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The function of replication and SCF complex during *Drosophila wing* development

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TABLE OF CONTENTS

1. ABSTRACT

Chromosomal DNA replication machinery functions in the growing cells and organs in multicellular organisms. We previously demonstrated that its knockdown in several tissues of *Drosophila* led to a rough eye phenotype, the loss of bristles in the eye and female sterile. In this paper, I investigated in detail the wing phenotype using RNAi flies, and observed that the knockdown not only of Mcm10 but also of some other prereplicative complex components including Cdt1, Polα-primase, RPA, Psf2 (partner of SLD five 2; a subunit of GINS (Go, Ichi, Nii, and San; five, one, two, and three in Japanese) and Rfc3 (replication factor C 3; a subunit of RFC complex) demonstrated wing phenotypes, using Gal4-driver flies. Surprisingly, some SCF complex components, which control cell cycle progression via protein degradation, also showed the wing phenotype. These results showed that the DNA replication machinery contributes to wing development independently of growth, probably through defects in DNA replication and protein degradation at specific places and times.

2. INTRODUCTION

DNA replication machinery is essential for cell proliferation (1). ORC (Origin recognition complex) is a platform for DNA replication initiation complexes. Cdc6 recruits Cdt1 (2, 3), which is considered to be a licensing factor leading the S phase, and Mcm 2–7 complex, which is a replicative helicase at the origin of DNA replication (4, 5). Furthermore, Polα-primase, which is associated with the Cdc45, Mcm2–7, and GINS (CMG) complex (6) (thought to be the DNA replication fork complex in the eukaryotic *Drosophila* embryo) is involved in the elongation phase of DNA replication. Moreover, the DNA polymerases Pol δ and Pol ε initiate DNA replication. Recently, using cryo-EM structures, it has been confirmed that each factor of the ORC-Cdc6-Cdt1-Mcm2–7 (OCCM) intermediate plays a characteristic role in the formation of a pre-replication complex (pre-RC) by ORC-Cdc6 and Cdt1 (7).

In contrast, in the G1-S and G2-M transitions, the activity of CDKs and other kinases is required and is regulated tightly to prevent inappropriate cell-cycle progression. The cell cycle is also regulated by SCF complex and the anaphase-promoting complex/cyclosome (APC/C), which are multisubunit RING finger E3s. SCF complex regulates cell-cycle progression by degradation of the proteins CycE, CDK inhibitor $p27^{Kip1}$, and others (8). SCF complex consists of Skp1, cullin-1 (Cul1), and F-box protein. These complexes are targeted to specific substrates by the F-box protein of SCF and by Cdc20 and Cdh1 activation of APC/C. Cul1 is an essential subunit of the SCF (Skpl, Cul1/Cdc53, F-box protein) ubiquitin E3 ligase complex that targets many phosphorylation

substrates, such as $p27^{Kip1}$, IκB, β-catenin, and Orc1 (9, 10). Although other cullin complexes are less well characterized, they all have the ability to bind the RING finger protein Roc1 or Roc2 (RBX/HRT), which recruits ubiquitin E2 conjugating enzymes for polyubiquitination and functions as ubiquitin E3 ligase. Furthermore, there are three orthologs of *Drosophila* Roc1 (Roc1a, Roc1b, and Roc2) (8). The finding that Roc1a silencing is sufficient to suppress the disappearance of Cdt 1 after irradiation (11) suggests that Cdt1 is specifically polyubiquitinated by the Cul4 complex and the interaction between Cdt1 and Cul4 is partially regulated by gamma irradiation (11).

Cell-cycle progression requires growth and development (12, 13). However, retinoblastoma protein (RB), which is a tumor-suppressor protein, has been shown to regulate cell-cycle control by binding to E2F–DP1 (14). The heterodimeric transcription factor E2F–DP1 drives G1 to S transition in the cell cycle. The complex produces many proteins, including Orc1, that advance the cell cycle (15). It is now clear that the RB/E2F pathway is important in regulating the initiation of DNA replication (14, 16).

