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A novel role for Skp2 in mitotic entry

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By

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A Novel Role for Skp2 in Mitotic Entry

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DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

"This thesis incorporates the outcome of a joint research undertaken in collaboration with Biju Vasavan under the supervision of Professor Andrew Swan. The collaboration is covered in Chapter 2 and Chapter 4 of this thesis. In all cases, the key ideas, primary contributions, experimental designs, data analysis and interpretations were performed by the author and the co-author".

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ABSTRACT

S phase kinase associated protein 2 (Skp2) is an E3 ubiquitin ligase, an established oncogene and an important G1-S regulator. The critical and well-studied target of Skp2 is the Cyclin dependent kinase inhibitor p27/Dap. Overexpression of Skp2 has been observed in a wide variety of cancer types and in most of these cancer types, downregulation of p27/Dap has also been observed. However, loss of *Skp2* in mammals and in *Drosophila* also results in polyploidy in mitotic tissues. Polyploidy resulting from overexpression of Skp2 has been widely studied but there is still no clear understanding on how loss of *Skp2* results in polyploidy. We found that loss of *Skp2* results in premature degradation of Cyclin A and other mitotic cyclins - possibly by the premature activation of APC-CDH1/Fzr resulting in mitotic failure. The cells then enter a G-like state and start endoreplicating, causing polyploidy. Our results show that the N-terminus of Skp2 interacts directly with Cyclin A and is required for rescuing polyploidy in *Skp2* null mitotic cells. We also showed that polyploidy resulting from overexpression of p27/Dap is different from polyploidy resulting from loss of *Skp2*. Our results show that the polyploid *Skp2* null cells which enter mitosis, delay in prometaphase/metaphase of the cell cycle with the activation of the Spindle Assembly Checkpoint (SAC). These cells frequently undergo double stranded DNA damage and activates apoptosis and autophagy mediated cell death. Our results argue that it is not polyploidy but the entry of polyploid cells into mitosis that activates the checkpoints that cause apoptosis for genome stability.

DEDICATION

To my mother
for teaching me the value of education

To my father
for fulfilling all my wishes

To my teachers
for always motivating me

To my husband
for always encouraging me and sharing all my responsibilities

To my kids
for helping me to stay focused

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LIST OF ABBREVIATIONS

ABC transporter	ATP-Binding Cassette transporter
APC	Anaphase Promoting Complex
ASL	Asterless
Atg	Autophagy-related genes
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
BRCA2	Breast cancer 2
BrdU	5-bromo-2'- deoxyuridine
BSA	Bovine serum albumin
BUB1	Budding uninhibited by benzimidazole-1
BUB3	Budding uninhibited by benzimidazole-3
BUBR1	Bub-1 related kinase
CAK	Cdk-activating kinase
CDC20	Cell division cycle 20
CDC25	Cell division cycle
CDC6	Cell division cycle 6
CDH1	CDC20 homologue 1
CDK	Cyclin-dependent kinase
CDK9	Cell division kinase 9
CDT1	Cdc10-dependent transcript 1
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CKI	Cyclin dependent kinase inhibitor
Cks1	Cyclin-dependent kinases regulatory subunit 1
CO2	Carbon dioxide
CoIP	Coimmunoprecipitation
CRL	Cullin ring E3 ligases
CRS	Cytoplasmic retention signal

CUL4	Cullin 4
D box	Destruction box
da-GAL4	daughterless GAL4
Dap	Dacapo
DChk1	*Drosophila* Checkpoint kinase 1
DChk2	*Drosophila* Checkpoint kinase 2
DDB1	DNA damage-binding protein 1
DDR	DNA damage response
DEST	Destination
DMEM	Dulbecco's Modified Eagle Medium
DN	Dominant negative
Dup	Double parked
E2F1	E2 factor 1
EDTA	Ethylenediaminetetraacetic acid
EDU	5-ethynyl-2'-deoxyuridine
Emi1	Early mitotic inhibitor 1
en-GAL4	engrailed GAL4
FBL	F- box and leucine-rich-repeat
FBS	Fetal Bovine Serum
FBW	F-box and WD40-domain protein
FISH	Fluorescent *in situ* hybridization
Fucci	Fluorescent ubiquitination-based cell cycle indicator
Fzr	Fizzy-related
Fzy	Fizzy
G1	Gap/Growth1
G2	Gap2
GFP	Green fluorescent protein
GST	Glutathione S-transferase
H2AV	Histone 2A variant
HA	Hemagglutinin
HEK293	Human Embryonic Kidney 293

Hid	Head involution defective
His	Histidine
HP	Hydrophobic patch
IAP	Inhibitors of apoptosis
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Isoleucine-Arginine
JNK	c-Jun N-terminal kinases
LC3	Microtubule-associated protein light chain 3
LRR	Leucine rich repeat
M	Mitosis
MAD1	Mitotic arrest deficient 1
MAD2	Mitotic arrest deficient 2
MAD3	Mitotic arrest deficient 3
MBP	Maltose binding protein
MCC	Mitotic checkpoint protein complex
MCM	Minichromosome maintenance protein complex
MKLP1	Mitotic kinesin-like protein 1
MPF	Maturation promoting factor
Mps1	Monopolar Spindle 1
NaCl	Sodium chloride
NEBD	Nuclear envelope breakdown
NES	Nuclear export signal
OD	Optical density
ORC	Origin recognition complex
Orc1	Origin recognition complex subunit 1
p130	protein 130
p21	protein 21
p27	protein 27
P27	protein 27
p57	protein 57
PAGE	Polyacrylamide gel electrophoresis

PBST	Phosphate Buffered Saline Tween-20
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PEI	Polyethylenime
Pim	Pimples
PLA	Proximity ligation assay
PLK1	Polo-like kinase 1
PMSF	Phenylmethane sulfonyl fluoride
PP1	Protein phosphatase-1
PP2A	Protein phosphatase-2A
PRC1	Protein Regulator of cytokinesis 1
ptc-GAL4	patched GAL4
Puc	Puckered
Rb	Retinoblastoma
Rca1	Regulator of cyclin A
RFP	Red fluorescent protein
rn-GAL4	rotund GAL4
ROS	Reactive oxygen species
RT	Room temperature
Rux	Roughnex
S	Synthesis
SAC	Spindle assembly checkpoint
SCF	Skp1 Cullin Fbox
SDS	Sodium dodecyl sulfate
Skp1	S phase kinase-associated protein 1
Skp2	S phase kinase-associated protein 2
TPR	Tetraricopeptide repeat
UBCH10	Ubiquitin-conjugating enzyme H10
Ube2S	Ubiquitin-Conjugating Enzyme E2S
WD	Tryptophan aspartic acid
WHS	Wing hair spacing

ZNC	Zone of non-proliferating cells
βTrcp	Beta transducin repeat containing protein

CHAPTER 1

INTRODUCTION

1.1. *Drosophila melanogaster* as a model organism

In early 1823, Swedish entomologist Carl Frederick Fallen first coined the name *Drosophila*, which means “lover of dew.” Two of the reasons why *Drosophila* caught scientists’ attention were because *Drosophila* were raised with little care and had a short generation time, usually 10 to 12 days at 25°C. In 1907, *Drosophila* were introduced to Thomas Hunt Morgan by Frank Lutz, and between 1910 and 1920 a significant amount of work was done on *Drosophila* in Morgan’s lab. Morgan discovered the *white* gene, the first sex-linked gene, which gave him the idea that genes are part of chromosomes. It was found out later that the white gene encoded a trans-membrane protein, ABC transporter. Members of ABC transporter protein family were found to be involved in drug resistance in cancer and other diseases. There were several examples of genes found in *Drosophila* long before the genes were known to have any connection to cancer or other diseases. Studies have shown that more than 50% of genes that cause human disease have orthologs in *Drosophila*.

The S phase kinase-associated protein 2 (Skp2) is a potential oncogene, meaning Skp2 has the potential to cause cancer in cells when overexpressed. Skp2 has been studied in mammalian systems since 1995; and interest in it has since grown. It has been observed to be overexpressed in most cancer-tissue types, causing the premature degradation of the critical Gap/Growth1-Synthesis (G1-S) phase regulator and tumour suppressor p27/Dap (Hao and Huang, 2015). A significant amount of work has been done on Skp2 using the mammalian cell culture system, despite the fact that the cell culture system has not provided the microenvironment that truly reveals the potential of a gene. I chose *Drosophila* as my model organism

to study Skp2 and to better understand its cell cycle role using a simpler *in vivo* system.

This introduction chapter focuses on the cell cycle role of some key regulatory proteins during Gap2-Mitosis (G2-M) transition, M (Mitosis) phase, G1-S transition and Gap/Growth 1 (G1) phase. Following a general discussion role of the cell cycle proteins, Skp2, Cyclin A and the activation of CDK will be reintroduced, later in the chapter and discussed in more detail. For ease of understanding, the literature review is separated into mammalian model systems and *Drosophila* model system. The genes and proteins with different names in Drosophila will be written as mammalian name/*Drosophila* name.

1.2 G2 regulation and mitotic entry in mammalian model systems

Cell cycle regulation in G2 phase, and its timely transition into M phase, is regulated by multiple protein complexes through overlapping or distinct pathways. Entry into mitosis depends on the activity of the maturation promoting factor, or MPF, which consists of a heterodimer composed of Cyclin-dependent kinase 1 (CDK1), and mitotic Cyclin B (Draetta and Beach, 1988; Ducommun et al., 1991; Krek and Nigg, 1991). The other important Cyclin during G2 regulation and mitotic entry is Cyclin A. Mammals have two Cyclin A genes, Cyclins A1 and A2, and two Cyclin B genes, Cyclins B1 and B2. Cyclins A2 (which will be referred to as Cyclin A) and B1 (which will be referred to as Cyclin B) are primarily involved in mitosis (Porter and Donoghue, 2003). There is another kind of cyclin known as Cyclin B3. Even though Cyclin B3 shares similarities with both Cyclin A and Cyclin B it is more closely related to Cyclin A than Cyclin B (Jackman et al., 1995; Jackman et al.,

2003). The synthesis of Cyclin B starts a little later than Cyclin A, in S phase and it reaches its peak activity as the cells enter mitosis. The subcellular localizations of Cyclin A and Cyclin B vary in interphase and mitotic cells. Despite Cyclin B's constant shuttling between the nucleus and the cytoplasm during interphase, the majority of Cyclin B is cytoplasmic during interphase, because the rate of nuclear export is more than the rate of nuclear import (Pines and Hunter, 1991; Toyoshima et al., 1998). During prophase, Cyclin B is rapidly imported into the nucleus, although there have been contradictory results in regards to the mechanism of its rapid import. Some studies suggest that during G2-M phase transition, Polo-like kinase 1 (PLK1) phosphorylates cytoplasmic Cyclin B at the N-terminus. The N-terminus of Cyclin B has a cytoplasmic retention signal (CRS), which has a nuclear export signal (NES) inside it. PLK1-mediated phosphorylation of the NES impairs the nuclear export, resulting in rapid Cyclin B import into the nucleus (Jackman et al., 2003; Pines and Hunter, 1994). However, some other studies suggested that PLK1-mediated phosphorylation of Cyclin B is not required for nuclear translocation. Instead, Cyclin B is autophosphorylated at its N-terminus in the cytoplasm, and is first detected as active on the centrosome. During prophase, the active cytoplasmic Cyclin B rapidly enters into the nucleus through changes in the nuclear import machinery, not through the accessibility of the nuclear import signal (Gavet and Pines, 2010; Jackman et al., 2003).

Cyclin A is mostly nuclear in interphase as well as in mitosis (Moore, 2013; Nigg et al., 1991). The most important function of Cyclin A during G2-M is to promote the activation of nuclear Cyclin B (Fung et al., 2007). Cyclin A with its

partner Cyclin dependent kinase (CDK1) phosphorylates Wee1 and inactivates it. This results in the reduced inhibitory phosphorylation of Cyclin B when it is shuttled to the nucleus, causing the activation of nuclear Cyclin B. Active Cyclin B then shuttles back to the cytoplasm to activate CDC25/String. This starts a positive feedback loop, causing more cytoplasmic Cyclin B to be activated (Brown et al., 2015; Fung et al., 2007; Margalit et al., 2005; Pagano et al., 1992). The low level of activation of Cyclin B-CDK1 begins 20 to 25 minutes before nuclear envelope breakdown (NEBD) in the cytoplasm. Cyclin B activation gradually increases, and full activation of Cyclin B is needed for NEBD (Boutros et al., 2007). Active Cyclin B-CDK1 phosphorylates nuclear lamins and other important structural proteins, and causes dissociation of the nuclear envelope (Gavet and Pines, 2010; Margalit et al., 2005). Even though Cyclin B is the critical cyclin needed for mitotic entry in human cells, the activity of Cyclin A is also important because it is needed for the activation of Cyclin B (Fung et al., 2007; Porter and Donoghue, 2003).

