RESEARCH ARTICLE

PIP degron-stabilized Dacapo/p21Cip1 and mutations in ago act in an anti- versus pro-proliferative manner, yet both trigger an increase in Cyclin E levels

Caroline Bivik Stadler1,*†‡, Badrul Arefin1,*‡, Helen Ekman1 and Stefan Thor1,2,‡§

ABSTRACT

During cell cycle progression, the activity of the Cyclin E-Cdk2 complex gates S-phase entry. CycE-Cdk2 is inhibited by CDK inhibitors (CKIs) of the Cip/Kip family, which include the human p21Cip1 and Drosophila Dacapo (Dap) proteins. Both the CycE and Cip/Kip family proteins are under elaborate control via protein degradation, mediated by the Cullin-RING ligase (CRL) family of ubiquitin ligase complexes. The CRL complex SCFFbxw7/Ago targets phosphorylated CycE, whereas p21Cip1 and Dap are targeted by the CRL4Cdt2 complex, binding to the PIP degron. The role of CRL-mediated degradation of CycE and Cip/Kip proteins during CNS development is not well understood. Here, we analyse the role of ago (Fbxw7)-mediated CycE degradation, and of Dap and p21Cip1 degradation during Drosophila CNS development. We find that ago mutants display over-proliferation, accompanied by elevated CycE expression levels. By contrast, expression of PIP degron mutant Dap and p21Cip1 transgenes inhibit proliferation. However, surprisingly, this is also accompanied by elevated CycE levels. Hence, ago mutation and PIP degron Cip/Kip transgenic expression trigger opposite effects on proliferation, but similar effects on CycE levels.

KEY WORDS: CKI, PIP degron, CNS proliferation, Protein degradation

INTRODUCTION

During embryonic development the cell cycle is precisely controlled to ensure proper tissue growth and organ size (Herranz and Milán, 2008). As cells progress through the canonical G1→S→G2→M cell cycle, the G1→S transition constitutes a key control point. Entry into S-phase is gated by the activity of the Cyclin E (CycE) and Cyclin-dependent kinase 2 (Cdk2) complex. CycE-Cdk2 activity is controlled by CDK inhibitors (CKIs) of the Cip/Kip family, which bind to and inhibit the CycE-Cdk2 complex (Besson et al., 2008; Lu and Hunter, 2010; Starostina and Kipreos, 2012). These include the mammalian p21Cip1, p27Kip1 and p57Kip2 proteins (Lu and Hunter, 2010), as well as the Drosophila Dacapo (Dap) protein (which, of the human Cip/Kip proteins, displays highest homology to p21Cip1) (de Nooij et al., 1996; Lane et al., 1996). CycE and Cip/Kip protein levels are controlled by ubiquitin-mediated proteasome degradation, mediated by E3 ligases of the Cullin-RING ligase (CRL) family. The CRL complex SCFFbxw7/Ago acts as a central CRL for CycE, and the interaction between CycE and SCFFbxw7/Ago occurs via a phospho-degron in CycE, phosphorylated by Cdk2 (Davis et al., 2011; Lane et al., 1996). The CRL degrading p21Cip1 is CRL4Cdt2, and their interaction occurs via the proliferating cell nuclear antigen (PCNA)-interacting peptide (PIP) degron in p21Cip1 (Abbas and Dutta, 2011; Havens and Walter, 2011). The PIP degron is different from the PIP box, found in a number of proteins that bind to PCNA but not to the CRL4Cdt2 complex (Tsanov et al., 2014). The PIP degron binds simultaneously to PCNA and CRL4Cdt2, thereby linking p21Cip1 degradation to DNA replication and S-phase entry (Abbas and Dutta, 2011; Havens and Walter, 2011).

In the Drosophila embryo, CycE-Cdk2 and the Cip/Kip protein Dap are important for embryonic development, and both necessary and sufficient for proper proliferation (de Nooij et al., 1996; Knoblich et al., 1994; Lane et al., 1996; Stern et al., 1993). In the developing CNS, both CycE and Dap play key roles in gating neuroblast (NB) and daughter cell proliferation (Baumgardt et al., 2014; Yaghmaei Salmani et al., 2018). Specifically, during embryonic NB lineage development most, if not all, NBs commence neurogenesis by sequentially budding off daughter cells that divide once, denoted Type I proliferation mode (Boone and Doe, 2008). Subsequently, many NBs switch to budding off daughters cells that differentiate directly, denoted Type 0 mode (Fig. 1D) (Baumgardt et al., 2014). The onset of Dap expression is a key trigger for the switch to Type 0, and Dap misexpression can trigger a premature switch to Type 0 (Baumgardt et al., 2014). In the ectoderm, Dap is degraded in S-phase (Swanson et al., 2015). Similarly, in the CNS, Dap cycles in NBs but is stabilized in daughter cells, allowing NBs to continue proliferating while daughter cells are blocked (Baumgardt et al., 2014). At the end of NB lineage progression, Dap plays a key role in gating the precision and cell cycle stage of the NB cell cycle exit (Baumgardt et al., 2014; Otsuki and Brand, 2019). CycE is crucial in promoting NB and Type I daughter cell divisions, and overexpression of CycE-Cdk2 is sufficient to override the Type 0 switch (Baumgardt et al., 2014). CycE shows dynamic expression in the NBs and their linages (Baumgardt et al., 2014). In addition, a genetic screen aimed at identifying genes involved in embryonic NB lineage progression identified the SCFFbxw7/Ago component archipelago (ago, Fbxw7 in mammals) as causing over-proliferation of the NB-6T lineage (Bivik et al., 2015). These findings in combination raise the question of how protein degradation of Dap and CycE is regulated during embryonic CNS development to ensure precise Type I→Type 0 daughter cell proliferation switches.

