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Role of Cks85A in cell cycle regulation

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by

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Abstract

The transition between different stages of the cell cycle needs the activation of Cdk/Cyclins. SCF^{Skp2} ubiquitin ligase complex plays a critical role in entry into the S-phase by activating the Cdk2/Cyclin E complex. Cks85A interacts with Skp2 and helps it in recognition of the SCF^{Skp2} ubiquitin ligase targets. Knocking out of *Cks85A* and *Skp2* has an effect on growth in Drosophila by reducing the cell volume in endoreplicating tissues. In this study, I have shown that the *Cks85A* and *Skp2* null mutant larvae are smaller due to DNA replication defects in endoreplicating cells. I have shown that there is polyploidy in brain and eye imaginal discs and this was confirmed by directly visualising the number of the chromosomes in *Cks85A* and *Skp2* mutant cells. I determined that the increased ploidy levels in the cells is due to re-replication of the DNA. I have shown that the expression level of Dup (Double parked), a protein that is important for initiation of DNA replication, increases in the *Skp2* mutant salivary glands and this might be because Dup is targeted by SCF^{Skp2} ubiquitin ligase complex. In addition, I have shown that Cks85A does not have a redundant role with Cks30A in cell cycle regulation.

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Chapter 1. **Literature Review**

1.1 Eukaryotic cell cycle regulation

The cell cycle is a very important process that involves a highly arranged progression of events to produce two genetically identical daughter cells. DNA replication must be highly controlled to keep the ploidy of the cells constant. The cell cycle is composed of two major parts: interphase and mitosis. Interphase includes G1, S and G2 phases and mitosis is composed of prophase, prometaphase, metaphase, anaphase and telophase. S-phase (DNA synthesis) and mitosis (when the cell divides) are the important parts of the cell cycle and G1 and G2 are the gaps between them. G1 occurs between M phase and the beginning of S phase. During G1, the cell is able to produce enough proteins for growth and DNA synthesis. During S-phase, DNA replication occurs. G2 is the second gap phase before the cell enters into mitosis. In this stage, cells ensure that DNA replication is complete and all the proteins needed for mitosis are synthesised. In mitosis, the nuclear envelope breaks down, centrioles migrate to the opposite poles of nuclei, the mitotic spindle forms and chromosomes segregate. After complete separation of the chromosomes, two daughter nuclei are formed and the cell undergoes cytokinesis (cytoplasmic division) where two daughter cells are produced.

1.2 Role of Cyclin/Cdks complex in cell cycle regulation

The transitions between different stages of the cell cycle are very important for regulation of the cell cycle. The major transitions during the cell cycle are from G1 to S-phase and metaphase to anaphase. How are these transitions controlled? This is a very important question that many researchers have worked on and know a lot about it but there is much more to be discovered. These transitions are mainly driven by the activity of Cyclin dependent kinases (Cdks). These

regulatory proteins need to bind to proteins called Cyclins to become active. They are called Cyclins because of their oscillating abundance during the cell cycle. The major role of Cyclins in cell cycle is the control of transition between the stages. There are different types of Cyclins and each has a specific role. Synthesis and destruction of the Cyclins are major events which control transitions.

A number of other proteins also regulate the activity of Cdks during the cell cycle. Two classes of Cdk inhibitors (Kip/Cip and INK4 families) inhibit Cdk activity in different ways, the most common way is by blocking ATP binding to the Cdk (Russo et al., 1996b; Serrano et al., 1993).

Cdks are a highly conserved family of protein kinase. They were first identified in budding yeast S*accharomyces cerevisiae* (Nurse and Bissett, 1981) and called Cdc28 and in fission yeast *Schizosaccharomyces pombe* (Beach et al., 1982) and called Cdc2. In higher eukaryotes there are several different Cdks which have roles in different stages of the cell cycle. There are several different Cdks in *Drosophila melanogaster* but Cdk1 (Cdc2), Cdk2 (Cdc2c) and Cdk4 are the critical ones in the Drosophila cell cycle (Lee and Orr-Weaver, 2003). Cell cycle regulation is not the only role of the Cdk/Cyclin complexes; they have other functions that include regulation of transcription, DNA repair, differentiation and apoptosis. Cdk7/Cyclin H is one such example, and has a role in basal transcription (Larochelle et al., 1998).

1.2.1 **G1 to S-phase transition**

In mammalian cells, transition from G1 to S-phase is controlled by the activity of the Cyclin dependent kinase Cdk4/Cyclin D, Cdk2/Cyclin E and Cdk2/Cyclin A complexes (Sherr, 1993).

In *Drosophila melanogaster,* Cdk4/Cyclin D and Cdk2/Cyclin E are responsible for controlling transition from G1 into S-phase (Dong et al., 1997; Lee and Orr-Weaver, 2003).

In mammalian G1 phase Retinoblastoma protein (Rb) is active, inhibiting Cdk2/Cyclin E, which is the major complex to trigger entry to S-phase (0.1). Rb binds to E2F and blocks E2F activity in G1. E2F is a transcription factor and it is required for the transcriptional activation of genes required for entry into S phase, including *Cyclin E* and *E2F* itself (Johnson et al., 1994). There are 2 E2F subunits and 2 Rb-related proteins (RBF1 and RBF2) in Drosophila. Knocking out of *E2F* in Drosophila result in block in G1 phase (Duronio et al., 1998; Royzman et al., 1997).

Cdk2/Cyclin E is crucial for transition into S-phase from G1. p27 binds to Cdk2/Cyclin E and inhibits its activity. As a result cells are blocked in G1 until they get signals from outside of the cell (mitogens, for example) or inside (systems monitoring cell growth, for example) which promote Cdk4/Cyclin D complex activity.

The activity of Cdk2/Cyclin A is required for entry into S phase and the control of DNA replication (Sugimoto et al, 2004). It has been shown that depletion of Cyclin A in Drosophila causes overreplication of DNA (Dong et al., 1997; Mihaylov et al., 2002; Sprenger et al., 1997). These findings, lead us to think about the role of Cyclin A for control of DNA replication but the exact role of Cyclin A is still unknown in Drosophila.

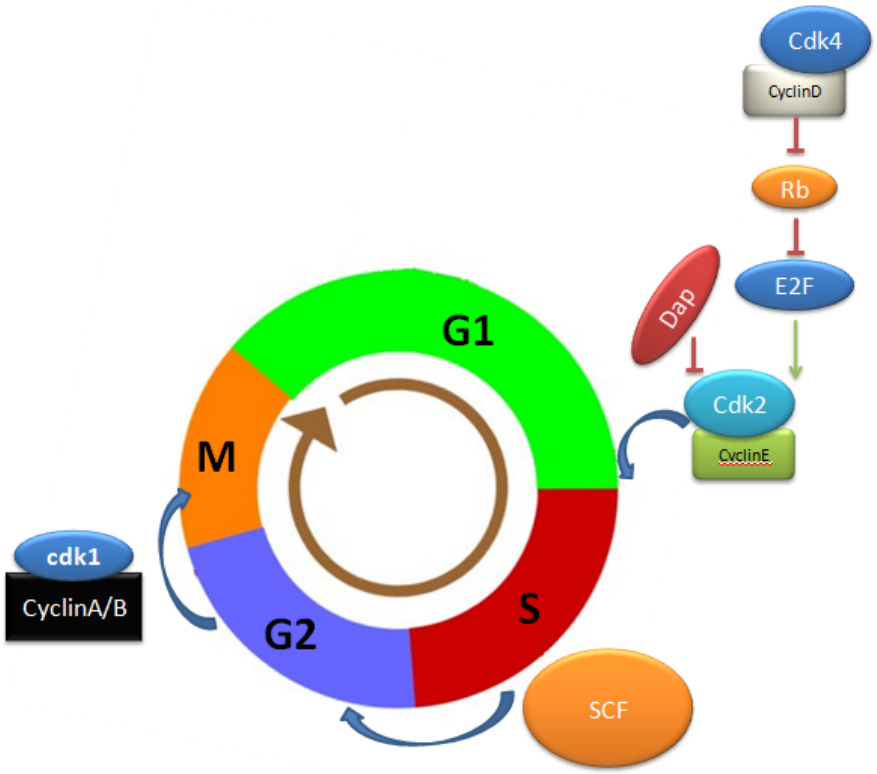


Figure 1.1 Mechanism of transition between different stages of the Cell cycle

1.3 G2/M transition

Cdk1 (also called Cdc2 in Drosophila) binds to Cyclin B at the end of G2 and it is required for entry into mitosis. Mitosis Promoting Factor (MPF) is a heterodimeric protein which includes structural proteins that are necessary for timing and control of transition from G2 to mitosis. Proteins of the nuclear pore complex (Macaulay et al., 1995), nucleolar proteins (Peter et al., 1990) and nuclear lamins (Dessev et al., 1991) are all substrates of Cdk1/Cyclin B. In flies, Cyclin A and Cyclin B are important for entry into mitosis (Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990; Knoblich and Lehner, 1993).

1.4 Regulation of Cdk/Cyclin complexes

Cdk/Cyclin activity is critical to control timing and transition between the stages of the cell cycle. There are three mechanisms that regulate the activity of Cyclin and Cdks: proteolysis, protein phosphorylation and regulation by inhibitory subunits.

1.4.1 **Regulation of Cdk/Cyclin complexes by inhibitory subunits**

The activity of Cdk/cyclin complexes during G1 and entry into S phase is regulated by Cdk inhibitory proteins (CKIs). The Cip/Kip and INK 4 families are CKIs in mammalian cells. The Cip/Kip family of CKIs includes p21, p27 and p57 which suppress Cdk2 and Cdk4 Cyclin complexes during G1 and G1/S phase transition (Sherr and Roberts, 1995). p27 binds to the phosphorylated Cdk2/Cyclin A complex and inhibits Cdk activity (Russo et al., 1996a). $p27^{kip1}$ directly binds to the ATP binding pocket of Cdk2, preventing ATP binding.

INK4 proteins inhibit Cdk4 activity when it is in a complex with Cyclin D and block cells in G1 phase (Hannon and Beach, 1994). $p16^{INK4}$, $p15^{INK4}$, $p18^{INK4}$ and $p19^{INK4}$ are different types of

INK4 and they bind to Cdk4 specifically and not the other types of Cdks (Serrano, 1997). In Drosophila, Rux (Roughex) play a similar role to INK4 proteins. Rux prevents the activation of Cdk1/Cyclin A in G1 and blocks the cell in G1 (Gönczy et al., 1994).

1.4.2 **Regulation of Cdk/Cyclin complexes by proteolysis**

The other way to control the activity of Cdk/Cyclin complexes is to degrade Cyclins via the ubiquitin-mediated pathway (Pagano, 1997). Two large multi-subunit complexes are responsible for degrading Cyclins in different stages of the cell cycle; Skp A, Cullin, F-box containing complex (SCF ubiquitin ligase complex) in S-phase and the Anaphase Promoting Complex (APC) in anaphase.

Cyclin B degradation is required for entry into anaphase. The APC ubiquitinates Cyclin A, B and B3 to prepare them for degradation by proteasome (King et al., 1995). Mitotic Cyclins have small sequence motif (RxxLxxIxN) followed by a lysine rich domain (destruction box) that is the target for APC recognition for ubiquitination (King et al., 1996).

The S-phase cyclins are also regulated by proteolysis. Cyclin E is important for entry into S-phase from G1 and its level controls by SCF^{Ago}. SCF^{Ago} is critical for Cyclin E degradation (Moberg et al., 2001). Archipelago (Ago) is an F-box protein in SCF ubiquitin ligase.

1.4.3 **Regulation of Cdk/Cyclin complexes by phosphorylation**

Activation of Cdks starts with binding to their Cyclins but this complex is still not fully active. For full activation of this complex, Cdk needs to be phosphorylated on a conserved threonine 161 residue (Krek and Nigg., 1992). When the cell is ready to divide, cdc25

dephosphorylates Cdk1 first at Thr14 and then at Tyr15 activating the Cdk1/Cyclin B complex (Honda et al., 1993).

Chapter 2. **History of Cks proteins**

The Cdc28 kinase subunit (Cks) proteins are very small (9-18kDa) and they play a very critical role in the eukaryotic cell cycle. The function of Cks is essential for Cdk activation. Cdc2 was identified first in fission yeast *Schizosaccharomyces pombe* (Nurse and Bissett, 1981) and mutations in this gene result in blocked cells in G1. Later studies on *Suc1* (*Cks* homolog in *Schizosaccharomyces pombe*) also show the same phenotype as *Cdc2* (Hayles et al., 1986). Cdc2 also interacts physically with Suc1 (Brizuela et al., 1987). Cks1 also binds to Cdc28 in *Saccharomyces cerevisiae* and *Cks1* mutations cause the cell cycle to arrest in G1/S and G2/M phases as well as budding. Cks homologes were discovered after that in other organisms (Table 1.1).

In humans, there are two Cks homologes which are called CksHs1 and CksHs2. *Drosophila melanogaster* also has two *Cks* genes, *Cks85A* and *Cks30A* (Swan et al., 2005). Cks85A is functionally close to Cks1, in its role in the cell cycle. Suc1 (*Schizosaccharomyces pombe*), Cks (*Saccharomyces cerevisiae*), Cks2 (Mouse) Xe-p9 (*Xenopus*) and Cks30A (*Drosophila*) are functionally related.

2.1 Interaction between Cdk and Cks

Cks was identified first as a high copy suppressor of temperature sensitive mutations of *Cdk* in *Saccharomyces cerevisiae* (Hadwiger et al., 1989). This study showed that this capability was due to physical interaction between Cks and Cdk proteins (Brizuela et al., 1987). Co-immunoprecipitation revealed interaction between Cks and Cdk1 in human cells (Draetta and Beach, 1988), frog eggs (Patra and Dunphy, 1996) and Drosophila (Swan et al., 2005).

	Organism	Common name/description	Cks protein/s	Length (Amino acid)	reference
Yeast	*Physarum polycephalum*	*Slime mould*	*Cksphy*	*84*	*(Birck et al., 1995)*
	Schizosaccharomyces pombe	*Fisson yeast*	*Suc1*	*112*	*(Hayles et al., 1986)*
	Saccharomyces cerevisiae	*Budding yeast*	*Cks1*	*150*	*(Hadwiger et al., 1989)*
plantae	*Arabidopsis thaliana*	*Arabidopsis*	*Cks1At*	*87*	*(De Veylder et al., 1997)*
	Ostreococcus tauri	*Marine unicellular green alga*	*Cks*	*77*	*(Robbens et al., 2005)*
	Triticum aestivum	*Wheat*	-	*90*	*(John et al., 1991)*
	Nicotiana tabacum	*Tobacco*	*Ntsuc1*	*55*	*(Qin et al., 1996)*
Animalae	*Caenorhabditis elegans*	*Nematode*	*Cks1* *Cks2*	94 118	*(Korf et al., 1998)*
	Drosophila melanogaster	*Fruit fly*	*Cks85A* *Cks30A*	*96* *74*	*(Finley and Brent, 1994)(Flybase.org)*
	Homo Sapiens	*Human*	*CksHs1* *CksHs2*	*79* *79*	*(Richardson et al., 1990)*
	Mus musculus	*Mouse*	*Cks1b* *Cks2*	*79* *79*	*(Carninci et al., 2005)*
	Patella vulgate	*Common limpet*	*Suc1*	*76*	*(Colas et al., 1993)*
protozoa	*Leishmania Mexicana*	---	*LmmCks1*	*99*	*(Grant et al., 1998)*
	Trypanosoma cruzi	---	*Tcp12Cks1*	*98*	*(Mutoz et al., 2006)*

In a number of organisms, studies showed that Cks can bind to the other types of Cdks: human CksHs1 can bind to Cdk1, Cdk2 and Cdk3 (Bourne et al., 1996)**,** Drosophila Cks85A binds to Cdk2 and Cdk1 and also Cks30A binds to Cdk1 (Swan et al., 2005).