The other mitotic cyclin, Cyclin B3, is not very well characterized in humans. It binds with Cyclin dependent kinase 2 (CDK2) instead of CDK1. It is required in meiosis but its mitotic function is not clear. Although overexpression of non-degradable Cyclin B3 shows premature entry into mitosis, these cells delay in anaphase, and an overall delay in mitotic exit. This indicates that Cyclin B3 might have an unknown role in mitotic entry and its degradation is needed for mitotic exit (Nguyen et al., 2002; Tschöp et al., 2006).

1.2.1 Regulation by Emi1

Apart from cyclins and Cyclin dependent kinases (CDKs), another key aspect of promoting timely mitotic entry is the regulation by the early mitotic inhibitor 1 (Emi1). The primary function of Emi1 is the inhibition of the E3 ubiquitin ligase Anaphase Promoting Complex (APC) in S and G2 phases. It competes with APC substrates to bind with APC co-activators Cell division cycle 20 (CDC20) and CDC20 homologue 1 (CDH1) (Miller et al., 2006). Details about APC and its co-activators will be discussed later. Emi1 can also block the elongation of the ubiquitin chain on substrates and inhibit the binding of the E2 ubiquitin ligase and APC (Wang and Kirschner, 2013). The knockdown of *Emi1* causes an upregulation of APC-CDH1 and APC-CDC20 activity, resulting in the premature degradation of APC substrates such as Cyclin A, Cyclin B, Geminin (Hsu et al., 2002; Machida and Dutta, 2007). In early prophase, Emi1 is phosphorylated by Cyclin B-CDK1 and Polo like kinase 1 (PLK1), and is targeted for destruction by Skp1 Cullin Fbox-beta transducin repeat containing protein (SCF-βTRCP), another important E3 ubiquitin ligase. (Hansen et al., 2004; Margottin-Goguet et al., 2003). This allows APC activation during M phase.

1.2.2 Regulation by CDC25

Cell division cycle 25 (CDC25) is a dual specificity serine/threonine and tyrosine phosphatase that plays a critical role in activating cyclin/CDK complexes. There are 3 different *CDC25* genes that have different functions in different phases of the cell cycle (Donzelli and Draetta, 2003). CDC25A is primarily active in G1-S, although it also shows some activity during the G2-M transition. Both CDC25B and

CDC25C play an active role in the G2-M transition. CDC25 is positively regulated by Cyclin B-CDK1, Cyclin A-CDK2, Cyclin E-CDK2, Aurora kinase A, and PLK1 through phosphorylation in the regulatory domain. During the G2-M transition, CDC25B activates the centrosome-associated Cyclin B-CDK1, while the nuclear Cyclin B-CDK1 is activated by CDC25C. (Boutros et al., 2007).

1.2.3 Regulation of the E3 ubiquitin ligase APC

The anaphase-promoting complex, or cyclosome (APC/C), is a 1.5 MDa protein complex with one or two copies of 19 different subunits. The complex also contains two tryptophan aspartic acid (WD) repeat-containing co-activators, CDC20 and CDH1 (Pines, 2011). Two APC subunits, APC 4 and APC 5, form a platform where the Tetraricopeptide repeat (TPR) sub-complex and the catalytic sub-complex bind. The subunits of the TPR sub complex consists of homodimers of APC 6, APC 7, APC 8, and APC 3. A Tetraricopeptide repeat domain is common to all the subunits of the TPR sub-complex and this domain is involved in co-activator interaction. The C-terminal motif as well as the C-terminal Isoleucine-Arginine (IR) tail on the co-activators interact with the TPR domain of the TPR sub complex. (Castro et al., 2005; Vodermaier et al., 2003). The catalytic sub complex consists of APC 2, APC 11, and APC 10. APC 2 and APC 11 have binding sites for E2 enzymes. APC 10 has an IR tail and is possibly needed for binding with the TPR domain of APC 3. The catalytic sub complex cooperates with E2 enzymes for protein ubiquitination (Pines, 2011). Human APC works with two E2 enzymes, UBCH10 and Ube2S (Wang and Kirschner, 2013). Structural studies on APC show that it is somewhat triangular with a cavity in the middle. In this cavity, the substrate

binds with the WD-40 domain of the co-activator and the DOC domain of APC 10 (Pines, 2011).

The primary function of APC is to identify substrates and ubiquitinate them for degradation (Chang et al., 2015; Pines, 2011; Vodermaier, 2004). APC is known to have over one hundred different substrates, and is only active during M phase and G1. The WD-40 repeat domain of the co-activators identifies their substrates through a consensus RXXLXXXXN motif, known as destruction box (D box), or a consensus KEN motif, known as KEN box. (Peters, 2006). The APC complex is kept inactive during late G1, S, G2, and beginning M phases to allow the accumulation of different substrates (Wang and Kirschner, 2013).

There are 43 phospho sites on APC, among which 34 are mitosis-specific and 4 are S phase-specific (Kraft et al., 2003). During interphase, phosphorylation by Cyclin A-CDK2 inactivates CDC20 as well as CDH1 and inhibits them from binding with the APC core complex. However, prior to mitotic entry, phosphorylation by Cyclin B-CDK1 activates APC core complex and CDC20. This facilitates CDC20 binding with the APC complex (Hein and Nilsson, 2016). This active APC-CDC20 is now kept inactive against most substrates by the mitotic checkpoint protein complex (MCC). MCC is a group of proteins that primarily bind to kinetochores that are not attached to spindle microtubules and generates a signal that inhibits the progression of cells from metaphase to anaphase (Sacristan and Kops, 2015). The details of this complex will be discussed later.

1.3 Completion of mitosis in mammalian model systems

Mitosis is a rapid and complicated cell cycle phase wherein a mother nucleus is carefully segregated into two daughter nuclei. Based on chromosomal arrangement, mitosis can be further divided into five distinct phases. These are prophase, prometaphase, metaphase, anaphase, and telophase. Cytokinesis is the last step and occurs after telophase to divide a mother cell into two identical daughter cells. The major events that happen during these phases are described below.

1.3.1 Prophase

The compaction of interphase chromatin is the first visible indication of prophase under the microscope. Cyclin B-CDK1 mediated phosphorylation of Condensin and other unknown proteins that are important for chromosome condensation start the process of chromatin compaction just prior to NEBD (Vagnarelli, 2012). There are two kinds of condensin complexes: condensin I and condensin II. The ratios of condensin I and II vary in different organisms. In mammalian cells, there is an equal ratio of condensin I and condensin II (Vagnarelli, 2012). Apart from condensin, Topoisomerase IIα, chromokinesin, and cohesin complexes are important for the condensation of chromosomes. Cohesin complexes maintain an attachment between sister chromatids until anaphase (Vagnarelli, 2012).

The phosphorylation of Histone H3 on serine 10 by the Aurora B kinase is an important modification of histone during prophase. It starts in the pericentromeric region of the chromosome in G2 and completes in prophase. There are several views regarding the significance of histone H3 phosphorylation during mitosis, but all studies point out that the phosphorylation of histone H3 causes its dissociation from

the DNA, and possibly allows the recruitment of other proteins that are needed for chromosome compaction during prophase (Prigent and Dimitrov, 2003).

Another important event during prophase is the phosphorylation of CDC25C by PLK1.This results in translocating CDC25C to the nucleus. In the nucleus, CDC25C activates Cyclin B /CDK1 as mentioned before (Boutros et al., 2007; Toyoshima-Morimoto et al., 2002).

1.3.2 Pro metaphase-Metaphase

The breakdown of the nuclear envelope marks the beginning of prometaphase. Astral microtubules radiate from the centrosomes toward the nuclear envelope during prophase, and with the help of the microtubule motor protein dynein, the nuclear envelope is pulled toward the centrosomes, creating several small invaginations. In prometaphase, the concerted actions of microtubules and motor proteins, break the nuclear envelope to integrate the cytoplasmic and nuclear proteins and organelles (Beaudouin et al., 2002). At this point, the kinetochore of the chromosomes attaches to the microtubules, which is important for segregation later in anaphase. The stability of the kinetochore microtubule attachments were maintained by Cyclin A and Aurora B kinase. During prometaphase, Cyclin A keeps the microtubules flexible enough to facilitate proper attachment with the kinetochore (Kabeche and Compton, 2013). Just after NEBD during prometaphase, Cyclin A is degraded by APC-CDC20 mediated ubiquitination (den Elzen and Pines, 2001; Pagano et al., 1992). After the degradation of Cyclin A Aurora B kinase localizes to the centromere and destabilizes erroneous kinetochore microtubule attachments. This

signals the spindle assembly checkpoint (SAC) to stop the cell from entering anaphase (Foley and Kapoor, 2013; Li et al., 2015).

1.3.3 *Spindle assembly checkpoint (SAC)*

The spindle assembly checkpoint monitors the microtubule kinetochore attachment status of a cell. As mentioned before, when the SAC is active, anaphase is prevented until the microtubules are properly attached with the kinetochores. The key components of SAC are MAD1, MAD2, MAD3/ BUBR1, BUB1, BUB3, and CDC20 (Sudakin et al., 2001). Among the many SAC proteins, BUBR1, MAD2 and MAD1 are the most essential (Lara-Gonzalez et al., 2012). When the SAC is active, BUBR1 prevents APC-CDC20 activation by binding to CDC20 as a pseudosubstrate. BUBR1 can also inhibit substrate recognition by moving CDC20 away from APC10. The other SAC protein MAD2 stabilizes the interaction between CDC20 and BUBR1 (Lara-Gonzalez et al., 2012; Sacristan and Kops, 2015). After the spindle microtubules are properly attached to the kinetochores, the SAC is silenced by the removal of SAC proteins from the kinetochore. SAC silencing has not been studied in detail in the mammalian model systems, but phosphatases such as PP2A and PP1 play important roles in SAC silencing (Lara-Gonzalez et al., 2012). BUBR1 also has a role in the alignment of chromosomes at the metaphase plate and recruits PP2A to the kinetochore. The BUBR1, PP2A complex then inhibits Aurora B kinase at the kinetochore as Aurora B kinase is no longer needed to destabilize erroneous microtubule kinetochore attachments (Sacristan and Kops, 2015; Xu et al., 2013).

1.3.4 Anaphase

Sister chromatid cohesin is established in S phase by assembly of cohesin complexes that hold the sister chromatids together. In human cells, cohesin complex proteins start to dissociate from the sister chromatid arms during prophase, but the chromatids are still held tightly until metaphase. During SAC activation, Securin stays bound to Separase and inhibits its protease activity. Soon after SAC is silenced, Securin is ubiquitinated by APC-CDC20 and this leads to Separase activation. Active Separase cleaves SCC1 and that causes sister chromatid separation and anaphase progression (Uhlmann, 2001; Waizenegger et al., 2000). In vitro experiments have shown that Securin can also be degraded by APC-CDH1 in a KEN-box dependent manner (Hagting et al., 2002). Following the degradation of Securin, Cyclin B is ubiquitinated by CDC20 (Clute and Pines, 1999). The degradation of Cyclin B and Cyclin A reduces the CDK1 activity and triggers the activation of APC-CDH1. Active CDH1 targets Aurora A kinase, Aurora B kinase, CDC20, and PLK1 for degradation during late anaphase (Floyd et al., 2008; Hyun et al., 2013).

1.3.5 Telophase and Cytokinesis

During anaphase, the framework for cytokinesis starts with the formation of the spindle midzone (Green et al., 2012). The spindle midzone is an array of interdigitating, bundled, central microtubules that do not attach to the kinetochores; they are formed between separating chromosomes. The formation of the spindle midzone requires the kinesin proteins MKLP1, KIF4 and PRC1, as well as the kinase activities of the chromosome passenger complex (CPC) and PLK1. Positioning of

the cleavage furrow is determined by the proteins that reside on the spindle mid - zone (Barr and Gruneberg, 2007; Green et al., 2012). Cytokinesis marks the exit from mitosis with the formation of two daughter cells.

1.4 G1 regulation in the mammalian model systems

Every stage of the cell cycle involves multiple proteins doing multiple activities, and G1 is no exception. The cell enters G1 with low CDK activity; this is crucial, because it sets the stage for many important cellular events. The pre-replication complex formation and activation of CDH1/Fzr are both triggered by low CDK1 activity.