To address Dap and CycE protein degradation in relation to NB lineage progression, we generated transgenic lines expressing...
Fig. 1. Dap/p21Cip1 PIP degron mutant proteins inhibit CNS proliferation. (A) Outline of the Drosophila Dap and human p21Cip1 proteins, showing the CDI (CDK inhibitor) and PIP degron domains, with the amino acid sequence of the PIP degrons. ψ denotes aliphatic and Θ hydrophobic amino acids, respectively. CRL4 and PCNA interaction sequences are underlined. Coloured residues were mutated to Ala. Synthetic, expression-optimized DNA constructs were inserted into the pUAS.attB vector, and integrated in the Drosophila genome at cytological position 89E, on chromosome 3. (B) Amino acid sequence of the Dap protein showing the amino acid residues mutated and tested in the UAS transgenes, from top to bottom (see Table S1 for a complete list of UAS transgenes). For several mutants, multiple residues were mutated simultaneously. (C) Staining for Pros, PH3 and Dpn, showing a dividing NB (PH3-positive, Dpn-positive, Pros-asymmetric) and a dividing daughter cell (PH3-positive, Dpn-positive, Pros-cytoplasmic). (D) Schematic depicting NB lineage progression, with the Type I → Type 0 daughter proliferation switch. (E-J) Embryonic nerve cord stained for Pros, PH3 and Dpn, showing dividing NBs and daughter cells, in control (wild-type), pros-Gal4/UAS-dap and pros-Gal4/UAS-dapPCNA segments T3-A1 at St13. Transgenic expression of dapPCNA results in fewer dividing daughter cells. (K-N) Quantification of dividing NBs and daughter cells, in control (wild-type) and pros-Gal4/UAS-thorax (T2-T3) and abdomen (A1-A2) segments at St14. Green lines show 30% above control (UAS-dap) mean; black lines show control mean; red lines show 30% below control mean. Kruskal–Wallis with Dunn posthoc; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001; n ≥ 10 segments; means ± s.d.
wild-type and mutant forms of Dap, targeting both the PIP degron and a number of putative phospho-degrons. For comparison, we also expressed both wild-type and PIP degron mutant versions of human p21\(^{\text{Cip1}}\). We found that of 21 mutant lines generated, only PIP degron mutant lines and a line with a mutation in the CDK inhibitor (CDI) domain showed robust anti-proliferation effects, specifically being able to trigger premature Type I→Type 0 daughter cell proliferation switches. Moreover, in contrast to the mild effects of transgenic overexpression of wild-type Dap in the developing wing imaginal discs (this study; Dui et al., 2013; Swanson et al., 2015), transgenic overexpression of PIP degron mutant Dap and p21\(^{\text{Cip1}}\) severely affected wing growth. Dap and p21\(^{\text{Cip1}}\) (Dap/p21\(^{\text{Cip1}}\) hereafter) PIP degron mutant proteins showed greatly increased stability, and an apparent lack of S-phase degradation. In spite of their anti-proliferative effects, PIP degron-stabilized Dap/p21\(^{\text{Cip1}}\) triggered greatly elevated CycE levels. Analysis of ago mutants in the CNS revealed both elevated NB and daughter cell proliferation, as well as elevated CycE levels. Hence, expression of PIP degron-stabilized Dap/p21\(^{\text{Cip1}}\), and mutation of ago, both result in elevated CycE levels, but with opposite effects on CNS proliferation. These results reveal that the protein degradation balance of CycE versus Dap/p21\(^{\text{Cip1}}\), mediated by the SCD\(^{\text{Flxw}/\text{Ago}}\) and CRL4\(^{\text{Cld}}\) CRLs, is key for gating the Type I→Type 0 daughter cell proliferation switch during CNS development.

RESULTS
Dapp21\(^{\text{Cip1}}\) PIP degron mutants inhibit CNS proliferation
Previous structure–function studies of Dacapo (Dap) involved mutating the PIP degron sequences (Swanson et al., 2015). Although this resulted in S-phase stabilization of the Dap protein, these \textit{UAS-dap} transgenes did not apparently block proliferation in the developing PNS, CNS or wing (Swanson et al., 2015). However, these constructs had an N-terminal fusion of GFP. To avoid potential issues with GFP fusion proteins, we generated a new series of \textit{UAS-dap} constructs without any GFP or other epitope tags added. Only one mRNA transcript has been identified from the \textit{dap} locus (\textit{dap-RA}), allowing us to focus on one single protein isoform of Dap (Dap-RPA). We further optimized the constructs for expression in \textit{Drosophila} by start-ATG optimization, codon optimization (resulting in a 24% DNA sequence mismatch to the endogenous Dap-RA transcript), and by using heterologous 5′ and 3′ UTRs (see Materials and Methods). Because most translational control involves the 5′ UTR (reviewed in Hinnebusch et al., 2016) and the majority of miRNAs target 3′ UTR sequences (reviewed in Brömmner and Hauser, 2014), our synthetic \textit{dap} constructs, which also include 24% mismatch in the coding sequence, should be unfeathered by most, if not all, posttranscriptional regulation. These constructs involved wild-type Dap and PIP degron mutants, which we mutated conserved residues involved in p21–PCNA interaction (denoted Dap\(^{\text{PCNA}}\)) and conserved basic residues flanking the PCNA-interaction domain, shown to be important for p21–CRL4\(^{\text{Cld}}\) interaction (denoted Dap\(^{\text{CRL4}}\)). We further generated a series of mutants with potential phospho-residues Ser, Thr or Tyr changed to Ala (Fig. 1.A,B). In some instances, we also replaced Ser with phospho-mimics (Glu). Additionally, we generated \textit{UAS} transgenes expressing the human Cip/Kip member p21\(^{\text{Cip1}}\), as well as transgenics with the p21 PIP degron sequence mutated, denoted p21\(^{\text{PCNA}}\) and p21\(^{\text{CRL4}}\) (Fig. 1.A). All \textit{UAS} transgenes were integrated into the same pre-determined genomic ‘landing site’ (89E), using phiC31 integrase technology, hence avoiding unwanted genomic position effects (Bischof et al., 2007).