Drosophila flies expressing transgenes in a tissue-specific manner can be obtained through crosses with flies producing Gal4 in a tissue-specific manner (i.e. with a Gal4 driver). When the antisense RNA of the target gene is expressed by using a tissue-specific Gal4 driver system, the RNA transcribed from the target DNA forms double-stranded RNA that is disrupted by the RNA interference (RNAi) machinery and depleted of the target gene product. Therefore, by using RNAi in *Drosophila* (17), we have compared the phenotype resulting from a specific gene knockdown in various tissues at various developmental stages (13) and have tried to clarify the role of the gene at those stages (13). Until now, we have knocked down the DNA replication machinery proteins by using *Act5C* and tubulin-p-Gal4 drivers expressed throughout the body. As a result, the target gene is knocked down throughout the body. Knockdown of Mcm2, Mcm4, Cdt1, and Cdc6 is lethal (13). In addition, Cul-4, dSkip-1/SkpA, Rbx1/Roc1, and Roc1b/Roc2 knockdown is lethal, and knockdown of Cul-1 and Elongin C results in severe proliferative defects (13).

In *Drosophila* tissues, chromosomal abnormalities, including gene amplification and endoreplication, occur in a developmentally controlled fashion (18). Recently, we showed that the DNA replication machinery required for the mitotic cell cycle is necessary for gene amplification and endoreplication[13]. Furthermore, the chromosomal DNA replication mechanism plays an active role in tissue development in *Drosophila* and contributes to physical development independent of proliferation (19).

In our previous study (13), a wing phenotype was observed at Mcm10 when we knocked down DNA replication machinery proteins by using the tubulin-p-Gal4 driver. From these phenomena, I hypothesized that the DNA replication mechanism directly affected wing development. First, I knocked down the DNA replication machinery proteins by using several Gal4 drivers for screening (13, 19). Previously, it was shown that knockdown of Mcm10 by using tubulin-p-Gal4 and *SD-Gal4* resulted in a wing phenotype (13). In contrast, knockdown of Mcm10 by using wing-specific Gal4-like *Vg* and *en*-Gal4 drivers did not lead to a wing phenotype. I knocked down several DNA replicators, including Mcm3 and Pol ε 255 kDa, by using *SD-Gal4*, but no wing phenotype was observed (13). These findings suggest that Mcm10 has other functions in addition to its function in DNA replication.

In this study, I first showed that some RNAi knockdowns of chromosomal DNA replication machinery can induce abnormal wing formation. Next, I demonstrated that such knockdowns not only affected DNA replication but also resulted in a rough wing phenotype, probably through defects in DNA replication. Finally, I revealed how the knockdown of DNA replication machinery, including Mcm10 and SCF complex components, led to novel phenotypes.

3. MATRERIALS AND METHODS

3.1. Fly stocks

Fly stocks were maintained under standard conditions. The RNAi knockdown lines were obtained from the National Institute of Genetics (Mishima, Japan) and the Vienna Drosophila RNAi Center (Vienna, Austria). *Tubulin-p-Gal4*, *SD-Gal4*, *Vg-Gal4*, *en-Gal4*, *ptc-Gal4*, *dll-Gal4*, and *dpp-Gal4 (yw)*, were obtained from the Bloomington Drosophila Stock Center (Indiana, USA).

3.2. Knockdown experiments

To investigate the function of DNA replication machinery in the rear of the wing imaginal disc, I knocked down the complex and SCF complex with *SD-Gal4* by means specific to tissue, time, and place (19). *SD-Gal4* predominantly expresses *Gal4* in developing imaginal wing discs. SD (*scalloped)* is expressed in the central and peripheral nervous systems of the developing larva, where it is required for the differentiation of sensory organs and effects on wing sensory organ distribution and at the wing margin (20). Expression of *ptc* (13), *dll*, and *dpp* differs from that of *SD-Gal4* in terms of such characteristics as timing and location.