1.4.1 Pre-replication complex (pre-RC) formation

During the late M phase of the cell cycle, origin recognition complexes (ORC), are formed to initiate the formation of a pre-RC. A pre-RC is formed by the assembly of ORC, CDC6, CDT1, and MCM (2 to 7) complexes (Nishitani and Lygerou, 2004). CDC6 and CDT1 are recruited to the ORC first; they then bring the MCM helicase to form the pre-RC. At this point the DNA becomes competent for replication and this event is referred to as replication licensing. Licensing starts at the end of the M and G1 phases, and chromatin stays licensed until the replication of the chromatin starts in S phase, with the transcription and activation of S phase cyclins. At the G1-S transition, with the activation of Cyclin E-CDK2 and Cyclin A-CDK2, some structural changes happen in the pre-RC. These changes allow the cyclins to load additional replication proteins to the pre-RC sites. DNA unwinds with the help of MCM helicase, which moves away from the pre-RC site. DNA polymerase is recruited to the pre-RC site and replication initiates. After replication

is initiated, the pre-RC factors are inactivated by proteolysis, phosphorylation, and translocation to the cytoplasm (Nishitani and Lygerou, 2004; Stoeber et al., 2001). CDT1 is degraded by E3 ubiquitin ligase SCF-Skp2, as well as CUL4-DDB1 in S phase. CDT1 is also inhibited by a protein called Geminin during the S, G2, and M phases. While Geminin inhibits CDT1, it also binds with CDT1 and prevents the degradation of it by SCF-Skp2, so that there is an accumulation of CDT1 during mitosis to start the next round of replication licensing (Ballabeni et al., 2004; Nishitani et al., 2006). At the end of M phase, Geminin is targeted by APC-CDH1. The degradation of Geminin frees CDT1 to bind with pre-RC to start another round of replication licensing (Benmaamar and Pagano, 2005; Skaar and Pagano, 2008).

1.4.2 Regulation by APC-CDH1

As mentioned before, APC-CDH1 is activated by the end of anaphase following the degradation of mitotic cyclins. During G1, CDH1 keeps the mitotic cyclin levels down through continuous ubiquitination of cyclins. It also targets Skp2 and Cks1 in G1 (Bashir et al., 2004). Cks1 is a small CDK-interacting protein that has been shown to bind with Skp2 and increase its affinity for substrates. (Ganoth et al., 2001; Spruck et al., 2001).

1.4.3 Regulation by Retininoblastoma (Rb)

Retinoblastoma (Rb) proteins are the gatekeepers of the G1 phase. Changes to the phosphorylation of Rb control its activity and a cell's advancement from G1-S phase. Rb is a member of a family of proteins that contains a pocket domain; they include Rb, p107, and p130. All of the pocket proteins can bind with the E2F family of transcription factors and represses their activity. (Henley and Dick, 2012).

Phosphorylation of Rb by Cyclin D/CDK4 and Cyclin A/CDK2 relieves its E2F repression, causing the transcription of genes needed for the G1-S transition, particularly *Cyclin E*, *Cyclin A*, *Skp2*, and *Emi1*. When the cells are in quiescence during G1, the cyclin-dependent kinase inhibitors (CKI) like p27 and p21 bind with Cyclin E-CDK2 and keep the cyclin-CDK complex inactive. During late G1, the Cyclin D-CDK4 complex sequesters CKIs from CDK2 and forms a ternary complex with CKIs. This event causes the activation of both CDK2 and CDK4. (Hsu et al., 2002; Ohtani et al., 1995; Schulze et al., 1995; Zhang and Wang, 2006). Active Cyclin E-CDK2 phosphorylates Rb and p27/Dap and Cyclin A-CDK2 phosphorylate proteins that promote replication (Girard et al., 1991; Ohtsubo et al., 1995; Zindy et al., 1992).

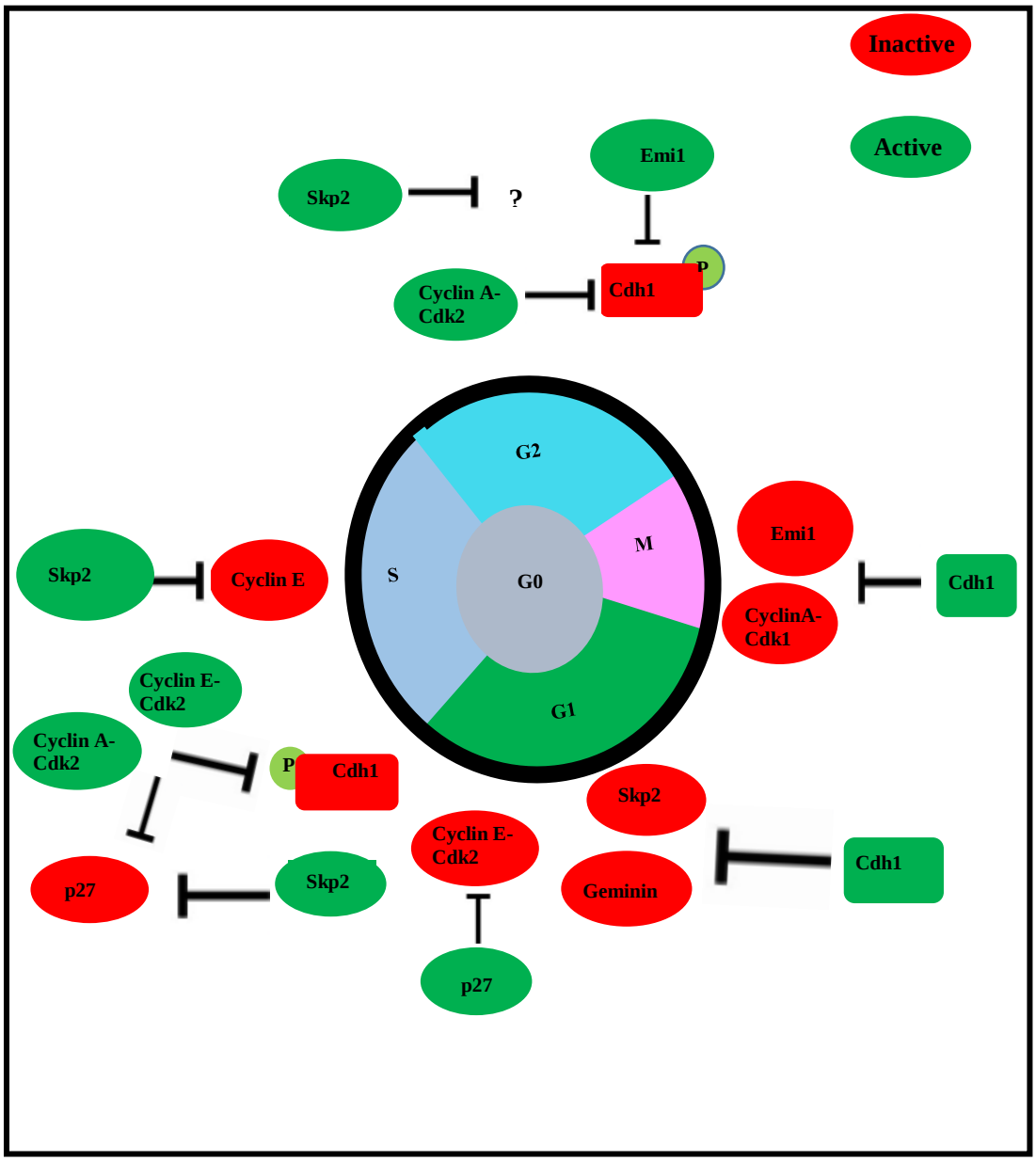

Fig. 1: Schematic diagram of cell cycle regulation of Skp2 and CDH1 in mammalian cell cycle.

1.5 G2 regulation and mitotic entry in *Drosophila melanogaster*

Extensive cell cycle studies have been done on *Drosophila* embryos. The first 13 cell cycles during *Drosophila* embryogenesis are driven only by S and M phases (Lee and Orr-Weaver, 2003). During these rapid cycles, the embryos use only maternally deposited proteins for their cell cycles. At the end of the 13th division, the embryos consist of multiple nuclei in a shared cytoplasm. Very little zygotic transcription occurs at this point. These are referred to as "syncytial embryos." During mitosis 10, each embryo's nuclei start migrating toward the embryo periphery. After the 14th S phase, a G2 phase is introduced with the cellularization of each nuclei; this creates the cellular blastoderm. By this time, the maternal deposit of cellular proteins has been depleted, and zygotic transcription has been initiated. This transition is known as a mid-blastula transition. At this stage, the embryo is referred to as "cellularized." During the 14th to 16th divisions, each cell undergoes an S-G2-M cycle. After mitosis 16, the epidermal cells exit the cell cycle, neuronal cells continue to undergo S-G2-M cycles, histoblasts arrest in G2, and cells that will differentiate into larval tissues undergo an S-G cycle to rapidly increase ploidy and cell size. At this point, the G1 phase is introduced to the imaginal disc cells. The imaginal discs later form the eyes, antennas, wings, legs, halters, and genitalia of an adult fly (Lee and Orr-Weaver, 2003). Syncitial embryos, cellularized embryos, wing imaginal discs, eye imaginal discs, and brains of 3rd-instar feeding or wandering larvae are widely used to study cell cycles.

The wing imaginal disc of each *Drosophila* originates from an embryonic primordium. Starting with only 50 epithelial cells in the first instar larval stage, the

cells grow to 50,000 in number by the third instar larval stage (Neufeld et al., 1998). At this stage, an area at the dorsal ventral boundary of the wing imaginal disc undergoes programmed cell cycle arrest. This area is known as the zone of non-proliferating cells (ZNC). In this zone, the cells are arrested into 2 stages: G1 and G2 (Fig. 2)(O'Brochta and Bryant, 1985).

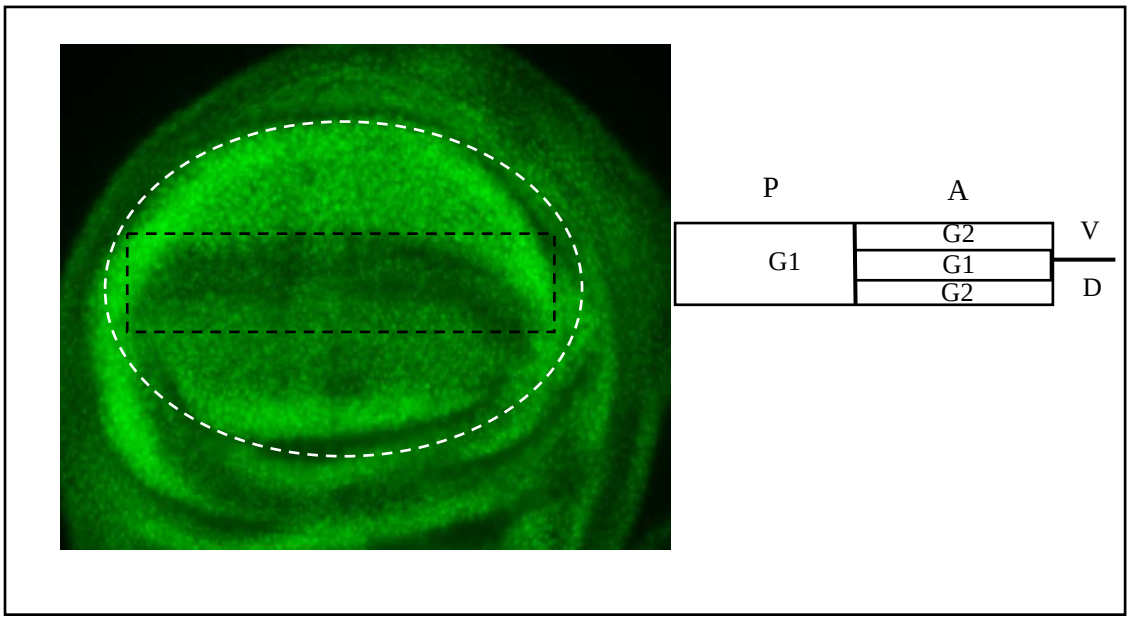

Fig. 2: Zone of non-proliferating cells (ZNC) in 3rd instar wing imaginal disc of *Drosophila*. The area within the white dotted line marks the wing pouch/blade. The area within the black dotted rectangle is explained in the cartoon. In the cartoon, A refers to anterior side of the wing disc, B refers to the posterior side of the wing disc, V refers to the ventral side of the wing disc and D refers to the dorsal side of the wing disc. G1 and G2 refers to the cell cycle phase the cells are arrested in the ZNC.