Cks homologes from one species can even bind to Cdks from other species. Both human Cks can bind to *S.cerevisiae* Cdc28 (Richardson et al., 1990) and also *S.cerevisiae* Cks can bind to the human Cdk2 (Elledge et al., 1992). This shows that the Cks structure is very similar in different organisms.

2.2 APC activation and Cyclin B destruction

To enter into anaphase, Cyclin B needs to be degraded. The APC is responsible for ubiquitinating Cyclin B and targets it for destruction by the proteasome (King et al., 1995). Cdc2 phosphorylates APC to activate it (Hershko et al., 1994) and it has been shown that Xe-p9 (Xenopus Cks2) is a part of this complex (binds to Cdc2) and it has a very critical role for APC activation. *Suc1* mutants in fission yeast cause the accumulation of Cdc13 (Cyclin B type) (Basi and Draetta, 1995) and similarly in budding yeast. Loss of *Cks1* causes a dramatic increase in the level of Clb2 (Cyclin B homolog) (Reynard et al., 2000). In Drosophila*,* the *Cks30A* null mutant also causes an accumulation of Cyclin A, B and B3 in female meiosis (Swan et al., 2005). Immunodepletion of the *Xe-p9* gene also causes Cyclin B accumulation (Patra and Dunphy, 1996). It seems APC activation depends on Cks activity and in the absence of this protein APC cannot ubiquitinate the Cyclins.

It has been shown that depletion of Xe-p9 in Xenopus egg extracts results in a high level of Cdc2 associated activity at the end of M phase, and also causes a decrease in ubiquitination of Cyclin B (Patra and Dunphy, 1998). It is not known whether Cks brings Cyclin A and Cyclin B

to the APC for degradation or it binds to the Cdc2, then this complex can activate APC at the beginning of anaphase. It has been shown that for destruction of Cyclin A and Cyclin B by APC, Cks brings Cyclins to the APC to be degraded (Wolthuis et al., 2008). According to the second model, Cks guides Cdc2-Cyclin B to the phosphorylated APC. This complex binds to the APC and induces further phosphorylation of APC and full activation at the end of M phase (Patra and Dunphy, 1998). Morris showed another very important and very different role of Cks in budding yeast which is the promotion of *Cdc20* transcription (Morris et al., 2003).

2.3 SCF^{Skp2} interaction and p27 destruction

Two studies in 2001 showed a novel role of Ckshs1 by interacting with Skp2 (F-box protein) which is a part of SCF Ubiquitin ligase complex. The SCF^{Skp2} plays a critical role in entry into S-phase by targeting $p27^{kip1}$ for degradation by the proteasome. Ckshs1 is critical to help Skp2 to recognize $p27^{kip1}$ for ubiquitination by SCF^{Skp2} (Ganoth et al., 2001; Spruck et al., 2001).

At G1 phase $p27^{kip1}$ act as a Cdk inhibitor by binding to Cdk2/Cyclin E and Cdk2/Cyclin A (Sherr and Roberts, 1999). Before entry into S-phase, Cdk4/Cyclin D accumulates (Sherr, 1993). Cdk4/Cyclin D starts outcompeting Cyclin E/Cdk2 for $p27^{kip1}$ which causes a partial activation of Cdk2 (Sherr and Roberts, 1995), Cdk2 also can help its own activation by phosphorylation of $p27^{kip1}$ on Thr187. Cdk2 deactivates Rb, which is the inhibitor of E2F. E2F is a transcription factor and promotes the activation of Cdk2 by activation of the transcription of *cyclin E* and genes encoding replication proteins. Phosphorylated $p27^{kip1}$ can be recognized by SCF^{Skp2} and ubiquitinated. Proteolysis of p27 by the proteasome is the next step for full activation of Cdk2/Cyclin E (Figure 2.2)

CksHs1 can only bind to the phosphorylated form of $p27^{Kip1}$ (Ganoth et al., 2001) and $p27^{Kip1}$ binds to the phosphate binding site of CksHs1 through Glu 185 and this interaction may be necessary for efficient binding of p27 to SCF^{Skp2}. Cks1 can associate with Cdks and this interaction may be important for binding the Cdk2/Cyclin A and E complex onto the SCF^{Skp2} ubiquitin ligase. An experiment done by Spruck in *vitro* showed that the ubiquitination level of $p27^{Kip1}$ in the presence of $Cdk1^{1N}$ (a mutated form of Cdk1 that cannot interact with Cks) is almost equal compared to the wild type Cdk1 background. This shows that the ubiquitination of $p27^{Kip1}$ is independent of the interaction between Cks1 and Cdk1 (Spruck et al., 2001).The crystal structure of Skp1/Skp2/CksHs1 bound to $p27^{Kip1}$ was identified by Hao (Hao et al., 2005). CksHs1 binds to the leucine-rich repeat (LRR) domain of Skp2 and C-terminal tail. The identification of Skp1-Skp2-CksHs1 complex associated with $p27^{Kip1}$ shows that Thr187 phosphorylation site of $p27^{Kip1}$ is recognized by phosphate binding site of CksHs1 and at the same time this $p27^{Kip1}$ binds to Skp2 from its Glu185 site. This model shows that both Skp2 and CksHs1 bind to $p27^{kip1}$.

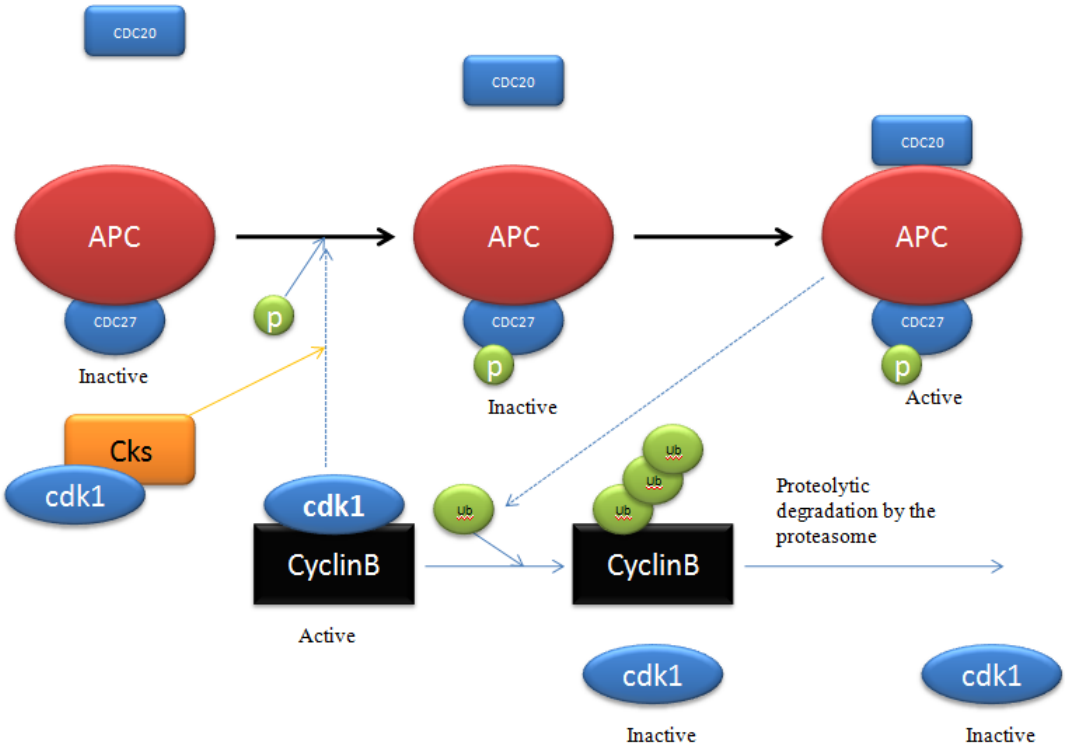


Figure 2.1 Role of Cks to mediate Cyclin B degradation by APC

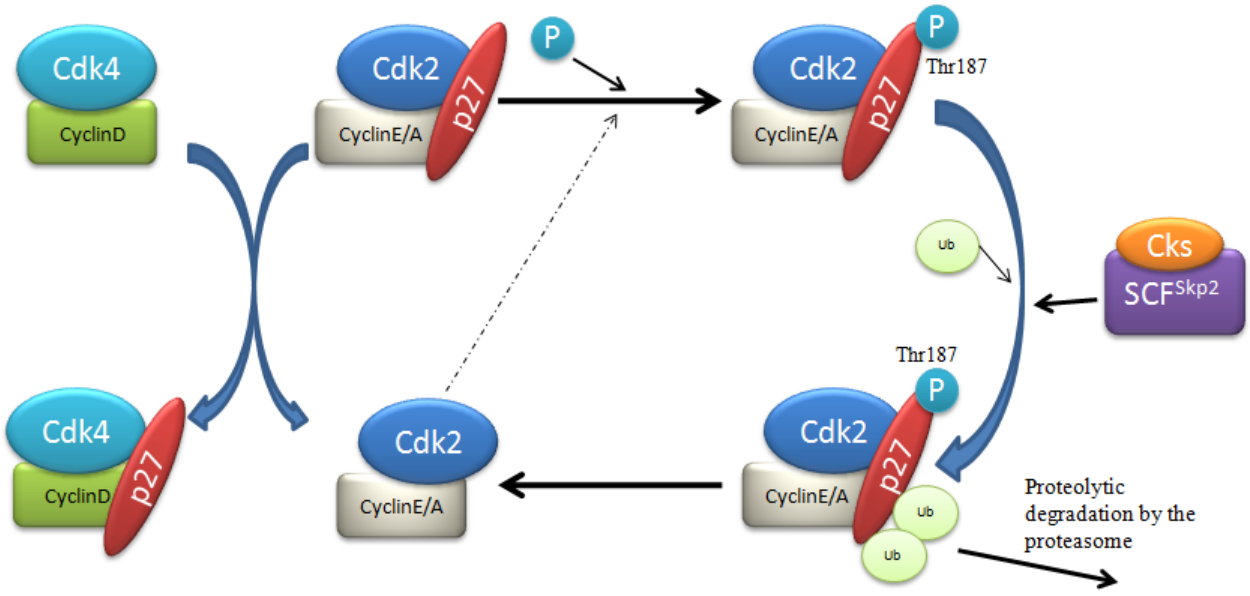


Figure 2.2 Role of Cks to mediate $p27^{Kip1}$ degradation by SCF^{Skp2}

Dacapo (Dap) is the $p27^{kip1}$ homolog in Drosophila and it is still unknown if it is targeted by SCF^{Skp2} for degradation. Dap controls the activity of Cdk2/Cyclin E in G1 phase and it plays a critical role in control of the endocycle (De Nooij et al., 2000). *dap* null mutations ($dap^{-/-}$) cause reduced endoreplication in nurse cells (Hong et al., 2007) by inactivation of Cyclin E/Cdk2 in G1. Cdt1 is another target of SCF^{Skp2} in mammalian cells (Nishitani et al., 2006). Cdt1 is important for DNA replication initiation in G1 phase and it is degraded right after DNA replication starts. Double parked (Dup) is the Cdt1 homolog in Drosophila and it plays the same role as in mammalian cells (Thomer et al., 2004). SCF^{Skp2} has been shown to target several other proteins including Cyclin E, c-Myc, E2F1, Orc1, p21 in mammalian cells (Nakayama and Nakayama, 2005).

2.4 DNA replication initiation control

DNA replication occurs in S-phase but the molecular process for initiation of DNA replication starts at G1 phase. Before starting DNA replication, the pre-replicative complex needs to be assembled on the origin recognition complex. For this reason, the origin recognition complex binds to the origin and then Cdt1 and Cdc6 proteins can in turn bind to the origin recognition complex. When this complex forms, they can recruit Mcm helicase to the origin recognition complex and thus the pre-replicative complex is complete. Following assembly of this complex DNA replication can start. After the start of DNA replication it is very important to disassemble the complex to prevent the re-replication of DNA. For this reason, Cdt1 and Cd6 are degraded and the origin recognition complex is phosphorylated in mammalian cells.

Cdt1 is ubiquitinated by SCF^{Skp2} in humans (Li et al., 2003) but depletion of Skp2 does not result in accumulation of Cdt1 in human cells because in the absence of Skp2, Cul4 ubiquitin ligase promote degradation of Cdt1 to prevent re-formation of pre-replication complexes

(Nishitani et al., 2006). Geminin is another protein which is important for preventing rereplication. Geminin binds to Cdt1 to inactivate it. In the absence of Geminin, cells undergo re-replication and become polyploid (Mihaylov et al., 2002).

Double parked (Dup) is the homolog of Cdt1 in Drosophila and it is also required for initiation of DNA replication (Whittaker et al., 2000). Dup needs to be degraded after S-phase to prevent the re-replication of DNA in Drosophila. It has been shown that Dup is degraded by Cul4 but it is still unknown if SCF^{Skp2} degrades Dup (Lin et al., 2009). Geminin also binds to Dup to inhibit its activity (Quinn et al., 2001). Overexpression of Dup causes re-replication of DNA and polyploidy (Thomer et al., 2004). Dup phosphorylation occurs by Cdk2/Cyclin E and it may be important for Dup degradation.

Cdk1 is critical for control of re-replication in yeast but its role in multicellular eukaryotes is still not clear. Mutations in *Cdc2* (*Cdk1*) cause polyploidy in Drosophila (Weigmann et al., 1997) but the main reason for this polyploidy is still not clear. Cdk1 is important for entering into mitosis and it is possible that in the absence of Cdk1 the cell does not undergo mitosis but instead it enters a second G1 and become polyploid. Alternatively, Cdk1 also inhibits Cdt1 by phosphorylation in mammalian cells. Therefore the loss of Cdk1 could lead to accumulation of Cdt1 and re-replication of the DNA by re-formation of pre-replicative complexes. Cyclin A silencing also causes re-replication of the DNA (Mihaylov et al., 2002).

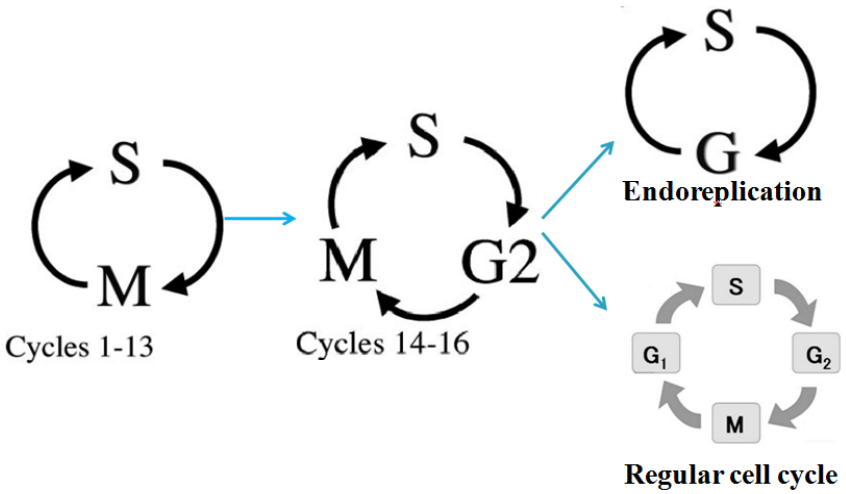
2.5 Cell cycle in Drosophila

The first 13 nuclear divisions in Drosophila embryos are promoted by maternal proteins and these cycles are independent of zygotic transcription (Figure 1). Starting at cycle 14, a G2 phase is added to the cell cycle. After cycle 16, cells undergo two different types of cell cycle;

endoreplication and the archetypal cell cycle (Lee and Orr-Weaver, 2003). Endoreplication happens in the cells which differentiate to form the tissues in larvae and endoreplication along with associated increases in cell size is responsible for the growth of larvae. Archetypal cell cycles occur in the tissue which will make adult tissues (Lee and Orr-Weaver, 2003). Drosophila development is composed of several stages. Embryogenesis takes one day. Larval development takes 5 days and is composed of three stages and this is followed by entry into the pupal stage. 3 days after pupariation the adult encloses.