3.3. Quantitative reverse transcriptase-PCR

Total RNA was isolated using Trizol Reagent (Invitrogen). Oligo dT primers and a Takara high

Table 1. Summary of phenotypes induced by knockdown of Mcm10 with each Gal4 driver

Gal4 driver	Phenotype	Abnormal wing/total flies (%)	Number
Tubulin-p-Gal4	Wing phenotype	59.6% [1]	129
	Abnormal wing folding	40.3%	
SD	Wing phenotype	Each wing: 22.5% Both wing: 1.9%	271
SD	Abnormal wing folding	3.0%	
ptc	Normal	1.8%	164
dll	Normal	0%	77
dpp	Normal	0%	31

[1]: Over 50% of flies showed wing phenotype

fidelity RNA PCT kit (Takara, Kyoto, Japan) were used for generation of complementary DNA. Then, real-time PCR was performed using a SYBR Green I kit (Takara) and the Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). RNA expression efficiencies decreased to 25% in every case (13).

3.4. Scanning electron microscope

Prepared specimens were examined using a fluorescence microscope (Olympus BX-50) equipped with a cooled CCD camera (Hamamatsu Photo ORCA-ER) and Aquacosmos image analysis software (Hamamatsu Photo ORCA-ER) (13, 19).

4. RESULTS

4.1. DNA replication machineries, including Mcm10, are potentially involved in wing formation

We showed previously that knockdown of Mcm10 by using tubulin-p-Gal4 and *SD-Gal4* resulted in a wing phenotype (13). These results suggested that the factor is involved in wing formation. The differences in effect among Gal 4-drivers in Table 1 indicate that differences in the timing and location of expression of each Gal4-driver contribute to the differences in knockdown. In this study, I performed knockdown of Mcm10 by using several Gal4-drivers involved in wing formation (Table 1). But similar wing phenotype was not observed when I used the *Vg* (13), *ptc*, *dll*, *dpp*, *or en-Gal4* (13) drivers (Table 1). These results indicated the specificity of *SD-Gal4*.

I also knocked down many other DNA replication devices by using *SD-Gal4*. knockdown of Mcm2, -4, and -5, Cdt1, Orc6, RPA70, Psf1 and -2 (a subunit of GINS), Rfc3 (a subunit of RFC complex) Polα-primase, and Pol δ—but not Cdc6, Mcm3, Orc5, and Polε—by using *SD-Gal4* resulted in a wing phenotype (Figure 1 and Table 2). Among these, knockdown of Cdt1, RPA70, Psf2, Rfc3, Polα-primase, and Pol δ resulted in lethality (Cdt1) or at least 50% of flies having the wing phenotype (Figure 1 and Table 2).

Surprisingly, these were the factors involved especially at the stage of elongation in DNA replication (Table 2 and Figure 2). As the CMG complex contains Mcm2-7 and Psf2, these findings suggest that a DNA replication apparatus containing Mcm10 functions not only in DNA replication but also in wing formation (Tables 1 and 2, and Figures 1 and 2).

4.2. Knockdown of the SCF complex by using *SD Gal4* drivers disturbs wing development

Degradation of proteins by the SCF complex and APC/C regulates cell-cycle progression at the G1 to S and G2 to M transitions (8, 10, 21). Previous studies have shown that CycA, CycE, Cdc 20, Cdh 1, and Rcal mutants are embryo-lethal (13). Knockdown of dSkip-1/SkpA, Rbx1/Roc1, and Roc1b/Roc2 by using *Act5C* and *Tubulin-Gal4* drivers is lethal (13). Moreover, Cul-1 and Elongin C knockdown results in marked proliferative defects (13). Using *SD-Gal4*, I performed knockdown of dSkip-1/SkpA, Rbx1/Roc1, and Roc1b/Roc2, but the results were normal (Table 2). Only Cul-4 knockdown by *SD-Gal4* results in a severe wing phenotype (Figure 1 and Table 2). This probably occurs via Cdt1 degradation.

