The Drosophila F-box protein dSkp2 regulates cell proliferation by targeting Dacapo for degradation

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ABSTRACT Cell cycle progression is controlled by a complex regulatory network consisting of interacting positive and negative factors. In humans, the positive regulator Skp2, an F-box protein, has been a subject of intense investigation in part because of its oncogenic activity. By contrast, the molecular and developmental functions of its Drosophila homologue, dSkp2, are poorly understood. Here we investigate the role of dSkp2 by focusing on its functional relationship with Dacapo (Dap), the Drosophila homologue of the cyclin-dependent kinase inhibitors p21cip1/p27kip1/p57kip2. We show that dSkp2 interacts physically with Dap and has a role in targeting Dap for ubiquitination and proteasome-mediated degradation. We present evidence that dSkp2 regulates cell cycle progression by antagonizing Dap in vivo. dSkp2 knockdown reduces cell density in the wing by prolonging the cell doubling time. In addition, the wing phenotype caused by dskp2 knockdown resembles that caused by dap overexpression and can be partially suppressed by reducing the gene dose of dap. Our study thus documents a conserved functional relationship between dSkp2 and Dap in their control of cell cycle progression, suggesting the possibility of using Drosophila as a model system to study Skp2-mediated tumorigenesis.

INTRODUCTION

In eukaryotes, cell cycle progression requires the activation of a series of cyclin-dependent protein kinases (CDKs) in combination with their partner cyclins at specific points (Morgan, 1995). For example, progression through the G1 restriction point in animal cells is controlled by the Cdk4/CyclD and Cdk6/CyclD complexes, and the transition from G1 to S phase is achieved through the Cdk2/CyclE complex (Vermeulen et al., 2003). CDK inhibitors (CKIs) are negative regulators of cell cycle progression by inhibiting the activity of CDK–cyclin complexes. Members of the Cip/Kip family of CKIs inhibit the G1 to S transition by associating with, and inhibiting the activity of, the Cdk2/CyclE complex (Wojda, 2000).

In humans, there are three Cip/Kip family members: p21cip1, p27kip1, and p57kip2. The activities of these proteins are subject to negative regulation by SCFSkp2 (Yu et al., 1998; Amati and Vlach, 1999; Carrano et al., 1999; Tsvetkov et al., 1999; Kossatz et al., 2004; Nakayama et al., 2004; Pagano, 2004; Bond et al., 2006; Pateras et al., 2006; Yung et al., 2007). SCFSkp2 is a ubiquitin E3 ligase complex, with its Skp2 subunit, an F-box protein, providing substrate specificity through direct protein–protein interactions. Skp2 has been shown to promote the G1 to S transition and proliferation in a mouse model (Zhu, 2010). Skp2−/− animals are viable, but cells from mutant mice contain markedly enlarged nuclei with polyplody and multiple centrosomes (Zhu, 2010). These cells also show reduced growth rate and increased apoptosis. As an important regulator of
Cell cycle control, Skp2 overexpression is a characteristic feature of a variety of cancers (Gstaiger et al., 2001; Signoretti et al., 2002; Ben-Izhak et al., 2003; Wang XC et al., 2012; Wang Z et al., 2012).

Cell cycle progression in Drosophila is believed to be controlled by highly conserved cyclins and CDKs (Follette and O’Farrell, 1997). Unlike humans, Drosophila has only one known Cip/Kip family member, Dacapo (Dap). Dap negatively regulates the G1 to S transition by inhibiting the CycE/Cdk2 complex, an action that is mediated by the conserved core CDI domain of Dap (de Nooj et al., 1996; Lane et al., 1996). Despite its critical role in cell cycle control, precisely how the stability of Dap protein is regulated is largely unclear. This is in part because Dap appears to have divergent phosphorylation sites critical for ubiquitination (Adams et al., 1996; Morisaki et al., 1997; Huang et al., 2006). Although it was proposed that CG9772 encodes Drosophila Skp2 (dSkp2; Moberg et al., 2004), the genetic and molecular functions of this gene were poorly studied. In a recent report, Ghorbani et al. (2011) presented genetic evidence that established a role for dSkp2 in maintaining diploidy of mitotic cells during development. However, they did not observe a role of dSkp2 in regulating Dap stability, raising the question of whether these two proteins might indeed exhibit a functional relationship that is conserved in cell cycle regulation. Here we describe genetic and molecular studies that specifically investigate the functional relationship between dSkp2 and Dap. Our results show that dSkp2 plays a role in targeting Dap for degradation and has a developmental function interacting with that of Dap in controlling cell cycle progression.