2.6 Drosophila eye imaginal disc structure

During larval development, the adult structures such as wings and eyes develop within structures called imaginal discs. Cells within imaginal discs undergo archetypal cell cycles. Cell cycle regulation in eye imaginal disc during developments is very different than the other tissues. Differentiation in the eye starts from the posterior side and progresses to the anterior side corresponding to the movement of a morphogenetic furrow from posterior to anterior (Figure 4) (Lee and Orr-Weaver, 2003). The eye disc cells divide until they enter the morphogenetic furrow. Cells arrest in G1 phase in the morphogenetic furrow. In the anterior side of morphogenetic furrow, some of the cells divide one more time (second mitotic wave) and then differentiate.



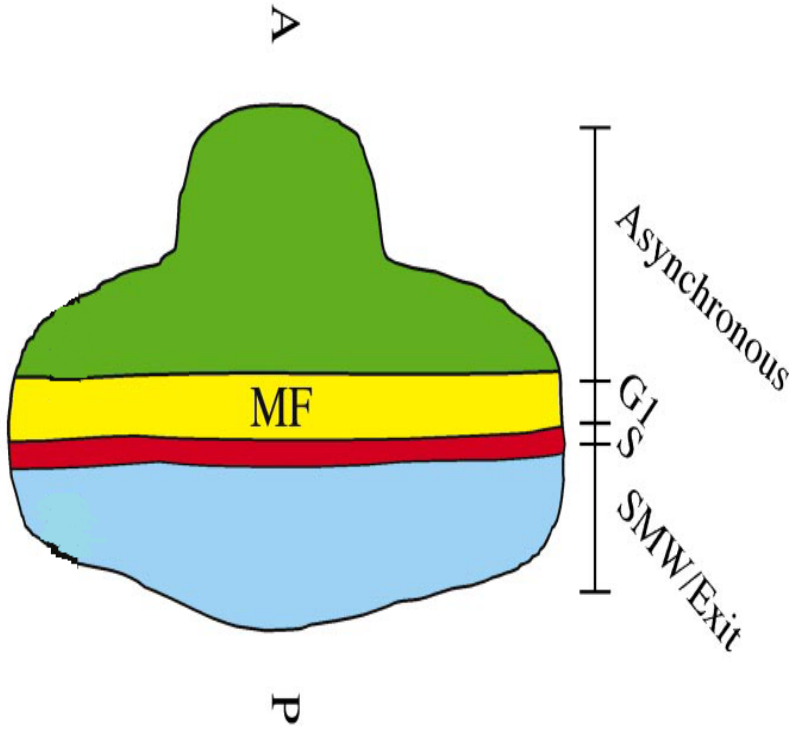


Figure 2.3 Drosophila melanogaster cell cycle

The cell cycle in Drosophila Embryo (A). Cell cycle regulation in eye imaginal disc in larvae (B). MF (Morphogenetic Furrow). Cells undergo diferentiation after Second Mitotic Wave (SMW).

Chapter 3. **Materials and Methods**

3.1 Fly genetic stocks

1- *Cks85A^{ex15}/TM6*

2- *Skp2^{ex9}/TM6*

3- wild type

4- *Cks85A^{ex15},Skp2^{ex9}/TM6*

5- Df(3R)BSC197*/TM6*

6- *UAS-HA-Skp2, UAS-Flag-Cks85A*

7- *UAS-HA-Skp2; UAS-Flag-Cks30A/TM6*

8- *nanos-Gal4*

9- *daughterless-Gal4*

10- *Cyclin B^{2} /CyOGFP;Cks85A^{ex15}/TM6*

11- *Cyclin B3^{L6540},Cks85A^{ex15}/TM6*

12- *Dup^{PA77} /CyOGFP;Cks85A^{ex15}/TM6*

13- *Dup^{PA77} /CyOGFP;Skp2^{ex9}/TM6*

14- *Cyclin A,Cks85A^{ex15}/TM6*

Cks85A (CG9790) and *Skp2 (CG9772)* null mutant flies were made in our lab. To make the *Cks85A^{ex15}*, we excised a P element and used PCR to identify imprecise excisions that removed *Cks85A* coding sequence (Swan et al., 2005). *Skp2^{ex9}* was made by the same method as *Cks85A^{ex15}* (Andrew Swan, unpublished)

3.2 Gal 4 system

UAS-Gal4 system is a method used to study gene expression and function in Drosophila. The system has two parts: the *gal4* gene, encoding the yeast transcription activator protein Gal4, and the *UAS* (Upstream Activation Sequence), a short section of the promoter region to which Gal4 specifically binds to activate gene transcription.

In Drosophila, there are different promoters to activate *gal4* expression in different tissues. *nanos-gal4* promotes expression in female germline. There are more *gal4* promoters and they are used for expressing specific proteins in the specific tissues. In some experiments *daughterless-gal4* was used. This gal4 line drives expression everywhere in Drosophila cells.

3.3 Measuring whole larva size

The Growth experiment was done using 3 different stocks of flies, *yw* (wild type), $Skp2^{ex9}$/ *TM6* and $Cks85A^{ex15}$/ *TM6*. In these stocks, flies with three different genotypes are possible: homozygous for *Cks85A* or *Skp2* genes (lethal in 3rd instar larval stage), homozygous for *TM6* (lethal in embryonic stage) and *Cks85A/TM6* or *Skp2/TM6* (viable). Flies were placed in egg lay chambers with apple juice plates supplemented with live yeast. Eggs were collected every 4 hours and kept on plates at 25^{o}C. Hatching started approximately 24 hours after plating. When ~ 20% of the eggs had hatched, larvae were removed from the plate and the plate was left in the incubator for an additional hour. Larvae were then collected and placed into a vial of food to grow for 3 days, 4 days, 4.5 days or 5 days. The larvae with the Skp^{ex9}/ $Skp2^{ex9}$ and $Cks85A^{ex15}$/ $Cks85A^{ex15}$ genotypes can be identified because they do not have the balancer chromosome with the dominant *Tubby* marker. The non-*Tubby* larvae were collected and fixed in 3.7% Formaldehyde (80µl Formaldehyde 37% in 520µl of PBST). After mounting they were measured

by tracing their outlines and their area was determined using Image J program (National Institutes of Health). This experiment was done 3 times.

3.4 Measuring the size of salivary gland, Wing imaginal disc and Brain

yw (wild type), *Skp2^{ex9}/TM6* and *Cks85A^{ex15}/TM6* stocks were used in this experiment. A certain number of flies were put into the vials of food and kept there for 4 hours laying eggs. The vials were kept at 25^{o}C for 5 days. Care was taken to not have a very low or very high number of eggs because both can affect the rate of larval growth. Non-Tubby larvae were taken randomly and prepared for dissection. They were washed with H_2O and put into the PBS for dissection and fixed in 3.7% Formaldehyde (540µl PBST + 60µl 37% Formaldehyde) for 30 minutes on a nutator. They were rinsed 3x with PBST followed by three 30 minute washes. Samples were incubated with RNase for 30 minutes and stained with Propidium Iodide (1/500) (Invitrogen) or Oligreen (1/1000) (Invitrogen) for one hour. Samples were mounted in 80% Glycerol and viewed by confocal microscope (Fluo View 1000, Olympus).

3.5 Brain Squashes

The same stocks were used for growth experiments. The 3rd instar non-Tubby larvae were dissected and incubated in 1mL PBS with 0.5µl Colchicine (40mg/ml) on the rotator for 1 hour. Samples were put into Sodium Citrate for 10 minutes. During these 10 minutes the brain was separated from the other tissues and the rest was discarded from solution. Brains were selected individually and put in solution composed of 550µl Acetic Acid, 550µl Methanol, and 100µl H_2O for 15 seconds. A drop of 45% Acetic acid was added to the slide. 4 brains were put on the slide and a cover slip was pressed on the slide by finger to squash the brains. Slides were flash frozen with liquid nitrogen and the cover slip was removed with a razor blade. Slides were then placed in methanol (-20^{o}C) for 10 minutes and dried. Propidium Iodide (1/500) and RNase were placed

on the sample and the cover slip was added again. After 1 hour the slide was washed by PBST and left to dry. Polymount or 80% Glycerol was used for mounting. 80% glycerol was better because Propidium Iodide fluorescence faded in Polymount.

3.6 Co-Immunoprecipitation

Either *UAS-HA-Skp2*; *UAS-Flag-Cks85A or UAS-HA-Skp2; UAS-Flag-Cks30A* or *yw* flies (as a control) were mated to the *nanos-Gal4* flies to overexpress the Skp2, Cks85A and Cks30A proteins in to the embryo. Embryos were collected after 4 hours. They were dechorionated with bleach, weighted and flash frozen.

The IP buffers used for immunoprecipitation were;

1- IP buffer: 10mM $CaCl_2$, 150mM NaCl, 50mM Tris-Hcl (PH=8), 0.1% Tween

2- IP buffer with inhibitors: above plus 1 tablet of Complete mini protease inhibitor (Roche) + 100µl of phosphatase inhibitor (Thermo scientific)

Samples were ground in IP buffer with inhibitors (30µl/mg of sample) with a glass grinder and spun for 10-20 minutes at high speed in cold room (4^oC). 60µl of the supernatant was taken and used as a control in western blots. 2µl of HA-beads (Roche Applied Science) and Flag-beads (Sigma Aldrich) per mg of embryo were used. Beads were washed 2 times with IP buffer and one time with the IP Buffer with inhibitors before used. Beads were added to the rest of supernatant and left on nutator for 2 hours. Samples were spun and the pellet was washed 3 times, every time 5 minutes in IP buffer with inhibitors and then last wash with just IP buffer. All of the steps were done in the cold room. At the end, 2X sample buffer was added to the beads and boiled. Sometimes, beads were eluted with the flag or HA peptide buffer which was made from;

7X IP buffer + 0.04X peptide (X= mg of embryo)

Samples were incubated with peptide on a nutator for 10 min and supernatant transferred in another tube and boiled with 5X sample buffer for 5 minutes.

3.7 Western blotting

7%, 10% and 15% bis-acyrlamide gels were used for western blotting. *yw, Cks85A*$^{ex15/ex15}$ *and Skp2*$^{ex9/ex9}$ 3rd instar larvae were used. Their brains (plus imaginal discs) and salivary glands were dissected out and stored separately in -80^{o}C for different experiment. For every 40 brains (plus imaginal discs) 80µl of 2X sample buffer was added in all of the samples. In every 30 salivary glands, 10µl of 2X sample buffer was added.

Standard procedures for transfer of proteins from gel to nitrocellulose membrane were followed. The membranes were blocked in 5% non-fat instant milk powder. Table 1 is the list of the primary antibodies that were used and table 2 is the list of secondary antibodies.

Title	Antibody name	Raised in	Concentration	Source
1	Cyclin A	Mouse	1/5	Developmental Studies Hybridoma Bank
2	Cyclin B	Mouse	1/20	Developmental Studies Hybridoma Bank
3	Actin	Mouse	1/250	Developmental Studies Hybridoma Bank
4	Dap	Rabbit	1/1000	Dr. Christian Lehner, University of Zurich
5	Dup	Guinea pig	1/1000	Dr. Terry L. Orr-Weaver, Massachusetts Institute of Technology
6	HA	Rat	1/1000	Roche Applied Science
7	Flag (M5)	Mouse	1/1000	Sigma Aldrich
8	Cdk1(PSTAIR)	Rabbit	1/1000	Santa Cruz Biotechnology
9	Cdk2	Rat	1/1000	Dr. Christian Lehner, University of Zurich
10	Skp A	Guinea pig	1/200	Dr. T. Murphy, Carnegie institution science
11	Cyclin E	Mouse	1/1000	Santa Cruz Biotechnology

Table 1. Primary antibodies used in these experiments

	Antibody name	Raised in	Concentration	Source
1	Rat	Goat	1/7000	Thermo scientific
2	Mouse	Goat	1/7000	Thermo scientific
3	Rabbit	Goat	1/1000	Invitrogen
4	Guinea pig	Goat	1/1000	Santa Cruz Biotechnology

Table 2. Secondary antibodies used in these experiments

Chapter 4. **Objectives**

SCF^{Skp2} ubiquitin ligase is important for growth in mammalian cells. In the absence of Skp2, growth was reduced in mouse and it was shown that there is an accumulation of p27 . CksHs1 is another proteins which is important for recognition and degradation of p27. CksHs1 interacts with SCF^{Skp2} ubiquitin ligase. Mammalian Skp2 is required in some cell types to prevent polyploidy. Cks85A is the homolog of CksHs1 protein and its role in Drosophila is still unknown. It has been shown that Cks85A can interact with Cyclins A, B and B3 and also Cdks1 and Cdk2. Null mutation in *Cks85A* cause lethality in 3rd instar larval stage. These findings lead us to design experiments in order to find the role of this protein in the cell cycle.

The following are my objectives:

1- Deterimination of the role of Cks85A in growth.

2- Confirmation of the effect of *Cks85A* and *Skp2* mutants on growth by using another allele of genes.

3- Characterization of the polyploidy caused by Cks85A and Skp2 depletion in Drosophila.

4- Characterization of Skp2 and Cks85A in SCF ubiquitin ligase complex.

5- Characterization of Cdks in SCF complex.

6- Determination of the interaction between Cks85A and Cks30A in cell cycle.

7- Identification of SCF^{Skp2} complex targets.

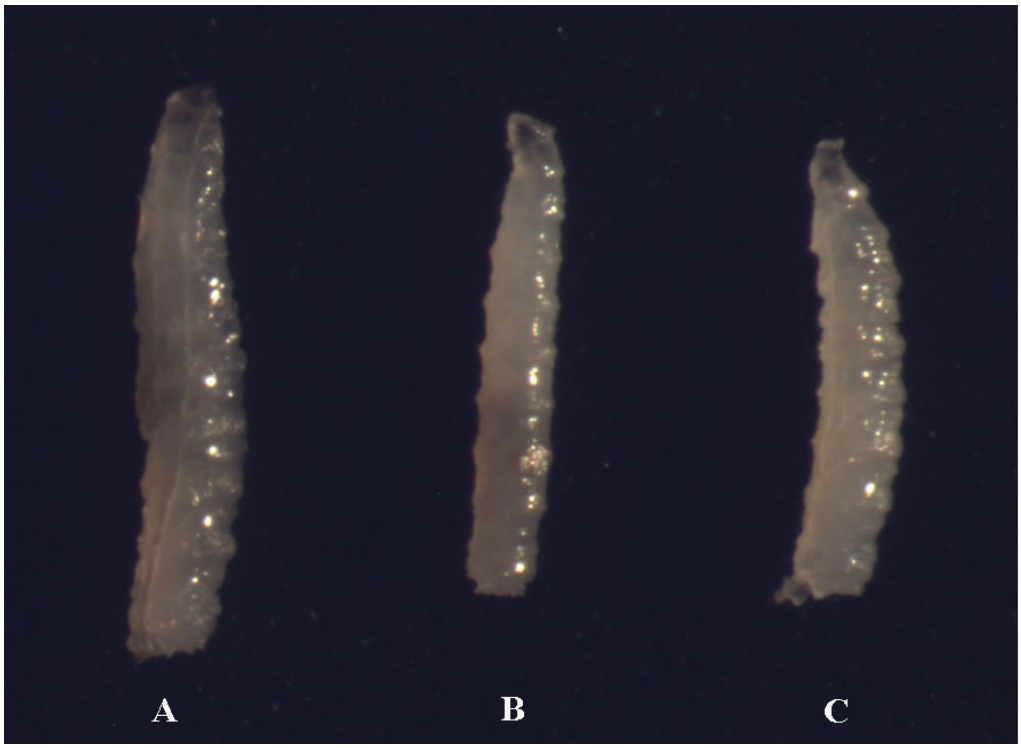
Chapter 5. **Results**

5.1 Role of Cks85A in growth

Skp2 and CksHs1 are important for growth control in mammalian cells and it has been shown that mice null for *Skp2* are smaller than the wild type (Ganoth et al., 2001; Spruck et al., 2001). This brings up the possibility that *Skp2* and *Cks85A* can also affect Drosophila growth. In Drosophila larval stage, growth depends largely on the endoreplicating tissues growth.