To test the effects of the \textit{UAS} transgenes we first turned to proliferation analysis in the embryonic nerv cord. We previously developed an approach allowing for the separate analysis of neuroblast (NB) versus daughter cell proliferation, which relies on the expression of phospho-Ser10-histone-H3 (PH3; detecting mitotic cells), Deadpan (Dpn) and asymmetric Prospero (Pros). Dividing NBs are PH3-positive, Dpn-positive and Pros-asymmetric, whereas dividing daughter cells are PH3-positive, Dpn-negative and Pros-cytoplasmic (Fig. 1C). This approach resulted in the identification of the daughter cell proliferation switch from Type I (daughters dividing once) to Type 0 (daughters directly differentiating) (Fig. 1D) (Baumgardt et al., 2014). Using this approach, we analysed NB and daughter cell proliferation at stage St13 and St14 in both thorax and abdomen (T2-T3 and A1-A2). In line with the tight control of Dap protein levels, we noted only minor effects on proliferation in \textit{pros-Gal4/UAS-dap} expressing embryos, only statistically relevant by pairwise comparison with control (wild type) (Fig. 1E-H,K-N; Fig. SIA-D). By contrast, expression of the PIP degron mutants \textit{dap}\(^{\text{PCNA}}\) and \textit{dap}\(^{\text{CRL4}}\) as well as the \textit{dap}\(^{\text{CDI}}\) mutant, all displayed reduced daughter cell proliferation (Fig. 1I-N; Fig. SIA-D). Similar results were observed for human p21, with \textit{UAS-p21} showing no apparent effects, whereas the PIP degron mutants \textit{p21}\(^{\text{PCNA}}\) and \textit{p21}\(^{\text{CRL4}}\) triggered reduced daughter cell proliferation (Fig. 1K-N; Fig. SIA-D). Neither the Dap/p21 PIP degron mutants, nor the \textit{dap}\(^{\text{CDI}}\) mutant, significantly affected NB proliferation. Several of the other \textit{UAS-dap} mutant transgenes trended downwards, but none of them showed significant effects on NB or daughter cell proliferation. Therefore, we focused on the Dap/p21 PIP degron mutants, as well as the \textit{dap}\(^{\text{CDI}}\) mutant.

To address the effect of \textit{UAS-dap} mutant transgenes at single-lineage resolution, we analysed the thoracic NB5-6 lineage, using the \textit{ibe(K)}-\textit{eGFP} transgenic marker (Ulvklo et al., 2012) (Fig. 2A). In control (\textit{pros-Gal4/+}; \textit{ibe(K)}-\textit{eGFP}+) at St13, around 25% of NB5-6 lineages display a mitotic NB and/or daughter cell (Fig. S2A-C) (Brahmamour and Thor, 2016; Baumgardt et al., 2014). In \textit{pros-Gal4/UAS-dap}, we observed a downwards trend in proliferation, albeit not significant (Fig. S2A,B). By contrast, \textit{UAS-dap}\(^{\text{CDI}}\), \textit{UAS-dap}\(^{\text{PCNA}}\) and \textit{UAS-dap}\(^{\text{CRL4}}\) all displayed significant reduction of daughter cell proliferation, and \textit{UAS-dap}\(^{\text{PCNA}}\) also triggered reduced NB proliferation (Fig. S2A,B). Hence, the single-lineage analysis was in agreement with the global analysis, revealing that PIP degron Dap mutants are particularly potent in blocking daughter cell proliferation.

Dapp21\(^{\text{Cip1}}\) PIP degron mutants are stabilized
Next, we analysed Dap expression from the \textit{UAS-dap} constructs, driven from \textit{pros-Gal4}, focusing on the thoracic segments, at St13. Surprisingly, in spite of the extensive modifications in our new synthetic transgenes (as outlined above), the \textit{UAS-dap} transgene displayed minimal, if any, elevation of Dap expression when compared to control (\textit{pros-Gal4/+}) (Fig. 2A,B). By contrast, \textit{UAS-dap}\(^{\text{CDI}}\), \textit{UAS-dap}\(^{\text{PCNA}}\) and \textit{UAS-dap}\(^{\text{CRL4}}\) all displayed striking elevation of Dap expression, both in NBs and daughter cells (Fig. 2C-E). To quantify the Dap levels, we focused on one specific NB lineage, NB5-6T, detected by the \textit{ibe(K)}-\textit{eGFP} transgene, and also upon the NB itself using Dpn as marker. Previous studies found that Dap was degraded in S-phase in embryonic ectodermal cells (Swanson et al., 2015), and that Dap also cycles during the NB cell cycle (Baumgardt et al., 2014). Therefore, to monitor signs of Dap cycling we measured Dap levels in both mitotic and non-mitotic NB5-6T neuroblasts at St13. Control and transgenic embryos were dissected, stained and scanned on the same slide, using identical confocal settings. Pixel intensity was multiplied with the NB cell volume to compensate for size, and this value was denoted.
integrated density’. The mean integrated density value from the control in each experiment was set to one. In line with the previously identified S-phase degradation of Dap and its cycling in NB5-6T, we observed a wide distribution of Dap expression in control NBs, from background levels to robust expression (Fig. 2F). This expression profile was not significantly altered in UAS-dap (Fig. 2F). By contrast, UAS-dapPCNA and UAS-dapCRL4 displayed robust elevation of Dap levels, whereas UAS-dapCDI was less elevated (Fig. 2F). Strikingly, for UAS-dapPCNA the mean elevation of Dap expression was almost sevenfold that of the control (Fig. 2G). In line with previous findings (Swanson et al., 2015) the PIP degron mutants appear not to cycle, as evidenced in the scatterplot, where the majority of data points for UAS-dapPCNA and UAS-dapCRL4 were above the highest points in control (Fig. 2F). Both endogenous and elevated Dap staining was primarily localized to the nucleus of NBs and daughter cells. The only apparent exception was DapCDI, which, although it was predominantly nuclear in NBs, displayed cytoplasmic staining in many daughter cells (Fig. 2A-E). We also quantified Dap levels in a subset of eight other Dap mutants, which showed a non-significant downward trend in the proliferation analysis. Of these, only DapT106A showed a weak but significant change in Dap levels (Fig. S3).