RESULTS
dSkp2 interacts with Dap and has a role in regulating Dap protein level in Drosophila

To investigate the question of whether there is a functional relationship between dSkp2 and Dap in Drosophila, we determined specifically whether dSkp2 and Dap can physically interact with each other. We transiently expressed tagged proteins 4xMyc-Dap and dSkp2-Flag in Drosophila S2 cells and performed communoprecipitation (coIP) assays. We used an anti-Flag antibody to precipitate dSkp2 from the cell extracts and an anti-Myc antibody in Western blots to detect the presence of Dap in the precipitated products. Our results show that 4xMyc-Dap was coimmunoprecipitated when, and only when, dSkp2-Flag was coexpressed in S2 cells (Figure 1A, lane 11; dSkp2-Flag interaction was enhanced by Cks85A, lane 12, a result to which we return in the Discussion). These results suggest a physical interaction between dSkp2 and Dap.

The following three sets of experiments performed in S2 cells further suggest that dSkp2 is a component of a conserved SCF E3 ligase complex, SCF*dSkp2, that has a role in targeting Dap for ubiquitination and proteasome-mediated degradation. First, our coIP assays using extracts from S2 cells expressing tagged proteins 4xMyc-SkpA and dSkp2-Flag show that, consistent with the recent report by Ghorbani et al. (2011), dSkp2 could physically interact with SkpA, a component of SCF complexes (see Figure 1B and legend for details). Second, the stability of Dap (as Myc-Dap fusion) in S2 cells was sensitive to the proteasome inhibitor epoxomicin (see Figure 1C, lane 3) but not the lysosome inhibitor chloroquine (lane 2; see control in lane 1). Of importance, coexpression of dSkp2 (as dSkp2-V5 fusion) reduced Dap protein levels in S2 cells (Figure 1D), whereas RNA interference (RNAi) against dSkp2 increased Dap protein levels (Figure 1E). These results suggest a role of dSkp2 in regulating Dap stability. Third, we expressed tagged proteins Flag-ubiquitin and 4xMyc-Dap in S2 cells, with or without coexpression of dSkp2-V5. Our results show that coexpression of dSkp2-V5 increased the amount of the polyubiquitinated species of Dap (Figure 1F, lanes 2 and 3), suggesting that dSkp2 has a role in targeting Dap for ubiquitination.

To extend our studies performed in S2 cells and further evaluate the molecular relationship between dSkp2 and Dap, we conducted experiments using Drosophila tissue extracts. Here our goal was to use fly tissues as a host to investigate whether these two proteins can also interact as seen in S2 cells. We generated transgenic flies containing UAS-expression constructs for tagged proteins hemagglutinin (HA)-dSkp2 and 4xMyc-Dap. We expressed these proteins in the eye using the GMR-Gal4 driver, and performed coIP experiments in extracts prepared from the fly heads. We used the anti-Myc antibody to precipitate Dap in head extracts, and used an anti-HA antibody in Western blots to detect the presence of Skp2 in the precipitated products. Our coIP results further confirmed the interaction between dSkp2 and Dap in Drosophila tissues (Figure 2A; see lane 9). To evaluate the role of dSkp2 in regulating Dap stability in host tissues of Drosophila, we determined the amount of 4xMyc-Dap in the extracts from fly heads with or without coexpression of HA-dSkp2. Our Western blotting results show that coexpression of HA-dSkp2 in the eye reduced the amount of 4xMyc-Dap (Figure 2B, lane 3; see lane 2 as control), whereas coexpression of dSkp2 RNAi led to an appreciable increase in the amount of 4xMyc-Dap (Figure 2C, lane 2; see lane 1 as control). These results provide further support to our suggestion that Dap stability is regulated by dSkp2.

dSkp2 plays a role in normal wing and eye patterning in Drosophila

To investigate the biological significance of the association of dSkp2 with Dap, we analyzed a null allele, Skp2<sup>ex9</sup>, generated in Andrew Swan’s lab (Ghorbani et al., 2011). Our results confirmed the reported early pupal lethality in homozygous animals (data not shown), demonstrating that dSkp2 is an essential gene for animal survival. To circumvent the lethality problem and facilitate tissue-specific analyses (see later discussion), we evaluated the effects of ubiquitous dSkp2 knockdown and compared them with those caused by dSkp2 mutation. Our results show that ubiquitous knockdown of dSkp2, using the drivers of daughterless-Gal4 (da-Gal4), tubulin-Gal4 (tub-Gal4), or actin-GAL4 (act-Gal4), led to similar early pupal lethality (data not shown). Of importance, such lethality can be rescued by coexpression of a wt dSkp2 transgene (data not shown), documenting the specificity of the RNAi lines used in this work.