To look at the larval growth rate in the absence of Skp2 and Cks85A proteins, $Skp2^{ex9/ex9}$ and $Cks85A^{ex15/ex15}$ mutant larvae were compared to the wild type at different times after egg hatching. Larvae were collected after 3, 4, 4.5 and 5 days after egg hatching (Figure 1A- D) and the area of 20-30 larvae was measured in each time point with the Image J program. *Skp2* and *Cks85A* mutant larvae were smaller than the wild type larvae. The difference in the larval size was apparent 3 days after egg hatching but the difference was more obvious after 4 days.

Comparison of early larvae showed that there is not a big difference between mutants and wild type larvae at 3 days after egg hatching. The size of *Cks85A* and *Skp2* mutant larvae was 67% of the size of wild type larvae at 3 days post-hatching and this number increased to 69% at 4- 4.5 days. In day 5 the *Skp2* and *Cks85A* mutant flies were 80% of the size of wild type flies indicating that there is not a big change in growth rate after 3 days. The difference between the *Cks85A* and *Skp2* mutants with wild type size between 4.5 to 5 days was because wild type larvae stopped growing after 5 days and subsequently enter the pupal stage while the mutants kept growing. These results showed that Cks85A and Skp2 proteins are important for growth in Drosophila.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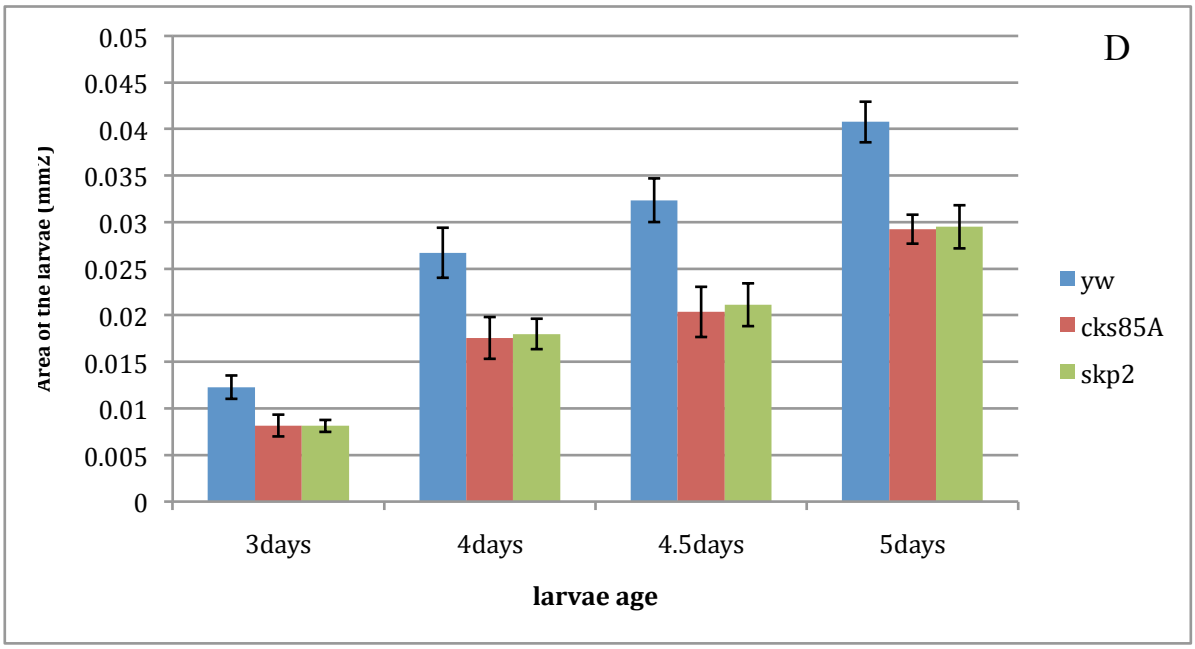

D
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Area of the larvae (mm2)
3days
4days
4.5days
5days
larvae age
yw
cks85A
skp2

Figure 5.1 Loss of *Skp2* and *Cks85A* cause reduced growth

Larval size in wild type (A), $Cks85A^{ex15/ex15}$ (B) and $Skp2^{ex9/ex9}$ (C) after 4.5 days of egg hatching. The graph shows the size of $Skp2^{ex9/ex9}$, $Cks85A^{ex15/ex15}$ (D) and wild type, measuring area by image J program. The Y axis is the size of the larvae in milimiter^2 and the X axis is the time points of experiments. This experiment was done three times and between 10-20 larvae were measured for each time point.

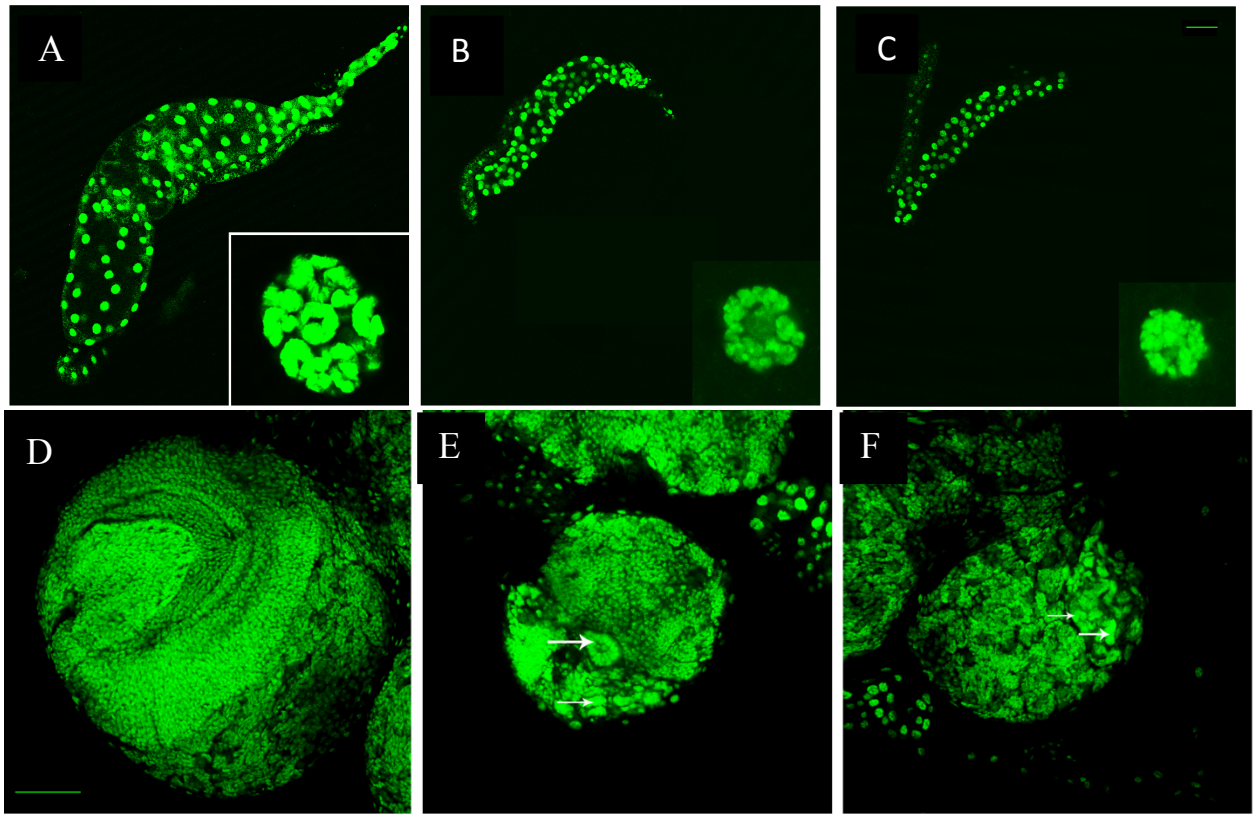
Drosophila growth depends on the growth in endoreplicating tissues such as salivary gland. To determine if null mutation in $Skp2^{ex9/ex9}$ and $Cks85A^{ex15/ex15}$ has an effect on endoreplicating cells, I looked at growth of the salivary gland. Salivary glands from $Skp2^{ex9/ex9}$ and $Cks85A^{ex15/ex15}$ larvae was dissected 5 days after egg hatching and compared to the wild type. This showed that the salivary gland in $Skp2^{ex9/ex9}$ and $Cks85A^{ex15/ex15}$ larvae are smaller than the wild type (Figure 2A- C).

The difference between the sizes of salivary glands could have two explanations; it could be either because of the difference between sizes of the cells or difference in number of cells. To distinguish between these possibilities, the number of cells in $Skp2^{ex9/ex9}$ and $Cks85A^{ex15/ex15}$ mutant salivary gland was counted and compared with the wild type in 3rd instar larvae. The experiment revealed that there are almost the same number of the cells in the $Skp2^{ex9/ex9}$ and $Cks85A^{ex15/ex15}$ mutants and wild type salivary glands (110 cells in wild type, 102 cells in $Cks85A^{ex15/ex15}$ and 98 cells in $Skp2^{ex9/ex9}$ mutant salivary gland), sent these results were obtained from 4 different salivary glands. From the same selected salivary glands, the size of nuclei were measured in mutant and wild type flies. Results revealed that the wild type salivary gland nuclei are 2-3 times larger than nuclei in $Skp2^{ex9/ex9}$ and $Cks85A^{ex15/ex15}$ mutants. It is possible that Skp2 and Cks85A play a role in endoreplicating tissues to promote DNA replication, similar to their role in mammalian diploid cells (Ganoth et al., 2001).

Two diploid tissues were examined to check if these tissues also affected in mutants. All the larvae were selected of the same age, 4.5 days post egg hatching. Examination of brains (Figure 2D- F) and wing imaginal discs (Figure 3A- B) showed an obvious difference in the sizes. Mutants brain and wing imaginal disc were smaller than the wild type. This experiment shows that Cks85A and Skp2 are important for growth in different cell types in Drosophila.

5.2 Confirming the effect of *Cks85A* and *Skp2* mutants on growth by using deficiency mutation of the genes.

The chromosome carrying the mutant allele that we made might have other mutations on it and using flies homozygous for one allele may cause phenotypes resulting from depletion of these other genes. $Cks85A^{ex15/Df}$ ($Cks85A^{ex15/+}$ over a small deficiency that removes *Cks85A* plus neighbouring genes) was used in this experiment. Mitotic tissues and salivary gland were smaller (Figure 3C-F) in $Cks85A^{ex15/Df}$ compared to the wild type. The $Cks85A^{ex15/Df}$ phenotype is the same as the phenotype of $Cks85A^{ex15/ex15}$ mutants. This experiment shows that the phenotype that seen in $Cks85A^{ex15/ex15}$ mutants is most likely because of the absence of Cks85A protein. This experiment needs to be repeated for $Skp2^{Df}$.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B
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D
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Figure 5.2 Mutation in *Skp2* and *Cks85A* gene cause reduced growth in Drosophila tissue.

Salivary gland in wild type (A), $Cks85A^{ex15/ex15}$ (B) and $Skp2^{ex9/ex9}$ (C) stained with Propidium Iodide. Individual salivary gland nuclei are shown in the insets. Brain in wild type (D), $Cks85A^{ex15/ex15}$ (E), $Skp2^{ex9/ex9}$ (F) stained with Propidium Iodide. Arrows indicate polyploid cells. Scale bars indicate 100 μm in salivary glands and 50 μm in brains.

