

Intestine (*ges-1p*)

C Hypodermis (LGI): *col-10p::TIR1::2A::BFP*

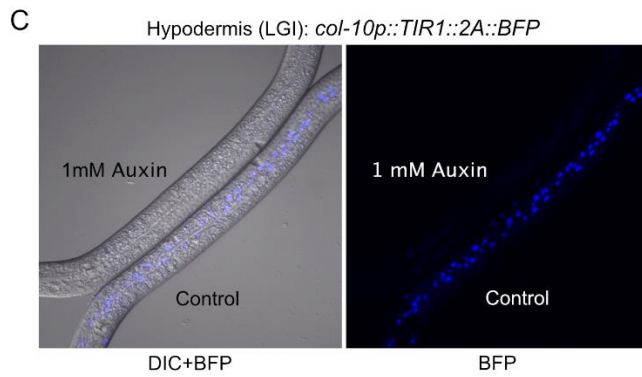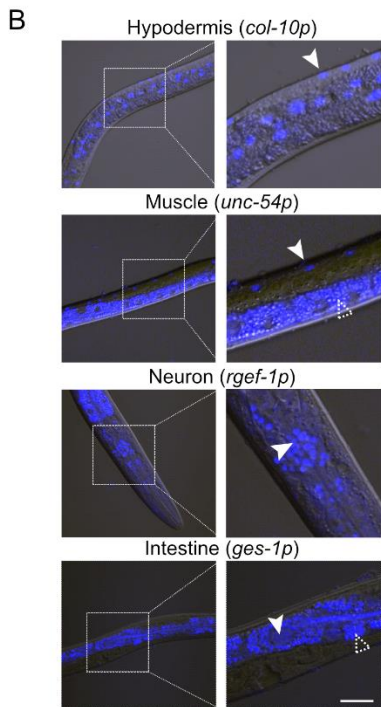1mM Auxin

Control

DIC+BFP

1 mM Auxin

Control

BFP

Figure 6. A new suite of TIR1 expression strains for tissue-specific depletion of AID-tagged proteins in *C. elegans*. A) Table describing new suite of TIR1::F2A::BFP::AID*::NLS strains. Strain names, promoter driving TIR1, tissue of expression, genotype, and insertion site are provided for each strain. The insertion sites are the genomic loci where the MosI transposon landed in the ttTi4348 and ttTi5605 insertion alleles. We note that our knock-ins were generated using CRISPR/Cas9-mediated genome editing in wild-type animals or in strains stably expressing Cas9 in the germline; there is no MosI transposon in these loci in these genetic backgrounds. B) BFP is detected in the expected nuclei of strains expressing TIR1 cassettes driven by *col-10p* (hypodermis), *unc-54p* (muscle), *ges-1p* (intestine), and *rgef-1p* (neurons). Representative BFP-expressing nuclei are indicated by solid arrows. Scale bars represent 20 μm. Note that the fluorescence signal at the bottom of the muscle image and surrounding the nuclei in the intestinal image is intestinal autofluorescence, indicated by an unfilled arrow with a dashed outline. C) Functional test of TIR1 activity in a *col-10p::TIR1::F2A::BFP::AID*::NLS* strain (DV3799). Hypodermal BFP expression is lost when animals are exposed to 1 mM auxin for three hours, but not when similarly grown on control plates.

All of the TIR1-expressing strains that we have deposited in the *Caenorhabditis* Genetics Center have detectable BFP expression that is lost when animals are shifted onto auxin plates, confirming TIR1 is active (Figures 2B, 6C, S2, and S4). However, an unanswered question is the importance of TIR1 expression levels for effective depletion of AID*-tagged proteins. Motivated by an interest in NHR-25 in gene regulation (Ward *et al.* 2013, 2014) and anchor cell (AC) invasion (Matus *et al.* 2015; Medwig and Matus 2017; Medwig-Kinney *et al.* 2020), we generated a strain to study early events in AC differentiation (DQM623; Figure 7). The *cdh-3* promoter drives transgene expression in the AC but not vulval precursor cells (VPCs) during early AC differentiation (Matus *et al.* 2015). The promoter becomes active in L4 VPCs and also drives expression in seam cells and neurons (Matus *et al.* 2015). We generated a *cdh-3p::TIR1::F2A::BFP::AID*::NLS* strain, but were not able to detect any BFP expression. To perform a functional test, we crossed the *nhr-25::GFP::AID*::3xFLAG*

allele into the strain harboring *cdh-3p::TIR1::F2A::BFP::AID*::NLS*. We had previously

demonstrated significant depletion of NHR-25 in ACs and VPCs using a strongly expressed

eft-3p::TIR1::mRuby2 transgene (Martinez *et al.* 2020). Strikingly, we observed auxin-

dependent depletion of NHR-25::GFP::AID*::3xFLAG in the AC but no depletion in the

adjacent VPCs (Figure 7). Thus, even if the presence of TIR1 is undetectable through BFP

reporter expression, there may still be a sufficient amount of TIR1 to deplete proteins of
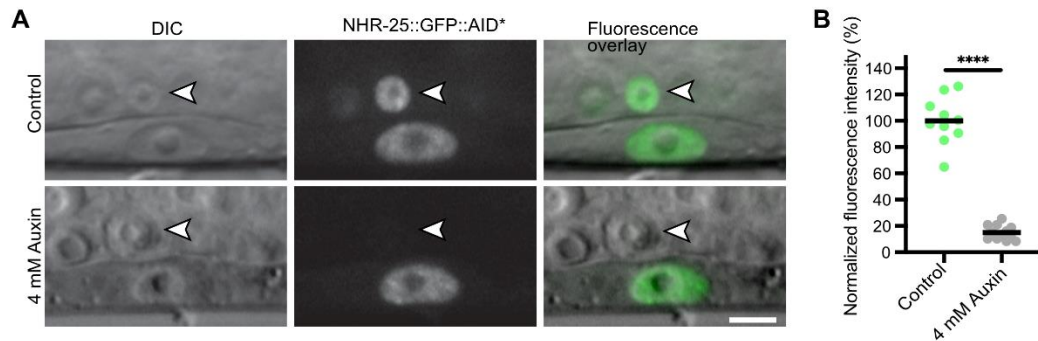
interest.

Ashley *et al.* (2020) Figure 7

Figure 7. NHR-25::GFP::AID*::3xFLAG can be depleted in a cell-specific manner in a strain with undetectable TIR1 expression via a BFP reporter. A) An anchor cell (AC)-specific TIR1 transgene (*cdh-3p::TIR1::F2A::BFP::AID*::NLS*) did not produce observable BFP in the AC. Crossing this strain to an *nhr-25::GFP::AID*::3xFLAG* allele resulted in depletion of NHR-25 in the AC when exposed to 4 mM auxin for 1 hr (indicated by white arrow with black outline). As expected, depletion of NHR-25 was not observed in the neighboring uterine cells or the underlying vulval precursor cells (VPCs). Scale bar represents 5 µm. B) Quantification of NHR-25::GFP::AID*::3xFLAG in ACs following auxin (K-NAA) treatment. Individual data points from a single replicate with more than 10 animals per condition are presented. The horizontal black bar depicts the mean for each condition; **** indicates *P* < 0.0001 by a two-tailed unpaired Student's t-test. *P* < 0.05 was considered statistically significant. Scale bars represent 5 µm

Vectors to generate FP::AID* knock-ins

There are two commonly used CRISPR/Cas9 editing strategies currently used in *C. elegans* (Dickinson and Goldstein 2016; Nance and Frøkjær-Jensen 2019). One uses injection of Cas9 ribonucleoprotein complexes and typically provides linear DNA repair templates (Paix *et al.* 2015; Dokshin *et al.* 2018; Ghanta and Mello 2020). The other approach uses injection of plasmids expressing Cas9 and sgRNA, and typically provides repair templates as plasmids with selectable markers (Dickinson *et al.* 2013, 2015; Norris *et al.* 2015; Schwartz and

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Jorgensen 2016). We created plasmids to facilitate assembly of repair templates for both of these genome editing strategies.