dSkp2 was also identified recently in a systematic phenotypic screening of F-box genes with multiple developmental roles (Dui et al., 2012). Tissue-specific knockdown of dSkp2, using two independent RNAi lines, dSkp2<sup>D3142</sup> and dSkp2<sup>2G10837</sup>, caused patterning defects in both the eye and the wing (see Supplemental Figure S1 for the RNAi knockdown efficiency and Supplemental Table S1). dSkp2 knockdown by RNAi in either the posterior region of the wing (driven by engrailed-Gal4/en-Gal4; Figure 3, C′ and C′′) or the entire wing (driven by nubbin-Gal4/nub-Gal4; Figure 3, B, B′, and B′′) led to a wing hair spacing phenotype. In particular, the space between wing hairs became larger, with irregular orientation and frequent clustering of wing hairs (see also Supplemental Figure S2). Furthermore, relative to wild-type or targeted regions, RNAi-expressing regions became smaller (see Figure 3, B′ and B′′, for effects on the entire wing and Figure 3, C′ and C′′, for effects on the posterior region). These phenotypes were completely rescued by coexpressing dSkp2 from the transgenes of either UAS-dSkp2 or UAS-HA-dSkp2 (Figure 3C and data not shown). Two additional independent RNAi lines, dSkp2<sup>2G1326</sup> and dSkp2<sup>4W300716</sup>, caused similar phenotypes,
dicer2, an RNAi-machinery protein (Supplemental Table S1). Taken together, these results suggest a role of dSkp2 in cell proliferation and patterning during Drosophila development. The role of dSkp2 although with different severity, likely reflecting differences in RNAi strength (Supplemental Table S1). RNAi strength for each of the four lines tested was enhanced by simultaneous overexpression of
in patterning was also detectable in the eye, where dSkp2 knockdown (driven by eyeless-Gal4/ey-Gal4) caused rough eyes (Figure 3, A, A’, and A”).

dSkp2 regulates cell cycle progression in Drosophila wing discs

To investigate the underlying defects of the wing hair spacing phenotype caused by dSkp2 knockdown, we determined whether cell number was altered in the adult wing. Supplemental Figure S2 shows that, relative to wild-type wings, dSkp2 RNAi-treated wings had a significantly reduced number of hairs within areas of the same size, indicating a decrease in cell density. Cell density change has generally been attributed to specific cell cycle alterations that underlie the posterior region of the wing disc with dSkp2 knockdown (driven by en-Gal4), cell density is significantly lower than the wild-type control (Figure 4, B and C).

Reduced cell density observed in Figure 4 can be explained by a defect in cell cycle progression caused by dSkp2 knockdown. An alternative (or additional) possibility is that a significant number of cells underwent apoptosis during wing development. It has been documented that cells containing a null allele of dSkp2, dSkp2ex9, exhibit enhanced apoptosis, in addition to other cell cycle defects (Ghorbani et al., 2011). The results shown in Figure 4, A–C, were obtained under a condition that resembles the null allele with respect to the presence of apoptotic cells induced by dSkp2 RNAi (data not shown). In these experiments, dicer2 was coexpressed with RNAi expression. We found that, without dicer2 coexpression, dSkp2 RNAi did not cause any detectable apoptosis (data not shown). To determine whether weaker perturbations caused by dSkp2 knockdown are sufficient to affect cell number without crossing the threshold of causing apoptosis, we performed a flip-out analysis (Neufeld et al., 1998) under the condition in which dicer2 was not coexpressed. As noted by Ghorbani et al. (2011), dSkp2 is located close to the centromere, precluding the use of mitotic recombination to generate dSkp2 mutant clones. Unlike the fluorescence-activated cell sorting technique, which monitors the snapshot behavior of a group of cells with regard to cell cycle progression, an advantage of the flip-out technique is that it quantifies the “accumulated” outcome of the behavior of cell cycle progression during a period of time. We reasoned that this “compounding effect” (due to time) may permit a sensitive detection of even potentially minor defects in cell cycle progression caused by weaker perturbations.