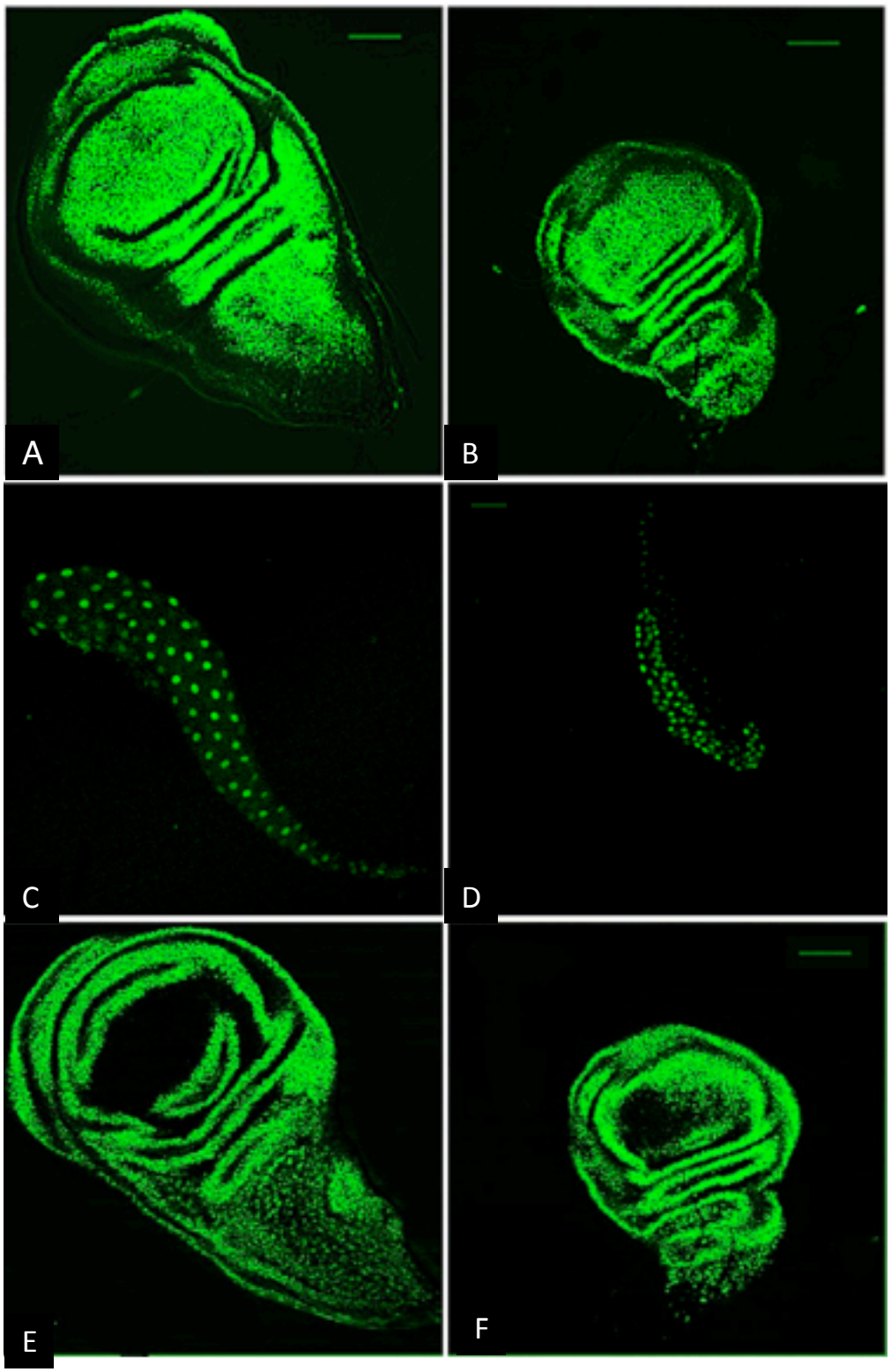
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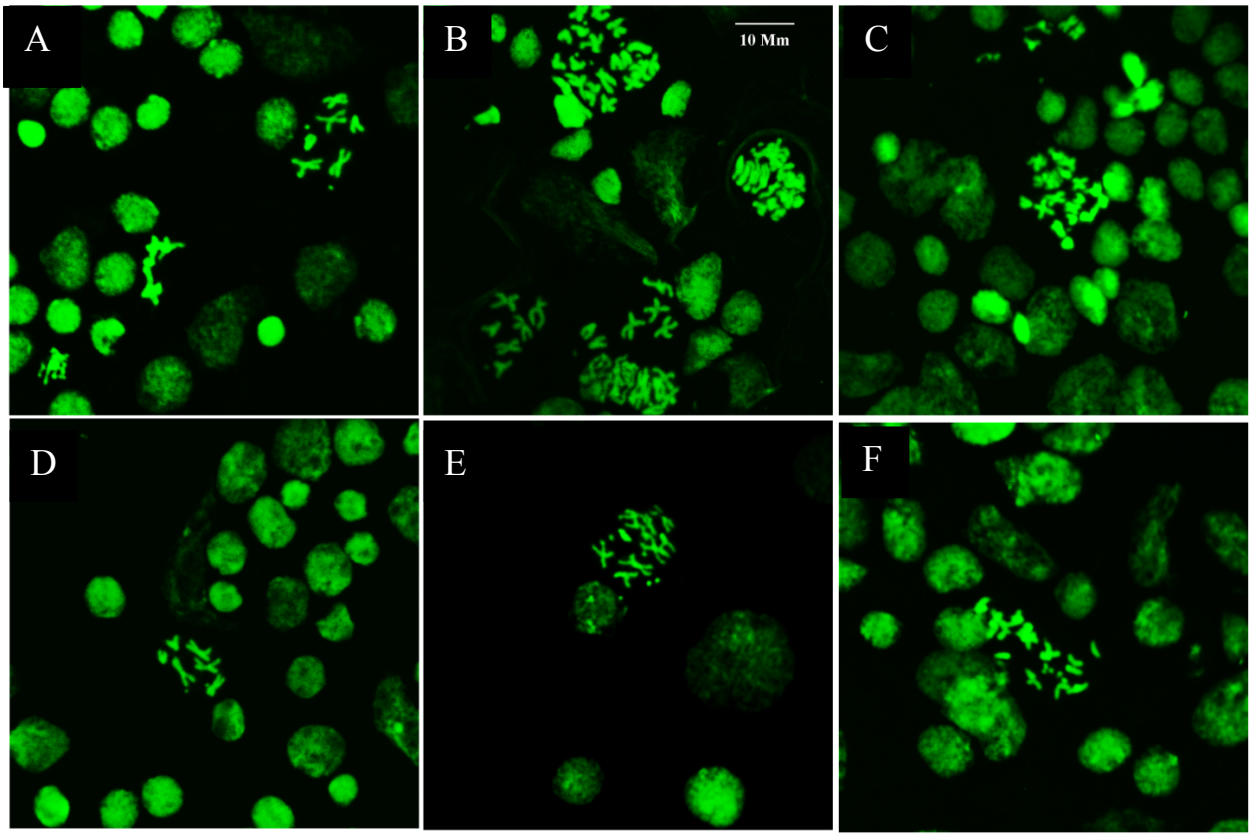
Figure 5.3 Mutation in Cks85A and Skp2 cause reduced in Drosophila tissue.

Wing imaginal disc in wild type (A), *Cks85A*$^{ex15/ex15}$ (B). Salivary gland in wild type (C), *Cks85A*$^{ex15/Df}$ (D) and wing imaginal disc in wild type (E), *Cks85A*$^{ex15/Df}$ (F) from 3rd instar larvae shows the *Cks85A* mutants wing imaginal discs and salivary glands are smaller than wild type. These experiments were repeated two times with *Cks85A*$^{ex15/ex15}$ and one time with *Cks85A*$^{ex15/Df}$ and *Skp2*$^{ex9/Df}$ mutants and each time with 5-8 salivary glands and wing imaginal discs. All of these larvae were selected 4.5 days after egg hatching (3rd instar wandering larvae). Scale bars indicate 100 μm in salivary glands and 50 μm in wing imaginal discs.

5.3 Cks85A and Skp2 depletion cause polyploidy in Drosophila

Skp2$^{ex9/ex9}$ and *Cks85A*$^{ex15/ex15}$ larval brains are smaller than the wild type and in addition there are some abnormally large nuclei in the brains (figure 2E- F). To determine if these large nuclei are polyploid, brains were squashed and individual chromosomes counted. This experiment showed that the big cells are polyploid (Figure 4A -F). This polyploidy could be due to the absence of mitosis in the cell cycle, which means cells undergo G1, S and G2 phases but not mitosis. If this happens after every round of cell cycle, DNA content increases two times and cells become polyploid. *Skp2*$^{ex9/ex9}$, *Cks85A*$^{ex15/ex15}$ and wild type 3rd instar larvae brains were used to examine this possibility. Brains were squashed and stained for their DNA with Propidium Iodide. Cells in mitosis and interphase in wild type, *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$ were counted (Figure 4G). In wild type 1.2% of cells were in mitosis and in *Cks85A* mutants 1% of cells were in mitosis and in the *Skp2* mutants 0.8% of cells were in mitosis. This is not a significant difference between wild type and mutants. The same experiment was done but this time cells were treated with Colchicine for 1 hour to block the cells in mitosis. Colchicine activates the spindle assembly checkpoint and prevents the progression of cells from metaphase to anaphase. In wild type 5.3% of the cells were in mitosis, *Cks85A* mutants 5.1% of cells were in mitosis and *Skp2* mutant 5.3% of cells were in mitosis and this difference is also not significant. This result showed that null mutation in these two genes does not effect entry into mitosis or arrest in mitosis. There are two other possibilities for seeing polyploid cells: re-replication of DNA in other stages or failure in cytokinesis. These possibilities are being studied currently in the lab.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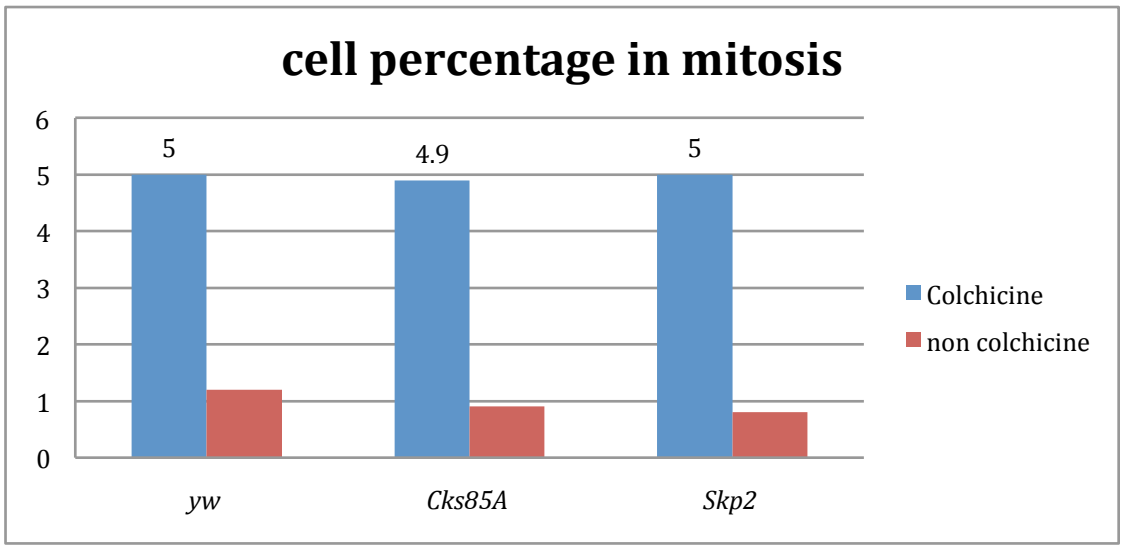

cell percentage in mitosis
6
5
4
3
2
1
0
5
4.9
5
yw
Cks85A
Skp2
Colchicine
non colchicine

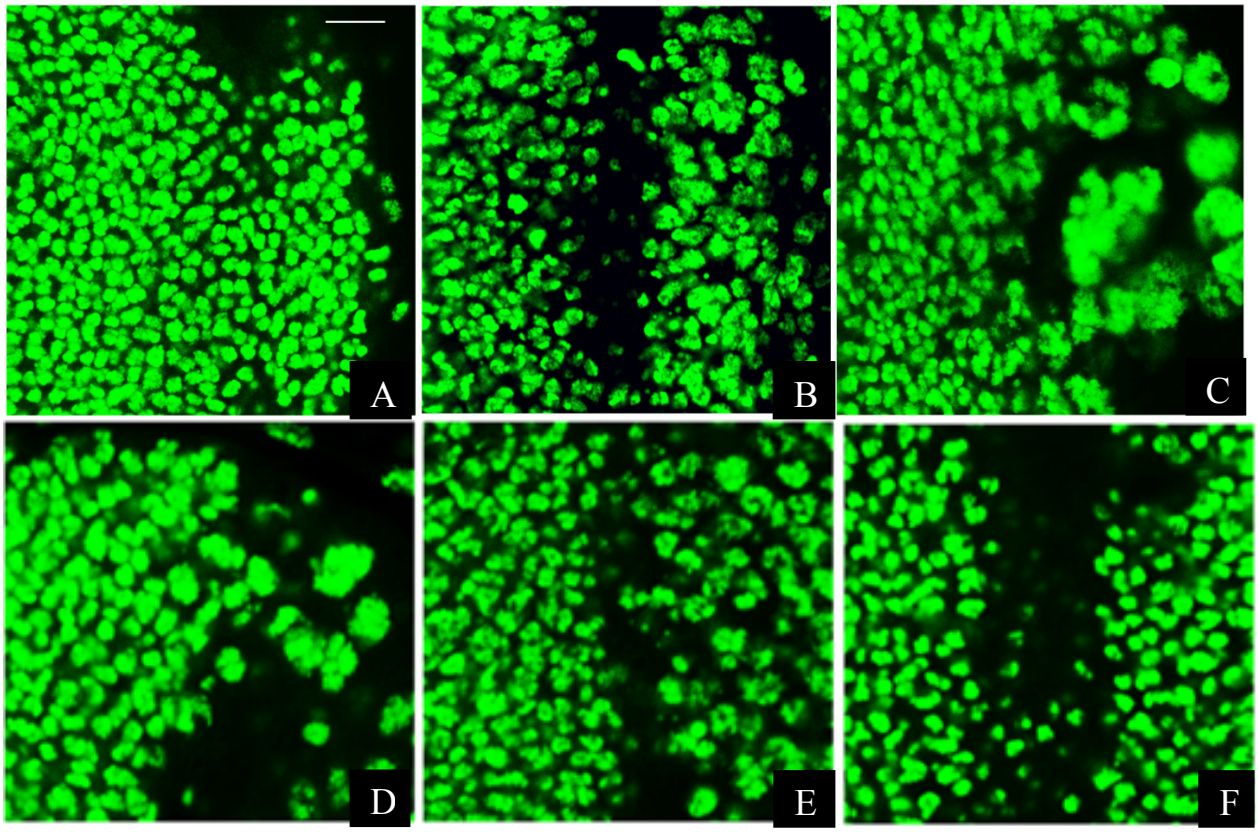
Figure 5.4 *Skp2* and *Cks85A* mutation cause polyploidy in brain cells.

Brain cells squash in wild type (A, D), $Cks85A^{ex15/ex15}$ (B, E) and $Skp2^{ex9/ex9}$ (C, F) stained with Propidium Iodide. A, B and C were treated with Colchicine and D, E and F without Colchicine. There are a normal number of chormosomes in the wild type (2 pairs of large autosomes, the sex chromosomes and a pair of dot-like small autosomes). Polyploid cells are obvious in the $Cks85A^{ex15/ex15}$ and $Skp2^{ex9/ex9}$ brains. The graph shows percentage of cells. These experiments were done 3 times and for every genotype at least 2000 cells were counted and every time around 700 cells were used in total.

To determine in which stage of larval development Cks85A and Skp2 proteins act, eye imaginal discs from *Skp2*$^{ex9/ex9}$ and *Cks85A*$^{ex15/ex15}$ 3rd instar larvae were observed. In both mutants, polyploidy was observed in the anterior side (Figure 5A- C). The morphogenetic furrow in the eye disc moves from posterior to anterior in the late 3rd instar larval stage. The cells in the anterior side differentiate at 3rd instar larval stage. Therefore it appears that polyploidy arises late in 3rd instar and only in cells that have not yet differentiated. The polyploidy was stronger in the anterior end of the *Skp2* eye imaginal discs compare to the *Cks85A* mutants. Looking at the *Cks85A* mutants eye imaginal disc from the larvae raised at 3 different temperature (18^{o}C, 25^{o}C, 29^{o}C) revealed that *Cks85A* is not necessary at low temperatures (18^{o}C) (Figure 5D- F) but Skp2 is necessary in all temperatures. It is possible that Cks85A protein only helps Skp2 to recognize the targets.

Transgenic flies were used (UAS-HA-Skp2 and UAS-Flag-Cks85A) to overexpress Skp2 and Cks85A protein in the *Skp2*$^{ex9/ex9}$ and *Cks85A*$^{ex15/ex15}$ flies. Daughterless (da)-gal4 driver was used to overexpress these proteins in 3rd instar larvae. Results showed that the overexpression of Skp2 and Cks85A protein in the *Skp2*$^{ex9/ex9}$ and *Cks85A*$^{ex15/ex15}$ larvae respectively rescues lethality. Normal eye imaginal discs were observed (Figure 6A-D). This experiment showed that the transgenic flies express the functional proteins and also *Skp2*$^{ex9/ex9}$ and *Cks85A*$^{ex15/ex15}$ phenotype (death in 3rd instar larval stage) is only due to the absence of Skp2 and Cks85A proteins.

These experiments show that knocking out of *Skp2* and *Cks85A* cause polyploidy in cells. Our observation in *Cks85A* and *Skp2* mutant brain and eye imaginal disc cells leads us to the question, how does mutation in these genes cause polyploidy in archetypal cells?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B
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F

Figure 5.5 Null mutation in *Skp2* and *Cks85A* genes cause polyploidy in eye imaginal discs.