First, we generated a set of vectors to create SEC-selectable plasmid repair templates. We took a set of vectors which use Gibson assembly to generate the final repair template (Dickinson *et al.* 2015) and introduced AID* sequences upstream of the 3xFLAG epitope. This set of vectors allows for tagging genes with mNeonGreen, GFP, YPET, mKate2, and TagRFP-T along with AID*::3xFLAG epitopes (Figure 8, Table S2). Methods in *C. elegans* using biotin ligases and biotin acceptor peptides have recently been described for protein affinity purification (Waaijers *et al.* 2016), proximity labeling (Branon *et al.* 2018), native chromatin purification (Ooi *et al.* 2010), and cell-type specific nuclei purification (Steiner *et al.* 2012). To support these approaches, we have made a set of FP^SEC^BioTag::AID*::3xFLAG vectors with GFP, TagRFP-T, and mKate2 (Figure 8, Table S2).

Another approach to generate plasmid repair templates with detectable markers is a Golden Gate assembly approach known as SapTrap (Engler *et al.* 2009; Schwartz and Jorgensen 2016). A library of SapTrap donor plasmids contain protein tags, selectable markers, and fluorescent proteins; these tags can be assembled in a desired order in a one-tube isothermal reaction to allow the creation of new repair templates (Schwartz and Jorgensen 2016; Dickinson *et al.* 2018) (Figure 8D). When trying to create SapTrap assemblies containing nine fragments, we encountered very poor cloning efficiencies. We therefore created a series of pre-assembled "multi-cassettes," where we combined fragments that we

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frequently use (Figure 8E, Table S2). These constructs contain inserts for SapTrap N-terminal

and C-terminal connector modules, as described by Schwartz et al. (2016) and include

various combinations of AID* cassettes, and epitopes for protein purification or detection

(3xMyc, 3xFLAG, BioTag). This approach restored high cloning efficiency. For our most

commonly used vectors, we have generated constructs containing full assemblies of the

knock-in epitope, lacking only the homology arms. PCR amplifying homology arms with SapI

sites and appropriate connectors allows high-efficiency generation of repair templates.

A

FP-SEC-AID* vector

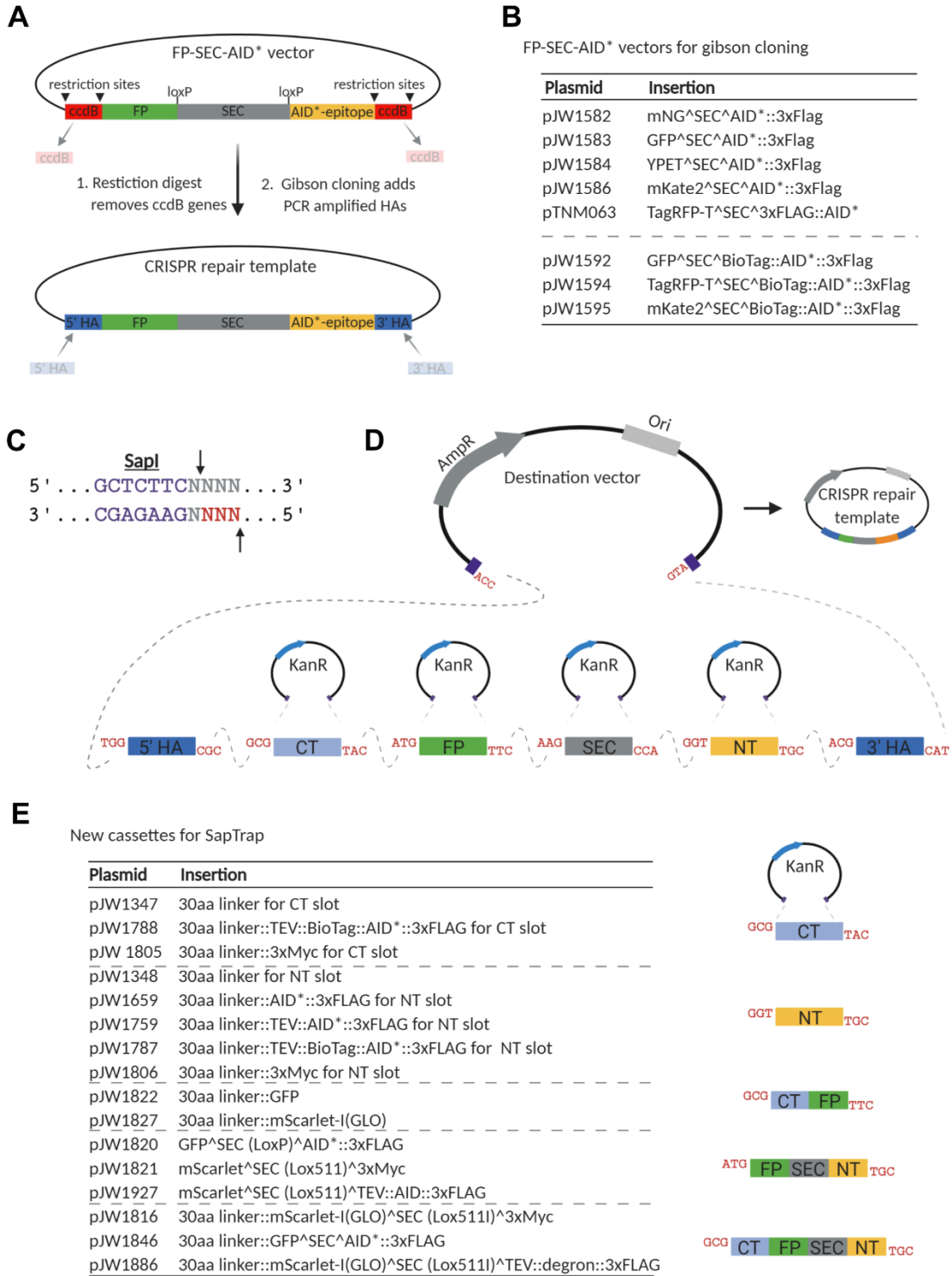restriction sites   loxP   loxP   restriction sites

ccdB | FP | SEC | AID*-epitope | ccdB

ccdB   ccdB

1. Restriction digest removes ccdB genes

2. Gibson cloning adds PCR amplified HAs

CRISPR repair template

5' HA | FP | SEC | AID*-epitope | 3' HA

5' HA   3' HA

B

FP-SEC-AID* vectors for gibson cloning

Plasmid	Insertion
pJW1582	mNG^SEC^AID*::3xFlag
pJW1583	GFP^SEC^AID*::3xFlag
pJW1584	YPET^SEC^AID*::3xFlag
pJW1586	mKate2^SEC^AID*::3xFlag
pTNM063	TagRFP-T^SEC^3xFLAG::AID*
pJW1592	GFP^SEC^BioTag::AID*::3xFlag
pJW1594	TagRFP-T^SEC^BioTag::AID*::3xFlag
pJW1595	mKate2^SEC^BioTag::AID*::3xFlag