4.3. Knockdown of E2F and RB by using *SD Gal4* drivers disturbs wing de and development

The Rb/E2F pathway is important in controlling the initiation of DNA replication (14). E2F1 and DP mutants, and knockdown of DP1 by using *Act5C-Gal4* and *Tublin-p-Gal4*, are lethal before the adult stage (13). In this research, I performed knockdown of DP1 by using *SD-Gal4*. In 100% of flies, knockdown of DP1 by *SD-Gal4* results in a wing phenotype (Figure 1 and Table 2). These results show that E2F-DP1 contributes to not only cell-cycle progression but also wing formation.

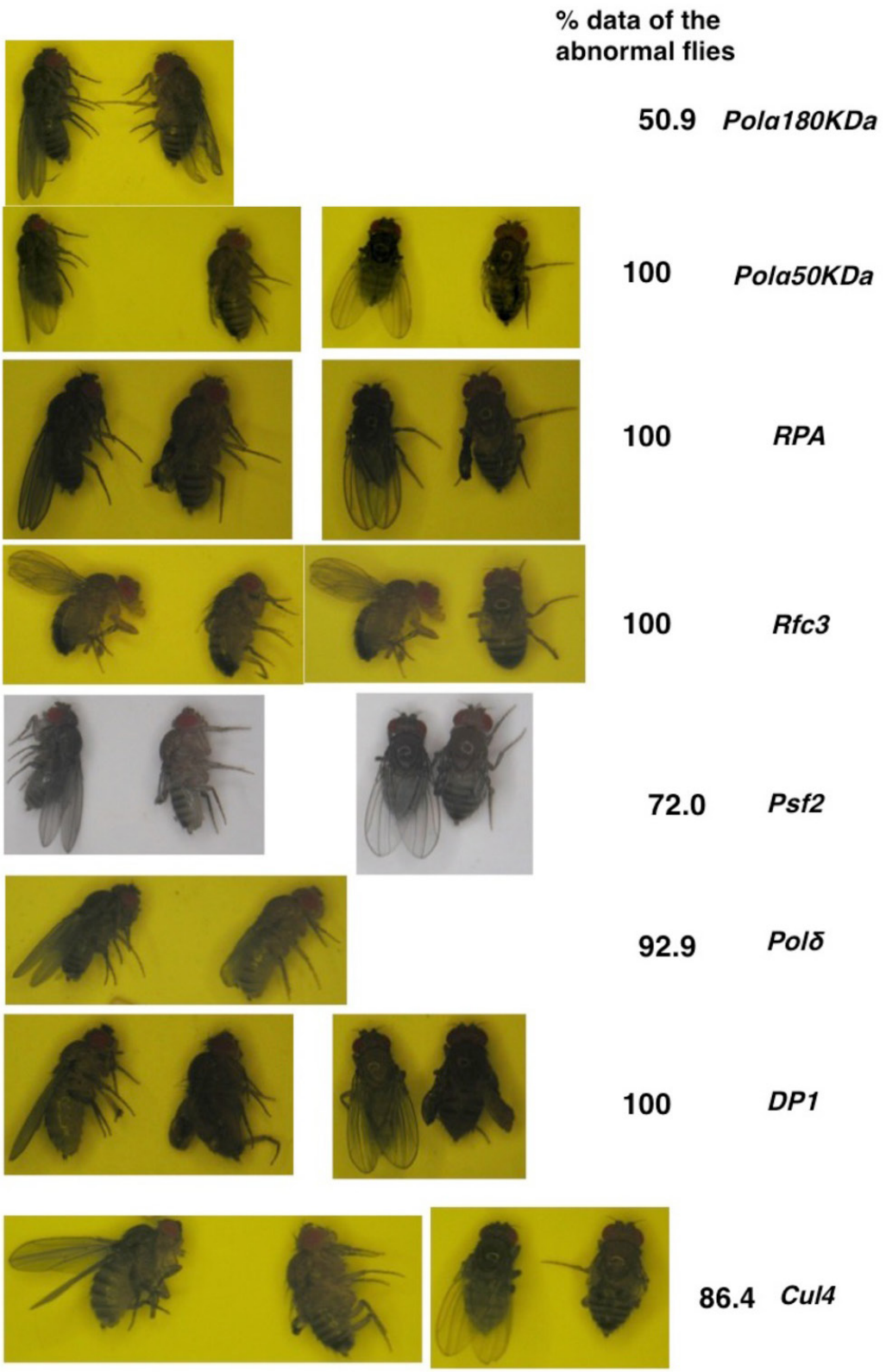

Figure 1. Knockdown of DNA replication machinery by SD-Gal4 drivers. Knockdown of other prereplicative complex components, including Cdt1, Polα-primase, RPA, Psf2 (a subunit of GINS), Rfc3 (replication factor C3), and Polδ, resulted in wing phenotypes. In one picture, the fly at right is the wild type and the fly at left is the knockdown fly. Knocked-down genes are shown at right in bold. Flies with knockdown of Polα-primase and Polδ are shown in the panels at one set picture; flies with the other knockdowns listed above are shown in the panels at two sets of pictures. % data of those abnormal flies should be shown.

Table 2. Phenotype of knockdown by *SD-Gal4*

Responder (UAS-IR)	Chromosome linkage	Wing phenotype/total flies (%)
Mcm2	III	32.2% (N = 202)
Mcm3	III	Normal (N = 21)
Mcm4IR-1	II	13.8% (N = 128)
Mcm4IR-2	III	17.7% (N = 141)
Mcm5IR-1	II	8.3 %(N = 72)
Mcm5IR-2	III	23.6% (N = 128)
Cdc6IR-3	II	Normal (N = 52)
Cdt1IR-1	II	Lethal (N = 18)
Cdt1IR-2	III	**69.0**% (N = 29)