1.5.1 Regulation by mitotic cyclins and CDC25/String

In *Drosophila*, the three mitotic cyclins are Cyclin A, Cyclin B, and Cyclin B3. In flies, unlike mammals, Cyclin A is localized exclusively in the cytoplasm during interphase of cellularized embryos. It enters the nucleus during prophase and is degraded by metaphase (Lehner and O'Farrell, 1989). The localization of Cyclin B and Cyclin B3 are similar to mammals. During interphase, the majority of Cyclin B can be found in the cytoplasm, Cyclin B accumulates in the nucleus in prophase; it disappears by anaphase (Lehner and O'Farrell, 1989, 1990). Cyclin B3 is localized in the nucleus during interphase. During prophase it has an intense accumulation with the chromatin, which fades during metaphase and disappears during anaphase. Unlike mammals, all three mitotic cyclins interact with only CDK1 as their kinase partner (Jacobs et al., 1998; Sigrist et al., 1995).

Among the three mitotic cyclins, *Cyclin B* and *Cyclin B3* mutants are viable; *Cyclin A* mutants are lethal, indicating that Cyclin A is critical among all the mitotic cyclins (Jacobs et al., 1998). However, single mutant studies have shown that in *Cyclin B* mutants mitotic spindle organization is disrupted and mitosis takes longer than wild type. *Cyclin A* mutants never enter mitosis 16, indicating a necessity for Cyclin A at this particular stage. No mitotic abnormalities have been seen in *Cyclin B3* mutants. To study the redundancy of cyclins, double mutants were made. *Cyclin B* and *Cyclin B3* double mutants showed a delayed entry into mitosis, combined with severe abnormalities in spindle formation and delays in prophase. In *Cyclin A* and *Cyclin B3* double mutants, spindle organization was normal, but chromosome condensation was severely affected. The double mutants did not enter mitosis 16

(Jacobs et al., 1998; Lehner and O'Farrell, 1989). However, the double mutants of *Cyclin A* and *Cyclin B* did not enter mitosis 15 (Knoblich and Lehner, 1993).

The expression of a non-degradable form of Cyclin A results in a metaphase delay with normal spindles. The expression of a non-degradable form of Cyclin B had shown normal chromosome condensation, but mitotic spindle defects and defects of chromosome arrangements were also observed on the metaphase plate (Sigrist et al., 1995). The cells did not proceed into anaphase or telophase. There was an enrichment of early anaphase chromosomes with separated sister chromatids. The expression of a non-degradable form of Cyclin B3 show a late anaphase delay with chromosome segregation (Jacobs et al., 1998; Sigrist et al., 1995).

In mitosis 14 of cellularized embryos, when maternal CDC25/String becomes depleted, cells are arrested at the G2 phase of the cell cycle. CDC25/String transcription is needed for cells to go into mitosis, implying that at this stage CDC25/String is an important regulator of mitotic entry (O'Farrell et al., 1989). Entry into mitosis 16 does not occur in *Cyclin A* mutant embryos despite the presence of cyclins B and B3, suggesting that at this stage the presence of Cyclin A is critical for mitotic entry with Cyclin B, having an intermediate effect. Cyclin B3 has the least effect on mitotic entry (Jacobs et al., 2001; Jacobs et al., 1998). As mentioned before, G1 is introduced for the first time after mitosis 16. The expression of non-degradable Cyclin B, non-degradable Cyclin B3, or CDC25/String did not restore mitosis 16 in *Cyclin A* mutant embryos. In these embryos, Cyclin B, Cyclin B3, and $CDC25^{String}$ disappeared prematurely (Reber et al., 2006). All these proteins have D boxes, which make them targets for APC-CDH1/Fzr (Sigrist and Lehner,

1997). Levels of Cyclin B, Cyclin B3, and CDC25/String were restored in *Cyclin A* and *CDH1*/*Fzr* double mutant embryos indicating that Cyclin A is needed to inhibit CDH1/Fzr mediated degradation of these proteins. (Reber et al., 2006). Apart from Cyclin A, Cyclin E can also inhibit CDH1/Fzr. However, Cyclin E disappears before mitosis 16 and is not able to inhibit CDH1/Fzr. Thus, at this stage the presence of Cyclin A is needed for CDH1/Fzr inhibition. Cyclin A can inhibit CDH1/Fzr even when all phosphorylation sites on CDH1/Fzr are mutated, suggesting that in flies, the phosphorylation of CDH1/Fzr does not cause its inactivation (Reber et al., 2006).

Emi1/Rca1 is an important regulator of the G2 phase in *Drosophila*. It is localized in the nucleus throughout the cell cycle. Similar to mammalian system, Emi1/Rca1 binds with CDH1/Fzr and inhibits it from becoming active in G2 (Grosskortenhaus and Sprenger, 2002). Loss of *Emi1/Rca1* results in premature overexpression of *CDH1/Fzr*, which causes a G2 arrest with the degradation of mitotic cyclins, and the inhibition of mitosis 16 (Grosskortenhaus and Sprenger, 2002; Sigrist and Lehner, 1997). Mutants of the other APC activator, *CDC20/Fzy*, arrest during the metaphase of mitosis 16, indicating that CDC20/Fzy is required during the metaphase to anaphase transition, not during G2 or mitotic entry (Sigrist et al., 1995).

1.5.2 Nuclear envelope breakdown

During interphase, in the early embryo when there is only the S-M cycle, the nuclear envelope (NE) forms around the periphery of the chromatin as a circular structure. As prophase begins with the condensation of chromosomes, the NE invaginates inwards, toward the condensing chromatin just beside the centrosomes.

Spindle microtubules can pass through the nuclear membrane and attach to the condensed chromosome. After metaphase, the nuclear lamina partially breaks down and disperses as granular structures in the cytoplasm (Fuchs et al., 1983; Paddy et al., 1996). Nuclear envelope breakdown in cellularized embryos has not been studied well.

1.6 Mitosis in *Drosophila melanogaster*

Similar to mammalian model system *Drosophila* mitosis is subdivided into 5 phases: prophase, prometaphase, metaphase, anaphase and telophase. As mentioned before, during prophase, Cyclin A enters the nucleus from the cytoplasm. Chromosome condensation follows right after Cyclin A entry, implying that Cyclin A might have a role in chromosome condensation (Dienemann and Sprenger, 2004). Cyclin B accumulates near the centrosomes just prior to nuclear entry. It enters the nucleus right before nuclear envelope breakdown and localizes throughout the mitotic spindle. During metaphase, Cyclin B localizes to the plus end of the interpolar microtubules. The degradation of Cyclin B is spatially regulated. In the syncytial embryos, spindle associated Cyclin B is degraded whereas cytoplasmic Cyclin B is not degraded. However, in cellularized embryos cytoplasmic as well as spindle associated Cyclin B are both degraded through APC-mediated ubiquitination. (Huang and Raff, 1999). Similar to mammals, CDC20/Fzy ubiquitinates mitotic cyclins in an ordered sequence, starting with Cyclin A. It then degrades Securin/Pim, Cyclin B, and lastly Cyclin B3 (Leismann et al., 2000; Sigrist et al., 1995) .

The main components of a *Drosophila* Spindle assembly checkpoint are BUBR1, BUB3, MAD2, MAD1, Rough Deal (Rod), Zw10, BUB1 and Mps1kinase.

Mps1 is upstream of the SAC proteins. Its function is to recruit SAC proteins to the kinetochore and phosphorylate BUBR1. The phosphorylation of BUBR1 results in recruitment of CDC20/Fzy to the kinetochore as well as the formation of the mitotic checkpoint complex. (Conde et al., 2013a). MAD2 stabilizes CDC20/Fzy and BUBR1 interactions in the kinetochore. Although flies have all the SAC proteins described in the mammalian system, SAC is not essential in flies. This indicates that the SAC proteins might have other essential roles that are not yet understood (Basto et al., 2004; Basu et al., 1999; Buffin et al., 2007; Conde et al., 2013b; Lopes et al., 2005; Orr et al., 2007).

1.7 G1 regulation in *Drosophila*

Similar to mammalian model systems, APC-CDH1/Fzr is an important regulator during the G1 phase. In *CDH1/Fzr* deficient embryos, mitotic cyclins re-accumulate after CDC20/Fzy ubiquitinates them in mitosis. These embryos do not enter G1; instead, they go for an extra replication phase (Sigrist and Lehner, 1997).

Roughex (Rux) is a *Drosophila*-specific G1 regulator that acts as a cyclin-dependent kinase inhibitor (CKI) specific to Cyclin A and Cyclin B (Foley et al., 1999). *Rux* mutant flies prematurely enter the S phase without maintaining G1 phase arrest within the morphogenetic furrow of the *Drosophila*'s eye imaginal disc. Rux can interact with Cyclin A and Cyclin B, and it inhibits its CDK1-mediated kinase activity in mitosis. While it acts primarily to inhibit CDK1 activity, at low levels it can promote CDK1 activity (Foley et al., 1999). Cyclin E-CDK2 phosphorylates Rux; this causes Rux degradation at the G1-S transition (Foley et al., 1999; Foley and Sprenger, 2001; Thomas et al., 1997).

Dacapo (Dap) is the other CKI in *Drosophila* that has homology with both p21 and p27/Dap in mammals. The overexpression of p27/Dap causes a G1 arrest in transgenic embryos. Bacterially expressed p27/Dap inhibits Cyclin E-CDK2 activity *in vitro*, but does not inhibit Cyclin A-CDK1 or Cyclin B-CDK1 activity (de Nooij et al., 1996; Lane et al., 1996).

1.8 G1-S transition in *Drosophila*

Unlike mammals, Cyclin D does not regulate G1-S transition in flies. Instead it regulates growth. Cyclin E is the sole regulator of the G1-S transition in *Drosophila melanogaster*. Cyclin E phosphorylates Rb and promotes its own transcription through the E2F transcription factor (Lee and Orr-Weaver, 2003).

1.9 Cyclins and cyclin-dependent kinases (CDKs)

The following section discusses CDK activation and detailed review about Skp2 and Cyclin A. Unlike cyclins, CDK protein levels remain constant throughout the cell cycle (Draetta and Beach, 1988). However, the activity of CDKs is periodic, based on their association with their regulatory and substrate recognition partner cyclins (Kaldis and Lim, 2013). *Drosophila* have 11 CDKs and 14 cyclins, whereas humans have 20 CDKs and 29 cyclins. Among them, only a fraction of cyclins and CDKs are involved in the cell cycle (Malumbres, 2014). Cyclins are a large family of proteins that vary in size from 35 to 90 kDa. Structurally, all cyclins have a variable N-terminus and a more conserved C-terminus. All cyclins have a cyclin box in their C-terminal domain (Nugent et al., 1991). This cyclin box is further divided into two stretches of approximately 100 amino acids, both stretches containing 5 alpha helices. In some cyclins, the stretches are referred to as the N-terminal cyclin box

(CBOX1) and the C-terminal cyclin box (CBOX2). The N-terminal cyclin Box is structurally conserved in all cyclins, and is necessary for CDK association (Morgan, 1997; Petri et al., 2007).

CDKs range in size from 250 amino acids to 1500 amino acids. The crystal structure of CDK2 shows it is a two-lobed protein. The N-terminal lobe (residues 1 to 85) contains beta sheets, while the C-terminal lobe contains alpha helices. ATP binds into the active site of the CDK, which is positioned between the two lobes (De Bondt et al., 1993). The C-terminal lobe contains the highly conserved PSTAIRE sequence which interacts directly with the cyclins. The C-terminal lobe also contain the inhibitory phosphorylation sites where Wee1 and Myt1 kinase adds the inhibitory phosphorylations on Thr 14 and Tyr 15 to inactivate the CDK. The CDK activating kinase (CAK) phosphorylates Thr 160 in the T-loop (residues 152-170). The T-loop blocks the active site and acts as an auto inhibitor for substrate binding (De Bondt et al., 1993; Jeffrey et al., 1995). CAK is a Cyclin H-CDK7 complex in mammals and flies (Merrick et al., 2008). In the steps toward the complete activation of CDK, cyclin binding is a critical step, apart from the phosphorylation of CDK (Ducommun et al., 1991). However, some studies have suggested that cyclin binding and CAK phosphorylation happen simultaneously for CDK1, but do not happen simultaneously for CDK2 (Merrick et al., 2008) . CDK2 is phosphorylated by CAK prior to cyclin binding (Fisher and Morgan, 1994; Merrick and Fisher, 2010; Russo et al., 1996b). During the Cyclin A-CDK2 interaction, the C-terminal helix of CDK2 rotates and moves toward the cleft, causing a tilt in the N-terminal beta sheet. The T-loop, the C-terminal PSTAIRE, and the N-terminal β sheet all interact with the N-

terminal cyclin Box of Cyclin A. Binding with Cyclin A causes a conformational change in the PSTAIRE and the T-loop. When cyclin is not bound with CDK2, the T-loop blocks the active cleft, but when CDK2 binds with Cyclin A, the T-loop undergoes a conformational change that relieves the blockade (Jeffrey et al., 1995).