C

<u>SapI</u>   ↓

5'...GCTCTTCNNNN...3'
3'...CGAGAAGNNNN...5'

↑

D

AmpR   Ori

Destination vector   →   CRISPR repair template

ACC   GTA

KanR   KanR   KanR   KanR

TGG 5' HA CGC   GCG CT TAC   ATG FP TTC   AAG SEC CCA   GGT NT TGC   ACG 3' HA CAT

E

New cassettes for SapTrap

Plasmid	Insertion
pJW1347	30aa linker for CT slot
pJW1788	30aa linker::TEV::BioTag::AID*::3xFLAG for CT slot
pJW 1805	30aa linker::3xMyc for CT slot
pJW1348	30aa linker for NT slot
pJW1659	30aa linker::AID*::3xFLAG for NT slot
pJW1759	30aa linker::TEV::AID*::3xFLAG for NT slot
pJW1787	30aa linker::TEV::BioTag::AID*::3xFLAG for NT slot
pJW1806	30aa linker::3xMyc for NT slot
pJW1822	30aa linker::GFP
pJW1827	30aa linker::mScarlet-I(GLO)
pJW1820	GFP^SEC (LoxP)^AID*::3xFLAG
pJW1821	mScarlet^SEC (Lox511)^3xMyc
pJW1927	mScarlet^SEC (Lox511)^TEV::AID::3xFLAG
pJW1816	30aa linker::mScarlet-I(GLO)^SEC (Lox511I)^3xMyc
pJW1846	30aa linker::GFP^SEC^AID*::3xFLAG
pJW1886	30aa linker::mScarlet-I(GLO)^SEC (Lox511I)^TEV::degron::3xFLAG

KanR

GCG CT TAC

GGT NT TGC

GCG CT FP TTC

ATG FP SEC NT TGC

GCG CT FP SEC NT TGC

**Figure 8. A collection of vectors to generate FP::AID* knock-ins through Gibson cloning and a suite of new vectors for the SapTrap cloning system.** A) Schematic of the AID*-containing vectors produced by modifying the set of vectors originally described by Dickinson *et al.* (2015). An AID* epitope was inserted downstream of the loxP-flanked SEC. New repair templates for CRISPR/Cas9-mediated genome editing can be produced by restriction digestion of the vector and Gibson cloning of PCR-derived 5' and 3' homology arms (5'HA and 3'HA), as described (Dickinson *et al.,* 2015). Counter-selection against the parent vector is provided by ccdB cassettes. B) A suite of new FP::AID* SEC plasmids. The vectors described in Dickinson *et al.* (2015) have been modified to insert an AID* or 23 amino acid biotin acceptor peptide (BioTag)::AID* cassette between the SEC and 3xFLAG cassette. C) SapI is a type II restriction enzyme that cuts one base pair and four base pairs outside of its binding site, allowing for the generation of programable 3 bp sticky ends. D) SapTrap cloning facilitates single-reaction cloning of multiple fragments, in the correct order, into a single repair template plasmid. Specific sticky ends are used for specific cassettes as described by Schwartz *et al.* (2016). E) Table of new vectors generated for the SapTrap CT and NT slots. Our initial assembly efficiencies were sub-optimal, and we found that reducing the number of fragments assembled improved our efficiencies. We have generated a set of multi-cassettes where partial assemblies (CT-FP, FP-SEC-NT, and CT-FP-SEC-NT) have been cloned, simplifying the SapTrap reactions and reducing the number of fragments required. 5'HA, 5' homology arm; 3'HA, 3' homology arm; FP, fluorescent protein; SEC, self-excising cassette; CT, C-terminal connector; NT, N-terminal connector.

Using Cas9 ribonucleoprotein complexes with linear repair templates is a common cloning-free approach in *C. elegans* (Paix *et al.* 2014; 2015), and recent refinements have further boosted editing efficiency (Dokshin *et al.* 2018; Ghanta and Mello 2020). We have gave generated repair templates to tag proteins with GFP, germline optimized mNeonGreen, and germline optimized mScarlet-I (Fielmich *et al.* 2018; Zhang *et al.* 2018) (Figure 9B, Table S2). All plasmids include a Myc or FLAG epitope for antibody-based detection, and we have versions with or without an AID* cassette. These vectors are suitable templates for PCR amplification to generate linear repair templates with short homology arms. FPs can be easily exchanged to generate new constructs by PCR linearization and Gibson cloning.

Linkers flanking the FP allow flexibility in targeting genes of interest and reduce functional

interference, permitting N-terminal, C-terminal, or internal tagging.

Ashley *et al.* (2020) Figure 9
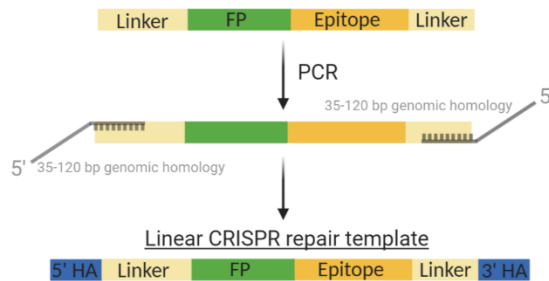
Figure 9. A collection of vectors to generate FP::AID* knock-ins using linear repair
templates. A) Schematic primer design to generate linear repair templates by PCR.
Primers with homology to the cassette and 5' homology to the desired integration site are
used to amplify a dsDNA repair template. 35-120 bp homology arms (HA in figure) are
recommended, as previously described (Paix *et al.* 2014; 2015; Dokshin *et al.* 2018). B) A
set of vectors to generate repair templates for Cas9 ribonucleoprotein complex (RNP)-
based genome editing. FP (fluorescent protein) and FP::AID* cassettes are flanked by
flexible linker sequences. A 30 amino acid sequence is at the 5' end of the cassette, and a
10 amino acid sequence is at the 3' end of the cassette. This design provides flexibility for
designing repair templates for N-terminal, C-terminal, or internal tagging. GLO=Germline
optimized using algorithm (Fielmich *et al.*, 2018); dpi=silent mutations to remove piRNA
binding sites (dpi) to promote germline expression (Zhang *et al.* 2018).

Discussion:

The original *C. elegans* AID system employed a TIR1::mRuby2 transgene, which was useful for visualizing TIR1 expression and cellular localization (Zhang *et al.* 2015). However, for applications where red fluorescent protein imaging is desired, the mRuby2 expression could increase background and hamper imaging analysis. We therefore developed a complementary construct containing a *TIR1::F2A::BFP::AID*::NLS* transgene (Figure 2A). TIR1 is unlabeled, and nuclear-localized BFP provides a readout for TIR1 expression (Figure 2, 3, 6, and S2). AID*-tagged BFP is degraded in the presence of auxin, confirming TIR1 activity via degradation of an internal control (Figure 2, 3 and S2). Recent work from our lab demonstrates that our new TIR1 strains are effective for experiments where red FP imaging is desired (Ragle *et al.* 2020).