Orc4IR-3	II	4.1% (N = 120)
Orc4IR-2	III	3.1% (N = 97)
Orc5IR-2	III	Normal
Orc6	II	17.3% (N = 103)
RPA70IR-1	II	**95.5**% (N = 133)
RPA70IR-2	III	**100**% (N = 21)
Psf1 IR-2	III	24.6 %(N = 57)
Psf2 IR-4	II	**72.0**% (N = 214)
Psf2 IR-1	III	20.5% (N = 215)
Rfc3 IR-9	II	**100**% (N = 158)
Polα180IR-1	III	25.5% (N = 55)
Polα180IR-3	III	**50.9**% (N = 228)
Polα50IR-1	II	**100**% (N = 12)
Polα50IR-2	III	**100**% (N = 146)
Polδ125KDa IR-1	II	**92.9**% (N = 84)
Polε255IR-1	II	Normal (N = 153)
Polε255IR-2	III	Normal (N = 180)
Cul-4	II	**86.4** %(N = 103)
Cul-1	II	Normal (N = 126)
dskp-1/SkpA	III	Normal (N = 7)
Roc1b/Roc2	III	Normal (N = 43)
Elongin C	III	Normal (N = 160)
DP1IR-2	III	**100**% (N = 82)

***** Bold indicates at least 50% lethality or 50% of flies having the wing phenotype.

5. DISCUSSION

5.1. Knockdown of DNA replication machinery, SCF complex, and E2F-DP1 by using *SD Gal4* drivers disturbs wing development

The tissue-specific knockdown of the genes in *Drosophila* is similar to that in the mouse *Cre-loxP* system (22, 23). In the cross between flies containing the Gal4 driver and flies with the hairpin RNAi transgene that suppresses the expression of specific genes, the RNAi target mRNA temporarily disappears in a spatial-, development-, and tissue-specific manner (22). I discovered that knockdown of several factors needed for the DNA replication machinery causes the rough eye phenotype (13, 19). Among them, Mcm10 may be important not only during wing formation and the growth phase but also during the differentiation stage and in DNA replication.