1.10 Cyclin A

Cyclin A was found to be present *Xenopus, Drosophila,* and *S.pombe* showing that Cyclin A is a conserved protein (Andrews and Measday, 1998; Lehner and O'Farrell, 1989; Minshull et al., 1990).

1.10.1 Cyclin A in the mammalian model systems

The human Cyclin A protein consists of 432 amino acids and has a predicted molecular mass of 58 kDa (Pines and Hunter, 1990). The crystal structure of Cyclin A was studied using residues 171 to 432. The crystal structure of Cyclin A shows it is a globular structure; the Cyclin Box is a compact domain of 5 α helices. α helix 1 is conserved among all cyclin families. As mentioned before, there are two cyclin Boxes (100 amino acids each) that consist of residues 199 to 306. Residues 208 to 303 consist of the N-terminal cyclin Box, and residues 309 to 399 consist of the C-terminal cyclin Box (Bourne et al., 1996; Brown et al., 1995).

The crystal structure complex of p27/Dap, CDK2, and Cyclin A show that p27/Dap can also separately bind with CDK2 and Cyclin A. The conserved RXLFG site of p27/Dap binds with the MRAILVDW site of Cyclin A. p27/Dap is brought to CDK2 after an initial binding with Cyclin A, where it inhibits the catalytic cleft of CDK2, which causes a block in ATP binding (Russo et al., 1996a).

1.10.2 Drosophila Cyclin A protein structure

Drosophila Cyclin A is 499 amino acids long (Lehner and O'Farrell, 1989). Cyclin A proteins shows 2 bands that are 61 kDa and 59 kDa; the 59 kDa band is the inactive form and the 61 kDa band is the active form (Lehner and O'Farrell, 1990). The relative abundance of the bands shows differences during the different developmental stages (Lehner and O'Farrell, 1989). The N-terminus of Cyclin A is 1-170 amino acids long, has two KEN boxes (KEN1 and KEN2), and has two D boxes (Dbs1 and Dbs2) (Fig. 3). The first degradation motif is KENPGIK (KEN2), which extends from 13 to 19 amino acids long. The second motif is RANFAVLNGN (Dbs2), which extends from 46 to 55 amino acids long. The third motif is KENHDVK (KEN1), which extends from 123 to 129 amino acids long. The last degradation motif is RSILGVIQS (Dbs1), which extends from 160 to 168 amino acids long. The Lehner lab shows that the deletion of 1 to 53 amino acids stabilizes Cyclin A (Jacobs et al., 2001). However, studies done by the Sprenger lab have shown that the deletion of 1 to 53 amino acids from Cyclin A delays its degradation but does not make it fully stable (Ramachandran et al., 2007). According to the Sprenger lab, with deletion of KEN2, Dbs2, another point mutation in D70, and the removal of the 5 Lysine residues K37, K40, K64, K85, and K86, are needed for complete stabilization of Cyclin A. Surprisingly, the deletion of the complete N-terminus (1-170), which included all the above sites, did not fully stabilize Cyclin A. This means that there is a region in the C-terminus that also contributes to degradation. Cyclin A-CDK1 interaction requires the amino acid phenylalanine 329 (F329) for its kinase activity (Fig. 3). Mutating this site can inhibit Cyclin A-CDK1

activity, but CDK1 can still bind with Cyclin A. Cyclin A can also be auto phosphorylated by CDK1 in the N-terminal (T145, S154, and S180) and in the C-terminal (T333 and T397) (Ramachandran et al., 2007).

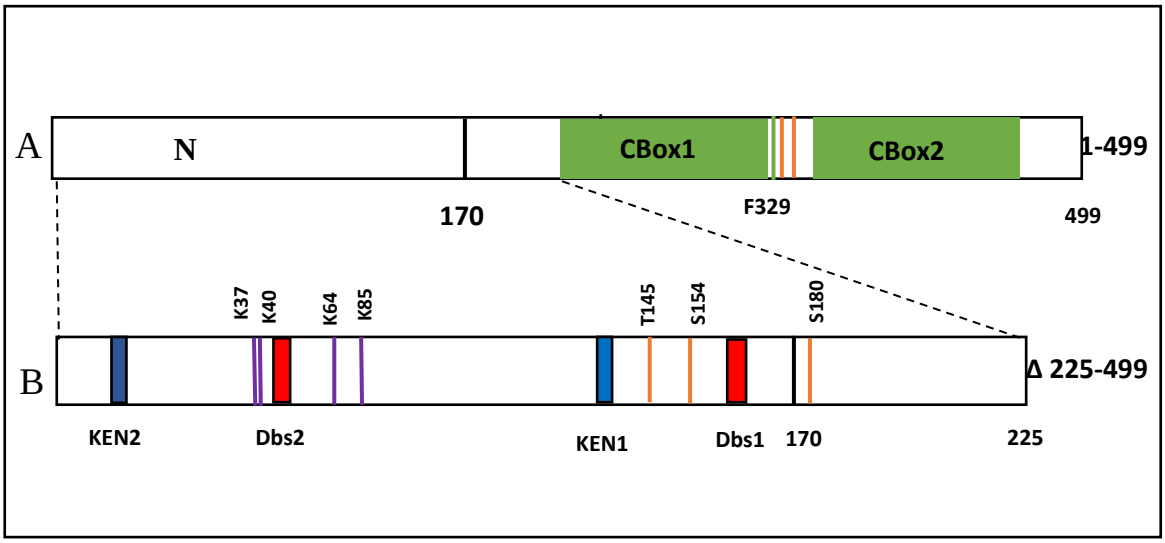

Fig. 3: Structure of *Drosophila* Cyclin A protein. A) Full-length Cyclin A, N-terminal 1–170 and C-terminal 171–499. Green boxes are cyclin boxes. F329 (shown as green box) is essential for CDK1 interaction. B) 1-225 Cyclin A. Blue boxes are KEN boxes, and red boxes are D boxes. Purple boxes are Lysine residues needed for ubiquitination. Orange boxes are autophosphorylation sites.

1.11 SCF Complex

The E3 ubiquitin ligase SKP1–CUL1–F-box-protein (SCF) complex is a well characterized RING Finger, type E3 ubiquitin ligase (Cardozo and Pagano, 2004). It consists of a complex of the three core proteins Cullin 1 (Cul1), S phase kinase-associated protein 1 (Skp1), and Ring-Box 1 (Rbx1). Cul1 acts as a scaffold protein that holds Skp1 on the N-terminus and holds Rbx1 on the C-terminus. An E2 ubiquitin ligase binds to Rbx1, F-box, and Skp1. The F-box protein is the co-activator of the SCF complex. There are several F-box proteins found in mammals that are classified into three categories: FBW, FBL, and FBX. FBW proteins have beta propeller structures on their carboxy terminus for substrate binding, FBL proteins have Leucine-rich repeats (LRR) on their carboxy terminus for substrate binding and FBX proteins have neither beta propeller structures nor LRR structures on their carboxy terminus for substrate binding, but do contain other protein-protein interacting motifs. There are approximately 78 F-box proteins in humans and approximately 30 F-box proteins in flies, but only a few have been studied (Cardozo and Pagano, 2004).

1.11.1 SCF-Skp2 in the mammalian model systems

Skp2 is an F-box protein of the SCF-ubiquitin ligase that was first identified by Beach Lab in 1995 as a protein that forms a complex with Cyclin A, CDK2, Skp1, and Cks1/Cks85A in both normal and transformed fibroblasts (Zhang et al., 1995). Skp2 is a protein of 435 amino acids with a calculated molecular mass of 48 kDa. The C-terminal half of Skp2 is composed of seven imperfect leucine-rich repeats (LRR) of 26 amino acids, and were involved in substrate interaction.

Crystal structure studies of Skp1-Skp2 also revealed that its structure forms a sickle shape (Schulman et al., 2000). The curved surface of the sickle is formed with the 40 amino acid F-box domain, followed by a 3 LRR (Leucine-rich region) linker domain and a 7 LRR substrate binding domain (Fig. 4). The linker domain connects the F-box to the protein-protein interaction domain. Skp1, which binds with the F-box domain, forms the handle of the sickle. All 10 LRRs have a β strand, and an α helix forms the sickle of Skp2. The C-terminal tail of 30 residues is loose and extends toward the first LRR of the linker domain. The tail lies on a concave surface that is formed due to the sickle shape of the LRR domain. In the crystal structure study, 88 residues from the N-terminus of Skp2 were truncated due to poor solubility and were not used (Schulman et al., 2000).

Skp2 cooperates with H-Ras^{G12V} to transform primary rodent fibroblasts to malignancy (Gstaiger et al., 2001). The overexpression of the *Skp2* mRNA and the protein has been frequently observed in almost all human cancer types (Chan et al., 2010; Frescas and Pagano, 2008; Wang et al., 2012). Skp2 overexpression was detected in 47.3% of adenocarcinomas (Shigemasa et al., 2003). A higher level of *Skp2* mRNAs was observed in 61% of ER-negative breast cancers compared to ER-positive breast cancers (Signoretti et al., 2002). The overexpression of Skp2 has been observed in small-cell lung carcinoma, lymphoma, gastric carcinoma, cervical cancer, prostate cancer, and glioblastoma (Chen et al., 2007; Latres et al., 2001; Masuda et al., 2002; Saigusa et al., 2005; Yang et al., 2002; Yokoi et al., 2002). Skp2's overexpression has been shown to be inversely correlated with p27/Dap downregulation (Carrano et al., 1999; Frescas and Pagano, 2008; Tsvetkov et al.,

1999). In late G1, Cyclin E-CDK2 phosphorylates p27/Dap on T187. This phosphorylated p27/Dap is identified by Skp2 and is ubiquitinated for degradation (Carrano et al., 1999; Tsvetkov et al., 1999). Cks1/Cks85A enhances Skp2's interaction with phosphorylated p27/Dap and is essential for p27/Dap ubiquitination (Ganoth et al., 2001).

The generation of *Skp2* null mice by Nakayama lab was very important in regards to understanding the role of Skp2 in an *in vivo* environment (Nakayama et al., 2000). *Skp2* null mice were significantly smaller than their litter mates, although their external size proportion was normal. These mice had an increased ploidy and multiple centrosomes. An accumulation of Cyclin E and p27/Dap was observed in the *Skp2* null mice, as Skp2 targets free Cyclin E and p27/Dap for degradation. The expression of the Skp2 phenotypes were tissue specific. It was only observed in hepatocytes, lungs, kidneys, testis, and embryonic fibroblasts. (Nakayama et al., 2000). The G1-S timing of Skp2-mediated degradation of p27/Dap was questioned by Nakayama lab. They reported that Skp2-mediated degradation of p27/Dap does not happen in G1-S; instead, Skp2 degrades p27/Dap during S-G2 phases. They showed that *Skp2* null cells fail to enter mitosis due to p27/Dap overexpression. p27/Dap binds with Cdk1 in *Skp2* null cells and that results in reduced kinase activity (Nakayama et al., 2004)

As mentioned before, over the years, research on Skp2 have emphasized p27/Dap as a critical target (Carrano et al., 1999; Ganoth et al., 2001; Nakayama et al., 2000; Sutterlüty et al., 1999; Tsvetkov et al., 1999). However, a later study in 2008 showed that in human melanoma cells *p27/Dap* knockdown in *Skp2* depleted

cells did not rescue the *Skp2* knockdown phenotype. The expression of nuclear-localized Cyclin B rescued the Skp2 phenotype (Hu and Aplin, 2008). This shows that knockdown of *Skp2* might have effect on other cell cycle regulators other than p27/Dap. It might also suggest that the effect of depletion of *Skp2* varies according to cancer types.

To better understand the binding of Skp2 to p27/Dap, and to better understand how Cks1/Cks85A helps in this interaction, a crystal structure of the quarternary complex of Skp1, Skp2, Cks1/Cks85A, and p27/Dap was determined (Hao et al., 2005). An N-terminal truncated Skp1 and Skp2, a truncated Cks1 (residues 5-73), and a 24-residue phosphopeptide of p27/Dap (residues 175 -197) were used for the study. Cks1/Cks85A binds with both the tail and the LRR domain of Skp2. Residues 181 to 184 of the short p27/Dap peptide bind with Skp2 alone, residue 185 binds with both Skp2 and Cks1/Cks85A, and residues 186 to 190 bind with only Cks1/Cks85A (Hao et al., 2005).