Our *eft-3p::TIR1::F2A::BFP::AID*::NLS* transgenes produced the same degree of depletion of AID-tagged substrates compared to an *eft-3p::TIR1::mRuby2* transgene (Zhang *et al.* 2015), but the depletion kinetics were slower (Figure 4, Figure 5). Initially concerned about the impact of this slower degradation rate, we tested whether it impacted the phenotypes observed following depletion of two AID*-tagged nuclear proteins (NHR-23 and NHR-25) and an AID*-tagged cytoplasmic protein (DAF-15). In all cases we observed comparable penetrance of expected phenotypes (Table 2, Table 3). Additionally, our *sun-1p::TIR1::F2A::BFP::AID*::NLS* transgene produced equivalent mNG::AID*::PAR-3 depletion and resultant synchronous cell division as compared to a *sun-1p::TIR1::mRuby2* transgene (Figure 2C and D). Similarly, using a *mex-5p::TIR1::F2A::BFP::AID*::NLS* transgene we

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observed complete sterility following NHR-23 or SPE-44 depletion, identical to that observed

using *pie-1p* or *sun-1p::TIR1::mRuby2* transgenes (Kasimatis *et al.* 2018; Ragle *et al.* 2020).

However, there may be cases where depletion rates affect phenotypic preference, so this

point should be taken into consideration when choosing which TIR1 transgene to use.

One open question is why did our BFP reporter slow the depletion of other AID*-tagged

substrates? Inefficient F2A processing could lead to a TIR1::2A::BFP fusion protein that could

be degraded. However, we were unable to detect such a fusion protein by western blotting,

arguing against this possibility (Figure 3B). Another explanation is that some component of

the system is limiting under these conditions. Possible candidates for the limiting factor

include TIR1 itself, the SCF ligase with which TIR1 interacts (Martinez *et al.* 2020), or

proteasomal activity. Pan-neuronal TIR1 expressed from an extrachromosomal array

produced much stronger depletion of an AID*-tagged substrate compared to the equivalent

TIR1 construct in single-copy (O. Hobert, personal communication). While this observation

needs to be explored further, it supports the idea that TIR1 is limiting in some cases. Strong

expression of both AID*::GFP and BFP::AID*::NLS could sequester the SCF ligase or

proteasome if they were limiting. This sequestration might be expected to produce

documented reduction-of-function phenotypes such as defects in cell fate and

differentiation, meiotic defects, or embryonic lethality (Kipreos *et al.* 1996; Nayak *et al.*

2002; Kamath *et al.* 2003). As we did not observe any such defects, we favor a model where

TIR1 is limiting in our system. Going forward, it will be important to test this model by

boosting TIR1 expression using tools such as site-specific integration of arrays or recently

described bipartite gene expression systems (Yoshina *et al.* 2016; Nonet 2020). Another

approach may be to fuse the Skp1 subunit of the SCF ubiquitin ligase to TIR1, which has

resulted in enhanced degradation efficiency of AID-tagged proteins (Kanke *et al.* 2011).

Determining which components of the system are limiting and boosting TIR1 activity has the

potential to address the reported issue of incomplete degradation of some AID-tagged

proteins and a failure to obtain null phenotypes (Patel and Hobert 2017; Serrano-Saiz *et al.*

2018; Duong *et al.* 2020)

We have compared the performance of natural auxin (IAA) with a synthetic, water-soluble

analog (K-NAA) using quantitative imaging and phenotypic assays. In many cases, they

produced identical results. For NHR-25::GFP::AID*::3xFLAG depletion, we observed more

rapid depletion with K-NAA in two cell types using our *eft-3p:: TIR1::F2A::BFP::AID*::NLS*

(Figure 5). However, interestingly K-NAA did not produce the larval arrest phenotype in

these animals, whereas IAA did (Table 2). It is unclear what is behind the discrepancy

between these results; we will need to determine in which cells NHR-25 is acting to promote

larval development and how IAA and K-NAA affect its depletion in this cell type.

Additionally, *daf-15::mNG::AID*animals grown on IAA arrested at an earlier larval stage

than animals treated with K-NAA (Table 3) consistent with faster and/or more robust

depletion (Duong *et al.* 2020). Our results do not indicate that one form of auxin is

consistently superior, but rather that one needs to empirically test each auxin, depending on

the AID-tagged gene, the TIR1 transgene, and the tissue in which depletion is occurring. As

the community tests IAA and K-NAA on more AID*-tagged factors, it will be important to

look for patterns that might emerge in order to better predict which auxin will perform

better in specific assays.

For many applications, the AID system offers a powerful method to conditionally degrade

proteins in specific tissues and at specific points in development. However, as the system

has gained popularity, particular challenges have emerged. While they do not dampen our

enthusiasm for the AID system, it is important to be aware of them. The first is the

previously discussed issue of incomplete degradation. The second issue is that auxin-

independent, TIR1-dependent degradation of certain AID-tagged proteins has been

documented in both human cells and in *C. elegans* (Zasadzińska *et al.* 2018; Sathyan *et al.*

2019; Martinez *et al.* 2020; Schiksnis *et al.* 2020). While there are recent solutions to this

issue, such as alternate AID systems (Li *et al.* 2019) or additional regulatory components that

block auxin-independent degradation of AID-tagged proteins (Sathyan *et al.* 2019), these

methods are not compatible with existing *C. elegans* strains using minimal AID tags.

Engineering an improved TIR1 that does not promote auxin-independent degradation of

minimal AID-tagged proteins would be the preferable solution. A strong candidate are

recently described TIR1 mutations that produce 670-1000x stronger binding to a modified

auxin, reducing the amount of auxin required for target knockdown (Nishimura *et al.* 2020;

Yesbolatova *et al.* 2020). This reagent would be compatible with and improve the

performance of the collection of minimal AID-tagged strains, which the *C. elegans*

community has already generated. Finally, AID-tagging in rare cases can disrupt protein

function. For example, an mNeonGreen::AID* tag caused a mild hypomorph of *unc-3* in the

absence of TIR1 and auxin, suggesting that the presence of the AID* tag was interfering with protein levels (Patel and Hobert 2017). More examples are required to determine rules for optimal AID tag placement in both structured and unstructured domains of proteins. As a precaution, we tend to use long 10-30 amino acid flexible linker sequences to space the AID* tag away from the protein of interest.

The ability to rapidly deplete proteins with temporal and cellular resolution allows precise dissection of the roles of gene products in developmental processes of interest. With the ever-increasing efficiency of genome editing and continued refinement of the AID system, one can envision creating libraries of FP::AID*-tagged genes covering the genome and a bank of TIR1 strains to allow depletion in virtually all cell types. Together, this collection of strains and vectors should facilitate efficient generation and depletion of new FP::AID*-tagged proteins.

Methods

Molecular Biology

In the below constructs, flexible linker sequences ranging from 5-9 glycine/serine residues are used to separate cassettes within constructs (i.e., AID*, 3xFLAG, 3xMyc, TEV protease recognition sites, etc.). Unless otherwise specified, pJW plasmids (Ward lab) were generated by Gibson cloning using an in-house made master mix, as described (Gibson *et al.* 2009). For two-fragment Gibson cloning, 0.63 µl of each DNA fragment was mixed with 3.75 µl of the

Gibson master mix and incubated for 1-4 hours at 50ºC. Longer reaction times were used for inefficient assemblies. Reactions were then transformed as described in the supplemental methods or stored at -20ºC. Detailed methods describing construct generation are provided in supplemental methods (Supplementary File S2). Oligos used to construct plasmids are listed in Table S1. All plasmids used are listed in Table S2. Primer design information for designing homology arms for Gibson and SapTrap cloning is provided in Supplementary File S3. Supplementary File S4 contains sequence files for all plasmids generated for this study.