At the growth/differentiation transition (GDT) point (24), differentiation signals are expected to enter the chromosomal DNA replication machinery (25). Mcm10 may be the endpoint of these wing formation signals. This may be revealed by screening transgenes and using various mutants.

Up to now, the interaction of DNA replication machinery has been investigated by *in vitro* analysis and in *Saccharomyces cerevisiae* or cell-culture

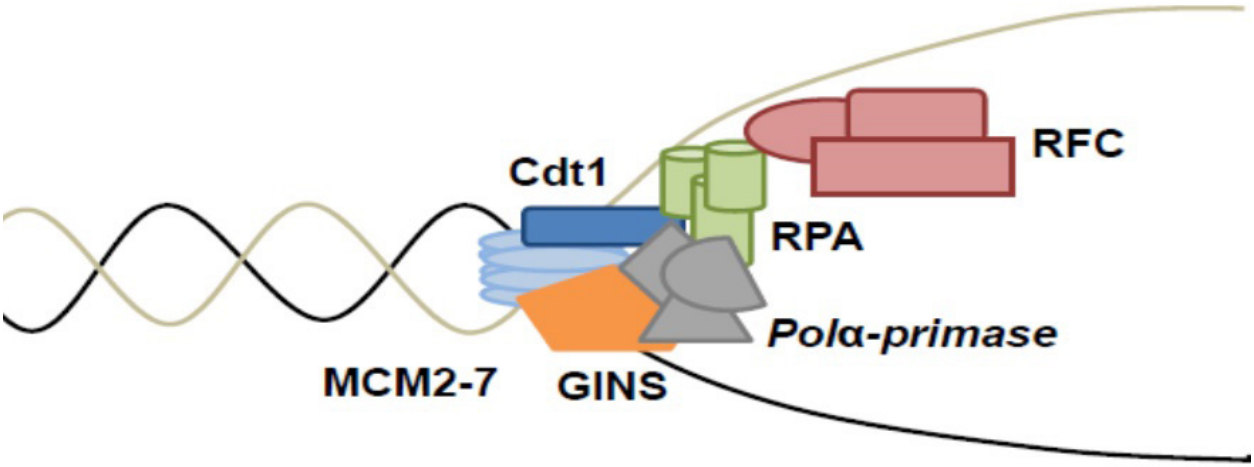

Figure 2. Creation of elongation complex in DNA replication. Mcm2–7, Cdt1, Polα-primase, RPA, Psf2 (a subunit of GINS), Rfc3 (replication factor C3) and Polδ are the factors involved especially at the elongation stage of DNA replication and were knocked down by using the *SD-Gal4* driver.

systems, but it has not been studied by morphogenesis of higher eukaryotes. I have revealed these interactions of *Drosophila in vivo* on the basis of phenotype (26).

In this study, I also knocked down many other DNA replication devices by using *SD- Gal4*. Knockdown of many of these DNA replication machinery components induced a wing phenotype. In some cases there was lethality (Cdt1), or at least 50% of the flies had the wing phenotype (Figure 1 and Table 2). Surprisingly, the factors that were knocked down were involved especially at the elongation stage in DNA replication (Table 2 and Figure 2). This suggests that the DNA replication machinery functions as an important key player during development and differentiation. In future, I would like to extend my analysis to other organisms.

Knockdown of Cul-4, one of the factors of the SCF complex, by using *SD-Gal4* yields a severe wing phenotype (Table 2 and Figure 2). However, in the case of dSkip-1/SkpA, Roc1b/Roc2, Cul-1, and Elongin C knockdown, no wing phenotype is observed. These results suggest that the Cul-4 knockdown phenotype results from Cdt1 degradation, because knockdown of Cdt1 results in a wing phenotype or lethality, even when *SD-Gal4* is used for knockdown (Figure 1 and Table 2).