In our analysis, we used the flip-out technique to generate control (wild-type) or RNAi clones that are marked by green fluorescent protein (GFP; Figure 4, D–F; see Materials and Methods for details; Neufeld et al., 1998). We induced RNAi clones at 38 h after egg deposition (AED) and allowed animals to develop for another 77 h. We then counted the cells in both wild-type and dSkp2-knockdown clones. Figure 4, D–F’, shows the distributions of clone size expressed as cell number in individual clones analyzed. The average clone sizes for wild-type, dSkp2ex9, and dSkp22G05142 clones were 143, 53, and 26 cells, respectively. On the basis of the measured clone size, we estimated the cell doubling time (see Materials and Methods). The estimated doubling times for wild-type cells, dSkp2ex9, and dSkp22G05142 cells were 11, 13, and 16 h, respectively. These results support the suggestion that dSkp2 knockdown can prolong the cell cycle time of wing disc cells.

dSkp2 and dap have functions that intersect in controlling cell proliferation

If Dap is indeed a target of SCFdSkp2, patterning defects caused by reduced dSkp2 activity may be reflective of, at least in part, an increased Dap level in wing cells. To evaluate this possibility, we analyzed whether overexpression of dap can cause defects similar to those caused by dSkp2 knockdown. For this purpose, we used two independent transgenic lines, UAS-4xMyc-dap (see earlier discussion) and UAS-dapC663 (Lane et al., 1996). Overexpression of dap in wing discs under the control of en-Gal4 led to a wing hair spacing phenotype (Figure 5A” and Supplemental Figure 3, C–C’ and

FIGURE 2: dSkp2 interacts with Dap and regulates Dap protein level in Drosophila tissues. (A) CoIP analysis detecting dSkp2-Dap interaction. Extracts were prepared from the adult heads of flies with the indicated genotypes: GMR>HA-dSkp2 (lanes 1, 4, 7, and 10), GMR>4xMyc-dap (lanes 2, 5, 8, and 11), and GMR>HA-dSkp2 + GMR>4xMyc-dap (lanes 3, 6, 9, and 12). The immunoprecipitates were pulled down with the anti-Myc antibody, and the anti-HA antibody was used in IB to detect the presence of HA-dSkp2. (B) dSkp2 regulates Dap stability. Extracts were prepared from the adult heads of flies as in A. Total protein level in the extracts was detected in IB using the indicated antibodies. Tubulin (lanes 7–9) is loading control. (C) dSkp2 knockdown in Drosophila eyes leads to accumulated Dap proteins in tissue extracts. Extracts were prepared from the adult heads of flies with the indicated genotypes: GMR>4xMyc-dap (lanes 1 and 3) and GMR>4xMyc-dap + GMR>dSkp22G05142 (lanes 2 and 4). Myc-tagged Dap protein level was determined by Western blotting using the Myc antibody. Tubulin (lanes 3 and 4) represents loading control.
Mediated cell cycle progression. This thus led us to examine whether dSkp2 and Dap have regulatory functions that interact in controlling cell proliferation. To further evaluate the functional relationship between dSkp2 and Dap in vivo, we performed genetic interaction studies. Here one copy of the mutation, dSkp2<sup>KK108837</sup> or dSkp2<sup>D</sup>, was introduced into flies that contain an en-Gal4, dSkp2<sup>KK108837</sup> chromosome (Figure 6, C and C'). Our results show that reducing the dSkp2 gene dose by half was able to partially suppress the wing hair spacing phenotype caused by the en-Gal4, dSkp2<sup>KK108837</sup> chromosome (compare Figure 6, C and C' with B). These genetic interaction results show that dSkp2 and Dap have functions that interact in controlling cell proliferation. This thus led us to examine whether dSkp2 could also genetically interact with other genes known to be involved in regulating Dap-mediated cell cycle progression.

CycE and Cdk2 are two regulators that promote normal cell cycle in Drosophila (Sauer et al., 1995). Knocking down either CycE or Cdk2 in the wing led to a wing hair spacing phenotype similar to that caused by dSkp2 knockdown (Supplemental Figure S4, C and D). Of importance, overexpression of CycE almost completely suppressed the wing hair spacing phenotype caused by dSkp2 knockdown (Figure 6, D and D'). Taken together, these results show that dSkp2 genetically interacts with both dap and other regulatory genes important for cell proliferation, further supporting a role of dSkp2 in promoting cell cycle progression.