Eye imaginal disc from 3rd instar larvae in wild type (A), $Cks85A^{ex15/ex15}$ (B) and $Skp2^{ex9/ex9}$ (C) 5 days after egg hatching and stained with Propidium Iodide. Eye imaginal disc from $Cks85A^{ex15/ex15}$ 3rd instar larvae raised at 29°C (D), 25°C (E), 18°C (E) show stronger polyploidy phenotype at higher temperature. Scale bar indicates 5 μm.

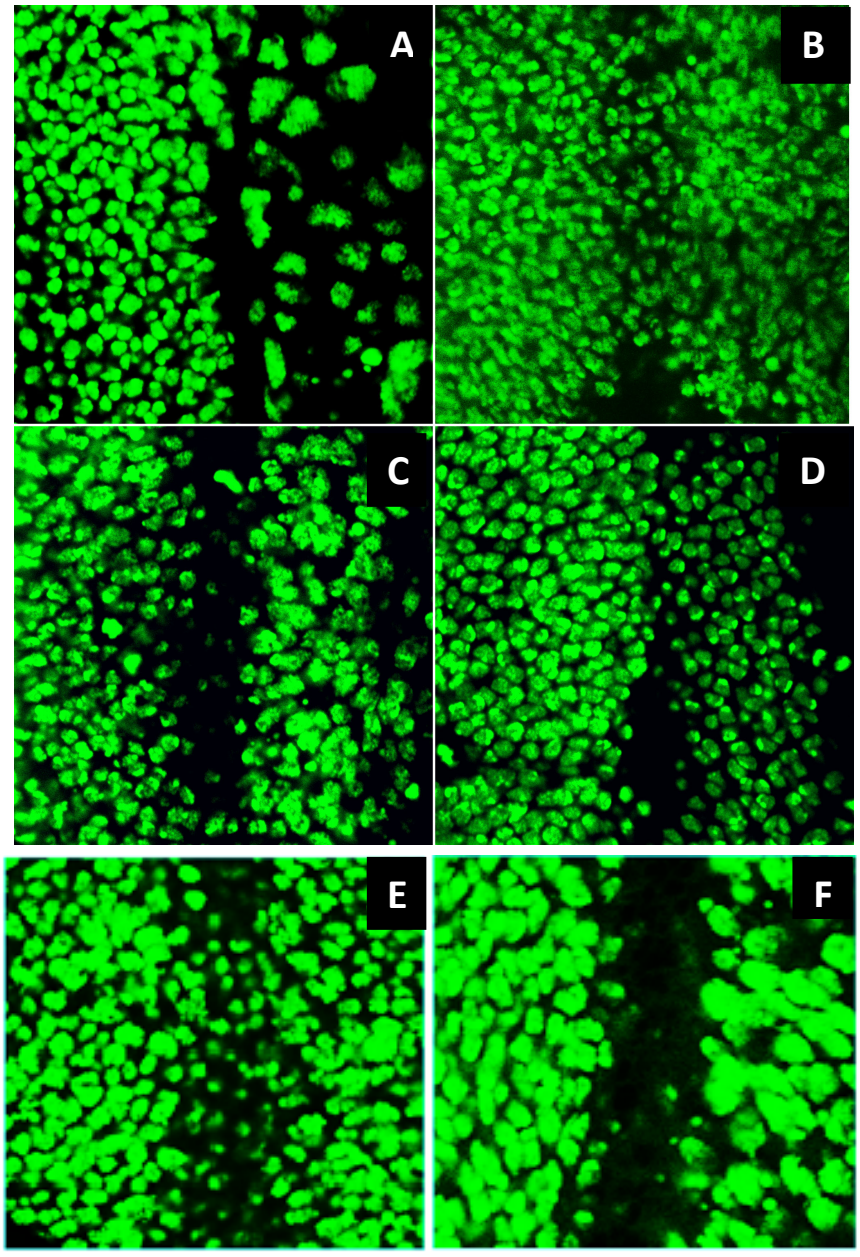

A
B
C
D
E
F

Figure 5.6 Overexpression of *Cks85A* and *Skp2* rescues the phenotype caused by *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$

Eye imaginal discs from *Skp2*$^{ex9/ex9}$ (A), *UAS-HA-Skp2; Skp2*$^{ex9/ex9}$ (B), *Cks85A*$^{ex15/ex15}$ (C) and *UAS-Flag-Cks85A; Cks85A*$^{ex15/ex15}$ (D) 3rd instar larvae. Overexpression of *Skp2* and *Cks85A* rescues the phenotype. Eye imaginal disc from *Cks85A*$^{ex15/ex15}$ (E) and *Cks85A*$^{ex15/ex15}$, *Skp2*$^{ex9/+}$(F) 3rd instar larvae 4.5 days after egg hatching shows that loss of one copy of *Skp2* enhanced the polyploidy caused by loss of *Cks85A*.

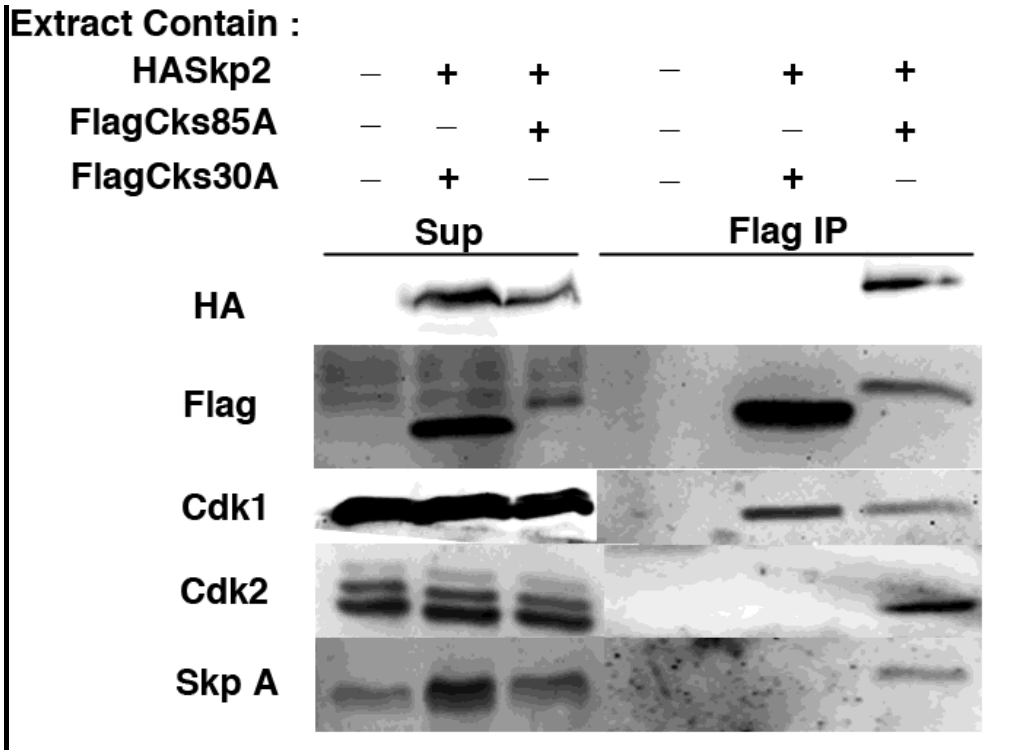
5.4 Characterization of Skp2 and Cks85A in SCF ubiquitin ligase complex

Mutation in *Skp2* and *Cks85A* cause polyploidy in archetypal cells. Genetic interaction between these two genes was examined to see if *Skp2* and *Cks85A* play role in a same pathway. The expression level of the *Skp2* gene was decreased in the $Cks85A^{ex15/ex15}$ flies to examine the possible redundant role between *Cks85A* and *Skp2*. The eye imaginal disc was chosen 4.5 days after egg hatching because phenotype is weak in $Cks85A^{ex15/ex15}$ flies at this time. Therefore in partial loss of *Skp2* in $Cks85A^{ex15/ex15}$ flies, we will see the polyploidy phenotype. The result showed that null mutation in *Skp2* enhances the phenotype of $Cks85A^{ex15/ex15}$ mutants (6E- F) which argues that these two proteins play a role either in a parallel pathway or in the same pathway.

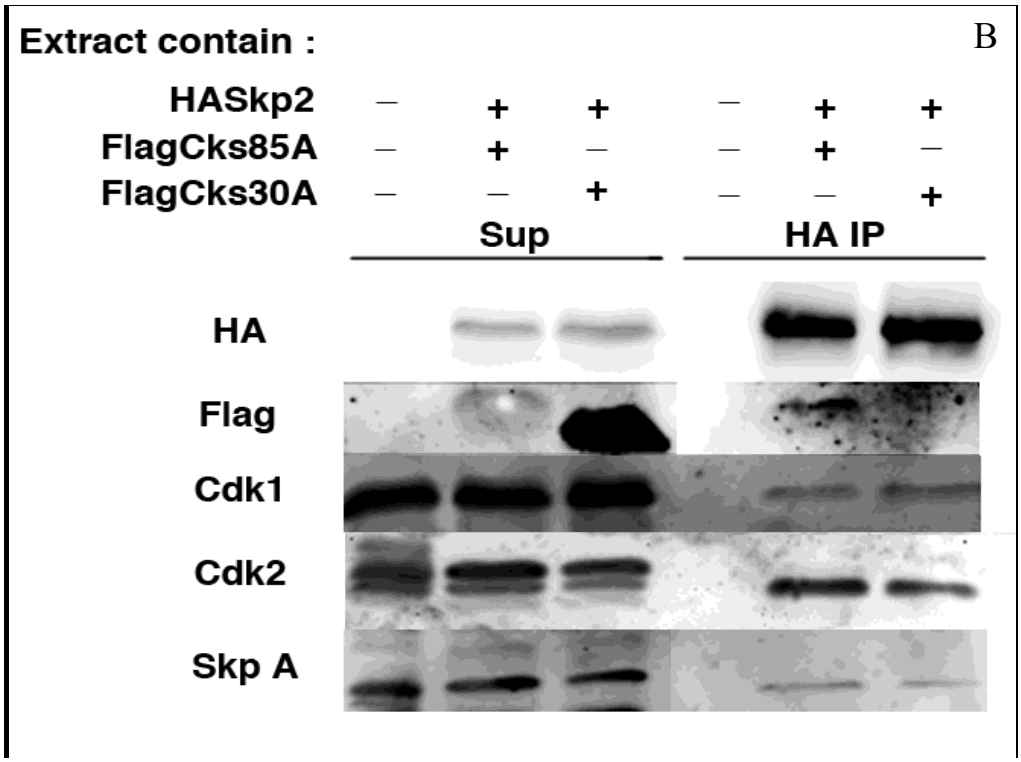
To determine if Cks85A and Skp2 are part of the same complex Co-IP experiment was designed. For this reason, HA-Skp2 and Flag-Cks85A proteins were overexpressed in the embryo by using *nanos-gal 4* driver. Embryos were collected for immunoprecipitation. Flag-Cks30A, HA-Skp2 transgenic flies was used as a control. Cks30A can act similar to Cks2 proteins in mammalian cells which have been shown to be important for activation of APC complex in mitosis (Swan et al., 2005).

Immunoprecipitation of HA-Skp2 revealed that Cks85A interacts with Skp2 (Figure 7A). SkpA (part of the SCF ubiquitin ligase complex) interacts with Skp2 but no interaction was observed between Cks30A and SkpA. These results were further confirmed by doing immunoprecipitation of Flag-Cks85A and Flag-Cks30A (Figure 7B). It revealed that Cks85A protein can interact with Cdks and Skp2. The importance of the interaction between Cks85A and Skp2 and interaction between Cks85A with Cdks in cell cycle will be clarified in the next experiment.

A


Extract Contain :
HASkp2 – + + – + +
FlagCks85A – – + – – +
FlagCks30A – + – – + –
Sup
Flag IP
HA
Flag
Cdk1
Cdk2
Skp A

B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HASkp2 – + + – + +
FlagCks85A – + – – + –
FlagCks30A – – + – – +
Sup
HA IP
HA
Flag
Cdk1
Cdk2
Skp A

Figure 5.7 Cks85A and Skp2 are in the same complex.

Immunoprecipitation of Flag-Cks85A and Flag-Cks30A (A) and HA-Skp2 (B). These proteins are overexpressed by using *nanos-gal4* driver which promotes protein expression in the female germline. The 1st and 4th lanes in both IPs are the control. 2nd and 5th lanes are using extract from transgenic flies which overexpress HA-Skp2 and Flag-Cks30A. 3rd and 6th lanes are using extract from transgenic flies which overexpress HA-Skp2 and Flag-Cks85A. The first three lanes are supernatent and lane 4-6 are IPs. Supernatant shows that the transgenic flies express all of the transgenes.

5.5 Characterization of Cdks in SCF complex

Cdk2 is important for initiation of S-phase and Cdk1 is critical for initiation of mitosis in Drosophila (Lee and Orr-Weaver, 2003). Cdk1/Cyclin A is also important for phosphorylation of Cdt1 in G2, thus maintainging a low level of Cdt1 in G2. It has been previously shown that Cks85A interacts with Cdk1, Cdk2, Cyclin A, B and B3 (Swan et al., 2005). As part of my studies I have shown that Cks85A interacts with Skp2 and also with SkpA which is the part of SCF^{Skp2} ubiquitin ligase complex (Figure 7).

Cdk1 and Cyclin A depletion can cause polyploidy in Drosophila similar to the phenotype caused by *Skp2* and *Cks85A* depletion. To determine whether *Cdk1* and *Cyclins* genetically interact with *Cks85A* and *Skp2*, the genetic interaction between these genes was examined.

To examine the genetic interaction between *Cks85A* and *cyclin A*, *Cyclin B* and *Cyclin B3,* the expression of the cyclins was depleted in $Cks85A^{ex15/ex15}$ flies and the influence of this depletion observed in the eye imaginal disc. $Cks85A^{ex15/ex15}$; $Cyclin\ A^{-/+}$, $Cks85A^{ex15/ex15}$; $Cyclin\ B^{2/2}$ and $Cks85A^{ex15/ex15}$; $Cyclin\ B3^{L6540/L6540}$ stocks were used to examine the genetic interaction between *Cks85A* with *Cyclin A*, *Cyclin B* and *Cyclin B3* respectively. Homozygous mutant of *Cyclin B* and *CyclinB3* were used because homozygous mutant flies for these two cyclins are viable. Homozygous mutant flies for *cyclin A* are lethal in embryonic stage thus only heterozygous flies could be used. The eye discs were observed from 3rd instar larvae at 4.5 days after egg hatching. The results show that *Cyclin A* and *Cyclin B3* (Figure 8C-D) can enhance the phenotype caused by *Cks85A* depletion, but no enhancement of the phenotype was observed following *Cyclin B* depletion (Figure 8B). The genetic interaction between *Cks85A* and *Cyclin A* suggests the possibility that *Cyclin A* might play a role in maintenance of the ploidy levels in a

similar fashion as *Cks85A* does. In the next section (5.6) it is shown that *Skp2* and *Cks85A* can influence the expression of *Cyclin A* (Figure 9A).