Next, we analysed p21 levels, using a monoclonal antibody to human p21. As anticipated from the limited primary amino acid
sequence conservation between human p21 and Dap (de Nooij et al., 1996; Lane et al., 1996), this antibody did not reveal any specific signal in control embryos (Fig. 2H). However, in pros-Gal4/UAS-p21 embryos we observed a robust signal (Fig. 2I). In line with the conservation of the PIP degron domain, both UAS-p21<sup>PCNA</sup> and UAS-p21<sup>CRL4</sup> showed a robust increase in p21 levels (Fig. 2J-L). The cellular appearance of p21<sup>PCNA</sup> staining was similar to p21, with mostly nuclear localization. By contrast, p21<sup>CRL4</sup> displayed predominantly cytoplasmic staining (Fig. 2L).

We conclude that mutation of the PIP degron stabilises both Dap and p21. Dap is also stabilized by the CDI mutation, but to a lesser degree than the PIP degron mutants.

**Dap/p21<sup>Cp1</sup> PIP degron mutants increase Cyclin E protein levels**

Next, we turned to analysing Cyclin E (CycE) and String (Stg; Cdc25) expression. Both CycE and stg mutants show severely reduced proliferation in the *Drosophila* embryo, including the CNS, and overexpression of CycE or stg can trigger increased proliferation (Baumgardt et al., 2014; Edgar and O’Farrell, 1989, 1990; Knoblich et al., 1994). Therefore, we addressed how the anti-proliferative effects of Dap and p21 overexpression might relate to CycE and Stg expression, using the same strategy as for Dap and p21 levels.

We found that *UAS-dap* triggered a minor but significant reduction in CycE expression (Fig. 3A,B,F). By contrast, all other constructs, apart from *UAS-dap<sup>CDI</sup>* triggered higher CycE levels (Fig. 3C,D,F). For *dap<sup>PCNA</sup>* and *dap<sup>CRL4</sup>* the effects were striking, with a more than fourfold increase in CycE levels (Fig. 3F). Both endogenous and elevated CycE staining primarily localized to the nucleus of NBs and daughter cells (Fig. 3A-E). Regarding Stg, only *dap* and *p21<sup>CRL4</sup>* induced a minor reduction in expression levels, whereas all other constructs displayed no effects (Fig. 3A-E).

We conclude that, in spite of their strong anti-proliferative effects, PIP degron mutant transgenes triggered a robust increase in CycE levels. By contrast, despite its anti-proliferative effects *Dap<sup>CDI</sup>* did not affect CycE levels.

**Dap PIP degron mutants do not elevate Cyclin E mRNA gene expression**

To determine whether the elevated CycE levels arise from transcriptional upregulation, we analysed the transcriptome using RNA-seq, focusing on the *UAS-dap* transgenes. RNA was extracted from control (*pros-Gal4/+*) and experimental (*pros-Gal4/UAS*) embryos at St15-St16, and two independent samples were analysed for each genotype. In line with the broad expression of CycE in many developing tissues, both of the control samples displayed robust expression of CycE, with an average reads per kilobase million (RPKM) of 3.6 (Fig. 3H). Similarly, both of the biological replicate RNA-seq samples of each the four *UAS-dap* transgenes (*dap*, *dap<sup>PCNA</sup>* *dap<sup>CRL4</sup>*, and *dap<sup>CDI</sup>* showed similar CycE levels, with no sign of significant up- or downregulation (Fig. 3I). As anticipated from the minimal effect of Dap overexpression on Stg levels, analysis of *stg* mRNA levels did not reveal any significant changes in the different genotypes (Fig. 3I). We conclude that expression of PIP degron-mutated Dap protein elevates CycE protein levels without affecting CycE mRNA levels.
Dap/p21<sup>Cip1</sup> PIP degron mutants affect wing and thorax development

To determine whether stabilized Dap can inhibit proliferation in other tissues, we turned to the developing wing. Previous studies did not find any proliferation effects of expressing wild-type or PIP degron-mutated Dap in the wing disc (Swanson et al., 2015). In addition, expression of a N-terminally, 4×Myc-tagged UAS-dap construct, or of the original UAS-dap transgene (Lane et al., 1996), resulted in mild phenotypes, with only a wing hair spacing phenotype (from en-Gal4, posterior disc driver) or wing hair spacing and partial loss of wing margin (from nub-Gal4, entire disc driver) (Dui et al., 2013). Here, we used the ap-Gal4 driver, which expresses robustly in the dorsal wing disc from second instar and onward throughout development (Fig. 4A). In line with previous studies expressing UAS-dap in the wing discs (Dui et al., 2013; Swanson et al., 2015), we found only weak effects of our novel UAS-dap transgene. This manifested as an apparent shortening of the wing, evident only after quantification of the adult wing: abdomen length ratio (Fig. 4B-D). In contrast to this mild effect, expression of PIP degron and Dap<sup>CDI</sup> mutants resulted in dramatic truncation of the wings and thorax (Fig. 4E-G). Similar effects were observed when we expressed PIP degron-stabilized p21 (Fig. 4I,J). Surprisingly, and in contrast to Dap, wild-type p21 also showed strong effects (Fig. 4H).