**FIGURE 3:** Tissue-specific knockdown of dSkp2 causes developmental defects. (A, A', and A'') Rough eyes induced by dSkp2 knockdown in the eye (with the use of the ey-Gal4 driver). Results from two independent RNAi lines are shown. (B', B'') nub-Gal4-driven expression of these two dSkp2 RNAi lines in the entire wing results in a similar wing hair spacing phenotype (WHS; see the text for details). Control is shown in B. (C', C'') dSkp2 knockdown in the posterior region of the wing (driven by en-Gal4) leads to the WHS phenotype in the posterior region only. This phenotype is fully rescued by a simultaneous overexpression of HA-dSkp2 (C).

D–D*). This phenotype is similar to that caused by dSkp2 knockdown, although with reduced severity (Figure 5A'; see Supplemental Figure S3 for high magnification). We also used other Gal4 drivers to further evaluate and confirm the phenotypic similarities between dSkp2 knockdown and dap overexpression. Figure 5, B' and B'', shows that dSkp2 knockdown or dap overexpression, driven by nub-Gal4 (in the entire wing), led to similar, although differing in severity, wing hair spacing phenotypes and partial loss of the wing margin. In addition, dSkp2 knockdown or dap overexpression, driven by vg-Gal4 at the D/V boundary of the wing disc, caused a similar wing margin loss phenotype (Figure 5, C and C'). Finally, notum-specific dSkp2 knockdown or dap overexpression, driven by pnr-Gal4, gave rise to similar dorsal closure defects and abnormal bristle patterning (Figure 5, D' and D*). Taken together, these results provide further support to the suggestion that dSkp2 and Dap have regulatory functions in a common pathway(s) during Drosophila development.

To further evaluate the functional relationship between dSkp2 and dap in vivo, we performed genetic interaction studies. Here one copy of the mutation, dap<sup>04454</sup> or dap<sup>B</sup>, was introduced into flies that contain an en-Gal4, dSkp2<sup>KK108837</sup> chromosome (Figure 6, C and C'). Our results show that reducing the dap gene dose by half was able to partially suppress the wing hair spacing phenotype caused by the en-Gal4, dSkp2<sup>KK108837</sup> chromosome (compare Figure 6, C and C' with B). These genetic interaction results show that dSkp2 and dap have functions that interact in controlling cell proliferation. This thus led us to examine whether dSkp2 could also genetically interact with other genes known to be involved in regulating Dap-mediated cell cycle progression.

For further information).
Low levels of the endogenous Dap in wild-type (wt) wing discs are difficult to detect by the antibody we used (de Nooij et al., 2000). In the experiments shown in Supplemental Figure S5, the uniformly low levels of Dap in wt wing discs are probably not beyond experimental background levels. This technical limitation is likely a contributing factor for the relatively modest effects of dSkp2 knockdown on Dap levels under the experimental conditions. To get around this technical limitation and increase the detection sensitivity for an improved evaluation of the role of dSkp2 in regulating Dap stability at a cellular level, we took advantage of UAS-4xMyc-dap transgenic lines and used an anti-Myc antibody in our immunostaining assays. We approached this problem by analyzing the effects of either knockdown or overexpression of dSkp2 on the detected signals for 4xMyc-Dap in wing discs. For each set of experiments, we ensured that all the experimental and imaging steps were performed side by side to allow effective comparisons. Figure 7, A–C′, shows immunostained wing discs that express, respectively, 4xMyc-dap alone, 4xMyc-dap together with dSkp2 knockdown, and 4xMyc-dap together with dSkp2 and Cks85A knockdown. Expression of 4xMyc-Dap alone led to accumulations of the protein in a nonuniform manner among the wing disc cells (Figure 7A). With dSkp2 knockdown, we detected a significant increase in both the mean number of fluorescence intensity clusters (referred to as intensity dots) within the 4xMyc-dap expression domain of the disc and the mean aggregate intensity of these dots (see Figure 7, D and E, for further increase caused by simultaneous knockdown of Cks85A). Conversely, coexpression of HA-dSkp2 led to significant reduction in these quantities (Figure 7, H and I). Taken together, these results provide further in vivo evidence supporting our suggestion that Dap is a substrate of SCF-dSkp2 in manifesting the patterning defects caused by reduction in dSkp2 activity during development.