C. elegans

C. elegans strains were cultured as originally described (Brenner 1974). All strains used in this study are listed in Table S3. The majority of genome editing was performed in N2 (wild type), EG9615 *oxSi1091[mex-5p::Cas9(smu-2 introns) unc-119+] II; unc-119(ed3) III,* or EG9882 *F53A2.9(oxTi1127[mex-5p::Cas9::tbb-2 3'UTR, Phsp-16.41::Cre::tbb-2 3'UTR, Pmyo-2::nls-CyOFP::let-858 3'UTR + lox2272]) III* animals (Table S3). EG9615 and EG9882 (unpublished) stably express Cas9 in the germline and are gifts from Dr. Matthew Schwartz and Dr. Erik Jorgensen. TIR1 was isolated by outcrossing and loss of the *Cas9* allele was confirmed by PCR or loss of *myo-2::NLS::OFP* expression. The *mex-5p, myo-2p* and *cdh-3p* strains were generated in specialized genetic backgrounds and then the TIR1 transgene was isolated by outcrossing. The loss of other alleles in the background was confirmed by PCR genotyping. Details are provided in Supplemental File 2 and Table S4. Specific details for the TIR1 strains (injection strain, Cas9 and sgRNA source, number of times outcrossed to an N2 background) are provided in Table S4. We note that we are reporting the final, SEC-excised

strains in this table. We have made the precursor strains containing the SEC available to the CGC for our *mex-5*p and *eft-3p* strains: JDW220 *wrdSi10[mex-5p::TIR1::F2A::BFP::tbb-2 3'UTR+SEC]* I:-5.32, JDW222 *wrdSi8[mex-5p::TIR1::F2A::BFP::tbb-2 3'UTR + SEC]* II:-0.77, and JDW224 *wrdSi22[eft-3p::TIR1::F2A:BFP::tbb-2 3'UTR+SEC]* I:-5.32. Following SEC-excision, chromosome I knock-ins can be PCR genotyped using oligos 2835+2836+3415 (Table S1). A wild-type locus produces a 623 bp band and the TIR1 knock-in produces an 881 bp band. Chromosome II knock-ins can be similarly genotyped post-SEC excision by PCR with oligos 273+274+3415 (Table S1). A wild-type locus produces a 684 bp band and a TIR1 knock-in produces an 881 bp band.

CRISPR/Cas9-based genome editing

All TIR1 strains were generated through SEC selection-based genome editing as previously described (Dickinson *et al.* 2015). Single-copy transgenes were inserted into the chromosome I and II loci where the ttTi4348 and ttTi5605 transposons are inserted for MosSCI (Frøkjaer-Jensen *et al.* 2008; Frøkjær-Jensen *et al.* 2012), respectively. The genetic map positions for these insertions are provided in the genotype information. In Table S5, we detail the genetic background in which injections were performed, which Cas9 and sgRNA plasmids were used, and how many times the strains were outcrossed against an N2 background. Repair templates were used at 10 ng/µl and Cas9+sgRNA or sgRNA plasmids were used at 50 ng/µl.

Auxin treatment

The Dickinson and Reiner labs routinely use 1 mM IAA, while the Matus and Goldstein lab routinely use 1 mM K-NAA for experiments. The Ward lab routinely uses 4 mM IAA as a stronger NHR-25 depletion phenotype (embryonic lethality) is observed at 4 mM IAA, but not at 1 mM IAA (Zhang *et al.* 2015). In addition, a sterility phenotype produced by depleting NHR-23 in the germline is more penetrant and reproducible on 4 mM IAA compared to 1 mM IAA (unpublished data). Therefore, the western blots (Figure 3B, Figure 4A) and phenotypic assays (Tables 2, 3, S5, S6) were performed using 4 mM IAA or K-NAA to provide the strongest possible depletion. For DQM623 imaging (Figure 7), we also used a higher concentration of auxin (4 mM K-NAA), as we could not detect BFP reporter expression from the *bmd176[cdh-3pTIR1::F2A::mTagBFP2::AID::NLS]* transgene and wanted to design the experiment to achieve the strongest possible depletion of NHR-25. Controls for experiments using IAA are NGM plates with an equivalent concentration of ethanol vehicle. Controls for experiments using K-NAA are NGM plates.

BFP::AID*::NLS reporter depletion experiments

For Figure 2B, LP630 *cpIs103[sun-1p::TIR1-C1::F2A::mTagBFP2-C1::AID*::NLS + SEC II:-0.77] II* L4 larvae were placed on standard NGM plates containing 1 mM Auxin or 4% ethanol (control) and kept at 22ºC overnight. Young adults were the mounted on 3% agar pads containing 10 mM sodium azide as a paralytic. Images were acquired on a Nikon TiE microscope equipped with a 405 nm diode laser for fluorescence excitation; a 20x air objective and 1.2x tube lens (total magnification of 24x); a CSU-X1 spinning disk head; and a

Hamamatsu ImageEM EM-CCD camera. Acquisition was controlled by MetaMorph software, and images were prepared for display by cropping, rotating and adjusting brightness and contrast using ImageJ. No other image manipulations were performed.

For DV3799 *reSi1[col-10p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32]*, DV3801 *reSi3[unc-54P::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32]*, DV3803 *reSi5[ges-1p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32]*, and DV3805 *reSi7[rgef-1p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32]* (Figure 6), we made an auxin (IAA) (Alpha Aesar, #A10556) stock solution (400 mM in ethanol) that was stored at 4˚C for up to one month. A 16 mM auxin working solution was then prepared freshly by diluting 1:25 in filtered water with 4% ethanol final concentration. 500 µL of 16 mM auxin was added to 60 mm NGM plates for a final concentration of 1 mM, with 4% ethanol as vehicle control (plates contain approximately 8 ml of agar). Plates were then seeded with OP50. Larvae were grown on NGM plates to the desired stage, then shifted onto auxin or vehicle plates for 3 hours before imaging. Animals were anesthetized with 5 mM tetramisole and images were acquired on a Nikon A1si Confocal Laser Microscope using a Plan-Apochromat 40x/1.4 DIC objective and DS-Fi2 camera. Images were analyzed using NIS Elements Advanced Research, Version 4.40 software (Nikon).

For JDW221 *wrdSi18[mex-5p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32]*, JDW225 *wrdSi23[eft-3p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32]*, JDW229 *wrdSi47[dpy-7p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32]* (Figure S4), 1-hour

auxin treatments were performed. Animals were synchronized at the L1 larval stage by

sodium hypochlorite treatment and moved to nematode growth media (NGM) plates

seeded with *E. coli* OP50. At the young adult stage, JDW221 worms were either kept on

OP50-seeded NGM plates (control) or moved to plates treated with 1 mM K-NAA (Phyto-

Technology Laboratories, N610) (Martinez and Matus 2020). At the mid-L3 larval stage,

JDW225 and JDW229 worms were either kept on OP50-seeded NGM plates or moved to

plates treated with 1 mM K-NAA. OP50-seeded NGM plates containing K-NAA were

prepared as described (Martinez and Matus 2020). Images were acquired on a custom-built

upright spinning-disk confocal microscope consisting of a Zeiss Axio Imager.A2 modified

with a Borealis-modified Yokogawa CSU-10 confocal scanner unit with 405 nm lasers and

488 nm lasers and equipped with a Hamamatsu Orca EM-CCD camera. Images shown for

JDW221 (pachytene region) were acquired using a Plan-Apochromat 40x/1.4 DIC objective.