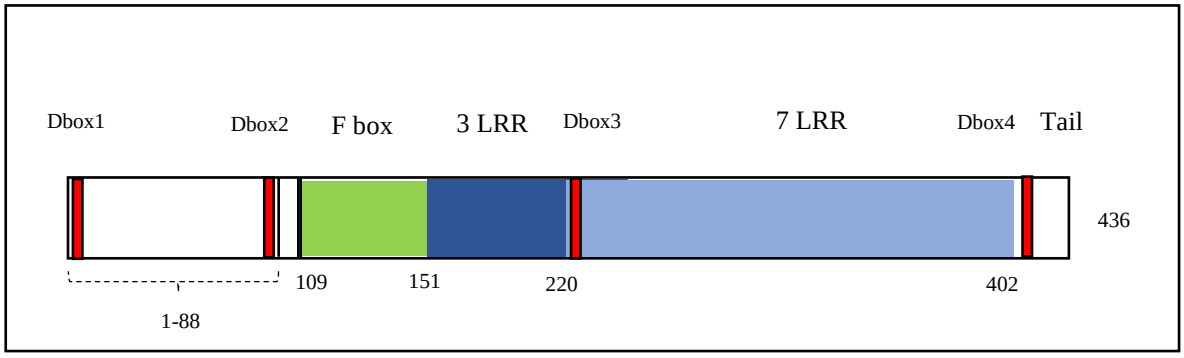

Fig. 4: Structure of human Skp2 protein. N-terminal 1–88 residues were deleted during crystal structure studies. Green boxes are cyclin boxes. 109-151 is the F box (shown in green). Red boxes are D boxes.

Apart from p27/Dap, Skp2 has other important targets. CDT1/Dup is an important target of Skp2. As mentioned before, CDT1/Dup is an important component of the pre-replication complex. Skp2 targets phosphorylated CDT1/Dup during the S and G2 phases and that prevents re-replication. Other than Skp2, CDT1/Dup is also ubiquitinated by DDB1-Cul4 for degradation during the S phase (Nishitani et al., 2006). Other targets of Skp2 are p21, p57, E2F1, Cyclin E, p130, BRCA2, Orc1, CDK9, cMyc, and FOXO (Kiernan et al., 2001; Kim et al., 2003; Li et al., 2003; Marti et al., 1999; Moro et al., 2006; Nakayama et al., 2000; Nakayama et al., 2004; Tedesco et al., 2002; Yu et al., 1998).

Skp2 is a substrate of APC-CDH1/Fzr. As mentioned before, CDH1/Fzr identifies its substrates through a D box or a KEN box. Skp2 does not have a KEN box, but has a total of 5 RXXL motifs in residues 3 to 6 (D box1), 84 to 87 (D box2), 234 to 237 (D box3), 294 to 297 (D box4), and 415 to 418 (D box5). The deletion of D box 1 causes the stabilization of Skp2 in the presence of CDH1/Fzr (Bashir et al., 2004).

1.11.2 SCF-Skp2 in Drosophila melanogaster

Skp2 in *Drosophila* is 33% identical and 59% similar to the human Skp2. To study the function of Skp2 in *Drosophila*, *Skp2* null flies were generated (Ghorbani et al., 2011). These flies were smaller than wild type flies without any compromise to their external size proportions. *Skp2* null flies died during the larval-pupal transition stage. *Skp2* null larvae were smaller than wild type larvae. The loss of *Skp2* increased ploidy in the mitotically dividing cells (brain, wing imaginal disc, and eye imaginal disc cells), but did not increase ploidy in endoreplicating cells (salivary

gland cells). Instead the ploidy in these cells was reduced compared to wild type (Ghorbani et al., 2011).

Flow cytometry on the wing imaginal discs of *Skp2* null showed a distinct tetraploid cell population (Ghorbani et al., 2011). *Skp2* knockdown in the posterior half of wing imaginal discs showed reduced cell density and increased wing-hair spacing compared to the anterior half (control). Each hair in the adult wing arose from a cell, and the distance between two hairs was constant in the wild type adult wing. If cells become bigger, the distance between two hairs increases (Dui et al., 2013; Ghorbani et al., 2011). The average doubling time of wild type wing imaginal disc cell is 11 hours, whereas for *Skp2* knockdown cells it takes 16 hours (Dui et al., 2013). It is also possible that the reduced cell density observed in *Skp2* knockdown was due to a high degree of apoptosis. Wing imaginal disc cells of *Skp2* null showed an increase in sub G1 population (Ghorbani et al., 2011). Although studies of Skp2 in flies are limited to few labs, p27/Dap has been identified as a Skp2 substrate (Dui et al., 2013).

1.12 Skp2-Cyclin A interaction in mammalian cells

In normal human fibroblasts, Cyclin A forms a complex with CDK2, p21, and PCNA, but in transformed cell lines Cyclin A changes its partner and complexes with Skp2, CDK2, Cks1/Cks85A, and Skp1 (Zhang et al., 1995). Interestingly, the interaction between Skp2 and Cyclin A occurs on a unique non-RXL site on the N-terminus of Cyclin A. This kind of binding site is unique because it does not maintain the traditional RXL-HP interaction between Cyclin A and non-CDK proteins (Ji et al., 2006). Two separate labs studied Skp2–Cyclin A interaction. Both

of their results confirmed that Skp2 is a substrate of Cyclin A-CDK2, and Cyclin A phosphorylates S76 of Skp2 (Ji et al., 2006; Yam et al., 1999). According to the Pagano lab, Skp2 interacts with Cyclin A to protect Cyclin A from p27/Dap mediated inhibition during the G1-S phase transition (Ji et al., 2006).

The Poon lab showed that the Skp2–Cyclin A interaction reduces the kinase activity of Cyclin A-CDK2 by 50% or more. The reduction in kinase activity is due to the conformational change of Cyclin A-CDK2 when it is bound to Skp2. Their results show that due to the conformational change, Cyclin A-CDK2 cannot be identified by CAK and Wee1. They also showed that Skp2 can also directly inhibit the kinase activity of Cyclin A-CDK2 by inhibiting substrate binding (Yam et al., 1999).

Skp2 interaction with Cyclin A points toward the possibility of an interesting non-traditional regulatory role of Skp2 that is independent of its role as E3 ubiquitin ligase. On one hand, Skp2 protects Cyclin A from p27 mediated inhibition during G1/S, but on the other hand it reduces the kinase activity of Cyclin A-CDK2 (Yam et al., 1999). This led to several open questions. Is this interaction only limited to G1/S or it is also essential during other phases, particularly G2/M? Is this interaction conserved in other organisms? If yes, then what is the significance of this interaction? We chose to study this interaction in *Drosophila Melaonogaster* where Cyclin A is strictly a mitotic cyclin and has no known role during G1/S phase transition. The major goal of this thesis is to explore the interaction between Skp2 and Cyclin A and we also wanted to understand how loss of *Skp2* results in polyploidy.

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CHAPTER 2

SKP2-CYCLIN A INTERACTION IS NECESSARY FOR GENOME STABILITY

2.1 Summary

S phase kinase associated protein Skp2 is the substrate recognition component of the SCF complex. Skp2 is a potential oncogene and is overexpressed in a variety of cancer types. Skp2 was first identified as a protein associated with Cyclin A, CDK2, Skp1 and Cks in transformed as well as normal cell lines. The critical target of Skp2 is Cyclin dependent kinase (CDK) inhibitor p27. Overexpression of Skp2 in cancer cells is directly related to premature degradation of p27. Previous studies on Skp2-Cyclin A interaction show that Skp2 can directly interact with Cyclin A and protect it from p27 mediated inhibition during G1-S phase transition. However, Cyclin A is not only a S phase cyclin it is also a mitotic cyclin and is therefore needed for mitotic entry. Accumulation of Skp2 and Cyclin A reaches its peak in G2 phase prior to mitotic entry. This raises an interesting possibility that Skp2 might also protect Cyclin A during G2-M transition. In this paper, we investigate Skp2 and Cyclin A interaction in *Drosophila melanogaster* where Cyclin A is strictly a mitotic cyclin. We showed evidence that Skp2 functions with Cyclin A in mitotic entry. We showed that loss of *Skp2* in mitotic cells results in loss of mitotic cyclins and causes cells to skip mitosis and instead endoreplicate. This results in polyploidy. We also showed that the N-terminus of Skp2 interacts with Cyclin A *in vitro and in vivo* and and is required for for its role in genome stability.

2.2 Introduction

The SCF ubiquitin ligase with its adaptor protein Skp2, is a critical regulator of the G1 to S transition and cell proliferation (Sutterlüty et al., 1999). Increasing evidence for the past two decades have established the role of Skp2 as a possible oncogene (Gstaiger et al., 2001). Overexpression of Skp2 has been linked to several different types of cancers (Abdou et al., 2012; Chiarle et al., 2002; Fagan-Solis et al., 2014; Latres et al., 2001; Lim et al., 2002; Masuda et al., 2002; Shigemasa et al., 2003; Shim et al., 2003; Signoretti et al., 2002; Wei et al., 2013; Yang et al., 2002). The best characterized target of SCF-Skp2 is the Cyclin dependent kinase (CDK) inhibitor, p27 (Dap in *Drosophila*) (Carrano et al., 1999; Nakayama et al., 2000; Sutterlüty et al., 1999; Tsvetkov et al., 1999). In most of the cancers, *Skp2* overexpression has been shown to have a correlation with premature degradation of p27/Dap (Chen et al., 2007; Chiarle et al., 2002; Fagan-Solis et al., 2014; Lim et al., 2002; Masuda et al., 2002).

Skp2 functions in tandem with CDK2, which first phosphorylates p27/Dap at T187, allowing p27/Dap to be recognized by SCF-Skp2 (Tsvetkov et al., 1999). p27/Dap destruction in turn leads to further CDK activation and S-phase entry. These findings have led to a growing interest in the possibility of suppressing *Skp2* function as a promising therapeutic (Chan et al., 2013; He et al., 2009; Sumimoto et al., 2005; Wang et al., 2012a; Wang et al., 2012b; Wei et al., 2013).

Interestingly, a study done by Nakayama lab in 2000, observed that *Skp2* knockout mice are polyploid and have reduced growth indicating a possible tumour

suppressive role of Skp2 (Nakayama et al., 2000). They found genetic evidence that suggests that both the reduced growth and polyploidy are due to elevated p27/Dap levels: *Skp2, p27/Dap* double mutants are largely rescued for both phenotypes (Nakayama et al., 2000; Nakayama et al., 2004). Studies in *Drosophila* also found that *Skp2* null flies are polyploid and have reduced growth, strengthening the idea that the tumour suppressive function of Skp2 is not limited to mammals but is conserved in flies as well (Ghorbani et al., 2011) . As it is in mammals, studies in flies also showed that p27/Dap is an important substrate of Skp2 (Dui et al., 2013).

According to Nakayama et al. 2004, Skp2 mediated degradation of p27/Dap is required in G2-M to activate CDK1 (Nakayama et al., 2004). In *Drosophila*, overexpression of p27/Dap has been shown to cause G1 arrest by inhibiting Cyclin E-CDK2 activity (de Nooij et al., 1996). p27/Dap has not been shown to bind or inhibit CDK1 function in flies and the role of p27/Dap in flies appears to be limited to G1-S transition and not G2-M (de Nooij et al., 1996; Lane et al., 1996). This led us to suspect that there might be some other way that Skp2 prevents polyploidy that still needs to be explored. It is important to understand this question of how Skp2 protects against polyploidy because a thorough understanding of the potential tumor suppressive role of Skp2 is essential.

The small CDK-associated protein, Cks1, (Cks85A in *Drosophila*) associates with Skp2 to form part of the p27/Dap binding interface of the SCF^{Skp2} complex. Cks1/Cks85A has a conserved phosphate-binding domain that may be largely responsible for the specific interaction of SCF^{Skp2} with the phosphorylated T187

residue on p27/Dap (Ganoth et al., 2001; Hao et al., 2005; Spruck et al., 2001). CDK1 and CDK2 have also been identified in complexes with the SCF-Skp2, linked via Cks1/Cks85A, but their importance for SCF-Skp2 function is not clear (Hao et al., 2005).

Skp2 was first identified as a protein that associates with Cyclin A-CDK2 in transformed as well as normal cells(Zhang et al., 1995). Later studies found that Skp2 interacts with Cyclin A to protect it from p27/Dap mediated inhibition (Ji et al., 2006). In *Drosophila*, Cyclin A is a mitotic cyclin and only interacts with CDK1, not CDK2 as in mammals (Hassel et al., 2014; Knoblich et al., 1994; Mihaylov et al., 2002; Sauer et al., 1995). It is not yet known if Drosophila Cyclin A interacts with Skp2, though interestingly, Cyclin A, like Skp2 seems to be required for preventing polyploidy. Skp2 has not yet been found to interact with mitotic cyclins and have a mitotic role apart from inhibition of p27/Dap (Nakayama et al., 2004).