**DISCUSSION**

Despite extensive studies of human Skp2 since its discovery more than a decade ago, the role of Drosophila Skp2 (dSkp2) has not been fully characterized. It is logical to infer that, for a highly conserved and fundamental process such as cell cycle control, the basic structure of the regulatory networks is most likely conserved among different species. However, it is unresolved whether the functional relationship between dSkp2 and its inferred target Dap is indeed conserved (see Introduction). In particular, a recent study raised the question of whether dSkp2 has a role in regulating Dap stability since its discovery more than a decade ago, the role of Drosophila Skp2 (dSkp2) has not been fully characterized. It is logical to infer that, for a highly conserved and fundamental process such as cell cycle control, the basic structure of the regulatory networks is most likely conserved among different species. However, it is unresolved whether the functional relationship between dSkp2 and its inferred target Dap is indeed conserved (see Introduction). In particular, a recent study raised the question of whether dSkp2 has a role in regulating Dap stability...
degradation (Ganoth et al., 2001; Mongay et al., 2001; Spruck et al., 2001; Wang et al., 2003, 2004; Hao et al., 2005; Yao et al., 2006). The Drosophila homologue of Cks1, Cks85A, was recently shown to interact with dSkp2 and play an important role in development (Ghorbani et al., 2011). In our study, knockdown of Cks85A and dSkp2 caused a similar wing hair spacing phenotype (Supplemental Figure S4B). Simultaneous knockdown of dSkp2 and Cks85A led to more pronounced accumulations of the endogenous Dap protein relative to dSkp2 or Cks85A knockdown alone (Supplemental Figure S5 and data not shown). Because the physical interaction between dSkp2 and Dap is enhanced by Cks85A (Figure 1A, lane 12), Cks85A most likely achieves its biological functions through a conserved molecular mechanism of strengthening the interaction between SCF^Skp2 and its substrates (Ganoth et al., 2001; Spruck et al., 2001).

The conservation between the Dap/p21-mediated cell cycle control networks in humans and flies extends to the function of Cdc kinase subunit 1 (Cks1) in regulating Dap/p21 protein level. Cks1 is a small accessory protein that facilitates SCF^Skp2 in targeting its substrates for degradation (Ganoth et al., 2001; Mongay et al., 2001; Spruck et al., 2001; Wang et al., 2003, 2004; Hao et al., 2005; Yao et al., 2006). The Drosophila homologue of Cks1, Cks85A, was recently shown to interact with dSkp2 and play an important role in development (Ghorbani et al., 2011). In our study, knockdown of Cks85A and dSkp2 caused a similar wing hair spacing phenotype (Supplemental Figure S4B). Simultaneous knockdown of dSkp2 and Cks85A led to more pronounced accumulations of the endogenous Dap protein relative to dSkp2 or Cks85A knockdown alone (Supplemental Figure S5 and data not shown). Because the physical interaction between dSkp2 and Dap is enhanced by Cks85A (Figure 1A, lane 12), Cks85A most likely achieves its biological functions through a conserved molecular mechanism of strengthening the interaction between SCF^Skp2 and its substrates (Ganoth et al., 2001; Spruck et al., 2001).

from that of Ghorbani et al. (2011), likely reflecting differences in detection method or sensitivity, as opposed to the way in which dSkp2 is perturbed. The dSkp2-null allele and the RNAi lines used in this study had identical phenotypes and, of importance, the dSkp2-knockdown phenotypes can be fully rescued by wild-type dSkp2. Because Skp2 has been associated with multiple cancers (see Introduction), our study thus suggests that Drosophila as a model system may benefit future studies of Skp2-mediated human tumors (Gstaiger et al., 2001; Signoretti et al., 2002; Ben-Izhak et al., 2003; Wang XC et al., 2012; Wang Z et al., 2012).

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enhanced apoptosis are two aspects of altered cellular behavior that respond to two distinct thresholds of dSkp2 activity alterations in the wing disc (Figure 4).

In humans, p21\(^{kip1}\) has been shown to undergo proteasome-mediated degradation through the actions of at least two E3 ligases, CRL4\(^{Cip1}\) and SCF\(^{Skp2}\). Whereas CRL4\(^{Cip1}\) predominantly regulates p21 levels during the S phase, SCF\(^{Skp2}\)-mediated degradation of p21 does not have such a temporal restriction. In Drosophila, Dap protein level is known to oscillate during ovarian endocycles (de Nooij et al., 2000). In our experiments, the heterogeneity in 4xMyc-Dap staining in the wing disc is not abolished by dSkp2 activity alterations (Figure 7), suggesting that Dap may also be targeted for degradation by multiple E3 ligases and that dSkp2 may not be primarily responsible for its oscillating property. Finally, the E3 ligase complex of Cul4-DDB1-Cdt2 is conserved in Drosophila (Zielke et al., 2011), and a loss of Cul-4 based E3 ligases causes a G1 arrest in a manner that is dependent on Dap (Higa et al., 2006). These results suggest that Dap stability may also be subject to regulation by Cul-4 E3 ligases. Understanding the complete fine structure of the regulatory networks for cell cycle control and their roles during development remains an interesting scientific problem and long-term challenge.