Images shown for JDW225 (uterine and vulval tissues) and JDW229 (hypodermal cells) were

acquired using a Plan-Apochromat 100x/1.4 DIC objective. MetaMorph software (version:

7.8.12.0) was used to automate image acquisition. Worms were anesthetized on 5% agarose

pads containing 7 mM NaN$_3$ and secured with a coverslip. Acquired images were processed

through Fiji software (version: 2.0.0- rc-69/1.52p).

For LP869 *cpSi171[vha-8p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32]*, LP870

cpSi172[myo-2p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32], and LP871

cpSi174[myo-3p::TIR1::F2A::mTagBFP2::AID*::NLS::tbb-2 3'UTR I:-5.32] Figure S4)*,* mixed

stage animals were transferred to 1 mM K-NAA NGM plates for 24 hours. Images were then

taken using the 60x objective on a Nikon TiE stand with CSU-X1 spinning disk head

(Yokogawa), 447 nm, 514 nm, and 561 nm solid state lasers, ImagEM EMCCD camera

(Hamamatsu). Worms were anesthetized and images were processed as described above.

AID*-tagged substrate depletion experiments

To examine depletion of mNG::AID*::PAR-3 in embryos (Figure 2), young adult worms were

treated with either 1 mM IAA or 0.25% EtOH in standard liquid culture conditions (Stiernagle

2006) for 1 hour. Then, embryos were dissected on poly-lysine-coated coverslips and

mounted in egg buffer with 22.8 µm beads as spacers. Confocal images were captured on a

Nikon Ti2 microscope equipped with 405 nm and 488 nm illumination lasers; a 60x, 1.4 NA

oil immersion objective; a Visitech VT-iSIM scan head; and a Photometrics PrimeBSI camera

controlled by µManager software. The total amount of mNG::AID*::PAR-3 present in each

embryo was measured in FIJI by drawing a box around each embryo, measuring integrated

pixel intensity, and then converting to a fluorescence intensity by subtracting off-embryo

background. Results were plotted using GraphPad Prism software.

To monitor depletion of GFP::AID in the vulval precursor cells (Figure 4B,C) CA1202 ieSi57

[eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)] II; ieSi58 [eft-3p::degron::GFP::unc-54

3'UTR + Cbr-unc-119(+)] IV and JDW185 *wrdSi54[eft-3p:TIR1:F2A:mTagBFP2:tbb-2 3'UTR, I:-*

5.32]; unc-119(ed3) III; ieSi58 [eft-3p::AID*::GFP::unc-54 3'UTR + Cbr-unc-119(+)] IV animals

were grown on NGM plates until early L3. A subset of animals were imaged at timepoint "0

min", and the remainder were shifted onto 1 mM K-NAA and imaged at the indicated

timepoints. Images were acquired using a Plan-Apochromat 100x/1.4 DIC objective on a

custom-built upright spinning-disk confocal microscope. This microscope consisted of a Zeiss

Axio Imager.A2 modified with a Borealis-modified Yokogawa CSU-10 confocal scanner unit

with 405 nm lasers and 488 nm lasers and equipped with a Hamamatsu Orca EM-CCD

camera. MetaMorph software (version: 7.8.12.0) was used to automate image acquisition.

Worms were anesthetized on 5% agarose pads containing 7 mM NaN$_3$ and secured with a

coverslip. Acquired images were processed through Fiji software (version: 2.0.0- rc-

69/1.52p). GFP was quantified as described in Martinez et al. (2020). Measurements of total

fluorescence intensity in the nucleus was measured, and fluorescence intensity immediately

outside the circumference of the nucleus was also measured to obtain the cytoplasmic

value. The ratio of these intensities is plotted in Figure S3. For Figure 4C, only the nuclear

intensity was plotted.

To monitor depletion of nuclear NHR-25::GFP::AID* in the vulval precursor cells and seam

cells using *eft-3p::TIR1* transgenes (Figure 5), JDW71 *ieSi57 [eft-3p::TIR1::mRuby2::unc-54*

3'UTR, cb-unc-119(+)] II; unc-119(ed3) III; nhr-25(wrd18[nhr-25::GFP::AID:3xFLAG]) X* and

JDW187 *wrdSi23[eft-3p::TIR1:F2A:mTagBFP2:: AID*::NLS:tbb-2 3'UTR, I:-5.32]; wrd52[nhr-*

25::GFP::AID::3xFLAG] X* animals were grown to L3 on NGM plates. Animals were auxin

treated, and they were imaged and analyzed as described above for AID*::GFP depletion

experiments. Experiments measuring NHR-25::GFP::AID* depletion using DQM623

bmd176[cdh-3pTIR1::F2A::mTagBFP2::AID::NLS] II; nhr-25(wrd10[nhr-*

25::GFP^AID*:3xFLAG]) X were similarly performed (Figure 7), except animals were imaged

one hour after exposure to control or 4 mM K-NAA plates.

Western Blot Analysis

For all western blots, animals were synchronized by sodium hypochlorite treatment, moved

to nematode growth media (NGM) plates seeded with *E. coli* OP50, and grown at 20°C for

~40 hours. L4 animals were collected untreated or exposed to 4 mM auxin (IAA) or ethanol

for the amount of time specified prior to collection in M9 buffer. Animals were washed

several times, pelleted, and freeze cracked in liquid nitrogen 2X. To each worm sample was

added house-made 4x Laemmli Sample Buffer, followed by 10 minutes boiling at 95°C and

centrifugation. The resulting supernatants were used for western blot analysis. The total

proteins were denatured for 10 min at 95°C. Equal amounts of protein samples were loaded

and separated by precast 4-20% Mini-Protean TGX Stain Free Gels (Bio-Rad) then

transferred to polyvinylidene difluoride membranes. The primary antibodies used were

rabbit anti-GFP antibody (Abcam, #ab290) (Figure 4 and Figure S1), and rabbit anti-tRFP

(Evrogen, #AB233) (Figure 3) both diluted at 1:1000. The secondary antibody used was goat

anti-Rabbit-HRP (Kindle Biosciences LLC, #R1006) diluted at 1:1000 in all cases. Images were

captured with a Bio-Rad ChemiDoc imaging system.

Phenotypic analysis

For all strains containing *nhr-23::GFP::AID* and nhr-25::GFP::AID** (Table 2, Table S5),

experiments were performed as previously described (Zhang *et al.* 2015). Briefly, adult

hermaphrodites were picked onto MYOB plates seeded with OP50 and containing 4 mM

IAA, 4 mM K-NAA, or EtOH (control). Adults were permitted to lay eggs for 4 hours at 25°C

prior to being removed and the resulting progeny were kept at 25°C and scored daily for

larval arrest and gonadal abnormalities for four days. For strains containing *daf-*

15::mNG::AID* (Table 3, Table S6), animals were synchronized at L1 by sodium hypochlorite

treatment and released onto MYOB plates seeded with OP50 and containing either 4 mM

auxin (IAA or K-NAA), or EtOH (control). Animals were grown at 20°C for 4 days and scored

for larval arrest. Larval arrest stage (L2 vs L3) was determined by animal size and confirmed

by imaging a subset of animals by DIC microscopy and evaluating gonadal development.