In this paper, we show that Skp2 has a critical role in mitotic entry. We show that N-terminus of Skp2 physically associates with Cyclin A to protect Cyclin A in G2 phase of the cell cycle. Cyclin A buildup then promotes entry into mitosis. The failure to enter into mitosis in *Skp2* or *Cyclin A* mutants resulted in reversion to a G1 state, leading to polyploidy. We have thus uncovered an important role of Skp2 with Cyclin A during G2/M transition in addition to its established role during G1/S transition.

2.3.1 Drosophila strains and genetics

All flies were maintained at 25°C. The following stocks were obtained from Bloomington Drosophila Stock Centre: *Cyclin* A^{H170} (9096), *Cyclin B2* (6630). *yw* was used as control unless otherwise indicated. The following stock was obtained from Vienna Drosophila Resource Centre: *Cyclin* $A^{v103595}$ *(Cyclin* A^{95}*).* The following stock was obtained from National Institute of Genetics (NIG-FLY): *Cyclin* $A^{5940R-1}$ *(Cyclin* A^{R1}*). Cyclin* $B3^{L6540}$ is a gift from Christian Lehner, University of Zurich. Fzr^{e4} is a gift from Tadmiri Venkatesh, the City College of New York. *UAS-HA-Skp2*, $Skp2^{ex9}$, $Cks85A^{ex15}$, $Skp2^{GD15636}$, $Skp2^{KK2101487}$ were described previously (Ghorbani et al., 2011) The following stocks were made in the Swan lab. *UAS-Venus-Cyclin* A^{21c}, *UAS-Venus-Cyclin* B^{31a} (Dhaliwal, unpublished), *UAS-Venus-CyclinB3* (Swan, unpublished).

2.3.2 Generation of UASp-HA- $Skp2^{\Delta 170}$ *transgenic line*

UASp-HA- $Skp2^{\Delta 170}$ was made by deleting 1-170 amino acids from the N terminal of *Skp2*. *Skp2* cDNA, *RE1525* was used as a PCR template. To attach the *attB* recombination sites on the *Skp2* gene a two-step PCR was performed. In the first step, the *Skp2* gene was amplified by using the following forward and reverse primers attached with partial *attB* sequences. The *Skp2* specific forward primer used is 5'-AAAGCAGGCTTAACGCATGGCCTACCGTTTGTCGACG - 3' and the *Skp2* specific reverse primer used is 5'- GAAAGCTGGGTATTAGTCGCGCGTGCGCAGACCCCA – 3'. In the second – step, PCR was performed to attach the full *attB* recombination sites (*attB1*

and *attB2*) to each end of the *Skp2* gene. The full *attB1* sequence is 5' – ACAAGTTTGTACAAAAAAGCAGGCT – 3' and the full *attB2* sequence is 5'-ACCCAGCTTTCTTGTACAAAGTGGT – 3'. In both the steps, Phusion high-fidelity DNA polymerase from Finnzymes was used. The PCR reaction conditions for the first-step PCR were as follows: initial denaturation at 98°C for 30 seconds 12 cycles of denaturation at 98°C for 10 seconds, annealing at 64.3°C for 30 seconds, and elongation at 72°C for 25 seconds, and final elongation at 72°C for 5 minutes. The PCR conditions for the second step PCR were as follows: initial denaturation at 98°C for 30 seconds, 12 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 30 seconds, and elongation at 72°C for 55 seconds, and final elongation at 72°C for 5 minutes. The PCR product was cloned into Invitrogen Gateway vector pDONR221. The reaction was performed using Invitrogen Gateway BP Clonase enzyme. Once the pENTRY vector was obtained it was then recombined with pPHW vector (UASp promoter N-Terminal 3xHA tag) from Drosophila Genomics Resource Center (DGRC) using Invitrogen Gateway LR Clonase enzyme to obtain the *UASp-HA- Skp2*$^{\Delta 170}$ transgene. Several different transgenic lines were obtained. *UASp-HA-Skp2*$^{\Delta 1702a}$ transgene was used for most experiments. This line when crossed to *daughterless-GAL4* (*da-GAL4*) showed similar levels of protein expression to *UASp-HA-Skp2*. The pENTRY vector was also recombined with pDEST 565 (Plasmid 11520 from Addgene) to get *GST-His- Skp2*$^{\Delta 170}$ plasmid.

2.3.3 Generation of GST-His-Skp2, and MBP-His-Cyclin A plasmid

For the *GST-His-Skp2* and *MBP-His-Cyclin A* plasmids the steps were followed as above. The pENTRY *Skp2* vector was recombined with pDEST 565 (Plasmid 11520 from Addgene) to get *GST-His-Skp2* plasmid and the pENTRY *Cyclin A* vector was recombined with pDEST- His-MBP (Plasmid 11085 from Addgene) For the generation of *MBP-His-Cyclin A* plasmid, *Cyclin A* cDNA (*LD44443*) was used.

2.3.4 GST-His-Skp2, GST-His-Skp2$^{\Delta 170}$ and His-MBP-Cyclin A protein expression and purification

To express the desired proteins for GST pull-down experiments between GST-His-Skp2 or GST-His-Skp2$^{\Delta 170}$ and His-MBP-Cyclin A, the plasmids were transformed into BL21-CodonPlus (DE3) RIL bacterial cells (a gift from Norah Franklin, University of Windsor). The steps used to express the proteins are as follows: A single colony was picked from the plated bacterial cells and inoculated overnight at 37˚C in 100ml of 2TY culture media supplemented with specific antibiotic. The following day 50 ml of the starter culture was added to 450 ml of 2TY media supplemented with appropriate antibiotic and grown in a 37˚C shaker until the OD600 reached 0.7-0.8. At this time IPTG (Thermo Fisher Scientific) was added to the culture to a final concentration of 0.05mM. The culture was then transferred to a 28˚C shaker for 4 hours. After 4 hours, the cells were pelleted at 4˚C at 4000 x g for 15 minutes. The supernatant was decanted and the pellet was resuspended in lysis buffer (50mMTris (8), 2mM EDTA, plus Protease inhibitor

cocktail tablets (Roche Diagnostics) in ice for 40 minutes. The cell suspension was then sonicated on ice in 10 second pulses 10 times and then centrifuged at 4˚C, 10000 x g for 15 minutes. 1ml aliquots of supernatant were collected in the cold room and saved at -80˚C for future use. To promote solubility some variations were done while expressing His-MBP-Cyclin A. IPTG concentration for induction of His-MBP-cyclin A was changed to 1mM when OD600 reached 0.5. In some cases the induced culture was transferred to a 25˚C shaker for 5 hrs instead of 28˚C for 4 hrs.

2.3.5 GST pull-downs

For GST pull-downs 500µl of His-MBP-cyclin A was pre- incubated at 30˚C for 30 minutes followed by incubation on ice for 5 minutes. 1ml of GST His Skp2 or I ml of 1x PBS for controls was added to the His-MBP-Cyclin A lysate and incubated on ice for 15 minutes followed by incubation at 30˚C for 15 minutes. The lysates were then added to 100µl of prewashed Pierce Glutathione Sepharose beads (Thermo Fisher Scientific) and incubated for 2 hours at 4˚C. After 2 hours, the lysate was centrifuged at 2000 rpm for 1 minute at 4˚C. The supernatant was collected and the pellet was washed three times with wash buffer (50mM Tris (8), 150mM NaCl, 0.05% Tween) and two times with wash buffer supplemented with Protease inhibitor cocktail tablets (Roche Diagnostics) at 4˚C. The supernatant and the pellet were boiled with 5x and 2x sample buffer respectively for 10 minutes and then centrifuged at 13,200 rpm for 10 minutes. Samples were then loaded on SDS PAGE gels for further detection and analysis.

2.3.6 Co-Immunoprecipitations (CoIPs) from Larvae and Embryos

HA-Skp2 or *HA-Skp2$^{\Delta 170}$* was crossed with *da-GAL4* to express the protein ubiquitously. 100 brains and imaginal discs of 3rd instar wandering larvae were dissected in 1xPBS and flash frozen in liquid nitrogen. The tissues were then lysed with lysis buffer (50mM Tris (8), 150mM NaCl, 0.05% Tween) supplemented with Halt phosphatase inhibitor cocktail (Thermo Scientific) and Protease inhibitor cocktail tablets (Roche Diagnostics). The lysate was then centrifuged at 14000 rpm for 20 minutes in 4˚C. CoIPs were performed using anti HA affinity agarose beads from Sigma Aldrich as in (Swan et al., 2005). The CoIP of embryos was performed as in (Ghorbani et al., 2011).

2.3.7 Western Blotting

Brains and imaginal discs of 3rd instar wandering larva were dissected in 1xPBS and flash frozen in liquid nitrogen. 1mg of brain and imaginal disc tissues were lysed in 20 µl of 2x sample buffer, boiled for 10 minutes and then centrifuged at 13,200 rpm for 10 minutes at room temperature. Western blotting of the samples was done according to standard techniques. The samples were probed using the following antibodies: mouse anti-Cyclin A antibody (A12) at 1/10, mouse anti-Cyclin B antibody (F2F4) at 1/20, mouse anti-actin antibody at 1/250, all from Developmental studies Hybridoma Bank, rabbit anti-Cyclin B3 antibody at 1/2000, (a gift from Christian Lehner), rabbit anti-Cdk1 (PSTAIRE) antibody from (Santa Cruz) at 1/1000, rat anti-HA antibody (Roche) at 1/1000, mouse anti-Cd2c antibody

at 1/40 (a gift from Christian Lehner). Chemiluminescence imaging and densitometry analysis was performed with an Alpha Innotech FluorChemTM HD2 imager.

2.3.8 Proximity Ligation Assay (PLA)

Duolink *in situ* starter kit from Sigma Aldrich was used for PLA. Experiments were conducted according to the manufacturer's protocol. Briefly, 3rd instar larval wing imaginal discs were incubated with anti-GFP antibodies to detect Venus-Cyclin A, and anti-HA antibodies to detect HA-Skp2. Then the wing imaginal discs were incubated with secondary antibodies provided in the PLA kit, which are conjugated with PLA specific oligonucleotide and a ligation mixture containing ligase and two "PLA probes" and fluorescently labeled nucleotides. If the two proteins of interest are in close proximity then the "PLA probes" will hybridize with oligonucleotides that are bound to the secondary antibody and a closed circular amplification will take place, which will incorporate the fluorescently labeled nucleotides. The amplified product formed as result of PLA specific reaction can be observed under a microscope due to fluorescent labeling.

2.3.9 Drosophila FUCCI system

Fucci (Fluorescent ubiquitination-based cell cycle indicator) system was developed by Zielke and his colleagues to study cell cycle profiles *in vivo* (Zielke et al., 2014). Briefly, the system consists of GFP tagged E2F and mRFP tagged Cyclin

B. To study the cell cycle profile, these two tagged transgenes are expresssed in the cells of intrest. From late M phase to end of G1, E3 ubiqutin ligase APC/C is active. mRFP-cyclin B will be degraded and therfore, the G1 cells will appear green due to accumulation of GFP-E2F. During S phase ubuquitin ligase $CRL4^{Cdt2}$ is active which ubiqutinate GFF-E2F. Therefore, cells appear red due to accucmulation of mRFP-cyclin B. Since APC/C and $CRL4^{Cdt2}$ are absent from G2 to late M phase, the cells appear yellow due to accumulation of both the transgenic proteins.

2.3.10 Cytology and Immunostaining

Third instar wandering larval wing imaginal discs were used for all the experiments unless otherwise stated. Third instar wandering larvae were inverted in 1xPBS and fixed in 4% formaldehyde in 1xPBST (0.2%Tween added to 1xPBS) for 20 minutes on a nutator at room temperature. The samples were then rinsed three times with 1x PBST followed by 3 washes in 1xPBST, 5 minutes each. Then they were blocked in 1x PBST plus 1% BSA for 2 hours on a nutator at room temperature. Primary antibody was added in presence of 1% BSA and nutated overnight at 4 ˚C. The primary antibodies used were rat anti-HA antibody (Roche) at 1/100, rabbit anti-phosphohistone H3 antibody (Santa Cruz) at 1/1000, rat anti-alpha tubulin antibody (Milipore) at 1/500, mouse anti-gamma tubulin antibody (Sigma) at 1/500, rabbit anti-GFP antibody (Abcam) at 1/10000, mouse anti-HA antibody (Abcam) at 1/1000. The following day, the inverted larvae were rinsed three times with 1x PBST followed by 3 washes in 1xPBST, 20 minutes each. They were then

subjected to secondary antibody in the presence of 1% BSA on the nutator in room temperature for 4 hours. Alexa fluor secondary antibodies were used at 1/1000 (Thermo fisher scientific). Finally the larvae were rinsed and washed as before and preserved in 80% glycerol in 4 ˚C. The discs were then dissected out of the inverted larvae and mounted on slides and sealed with nail polish. EDU labelling was performed for detection of S phase cells as in (Ghorbani et al., 2011).