Dap/p21<sup>Cip1</sup> PIP degron mutants affect wing imaginal disc development

The striking effect on wing development of PIP degron-mutated UAS-dap and -p21 transgenes prompted us to address Dap and CycE expression, as well as proliferation, in the developing wing imaginal disc. Previous studies have revealed that endogenous Dap levels are low and uniform in the wing disc (de Nooij et al., 2000; Dui et al., 2013), and we observed a similar staining profile in control third instar larval wing discs (Fig. 5A). In control flies, CycE expression showed a similar profile, with low and apparently uniform levels, and PH3 revealed an even distribution of mitotic cells throughout the disc (Fig. 5A). Expression of UAS-dap from ap-Gal4 resulted in elevated Dap levels in the dorsal disc, combined with elevated CycE levels (Fig. 5B,J). PH3 staining was patchy, and a clustering of mitotic cells was observed at the edges of high Dap and CycE expression (Fig. 5B, arrows). UAS-dap<sup>PCNA</sup>, UAS-dap<sup>CRL4</sup> and UAS-dap<sup>p21</sup> all displayed strong Dap and CycE expression, co-localized in dorsal patches (Fig. 5C-E). These regions were largely devoid of PH3-positive cells in UAS-dap<sup>PCNA</sup> and UAS-dap<sup>CRL4</sup>, and in cells where PH3 was expressed, expression localization instead abutted the patches of Dap and CycE, whereas UAS-dap<sup>p21</sup> showed a more intermingled profile (Fig. 5C-E, arrows). Similar effects were observed when we expressed wild-type or PIP degron-stabilized p21, with elevated CycE levels and largely a complete absence of PH3 in the ectopic CycE regions (Fig. 5F-I). Furthermore, all transgenes apart from UAS-dap triggered malformation of the developing wing discs, when compared to control (Fig. 5A-I). We conclude that the severe adult wing and thorax malformations caused by expression of wild-type and mutant Dap/p21 apparently have their origin in the striking anti-proliferative effects observed in the developing wing imaginal disc. These anti-proliferative effects appear to cause severe disorganization of the developing dorsal wing discs. Similar to the CNS results, these effects are accompanied by elevated CycE expression.

Fig. 4. Dap/p21<sup>Cip1</sup> PIP degron mutants exhibit reduced wing size. (A) Expression of UAS-nmEGFP (nuclear-myc-tagged EGFP) driven from ap-Gal4. Expression is evident in the entire dorsal region of the disc. a, anterior; p, posterior; d, dorsal; v, ventral. (B,C) Image of adult control wings (B), and adult ap-Gal4/UAS-dap wings (C). (D) Quantification of the ratio between wing (green line in B) and abdomen (red line in B) length in ap-Gal4/+ (control) and ap-Gal4/UAS-dap flies. The wing was measured from the tip to the scutellum vein. Student’s two-tailed t-test; **P≤0.01; n≥26 adults; mean±s.d. (E-J) Representative images of adult wings in other ap-Gal4/UAS- genotypes, showing severe effects upon wing and thorax development. Note that control images in A are reproduced for reference in Fig. 5J.
archipelago mutants show increased proliferation and elevated CycE levels

In our recent screen for genes involved in NB lineage development (Bivik et al., 2015), in addition to dap, we identified a new allele of archipelago (ago), the Drosophila orthologue of the mammalian Fbxw7 gene (Moberg et al., 2001). ago mutants display an increase in the number of cells generated in the NB5-6T lineage (Bivik et al., 2015), pointing to an over-proliferation effect.

To address the effect of ago on proliferation in the CNS in more detail, we analysed NB and daughter cell proliferation at St15 in control and ago1/agoDf hemizygous mutants [ago1 is a nonsense mutation truncating the Ago protein at amino acid 1195; agoDf is a deletion of the entire ago locus; Df(3L)Excel9000] (Moberg et al., 2001). This analysis revealed a strong increase in the number of dividing NBs and daughter cells (Fig. 6A-D,I-J). The expression of Dpn (NB marker) furthermore indicated an increase in NB numbers in ago mutants (Fig. 6A,B,E,F). Quantification confirmed this, revealing an increase in NB numbers in ago mutants at St15 (Fig. 6K).

Previous studies showed elevated CycE expression in ago mutant wing discs (Moberg et al., 2001). We analysed CycE expression in the developing nerve cord, and observed apparently elevated expression (Fig. 6A-H). Quantification of CycE levels within mitotic NBs confirmed this, showing a striking twofold increase in CycE (Fig. 6L). We also analysed Dap levels in ago mutants, but found no evidence for elevated levels (Fig. 6E-H,M).