Statistical analysis

Statistical significance was determined using a two-tailed unpaired Student's t-test. *P* < 0.05

was considered statistically significant. **** indicates *P* < 0.0001

Data availability

Strains in Figure 6A and Table S4 are available through the *Caenorhabditis* Genetics Center

with the exception of DQM526 which can be obtained upon request from Prof. David Matus.

Other strains and plasmids can be requested directly from the authors. The data that

support the findings of this study are available upon reasonable request. The indicated

plasmids in Table S2 will be made available through Addgene. Supplemental files will be

available on figshare at https://figshare.com/s/2e570e4c83c897462926.

AUTHOR CONTRIBUTIONS

D.J.D. designed the built-in F2A::BFP::AID*::NLS reporter strategy for TIR1 expression and activity. G.A, T.D., M.L., M.A.Q.M., L.C.J., J.D.H, N.J.P., D.Q.M., D.J.D., D.J.R., and J.D.W. conceived and designed the experiments. G.A. T.D., R.D., J.D.H., W.Z., T.N.M.-K., S.S.S., D.Q.M., D.J.D., D.J.R., and J.D.W. designed the constructs. T.D., J.D.H., R.D., N.J.P., J.M.R, and D.J.D. performed the microinjections. G.A., T.D., M.L., M.A.Q.M., J.D.H., N.J.P., and D.J.D. performed the crosses and characterized strains. G.A., T.D., M.L., R.D., M.A.Q.M., L.C.J., J.D.H., H.N.S., N.J.P., R.M., B.D., J.M.R., and D.J.D. performed the experiments. G.A., T.D., M.A.Q.M., J.D.H., N.J.P., D.Q.M., D.J.D., D.J.R., and J.D.W. analyzed and quantified the data. G.A. and J.D.W. wrote the manuscript with contributions from the other authors. The authors declare no competing interests.

Supplemental Figures:

A

wild type   eft-3p::TIR1::2A::BFP;
              AID*::GFP

75 kDa   *

50kDa   anti-GFP

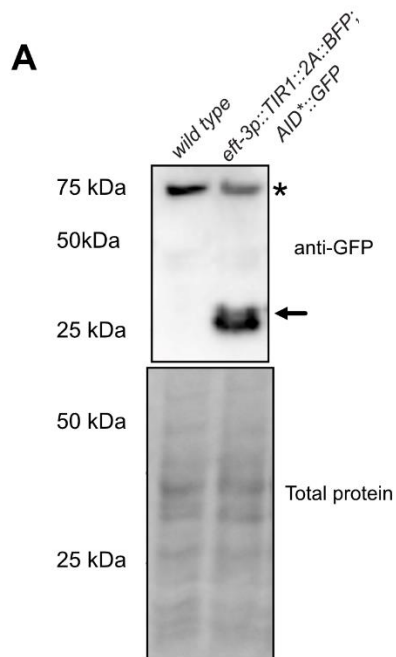25 kDa   ←

50 kDa

              Total protein

25 kDa

Figure S1. Anti-GFP western blot with wild type (N2) control. A) Anti-GFP western blot performed in wild type (left) and in an AID*::GFP reporter strain containing the *eft-3::TIR1::F2A::BFP::AID*::NLS* insertion. Anti-GFP blot (top) showing background band (marked with *; see Figure S1) and a doublet at approximately 27 kDa, (arrow) consistent with the predicted size of GFP. Stain-free (Bio-Rad) analysis of total protein on the blot is provided as a loading control. Marker size (in kilodaltons) is provided
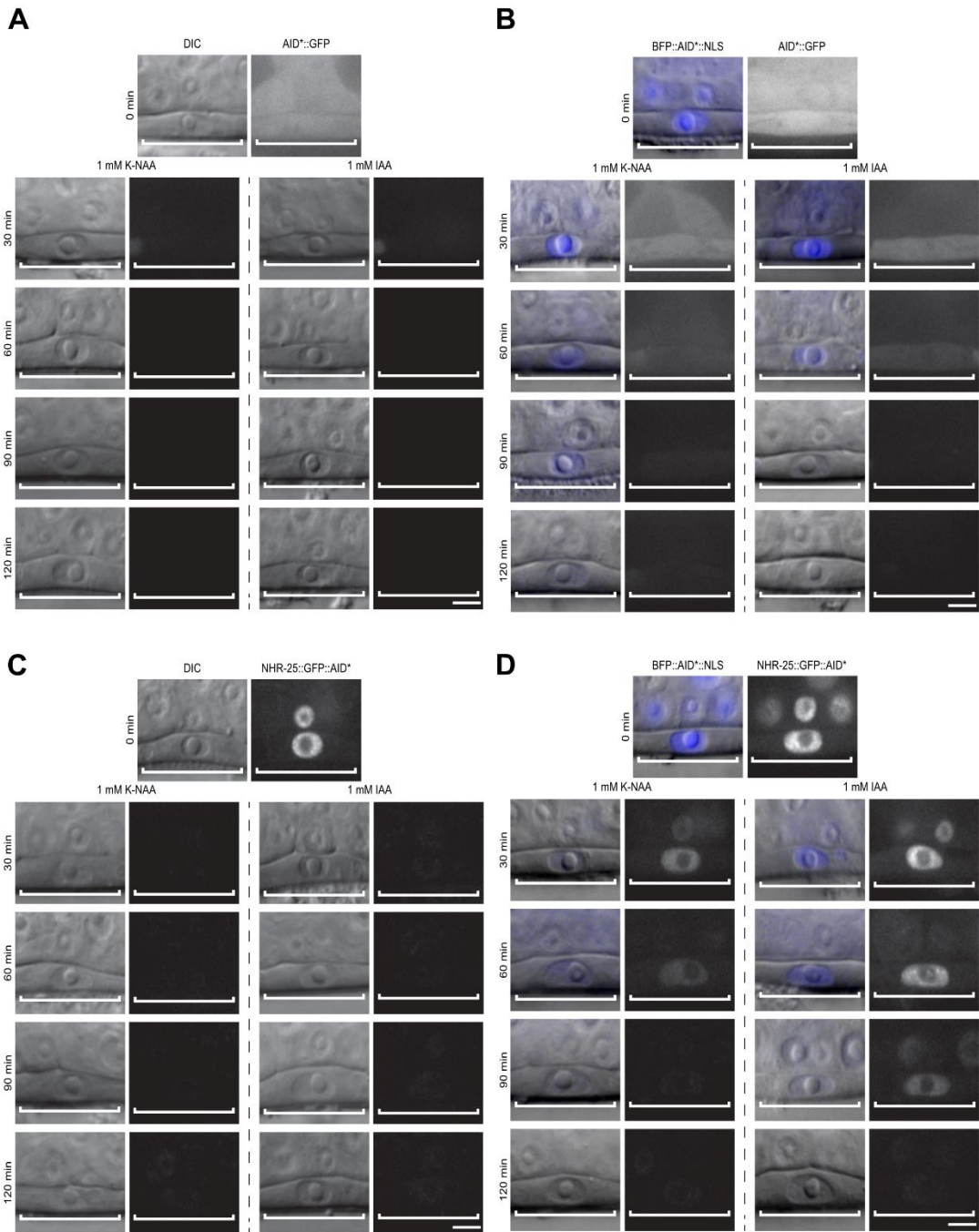
Figure S2. Representative images of AID*::GFP and NHR-25::GFP::AID* depletion in animals expressing either *eft-3p::TIR1::mRuby2* or *eft-3p::TIR1::F2A::BFP::AID*::NLS*. A-D) DIC and corresponding GFP images of VPCs (brackets) from L3 larvae at the P6.p 1-cell stage. For animals expressing *eft-3p::TIR1::F2A::BFP::AID*::NLS*, an overlay of DIC and BFP images were used to demonstrate BFP internal control expression in VPCs. Animals expressing *eft-3p::TIR1::mRuby2* (A,C) and *eft-3p::TIR1::F2A::BFP::AID*::NLS* (B,D) were treated with 1 mM K-NAA or IAA to visualize loss of AID*::GFP (A,B) and NHR-25::GFP::AID* (C,D) over time.
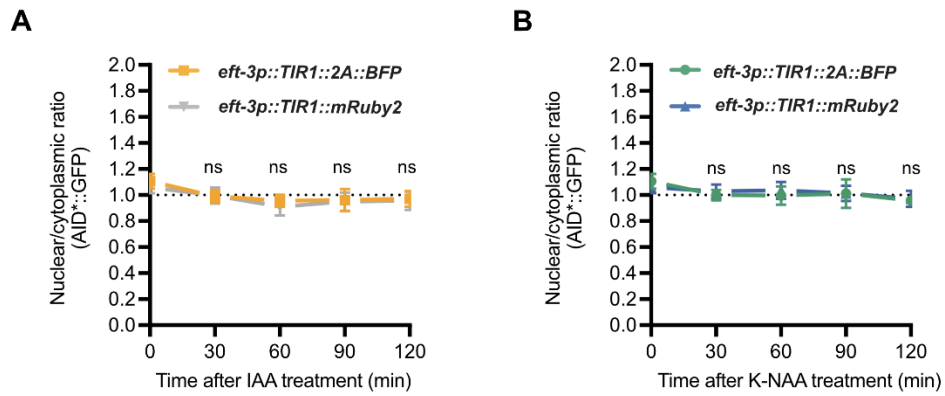
A