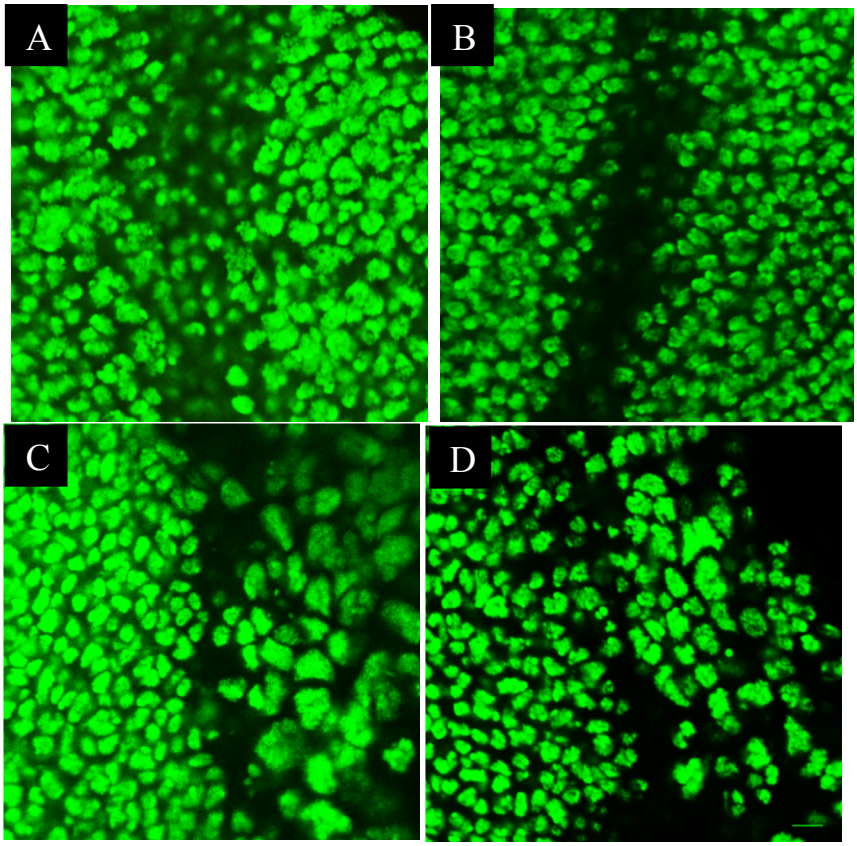

A
B
C
D

Figure 5.8 *Cks85A* genetically interact with *Cyclin A* and *Cyclin B3*.

Eye imaginal disc of $Cks85A^{ex15/ex15}$ (A), $Cks85A^{ex15/ex15};CyclinB^{2/2}$ (B), $Cks85A^{ex15/ex15},CyclinB3^{L6540/L6540}$ (C) $Cks85A^{ex15/ex15},CyclinA^{-/+}$ (D) dissected from 3rd instar larvae 4.5 days after post egg hatching, and labeled with Propidium Iodide indicating that *Cyclin A* and *Cyclin B3* enhance the polyploidy phenotype of null mutation in *Cks85A*.

5.6 Interaction between Cks85A and Cks30A in cell cycle

Cks30A and Cks85A are very similar in amino acid sequence. It already has been shown that the overexpression of *Cks30A* in $Cks85A^{ex15/ex15}$ cannot rescue the lethality of the flies. It is still possible that these two genes have a redundant role in cell cycle.

Double mutant flies ($Cks30A^{KO/KO}$; $Cks85A^{ex15/ex15}$) were used to examine if depletion of *Cks30A* can enhance the polyploidy of $Cks85A^{ex15/ex15}$. $Cks85A^{ex15/ex15}$ flies were used as a control. $Cks30A^{KO/\ KO}$ did not show any phenotype in the eye imaginal disc (Figure 9A- C). The results showed that the phenotype did not enhance in $Cks30A^{KO/KO}$; $Cks85A^{ex15/ex15}$ flies compared to the control but milder phenotype was observed compare to the $Cks85A^{ex15/ex15}$ flies (Figure 7A-C) indicating there is not any redundant role between these two genes.

The next experiment was designed to determine if overexpression of *Cks30A* in the $Cks85A^{ex15/ex15}$ flies can rescue the phenotype of *Cks85A* mutant flies. The transgenic flies (*Flag-Cks30A;da-gal4,*$Cks85A^{ex15/ex15}$) were used in this experiment. Eye imaginal discs from 3rd instar larvae 5 days after egg hatching were studied (Figure 9D, E). The results showed that overexpression of *Cks30A* cannot rescue the phenotype caused by *Cks85A* null and in fact this overexpression enhanced the phenotype cause by $Cks85A^{ex15/ex15}$. These results showed that there is no redundant role between *Cks85A* and *Cks30A* genes. The genes appear to act antagonistically in the cell cycle.

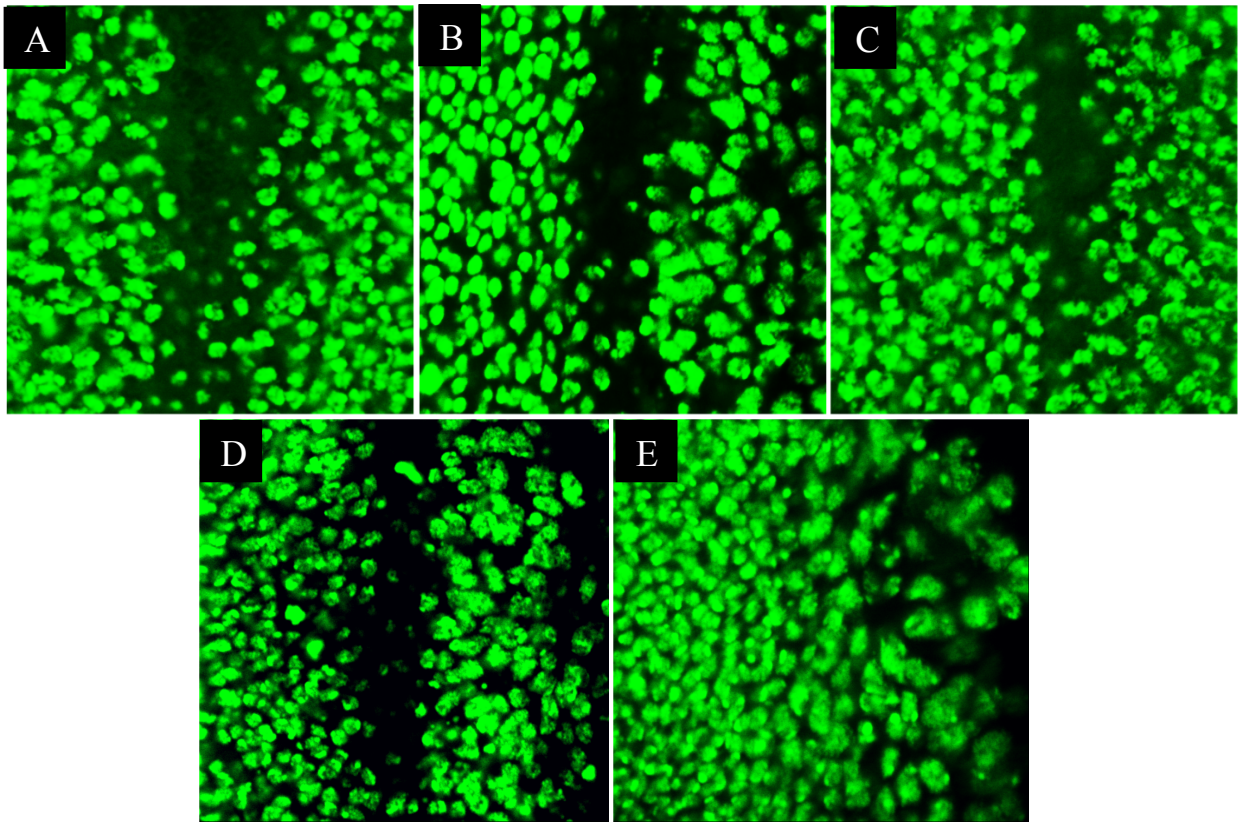

A
B
C
D
E

Figure 5.9 There is no redundancy between *Cks85A* and *Cks30A*.

Eye imaginal disc dissected from $Cks30A^{KO/KO}$ (A), $Cks85A^{ex15/ex15}$ (B) and $Cks85A^{ex15/ex15}$; $Cks30A^{ko/ko}$ (C) larvae after 4.5 days, stained with Propidium Idodide, the double mutant does not show any increase in polyploidy compare to the $Cks85A^{ex15/ex15}$ mutant. $Cks85A^{ex15/ex15}$ (D), *UAS-Flag-Cks30A*; $Cks85A^{ex15/ex15}$ (E) eye imaginal discs from 3rd instar larvae 5 days after egg hatching and stained with Propidium Iodide. Overexpersion of *Cks30A* could not rescue the polyploidy in $Cks85A^{ex15/ex15}$.

5.7 Identification of SCF^{Skp2} complex targets

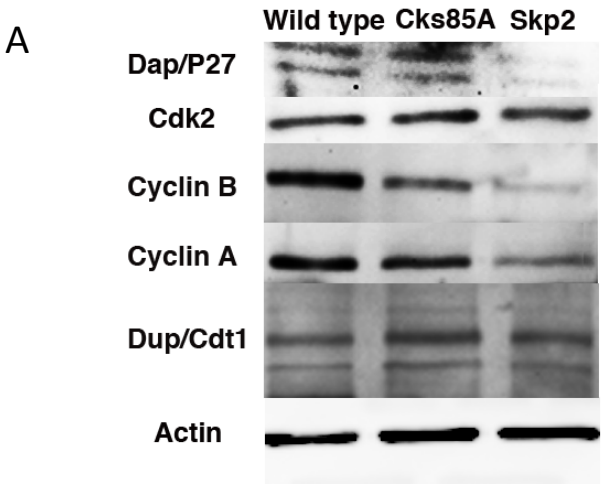
SCF^{Skp2} targets several proteins for degradation such as c-Myc, Cdt1 and $p27^{kip1}$. $p27^{kip1}$ is the best characterized and possibly most important target in mammalian cells. Based on observed phenotypes $p27^{kip1}$/Dap is a suitable candidate for Skp2 because it has been shown to play a role in initiation of DNA replication. Dup is also a possible target of SCF^{Skp2} and must be degraded in order for the cell to enter into the S-phase. It is possible that *Skp2* null cells become polyploid because Dup is not degraded after S-phase in the cell thus allowing the pre-replication complex to reform.

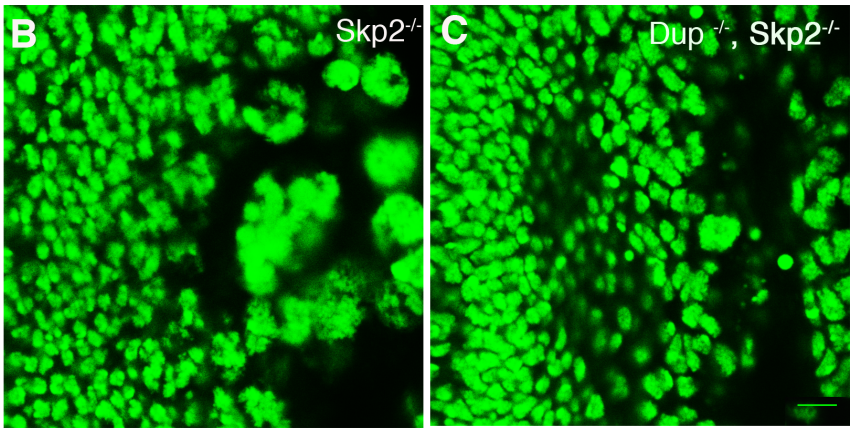
In the absence of Skp2 protein, the levels of its targets should increase. 3rd instar larvae were used to measure the level of candidate targets of Skp2. This experiment was done in 2 different types of tissue separately, mitotic cells from brains and imaginal discs, and in endoreplicating cells from salivary glands. The main reason for doing these separate tests is that the mechanism of cell cycle is completely different in endoreplicating tissues compared to the archetypal tissues, and there due to potential might be a difference targets for Skp2 in these two different cell types. In imaginal discs and brain, the level of Dap and Dup was tested but increase in the level of either was not observed in the $Skp2^{ex9/ex9}$ and $Cks85A^{ex15/ex15}$ mutant cells.

If the polyploidy in $Skp2^{ex9/ex9}$ and $Cks85A^{ex15/ex15}$ mutant cells was due to overaccumulation of a specific protein, it may be possible to suppress the polyploidy by genetically reducing the expression of the target protein. To reduce the expression of *Dup* in the $Skp2^{ex9/ex9}$ mutant background the viable hypomorphic allele of *Dup*, Dup^{PA77} was used. The results shows that in the eye imaginal discs taken from $Dup^{PA77/PA77}$, $Skp2^{ex9/ex9}$ double mutant 3rd instar larvae the phenotype is milder compared to the $Skp2^{ex9/ex9}$ phenotype (Figure 10B-C) . This result can indicate that the reduction of Dup can suppress the polyploidy caused by knocking out of the

Skp2 protein and therefore it may be a target of SCF^{Skp2}. This genetic interaction experiment result is not consistent with results obtained from examining the Dup level by western blotting. one possibility for this observation is that Dup is not a direct target of the SCF^{Skp2} but it is a cofactor. The same experiment was tried with Dap but there was a problem with the stock as they were lethal in the embryonic stage and it was therefore not possible to obtain double mutant larvae for western blotting.

To test if these proteins are the target of SCF^{Skp2} ubiquitin ligase complex in endoreplicating tissue, levels of the proteins were measured in salivary glands (Figure 10D). 3rd instar feeding larvae were used because in later stages salivary glands do not undergo endoreplication. Dup protein has 3 different isoforms in Drosophila and using the antibody revealed two bands, at 80 and 105-106 kDa (Thomer et al., 2004). An increase in the level of the 80 kDa isoform in *Skp2* mutant was observed (Figure 10D) but no change was observed in 105-106 kDa isoform. Dup level was not changed in $Cks85A^{ex15/ex15}$. This experiment needs to be repeated using other antibodies to make sure the band that we observed in 80 kDa is a right band and it is not a non-specific band. By using another antibody which only detected 80 kDa isoform, a very obvious increase in the level of this protein was observed in $Skp2^{ex9/ex9}$ extract but the level did not change (more likely decreased) in $Cks85A^{ex15/ex15}$ which is the same result as we observed by using the first antibody (data not shown). Dup could therefore be a target of SCF^{Skp2} complex in endoreplicating tissues. Further experiments are needed to determine if Dup is a target of SCF^{Skp2} complex in mitotic tissues.


A
Wild type
Cks85A
Skp2
Dap/P27
Cdk2
Cyclin B
Cyclin A
Dup/Cdt1
Actin


B
Skp2-/-
C
Dup -/-, Skp2-/-

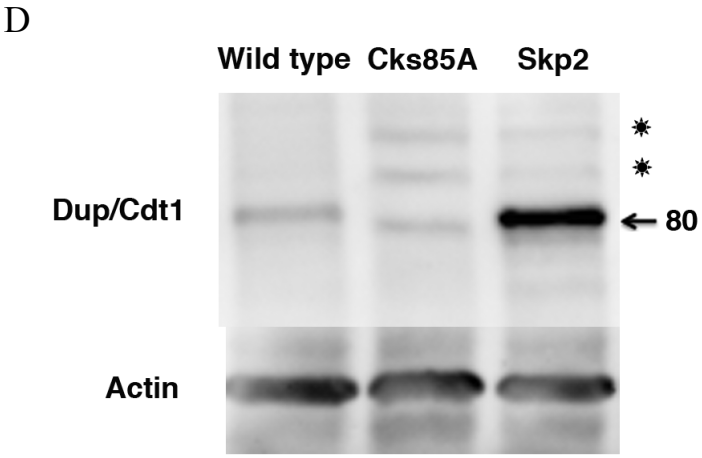

D
Wild type
Cks85A
Skp2
Dup/Cdt1
Actin
80

Figure 5.10 Dup is a possible target of SCF^{Skp2} ubiquitin ligase complex.