**DISCUSSION**

Resilient NBs versus sensitive daughter cells

We find that mutation of the PIP degron stabilises Dap and p21Cip1 and has strong effects on CNS daughter cell proliferation. In spite of the increased stability of the Dap/p21Cip1 PIP degron transgenes there was no significant effect on global NB proliferation, the only exception being UAS-dapPCNA in NB5-6, which triggered a reduction in NB proliferation. However, the resilience of NBs to expression of PIP degron-stabilised Dap/p21Cip1 is perhaps not surprising. In the embryonic nerve cord, most NBs commence cycling in the Type I mode i.e. generating daughter cells that divide once. Subsequently, many NBs switch to Type 0 mode, generating non-dividing daughter cells, which directly differentiate. One of the key triggers for the Type I→Type 0 switch is the activation of Dap expression (Baumgardt et al., 2014). However, NBs continue cycling in the Type 0 mode even after Dap expression has commenced. While Dap protein appears to be stable in daughter cells, immunostaining of NBs revealed frequent loss of Dap staining, interpreted as an effect of S-phase degradation of Dap (Baumgardt et al., 2014). In line with this, S-phase degradation of Dap was previously observed in embryonic ectodermal cells (Swanson et al., 2015). Our results support this notion, showing a wide range of Dap expression levels in NBs, in both control and pros-Gal4/UAS-dap flies, ranging from background levels to robust expression. Hence, Dap expression is activated in many NBs during lineage progression, but cycles during the cell cycle, hence allowing for continued NB divisions. By contrast, Dap is stable in daughter...

---

**Fig. 5.** Dap/p21Cip1 PIP degron mutants exhibit affected wing disc development. (A-I) Representative images of wandering third instar larvae wing imaginal discs, stained for Dap, PH3 and CycE (A-E), or PH3 and CycE (F-I). Arrows highlight areas with clusters of PH3-positive cells in A-E. (J) Expression of UAS-nmEGFP (nuclear-myc-tagged-EGFP) driven from ap-Gal4 (note that these control images are reproduced from Fig. 4A). See text for details.
cells, hence triggering the Type 0 switch i.e. stopping daughters from dividing. Against this backdrop, it is surprising that the PIP degron mutants, which show robust expression levels in all NBs measured and hence do not appear to cycle, are still unable to stop NB proliferation. However, NBs have a stronger mitotic ‘drive’ than the ‘sensitive’ daughter cells, which even in the Type I mode will only divide once. This NB ‘drive’ stems from the asymmetric cell division that NBs undergo, which acts to deposit key cell cycle...
mutations resulted in a significant reduction in proliferation, phospho-degron residues in Dap. Kinases and ubiquitin ligases, have also been identified (reviewed in Degradation of Dap/p21Cip1 can occur via the PIP degron. But for PIP degron is a key degron for Dap/p21Cip1 inhibitors, e.g. Prospero, which downregulates CycE expression, to the daughter cells, while the NBs retain ‘stemness’ factors (reviewed in Knoblich, 2010; Sousa-Nunes et al., 2010). Strikingly, the resulting NB ‘drive’ appears to be able to overcome even the transgenic expression of PIP degron mutant, S-phase stabilized Dap/p21Cip1.

In contrast to the selective effect of PIP degron mutant-stabilized Dap/p21Cip1 on daughter cells and not NBs, we observed more global effects in developing wing imaginal discs, resulting in striking defects in the adult thorax and wings. To our knowledge, in contrast to the CNS, there are no reports of asymmetric cell divisions in developing wing disc cells, and thus all cells, both mothers and daughters, may be sensitive to stabilized Dap/p21Cip1. Also, in contrast to the narrow time window in the nver cord experiments (onset of pros-Gal4 at St10 and assaying at St13-14), using ap-Gal4 as driver, we transgenically expressed Dap/p21Cip1 over several days; from second instar larvae and into pupariation, hence allowing for a much longer window within which Dap/p21Cip1 can act.

One peculiar effect was that although wild-type Dap had a minimal effect on wing development (apparent only after measuring the adult wing size), wild-type p21 showed strong effects, both in the wing disc and in the adult wing and thorax. This contrasts with the CNS phenotype, where wild-type p21 did not significantly affect proliferation; only PIP degron mutants did. Given the limited primary amino acid sequence conservation of human p21 to Drosophila Dap (de Nooij et al., 1996; Lane et al., 1996) it is tempting to speculate that human p21 may escape non-PIP degron degradation pathways in Drosophila, possibly acting in the wing disc.

**PIP degron is a key degron for Dap/p21Cip1**

Degradation of Dap/p21Cip1 can occur via the PIP degron. But for p21Cip1, a number of phospho-degrons, targeted by several different kinases and ubiquitin ligases, have also been identified (reviewed in Lu and Hunter, 2010). Because only the PIP degron was previously studied in Dap, we mutated a number of potential (Ser, Thr or Tyr) phospho-degron residues in Dap.

Surprisingly, apart from the PIP degron, only the DapCDI mutations resulted in a significant reduction in proliferation, accompanied by increased protein stability. In contrast to PIP degron mutants, DapCDI did not affect CycE or Stg levels. The residues that we mutated in DapCDI are three adjacent Ser-Ala kinases and ubiquitin domains, have also been identified (reviewed in Lu and Hunter, 2010). Because only the PIP degron was previously studied in Dap, we mutated a number of potential (Ser, Thr or Tyr) phospho-degron residues in Dap.

Is the PIP degron itself also a phospho-degron? Phosphorylation of S114 in p21 by GSK3β is involved in CRL3Cdk2 binding to p21, and its degradation upon DNA damage (Abbas et al., 2008; Bendjennat et al., 2003; Lee et al., 2007). However, it is unclear whether S-phase binding of CRL4Cdk2PCNA to p21, and the accompanying S-phase destruction of p21, requires phosphorylation. In line with this notion, the DapT188A mutant tested herein, located inside the PCNA-interaction region, did not show significant proliferation effects.