B

Figure S3. Nuclear/cytoplasmic ratios of AID*::GFP in VPCs with activated TIR1. These ratios were calculated by dividing AID*::GFP levels in the nucleus by AID*::GFP levels in the cytoplasm. L3s expressing *eft-3p::TIR1::F2A::BFP::AID*::NLS* and *eft-3p::TIR1::mRuby2* animals exposed to 1 mM IAA (A) or K-NAA (B) were analyzed. In both instances, the mean ratio of normalized fluorescent intensity from the nucleus and cytoplasm is shown from a single replicate of 10 or more animals. Error bars represent standard deviation
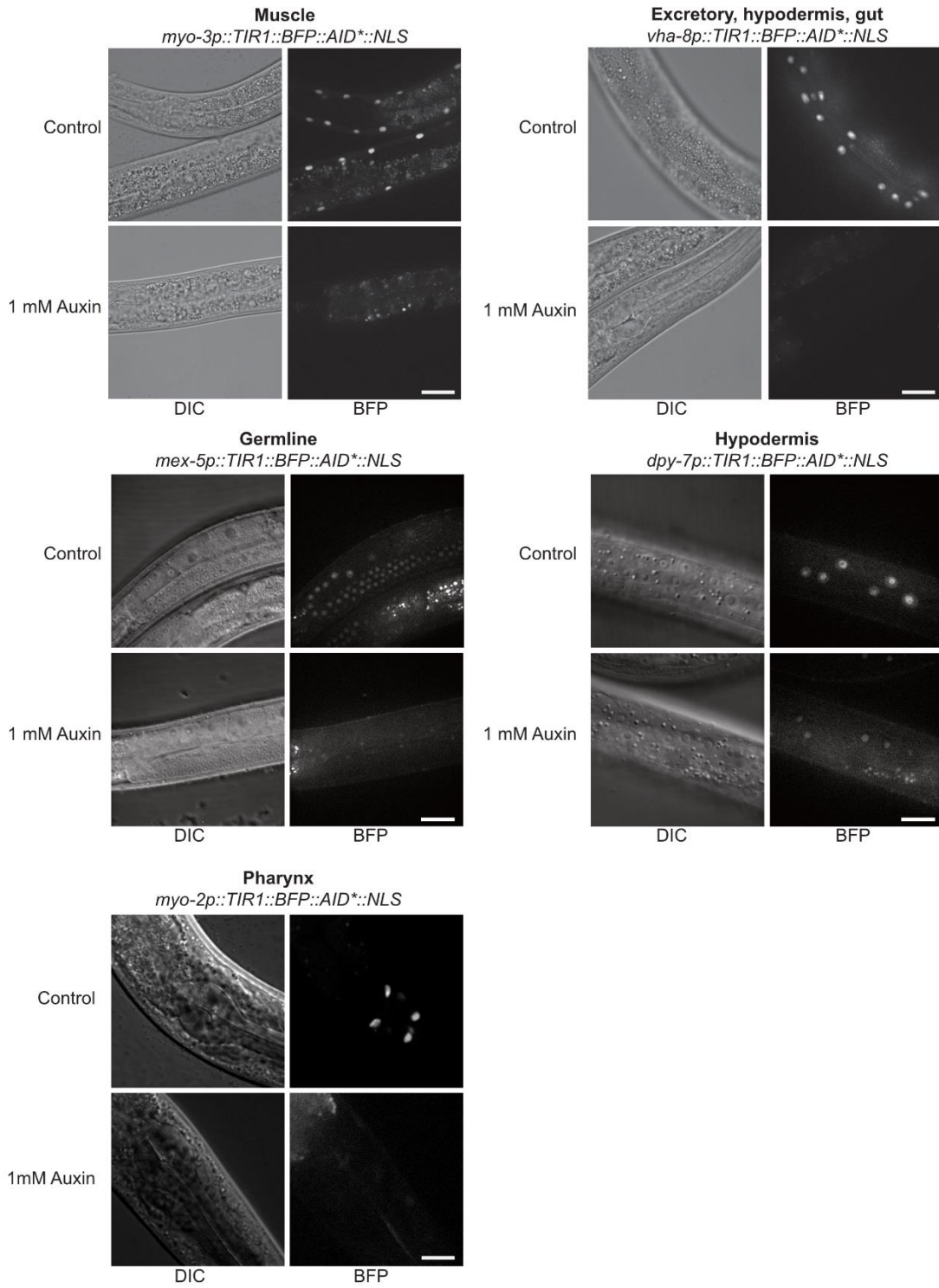
Muscle
myo-3p::TIR1::BFP::AID::NLS*

DIC   BFP

Excretory, hypodermis, gut
vha-8p::TIR1::BFP::AID::NLS*

DIC   BFP

Germline
mex-5p::TIR1::BFP::AID::NLS*

DIC   BFP

Hypodermis
dpy-7p::TIR1::BFP::AID::NLS*

DIC   BFP

Pharynx
myo-2p::TIR1::BFP::AID::NLS*

DIC   BFP

Figure S4. Functional test of new TIR1-expressing strains. Strains of the indicated genotypes were grown on 1 mM water-soluble, synthetic auxin (K-NAA) for ~24 hours (*myo-2, myo-3p* and *vha-8p)* or for 1 hour (*mex-5p* and *dpy-7p*) before imaging. Animals were similarly grown on control seeded NGM plates lacking K-NAA. For all strains, the expected BFP expression pattern was observed in control animals, and BFP expression was reduced or lost in auxin treated animals. Scale bars represent 20 μm (*myo-2, myo-3p, vha-8p*), 15 μm (*dpy-7p*), and 40 μm (*mex-5p*).

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