The present study, which documents the dSkp2–Dap connection, represents an important step toward meeting this challenge through the use of a genetic model system.

It is well documented that E3 ligases can have multiple substrate proteins and a given protein can be targeted for degradation by multiple E3 ligases (Skaar et al., 2009a,b; Dui et al., 2012). These particular features of the E3 ligase–substrate relationship contribute significantly to the richness of the biological regulatory systems. In addition to p21\(^{kip1}\)/p27\(^{kip2}\)/p57\(^{kip2}\), human Skp2 can also target other proteins for degradation, including Cyclin A (Yam et al., 2001), Cdk9 (Kiernan et al., 2001), CycE (Yeh et al., 2001), Rb130 (Tedesco et al., 2001), Myc (Kim et al., 2003; von der Lehr et al., 2003), and Cdt1 (Li et al., 2003). Thus it is likely that dSkp2 may also have multiple substrates to execute the various biological functions of dSkp2. For example, it remains possible that, in addition to Dap, dSkp2 may also have a role in regulating the activity and/or stability of Cdt1/Dup in Drosophila and contribute to the development of polyploidy cells. It has been reported that overexpression of Drosophila Cdt1/dup (double-parked) is sufficient to induce rereplication (Whittaker et al., 2000; Thomer et al., 2004; May et al., 2005; Lin et al., 2009). Future studies with sensitive tools are required to further investigate whether there might be a functional relationship between dSkp2 and Cdt1/dup and, if there is, the disentangled molecular and genetic relationships among all the relevant regulators in maintaining diploidy in mitotic cells (Ghorbani et al., 2011). We note that, despite the complexity of the regulatory roles of dSkp2 during development, an important finding of our study is that the defects in cell cycle progression and

**MATERIALS AND METHODS**

_Drosophila_ strains used in this study are listed according to their sources (see foregoing text for references to individual stocks/mutations):

- Vienna _Drosophila_ RNAi Center (Vienna, Austria): dSkp2\(^{205142}\), dSkp2\(^{KK108837}\), Cks85A\(^{GD13737}\).
- Bloomington _Drosophila_ Stock Center (Bloomington, IN): w\(^*\)/dap\(^{CyO}\), dap\(^{4454}/CyO\), dSkp2\(^{KK108837}\), dSkp2\(^{01306}\), Cdk2\(^{MS2074}\), w\(^*\)/UAS-CycE.R, w\(^*\)/UAS-CycE.R, UAS-dcr2\(^{w}\), en-Gal4\(^{UAS-GFP}\), en-Gal4\(^{UAS-dcr2}\) w\(^{1118}\), en-Gal4\(^{UAS-GFP}\), UAS-dcr2\(^{w}\) nub-Gal4, UAS-dcr2\(^{w}\) pnrgal4.
- National Institute of Genetics (Mishima, Japan): CycE\(^{IR}\).
- Christian F. Lehner (University of Zurich, Switzerland): UAS-Dap\(^{CL659}\) and UAS-Dap\(^{CL640}\) transgenic flies.
- Andrew Swan (University of Windsor, Canada): dSkp2\(^{949}\) mutant.

Flies were reared under standard condition at 25°C unless stated otherwise.

**Generation of transgenic flies**

To construct pUAST-dSkp2, two PCR fragments amplified from dSkp2/CG9772 genomic DNA were obtained using the following primers: 5’-ataagaattGGGCCGCCAGCCGAAATTGTGTGGTT-3’.
FIGURE 7: dSkp2 has a role in regulating Dap protein level in vivo. (A–E) Knockdown of dSkp2 leads to an increase in fluorescence signals for 4xMyc-Dap in wing discs. 4xMyc-dap\(^{51}\) was expressed in the posterior regions of the wing discs under the control of en-Gal4 either alone (A, A') or with the dSkp2 knockdown (B, B') or with a simultaneous knockdown of dSkp2 and Cks85A (C, C'). The confocal images shown were from side-by-side experiments and were captured under identical imaging settings. (D, E) Mean number of clusters of fluorescence signals (referred to as dots) per disc and mean aggregate intensity (in arbitrary units), respectively, of the detected dots. Error bars, SD calculated across different discs in a group. \(n = 20\) discs for each genotype.