Our PIP degron Dap/p21Cip1 mutants can stop mitotic proliferation, in both CNS daughter cells and in wing discs. By contrast, previously published work did not show any effect of PIP degron Dap mutants on mitotic diploid proliferation in the CNS or wing discs, only blocked endocycles of polyploidy midgut cells (Swanson et al., 2015). Swanson and colleagues generated Dap PIP degron mutants with six key residues converted to Ala, which would probably have similar stabilising effects as our PIP degron mutations. Therefore, we think it is more likely that the differences in effects upon cell proliferation between our study and the previous one pertains to the fact that they based their PIP degron mutations upon a GFP N-terminally tagged version of Dap, which probably resulted in reduced activity of the Dap protein.

**Dap/p21Cip1 and CycE stability**

How does expression of PIP degron-stabilized Dap/p21Cip1 trigger increased levels of CycE? We questioned whether this increase occurs at the level of CycE mRNA or protein. We observed elevated CycE protein expression in Dap/p21Cip1 PIP degron mutants but did not find any significant change in CycE mRNA expression in the RNA-seq data, indicating that the CycE increase is at the protein level. p21Cip1 binds to both CycE and Cdk2 via its CDI (CDK inhibitor) domain, and inhibits Cdk2 activity (Besson et al., 2008; Lu and Hunter, 2010; Starostina and Kipreos, 2012). Given that the CDI and PIP degron domains are located N- and C-terminally, respectively, we would predict that the PIP degron mutations described herein could still interact with CycE-Cdk2. Studies of PIP degron mutant Xic1 (Xenopus Cip/Kip) protein indicate that it can still interact with CycE-Cdk2 (Chuang and Yew, 2005). One of the major phospho-degron sites in CycE is located in the C-terminal, whereas Cdk2 is known to phosphorylate CycE on T380 and S384, triggering interaction with Fbxw7, and subsequent ubiquitin-mediated proteolysis by the SCF(Fbxw7) ligase (Davis et al., 2014; Tan et al., 2008; Wang et al., 2012). Since p21 inhibits Cdk2, it is tempting to speculate that binding of Dap/p21Cip1 to CycE-Cdk2 inhibits CycE phosphorylation by Cdk2, hence reducing ubiquitination and degradation of CycE by SCF(Fbxw7/AgO) (Fig. 7). Alternatively, Dap/p21Cip1 might sterically hinder the CycE-Fbxw7 interaction, although, to our knowledge this has not been previously demonstrated.

**ago controls CycE stability**

In spite of extensive forward genetic screens, the spectrum of CRL3-ligases, phospho-degron kinases and phosphatases involved in cell cycle degradation control in the Drosophila embryo is not well characterized. One of the primary reasons for this gap in our knowledge is probably that many of these components, such as Cdk2 and Cdt2, are maternally loaded (Sloan et al., 2012; Stern et al., 1993). In addition, other cell cycle components, such as CycB and CycB3, display genetic redundancy (Jacobs et al., 1998). Both of these issues were echoed in our previous mutant analysis of 21 cell cycle genes, which only identified robust effects for CycE, dap, sig and E2f1 on NB lineage progression (Baumgardt et al., 2014). Similarly, an extensive forward NB genetic screen only identified CycE, CycT, E2f1 and ago (Bivik et al., 2015). Against this backdrop, the ago mutants provide a unique window into the role of protein degradation during Drosophila embryonic development.

We find that ago mutants display extra NBs, a well-known Notch pathway loss-of-function phenotype. The supernumerary generation of NBs in ago mutants is interesting when compared with previous findings on Ago/Fbxw7 and the Notch pathway. In mammals, Fbxw7 is involved in the degradation of both Notch1 and Notch4 (Mao et al., 2008; Welcker and Clurman, 2008). By contrast, previous studies in the developing Drosophila eye disc did not reveal apparent changes in Notch protein levels in ago mutant cells, although the Notch target gene E(spl)mß was indeed
upregulated in ago mutant cells, indicating elevated Notch signalling (Nicholson et al., 2011). We observed extra NBs in ago mutants, which represents a typical Notch loss-of-function phenotype. This suggests that the interplay between ago and Notch signalling may be different in the developing eye versus the embryonic neuroectoderm.

Regarding proliferation control, we find that ago mutants show over-proliferation of both NBs and daughter cells. Because ago mutants also display generation of extra NBs, the increased NB and daughter cell proliferation probably, at least to some extent, stems from supernumerary NBs. However, whereas the increase in NB number is ~60%, the increased daughter cell proliferation is close to 200%. In addition, and in line with previous studies in Drosophila wing and eye imaginal discs (Moberg et al., 2001; Nicholson et al., 2011) and mammalian cells (Davis et al., 2014; Tan et al., 2008; Wang et al., 2012), we find greatly elevated CycE levels in ago mutants. The elevated CycE levels should, based upon previous misexpression analysis (Baumgardt et al., 2014; Knoblich et al., 1994), be sufficient to trigger aberrant NB and daughter cell proliferation. Hence, we believe that the over-proliferation of NB and daughter cells in ago mutants probably results from both extra NBs and extra proliferation of NBs and daughter cells, chiefly triggered by increased CycE protein levels.

In summary, the PIP degron-mediated degradation of Dap/p21<sup>1054</sup> and the Ago-mediated degradation of CycE reveals that the control of cell cycle factor protein stability is precisely gated to ensure fidelity in the Type I→Type 0 daughter cell proliferation switch and NB cell cycle exit.

**MATERIALS AND METHODS**

**Fly stocks**

*pros-Gal4* on chromosome III (provided by Chris Q. Doe, University of Oregon, OR, USA); *ap<sup>med4</sup>* (referred to as ap-Gal4; Bloomington Drosophila Stock Center BL#3041); ago<sup>1</sup> (provided by Kenneth Moberg, Emory University School of Medicine, Atlanta, GA, USA); ago<sup>1054</sup>-Df(3L)Exc9000 (Bloomington Drosophila Stock Center BL#7921); lbe(K)-eGFP (Ulkvlo et al., 2012); UAS-nls-myc-eGFP (referred to as UAS-nmEGFP) (Allan et al., 2003). Mutants were maintained over GFP- or YFP-marked balancer chromosomes. As control, Oregon-R or Gal4<sup>+/+</sup> were used. Staging of embryos was performed according to Campos-Ortega and Hartenstein (1985).