Western blot using 3rd instar larval brain (A) first lane is the wild type, second lane is *Cks85A*$^{ex15/ex15}$ mutant and 3rd lane is *Skp2*$^{ex9/ex9}$ mutants. Eye imaginal disc from 3rd instar larvae 5 days after egg hatching, *Skp2*$^{ex9/ex9}$ (B) and *Dup*$^{PA77/PA77}$,*Skp2*$^{ex9/ex9}$ (C) stained with Propidium Iodide. Western blot by using salivary gland from 3rd instar larvae 4 days after egg hatching (D).The first lane is wild type, second lane is *Cks85A*$^{ex15/ex15}$ mutant and 3rd lane is *Skp2*$^{ex9/ex9}$ mutants. Salivary glands was used from 3rd instar larvae 4 days after egg hatching. Scale bar indicates 5 μm.

Chapter 6. **Discussion**

6.1 Cks85A and Skp2 are important for growth in Drosophila

Drosophila growth depends on the growth of endoreplicating tissues. Endoreplication occurs in some tissues like salivary gland where S-phase occurs normally and the cells becomes bigger without mitosis. I have shown that *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$ mutant larvae are smaller than wild type and have smaller salivary glands. Salivary gland and other endoreplicating tissues undergo endoreplication after cycle 16 of embryogenesis and cell number stays constant in these tissues. The cell number was not affected in *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$ null mutants but the cell volume was apparently different. The DNA content in the *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$ mutants was also diminished compared to the wild type.

Endoreplication occurs by an oscillation in expression of Cyclin E and Dup/Cdt1. Cyclin E is necessary for entering S-phase and onset of DNA replication (Knoblich and Lehner, 1993) while Cdt1 is critical for control of initiation of DNA replication in G1 (Whittaker et al., 2000). Cyclin E degradation is important at the end of S-phase to get a normal endocycle and Cyclin E overexpression blocks progression through endoreplication (Weiss et al., 1998). Cyclin E levels in the endocycle are regulated by different mechanisms. SCF^{Ago} is critical for Cyclin E degradation (Moberg et al., 2001). E2F is a transcription factor which is important to control the expression levels of Cyclin E and E2F expression levels increase in G1 to activate transcription of *Cyclin E*. E2F is degraded by SCF^{slimb} ubiquitin ligase (Heriche et al,. 2003).

The Salivary gland phenotype in *Skp2* and *Cks85A* mutants is similar to the phenotype caused by over accumulation of Cyclin E. It is possible that Cyclin E is being targeted by SCF^{skp2} ubiquitin ligase. We tried to investigate the levels of Cyclin E in *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$ by

western blotting. This experiment was not completed because the available Cyclin E antibody was not stringent enough and detects several of non-specific bands.

Dup/Cdt1 is important for initiation of DNA replication in both endoreplicating and archetypal tissues. Depletion of Dup in salivary glands causes a dramatic decrease in cell size and DNA content (Park and Asano, 2008; Whittaker et al., 2000). The same phenotype was observed in *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$ null mutants. Dup/Cdt1 levels were quantified in *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$ mutant salivary glands. The preliminary results showed an increase in the levels of Dup/Cdt1 in *Skp2* mutants but not in *Cks85A*. Dup/Cdt1 has 3 isoforms in Drosophila and only one of the isoforms (80 kDa) increased in Skp2 mutant salivary glands. To ensure that these 3 bands are not non-specific bands, other types of Dup antibody were used. The second antibody showed a similar result but could only detect one of the isoforms (80 kDa) (Ghorbani, unpublished). These results showed that Dup/Cdt1 can be the target of SCF^{Skp2} ubiquitin ligase for degradation. This is a preliminary result that needs to be confirmed by other experiments. This result will be further confirmed by doing western blot on extracts obtained from knocking down of the *Dup* by *Dup-RNAi* in the *Skp2*$^{ex9/ex9}$ mutants to make sure the bands that are detected in the *Skp2*$^{ex9/ex9}$ mutants are the right bands. This extract depleted for Dup can be western blotted along side Cks85A and Skp2 mutant extracts see which band is not Cdt1/Dup protein. Another way to confirm this result is to carry out immunostaining against Dup in salivary gland to see if Dup levels increase in the salivary gland cells. The reason why the *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$ mutant larvae are smaller is still unknown but it is possible that the accumulation of Dup in salivary gland cells causes this phenotype by interrupting DNA replication. Other experiments also show that DNA replication is slower in *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$ mutant larvae (Biju Vasavan, unpublished) but this is still a preliminary result.

The mitotic tissues, such as wing imaginal disc and brain were also smaller in *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$ compared to wild type 3rd instar larvae. There is no elevation in the levels of Dup/Cdt1 in the mitotic tissues in *Cks85A* and *Skp2* mutants arguing that Skp2 and Cks85A proteins are not important for degradation of the Cdt1/Dup in mitotic tissues. It seems the growth rate in *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$ mutants is slower than wild type but the main reason for this is still unknown. In mitotic tissues from *Cks85A*$^{ex15/ex15}$ and *Skp2*$^{ex9/ex9}$, some of the cells undergo apoptosis (Emona Kraja, unpublished) and this can be another reason explaining why we see smaller mitotic tissues in *Cks85A* and *Skp2* mutants.

6.2 Cks85A and Skp2 are critical to prevent re-replication of DNA

The pre-replicative complex forms in G1 where Dup/Cdt1, Cdc6 and Mcm helicase proteins bind to the origin recognition complex to complete the pre-replicative complex. Completion of the pre-replicative complex is necessary to start DNA replication. It is very important this complex disassembles after onset of DNA replication to prevent re-replication of the DNA and polyploidy. Dup/Cdt1 and Cdc6 are degraded after initiation of DNA replication to prevent re-formation of the pre-replicative complex.

The cells in wing imaginal disc and brain of *Cks85A*$^{ex15/ex15}$ *and Skp2*$^{ex9/ex9}$ mutants are larger compared to wild type. We have shown that these cells are polyploid both by brain squashes and by flow cytometry (Emona Kraja, unpublished). These findings suggest that *Cks85A* and *Skp2* null mutations cause polyploidy in mitotic cells and it is possible that these proteins play a role in preventing the formation of the pre-replicative complex in other stages (G2 or mitosis) of the cell cycle. In mammalian cells SCF^{Skp2} ubiquitin ligase is important for Cdt1 degradation and CksHs1 is also necessary for this action. Knockout of *Skp2* in mammalian cells causes polyploidy. It is

possible that Drosophila Cks85A and Skp2 are also necessary for Dup/Cdt1 degradation and in the absence of these proteins accumulation of Dup/Cdt1 cause re-replication of the DNA.

Cks85A and Skp2 might play a role in degradation of Dup/Cdt1 after initiation of DNA replication. The Western blot with mitotic tissues has shown that the level of Dup does not change in *Skp2* and *Cks85A* mutants. In other experiments, the phenotype in $Dup^{PA77/\ PA77}$,$Skp2^{ex9/ex9}$ eye imaginal disc and $Skp2^{ex9/ex9}$ were observed at the same time. The phenotype caused by *Skp2* null mutants was almost rescued by the depletion of Dup. The western blot already showed that Skp2 is not important to control the degradation of Cdt1/Dup in mitotic cells but it is possible that Cdt1/Dup is a co-factor that helps Skp2 in degradation of the unknown target. It is also possible that Cul4 ubiquitin ligase plays a role in degradation of Dup/Cdt1 in the absence of Skp2 as has been shown (Nishitani et al., 2006). This would also explain the failure to observe an increase in the levels of Cdt1 in western blot.

Another reason diploid cells can become polyploid can be due to the absence of M-phase. In the absence of M-phase, replication of DNA occurs without cell division and the cell ploidy increases. To test this possibility, an experiment was done to compare the frequency of cells in mitosis in $Cks85A^{ex15/ex15}$ *and* $Skp2^{ex9/ex9}$ mutants to wild type. Results showed that the frequency of mitotic cells in wild type and both mutants are very similar. It seems therefore that knocking out of *Cks85A* and *Skp2* does not affect entry into mitosis. If a cell fails to enter mitosis, it is expected that it will accumulate extra centrosomes. However no extra centrosomes were observed, further arguing that cells have normal mitosis (Biju Vasavan, unpublished).

Cdk1 binds to Cyclin A in G2 to phosphorylate Cdt1 in mammalian cells. Phosphorylated Cdt1 is ubiquitinated by SCF^{Skp2} and then degraded by the proteasome (Li et al, 2003). It has been shown that Cks85A interacts with Cdk1 and Cyclin A (Swan et al., 2005) so it is possible that Cks85A also plays a role in G2 by binding to Cdk1 and Cyclin A to phosphorylate Dup/Cdt1. The genetic interaction between Cyclin A and Cks85A supports this model. Therefore formation of a pre-replication complex might happen in mitosis in the mutants because Cks85A and Skp2 are necessary for degradation of Cdt1/Dup. Another experiment was designed to examine this hypothesis. In this experiment, BrdU was used as an S-phase. Cells were also stained with a G2 marker (Cyclin B) or a mitosis marker (PH3) (Biju Vasavan, unpublished). If both the BrdU and the G2 or mitosis markers are observed in the same cell, it would indicate that DNA replication occurs in G2 or mitosis respectively. Preliminary results from this experiment showed double labelling for S-phase and G2 phase markers, arguing that DNA replication happens in G2. This experiment needs to be repeated.

The completion of chromosome segregation in anaphase is very important to ensure that a proper number of chromosomes go into every daughter cell. Any problem that causes abnormal chromosome segregation can lead to polyploidy. To examine the possibility of the 2nd hypothesis, we tried to look at chromosome segregation by immunostaining (Biju Vasavan, unpublished) and we saw that chromosomes sometimes mis-segregate leading to anneuploidy

Therefore it seems that polyploidy that occurs upon loss of *Cks85A* and *Skp2* happens by inhibition of Dup/Cdt1 degradation leading to re-replication of the DNA. Double labelling for S phase and G2 also supports the idea of DNA re-replication in G2 possibly caused by over accumulation of Cdt1/Dup in this stage. Chromosome mis-segregation can be another cause of

polyploidy but it is still unknown if this abnormality causes polyploidy in cells or abnormal number of chromosomes causes chromosome mis-segregation.

6.3 Role of Cks85A in SCF^{Skp2} complex

Cks85A interacts with both Skp2 and Skp A and thus can be part of the SCF ubiquitin ligase complex. In mammalian cells Cks1Hs binds to Skp2 to help in recognition of $p27^{kip1}$ (Ganoth et al., 2001; Spruck et al., 2001). Cks85A seems to play a similar role with the SCF^{Skp2} complex in Drosophila but it is not involved in degradation of Dap ($p27^{kip1}$), but rather for degradation of other unidentified proteins. The phenotype caused by knocking out of *Cks85A* is milder than that from knockout of *Skp2* and it is possible that Cks85A assists Skp2 to recognize its targets. We have shown that the overexpression of *Skp2* in the $Cks85A^{ex9/ex9}$ mutant flies causes partial rescue after pupal stage in $Cks85A^{ex9/ex9}$ mutants (Andrew Swan, unpublished). Furthermore, *Cks85A* is not as critical for preventing polyploidy at lower temperatures. This experiment also supports the idea that Cks85A is not necessary for Skp2 activity, but can help increase activity of this protein. Another possibility is to say that these two proteins are important for DNA replication in the cell cycle but they are not part of the same pathway. They might play a role in two parallel pathways which are important to control the replication of DNA.

6.4 Cdk1 might play a role in regulation of DNA replication

Reduction of Cyclin B3 and Cyclin A protein levels in the *Cks85A* mutant flies enhanced the polyploidy phenotype in eye imaginal disc. It has been shown that the Cks85A interacts with these two proteins physically (Swan et al., 2005). Cyclin A plays a role in entry to S-phase by interacting with Cdk1 and also plays a role in G2 by binding to Cdk1. *Cyclin A* mutation causes polyploidy in Drosophila (Sauer et al., 95, Mihyalov et al, 2002). It is possible that Cks85A also play a role in G2 by interacting to Cdk1 and Cyclin A to prevent polyploidy.

Preliminary results from our lab show that the interaction between Cdk1 and Cks85A is important to prevent polyploidy in the cell cycle (Carla Perissinotti, unpublished). In this experiment, $Cdk1^{1N}$ mutants were used. This mutation interrupts the interaction between Cdk1 and Cks85A. $Cdk1^{1N}$ was overexpressed in the flies that are homozygous mutant for endogenous *Cdk1* and they were lethal in 3rd instar larval stage. Polyploid cells were observed in brain and discs. In another experiment, we also examined the importance of interaction between Cks85A and Cdk2 in genome stability. A set of *Cks85A* mutants were made that interrupt the interaction between Cks85A and Cdk2 protein. This transgene was injected into the flies and they are ready for the experiment.

Physical interaction between Cks85A and Cdk1 seems to be critical to prevent polyploidy in cells. Cks85A, Cdk1 and Cyclin A might play a role in G2 to phosphorylate Cdt1/Dup which is important to prevent the formation of pre-replicative complex.

6.5 Cks85A and Cks30A have distinct role in cell cycle

Cks30A is a homolog of Cks85A. Cks30A plays a role in meiosis by interacting with Cdk1. In budding and fission yeast there only one Cks. There might be some redundant role between the two Cks proteins in Drosophila because they are very close in amino acid sequence. It has already been shown that *Cks85A* is not redundant with *Cks30A* in meiosis (Swan et al., 2005). I found that overexpression of *Cks30A* in $Cks85A^{ex15/ex15}$ mutants did not rescue the eye phenotype. Interestingly, when *Cks30A* was overexpressed in $Cks85A^{ex15/ex15}$ null background the phenotype was stronger than the $Cks85A^{ex15/ex15}$ on its own. The phenotype in $Cks30A^{ko/ko}$,$Cks85A^{ex15/ex15}$ mutant was milder than the phenotype in $Cks85A^{ex15/ex15}$ mutant. This might be because these two proteins compete to bind to Cdk1. In overexpression of *Cks30A* in *Cks85A* mutant flies, Cks30A binds to Cdk1 in the cells, and Cks85A cannot bind, so we see the phenotype is stronger. In the

double mutant of C*ks30A* and *Cks85A,* the phenotype is milder because there are lower levels of Cks30A in the cell and thus Cks85A can bind to more Cdk1 resulting in a milder phenotype. This result shows that there is no redundant role between *Cks85A* and *Cks30A*.

6.6 Conclusion

Cancer cells undergo uncontrolled growth, loss of genome stability and abnormal number of chromosome are observed similar to *Skp2* and *Cks85A* mutant cells. Study of the importance of these genes in cell cycle will help us to understand more about how cells become cancerous.

We have shown that knocking out of *Skp2* and *Cks85A* causes polyploidy in mitotic cells and this might be because of the accumulation of the pre-RC factor Dup/Cdt1. These proteins might play this role by interacting with the SCF ubiquitin ligase complex or in two different parallel pathways. Cdks and Cyclin A are also important to prevent polyploidy. Physical interaction between Cks85A with Cdk1, Cdk2 and Cyclin A also drive us to think about the pathway which is important to prevent ploidy. In one of the possible pathways which occurs in G2, the interaction between Cks85A with Cdk1 and Cyclin A could be critical to phosphorylate Cdt1/Dup which is important for recognition and ubiquitination by SCF^{Skp2} ubiquitin ligase. Cks85A and Skp2 are also important for growth in Drosophila by controlling the DNA replication in endoreplicating cells.

To date, the main targets of Skp2 and Cks85A are still unknown and it is still not quite clear what causes of polyploidy in *Skp2* and *Cks85A* mutants. The experiments in this thesis give us some preliminary answers to these questions.

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