E2F–DP1 plays an important role in progression of the cell cycle, and Buttitta *et al*. (27) showed that crosstalk between E2F and cyclin/Cdk activity is suppressed as cells terminally differentiate in *Drosophila* wings. Therefore, the activity of both E2F and G1 cyclin/Cdk must be simultaneously increased so that these cells bypass the exit of the cell cycle or re-enter the cell cycle after differentiation. In the wing epithelium, a further unknown mechanism may contribute to reduced activity of E2F and G1 cyclin/Cdk, in part through the differentiation of Orc1 expression (9, 15, 28). I also found that knockdown of DP1 induced a severe wing phenotype. These results show that E2F-DP1 contributes to wing formation (Figure 1 and Table 2). But this mechanism remains unclear. As a next step in the study, I need to investigate the relationship between E2F function and terminal differentiation.

5.2. Cell-cycle progression via protein degradation

Control of cell-cycle progression occurs is harmonious. Therefore, proteins that are no longer needed quickly lose their function and are degraded. Through phosphorylation by G1 cyclin/Cdk, SCF complex adds ubiquitin to unnecessary proteins (substrates); proteasome then decomposes and removes the proteins. Cyclin E, a G1 cyclin, is also degraded by SCF complex. SCF complex has a variety of substrate-recognizing F-box protein combinations (Table 3).

For example, Cdc6 is broken down at the time of prevention of DNA re-replication. In addition, Cdt1 is a factor required for the replication origin loading of Mcm complex, which is thought to be a replicative helicase. Also, the activity of Cdt1 is regulated by proteolysis via SCF complex. The cell cycle progresses by decomposition of the CDK inhibitors p27 or p21 through the action of SCF complex (Table 3).

Although E2F expressing the protein necessary for S phase is also inactivated by phosphorylated Rb, E2F-DP1 functions, and exerts control as a transcription factor, by phosphorylation of Rb, but it is decomposed by the action of SCF complex (Table 3), with cell-cycle progression and escape from S phase. Because abnormality of the cell cycle causes cell death by the checkpoint mechanism, part of my

Table 3. Protein degradation

Key players of protein degradation	Target substrate
SCF complex	
Skp2	c-Myc (31), p27 (32), Cyclin E (33)
Fbw7/Cdc4	c-Myc (34), Cyclin E (35), Cdc6 (36)
β-Trcp	IkB, β-catenin (37), PR-Set7/Set8 (38)
NRBE3	Rb (39)
Ddb1-Cul4a-Roc1	Cdt1 (40)
Cul1-Roc1	E2F (41)
CRL4-CDT2	p21 (42, 43), POLH-1 (pol eta) (44), PR-Set7/Set8 (45), Gcn5 (46), RFC1, Ctf18 (47)
CRL1- Fbxo11	Cdt2 (48)
Cyclin F	Cdc6 (49)
Pirh2,	p53 (50)
Others	
Mdm2	p53, Mdm2 (51)
KPC complex	p27 (52)
APC/C	Cdc6 (53), geminin (54)□Orc1 (9)

results may be due to knockdown of the SCF complex: proteolysis functions not only in the cell cycle, but also in differentiation and development.

Indeed, an E3 ubiquitin ligase Nedd4 analysis of embryonic and adult fly hearts has revealed that Nedd4 protein regulates heart development in Drosophila larval fly hearts overexpressing *miR-1*, a small noncoding RNA molecule that modulates gene expression in heart and skeletal muscle (29). Also, Velentzas et al. (30) showed proteasome/ubiquitin proteolytic activity is required in the normal course of eye and wing development

In future, cell-cycle progression via protein degradation in various organs may be reported elsewhere.

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