**UAS transgenes**

Sequences coding for Dap and human p21<sup>1054</sup> were optimized for *Drosophila* expression (Genscript). A consensus start codon (Cavener and Ray, 1991) and an EcoRI site were added to the 5′ end. Stop codons [UAG (amber), UAA (ochre), and UGA (opal)], followed by an XbaI site were added to the 3′ end (see Table S1 for DNA sequences).Gene synthesis was used to generate the DNA sequences (Genscript), which were subsequently cloned into pUASattB (provided by Konrad Basler and Johannes Bischof, University of Zurich, Switzerland) (Bischof et al., 2007), as EcoRI/XbaI fragments. DNA constructs were injected (BestGene) into landing site strain y<sup>1</sup>, w<sup>1118</sup>; PBac{y+-attP-9A}VK00027 (Bloomington Drosophila Stock Center BL#9744) (chromosome 3, position 89E). Transgene integration was mediated by X-chromosome expression of the integrase PhiC31, driven from the *vasa* promoter (vas-int) (Bischof et al., 2007).

**Immunohistochemistry**

Immunohistochemistry was performed as previously described (Bahnampour et al., 2017; Baumgardt et al., 2009). Primary antibodies used were: guinea pig anti-Dap (1:1000) (Baumgardt et al., 2014); guinea pig anti-Dnp (1:1000); rat anti-Dnp (1:500) (Ulkvlo et al., 2012); rabbit anti-phospho-histone H3-Ser10 (PH3) (1:1000; #06-570, Upstate/Millipore); rat anti-PH3-Ser28 (1:1000; ab10543), rabbit monoclonal anti-PH3-Ser10 (1:1000; ab177218), rabbit monoclonal anti-human-p21<sup>1054</sup> (1:1000; ab109520) (Abcam); mouse monoclonal anti-Dap (1:500; NP1) and monoclonal anti-Prox MR1A (1:10; MR1A) (Developmental Studies Hybridoma Bank). Rabbit anti-CycE (1:500; #sc-33748, Santa Cruz Biotechnology); rat anti-Stg (1:500) (Bivik et al., 2016). Secondary antibodies were AMCA-<sup>+</sup>, FITC-<sup>+</sup>, Rhodamine Red-X<sup>+</sup> and Cy5<sup>+</sup>Alexa Fluor 647-conjugated donkey antibodies (1:200; Jackson ImmunoResearch Laboratories).

**Confocal imaging and data acquisition**

Zeiss LSM700 or Zeiss LSM800 confocal microscopes were used for fluorescence imaging. Confocal stacks were merged using LSM software (Zeiss) or Fiji software (Schindelin et al., 2012). Images and graphs were compiled in Adobe Photoshop and Adobe Illustrator. Adult wings were photographed using an iPhone 4S, and compiled using Adobe Photoshop.

**Statistical analysis**

GraphPad Prism software (v7.04) or Excel was used for statistical calculations. For comparisons between two groups, Student’s t-test was used for normal distributed data, and nonparametric Mann–Whitney U-test was used for non-Gaussian distribution. Comparing more than two groups, for normal distributed data, and nonparametric Mann–Whitney U-test was used, and for non-Gaussian distribution, Kruskal–Wallis with Dunn’s posthoc test was used. To evaluate cell cycle protein levels, control and overexpression embryos were dissected in at least three different separated experiments. Staining intensity was measured in mitotic NBs (PH3+), using Imaged Fiji software (Schindelin et al., 2012), and multiplied with the estimated cell volume to compensate for size, denoted ‘integrated density’. The integrated density values from the control in each experiment were set to one, and the whole dataset was analysed using Student’s t-test.
RNA sequencing and analysis

RNA was extracted from control (pros-Gal4/+ ) and experimental (pros-Gal4/UAS) embryos at St15-St16, using RNaseasy Mini Kit (50) (Qiagen). Two independent samples were analysed for each genotype. Yields ranged from 10.7 µg to 17.8 µg of total RNA. Transcriptome sequencing of poly(A) purified RNA was performed by GeneWhiz on one lane of the HiSeq2500 with a 1×50 bp single-read sequencing configuration. This yielded from 20.7 to 43.4 million reads per sample. RNA expression profiling and fold-change comparison was performed using DESeq2.0, 0.80, SeqMan NextGen software. Gene expression data is available upon request.

Acknowledgements

We are grateful to Kenneth Moberg, Chris Q. Doe, Johannes Bischof, Konrad Basler, the Developmental Studies Hybridoma Bank at the University of Iowa, and the Bloomington Stock Center for sharing antibodies, fly stocks and DNA. We thank Tony Hunter for advice. We thank Julia Pagan, Marco Milan and Olle Sangfelt for critically reading the manuscript. Annika Starkenberg and Carolin Jonsson provided excellent technical assistance.

Competing interests

The authors declare no competing or financial interests.

Author contributions


Funding

We thank the Swedish Vetenskapsrådet (grant number 2017-04061), Knut och Alice Wallenberg Stiftelse (grant number KAW2012.0101), Cancerfonden (grant number CAN2017/257), and Royal Swedish Academy of Sciences for funding to S.T.

Data availability

RNA-seq data have been deposited in GEO under accession number GSE133825.

Supplementary information

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.175927.supplemental

References