(F–I) Coexpression of HA-dSkp2 leads to a reduction in fluorescence signals for 4xMyc-Dap in wing discs. 4xMyc-dap\(^{10–1}\) was expressed in the posterior region of the wing discs under the control of en-Gal4 either alone (F, F') or with HA-dSkp2 together (G, G'). The confocal images shown were based on side-by-side experiments with images captured under identical settings. (H, I) Mean number of fluorescent dots detected per disc and mean aggregate intensity (in arbitrary units) of the detected dots, respectively. \(n = 13\) for 4xMyc-dap\(^{10–1}\) discs and 19 for 4xMyc-dap\(^{10–1}\) & HA-dSkp2 discs. ** \(p < 0.01\) (Student's t test). Scale bar, 75 μm.
and pUAST-4xMyc-Dap were used for microinjection to generate transgenic flies, using standard protocols (Xu et al., 2009; Chen et al., 2010).

**Constructs for protein expression in S2 cells, transfection, and RNA interference assays**

The pAc5.1/V5-His vector with the Drosophila actin 5C promoter was used for protein expression in S2 cells (Liu and Ma, 2011). Plasmids of pAc5.1-dSkp2-FLAG, pAc5.1-dSkp2-V5, pAc5.1-4xMyc-Dap, pAc5.1-4xMyc-SkpA, and pAc5.1-Cksδ5A-V5 were constructed by standard cloning methods (cloning strategies are available upon request). Drosophila S2 cells were cultured in Hyclone serum-free insect cell culture medium (Roche, Indianapolis, IN). Transfection of S2 cells was performed using FuGENE HD transfection reagent (Roche) according to the manufacturer’s instructions. For treating S2 cells, the pseaeosome inhibitor epoxy(micin (final concentration 300 nM; Sigma-Aldrich, St. Louis, MO), the lysosome inhibitor chloroquine diphosphate (final concentration 500 μM; Sigma-Aldrich), and the translation inhibitor cycloheximide (final concentration 100 μg/ml; Sigma-Aldrich) were used. For RNA interference, double-strand RNA (dsRNA) was prepared with the RibopMAX large-scale RNA Production System-T7 Kit (Promega, Madison, WI). The primers for GFP control were 5′-ttataacgtctacatgaggagggagaCTC-3′ (forward) and 5′-ttataacgtctacatagggagaCTTGTCG-3′ (reverse). The primers for dskp2 RNAi were 5′-aatttaacgtctacatagggaggaCTC-3′ (forward) and 5′-aatttaaactagctcattagggagagagacGTGAG-3′ (reverse). The primers for dskp2 RNAi were 5′-aatttaacgtctacatagggaggaCTC-3′ (forward) and 5′-aatttaaactagctcattagggagagagacGTGAG-3′ (reverse). The primers for dskp2 RNAi were 5′-aatttaacgtctacatagggaggaCTC-3′ (forward) and 5′-aatttaaactagctcattagggagagagacGTGAG-3′ (reverse). The primers for dskp2 RNAi were 5′-aatttaacgtctacatagggaggaCTC-3′ (forward) and 5′-aatttaaactagctcattagggagagagacGTGAG-3′ (reverse). The primers for dskp2 RNAi were 5′-aatttaacgtctacatagggaggaCTC-3′ (forward) and 5′-aatttaaactagctcattagggagagagacGTGAG-3′ (reverse). The primers for dskp2 RNAi were 5′-aatttaacgtctacatagggaggaCTC-3′ (forward) and 5′-aatttaaactagctcattagggagagagacGTGAG-3′ (reverse).

**Quantification of the fluorescence signals for 4xMyc-tagged Dap in wing discs**

Quantification of the fluorescence signals for 4xMyc-tagged Dap was performed to evaluate both the number of fluorescent clusters (referred to as dots) in a disc and the aggregate intensity (in arbitrary units) of the detected dots. Briefly, for each image, we first used DAPI counterstain signals to mask the disc. Whenever necessary, we performed manual cropping to remove the conjugated, unwanted tissues or other discs present in an image. We then used the Otsu thresholding algorithm (Otsu, 1979; Cheung et al., 2011) to define a threshold. Clusters of fluorescence intensity signals above this threshold were identified, and the number was determined for each disc. The aggregate intensities (in arbitrary units) of individual dots were also calculated, and a mean was calculated for all the detected dots in each disc. All calculations were performed on MATLAB (MathWorks, Natick, MA).

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