2.3.11 Confocal microscopy

Immunostained imaginal discs were imaged using an Olympus FluoView FV1000 laser scanning confocal microscope. Images were analyzed in Olympus Fluoview software version 1.5. The images were modified (brightness and contrast) and compiled through Adobe Photoshop 2014.

2.3.12 Image J analysis of M phase and S phase indices

Images taken on the confocal microscope were analyzed with image J version 1.49. To measure the mitotic index and S phase index of different genotypes, the scanned images were changed to 8 bit images. The threshold of the image was kept at a default setting. The image particles were analyzed by setting the size of the particles between 70-infinite pixel areas. The image particles were measured from a predefined area within the domain where transgenes were expressed. Using Image J summarize option, the measurement of particles in a 100 pixel area is calculated. The measurements of particles were plotted on Microsoft Excel 2013 to calculate the

standard deviation between different sample measurements. This way of calculating cell cycle phase specific indexes was chosen to essentially determine the area instead of number of cells within an area. The reason for this is that different genotypes have different cell sizes. Thus in genotypes in which cell size is greater, total number of mitotic cells etc would be underestimated. The fucci results of G2-M indexes of different genotypes were calculated by adding RG2B plugin to Image J 1.49. The threshold of red channel and green channel was kept to auto threshold.

2.4 Results

2.4.1 Skp2 is required for entry into mitosis

Loss of Skp2 results in polyploidy in a number of experimental systems (Dui et al., 2013; Ghorbani et al., 2011; Nakayama et al., 2004). The exact cause of polyploidy, however, is not clear and it is possible that this phenotype can arise by more than one means depending on the cell type. In cell culture models, it has been shown that loss of *Skp2* results in elevated Cdt1/Dup levels resulting in aberrant origin firing in G2 of the cell cycle (Li et al., 2003; Nishitani et al., 2006). To determine if the loss of *Skp2* in *Drosophila* leads to aberrant DNA replication in G2, we examined the G2 arrested cells in the wing margin. These cells can be identified based on their expression of Achaete. To determine if these G2 cells replicate, we colabelled these cells for GFP-PCNA, a marker of DNA replication. While other S-phase cells show expression of GFP-PCNA, we did not detect any PCNA in these cells in which *Skp2* was knocked down (Fig. 1A, B) We also examined the cells posterior to the morphogenetic furrow of the eye that normally arrest in G2 to see if these cells incorporate the nucleotide analogue, EDU, a marker of DNA replication. In eye imaginal discs, in which Cdt1/Dup is overexpressed, cells posterior to the furrow continue to incorporate EDU, suggesting that they are re-replicating in G2 (Thomer et al., 2004). In contrast, when we examined eyes from *Skp2* mutants, these showed no sign of re-replication beyond the programmed S-phase in the morphogenic furrow (Fig. 1C). Together these results argue that *Skp2* null cells do not undergo re-replication in G2.

The failure to complete cytokinesis can lead to polyploidy. Due to the fact that cytokinesis occurs after complete sister chromatid segregation in anaphase, the failure of cytokinesis would result in cells having double the number of distinct chromosomes. We performed fluorescent *in situ* hybridization (FISH) to a single chromosome, the X- chromosome, to determine if this is the case in *Skp2* mutants. In wild type males we observe a single FISH signal at all phases of the cell cycle except anaphase when sister chromatids separate (Fig. 1D). In *Skp2* males, we also detect only a single FISH signal per cell, though the FISH signal often appears to be larger than in wild type (Fig. 1D). This indicates that polyploid *Skp2* null cells retain sister chromatid cohesion (at least along peri-centric regions corresponding to the site recognized by the FISH probe). We conclude that cytokinesis failure is not the cause of polyploidy in *Skp2* mutants.

The failure to enter mitosis can also lead to polyploidy (Weaver and Cleveland, 2005). This would be detectable as an overall reduction in the frequency of mitosis. Indeed, the mitotic index for *Skp2* null imaginal discs is lower compared to controls (Figs. 1E, G, I) suggesting that *Skp2* null cells are specifically delayed in the entry into mitosis. The frequency of S phase also appears to be decreased in *Skp2* null cells compared to controls (Figs. 1J-L). This might be due to the up-regulation of p27/Dap in *Skp2* null cells, since upregulation of p27/Dap has been shown to arrest cells in G1 (de Nooij et al., 1996; Dui et al., 2013; Zielke et al., 2014). We also employed the fly FUCCI system to distinguish cell cycle phases *in vivo* (Figs. 1M-P). This reveals that the G1 population is increased in *Skp2* null cells (Fig. 1P). The

G2/M population in *Skp2* null cells is similar to that of wild type cells (Fig. 1P). If we consider that fewer cells go into mitosis in *Skp2* null, we can conclude that the G2 population is at least somewhat greater in the *Skp2* mutant. We conclude that *Skp2* mutants are delayed in G2 and may bypass mitosis.

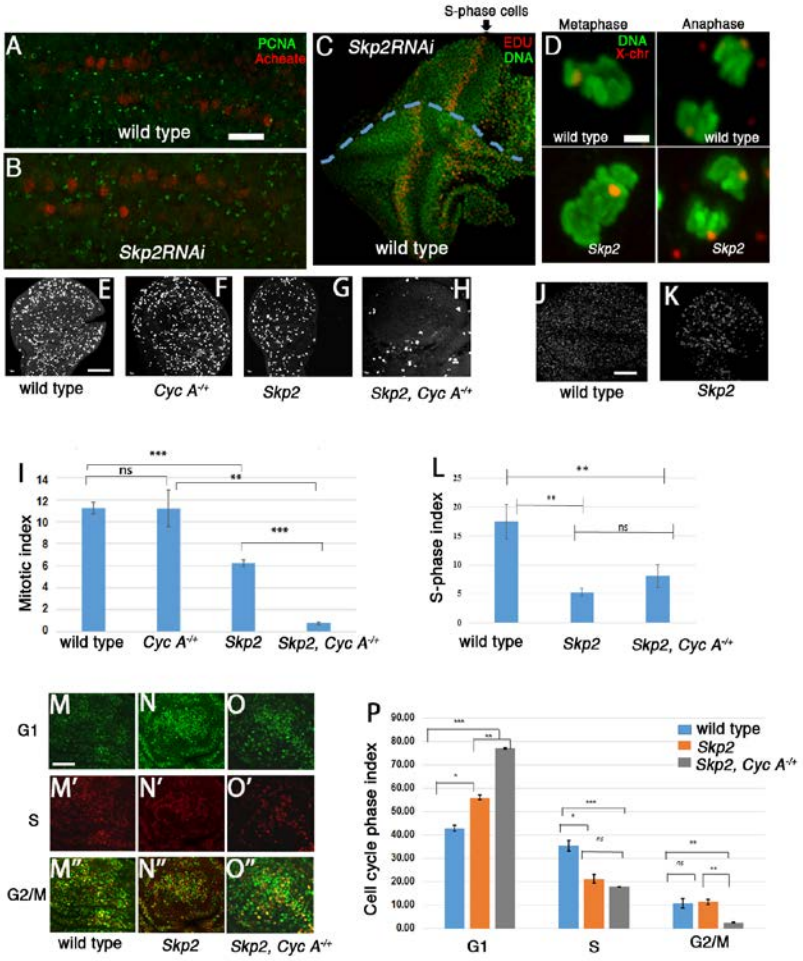

Fig. 1: Skp2 is required for entry into mitosis

Fig. 1: Skp2 is required for entry into mitosis. A-B) Co-labeling of GFP-PCNA (S-phase marker shown in green) and Achaete antibody (G2- phase marker, shown here in red) in the above mentioned genotypes. C) Eye imaginal discs of 3rd instar wandering larvae probed with EDU to label cells in S-phase (red) and with Oligreen to mark DNA (green). *Skp2* was knocked down in the dorsal compartment of these eye imaginal discs. D) Wing imaginal discs of 3rd instar wandering larvae were probed with X –Chromosome FISH probe (red) and stained with Oligreen to mark DNA (green). Metaphase cells in wild type and *Skp2* show a single dot while anaphase in both genotypes have two distinct dots. 1E-H) Representative wild type (E), *Cyclin* $A^{H170+/-}$ (F) $Skp2^{ex9}$ (G) and $Skp2^{ex9}$, *Cyclin* $A^{H170+/-}$ (H) wing imaginal discs from 3rd instar wandering larvae immunostained with phospho-Histone H3 antibodies. I) M phase index of above mentioned genotypes. J-K) Representative wild type (J) and $Skp2^{ex9}$ (K) wing imaginal disc from 3rd instar wandering larvae stained with EDU (I hour incubation). L) S phase index of above mentioned genotypes. M-O'') Representative FUCCI results for wing imaginal discs of different genotypes as shown. M-O) G1-G2-M cells are in green. M'-O') S-G2-M cells are in red. M''-O'') G2-M cells are in yellow. P) Cell cycle phase indices of above mentioned genotypes based on fucci results. Scale bar in E =20 µm, applies to F-H, J-K. Scale bar in M =10 µm, applies to N-O''.

2.4.2 Skp2 is required to maintain mitotic Cyclin levels

The combined effects of mitotic Cyclin build up and activation of CDK-Cyclin complexes determines mitotic entry. To determine how Skp2 could affect mitotic entry we first examined the levels of mitotic cyclins in *Skp2* null cells. We found that loss of *Skp2* results in a reduction in protein levels of all three mitotic Cyclins: A, B and B3 in larval mitotic tissues (Fig. 2A-B). This effect on cyclin level appears to be at the level of protein stability as transgenic Cyclin A, lacking native promotor or UTR sequences and thus refractory to transcriptional and translational control, also accumulates at lower levels in the *Skp2* null background (Fig. 2C-D).

We predict that the reduced level of mitotic cyclins, particularly mitotic Cyclin A, might at least partially explain the polyploidy seen in *Skp2* mutants. As mentioned before, loss of *Cyclin A* has been shown to cause increased ploidy in mitotic cells (Hassel et al., 2014; Mihaylov et al., 2002; Sallé et al., 2012; Sauer et al., 1995).We used two different RNAi lines against *Cyclin A* (*Cyclin* A^{95} and *Cyclin* A^{R1}) to examine the effects of *Cyclin A* loss in the wing imaginal disc. Both lines result in significant knockdown of *Cyclin A*, as judged by western blotting (Figs. 2E-F), and both result in polyploidy in wing imaginal discs (Figs. 2G-I). They also cause increased wing hair spacing in the adult wings (Figs. 2J-L). Each cell of the wing gives rise to a single hair. If the cell becomes polyploid the distance between the wing hairs increases as well. Further, the degree of knockdown correlates with severity of these phenotypes: *Cyclin* A^{95} (Figs. 2F, I, L) results in a greater reduction in protein levels and a more severe wing phenotype compared to *Cyclin* A^{R1} (Figs.

2F, H, K). In addition to the increase in wing hair spacing, *Cyclin* A^{95} wings have an uneven spacing and aberrant orientation of wing hairs. In addition, the area of the wing corresponding to *Cyclin A* knockdown appears severely reduced compared to wild type or *Cyclin* A^{R1}, suggesting that cell viability is compromised.

To test if it is the reduction in Cyclin A levels that is responsible for the polyploidy in *Skp2* null cells, we first asked if further decreasing the levels of Cyclin A (or the other major mitotic cyclins, Cyclin B and Cyclin B3) leads to enhancement of the *Skp2* null phenotype. As previously shown, RNAi against *Skp2* leads to an increase in wing hair spacing and this correlates with increased ploidy in the wing imaginal disc (Dui et al., 2013; Ghorbani et al., 2011). To determine the effect of cyclin dose on this phenotype we expressed *Skp2 RNAi* in a background heterozygous for either *Cyclin A, Cyclin B* or *Cyclin B3* null mutants (Figs. 2M-Q). The reduction of one copy of *Cyclin B, Cyclin B3* or *Cyclin A* did not show an increase in wing hair spacing compared to *Skp2 RNAi* phenotype (Fig. 2O- Q). However, the overall size of the posterior wing is reduced in *Skp2 RNAi, Cyclin* $A^{H170+/-}$ and that led to a difficulty in assessing the changes in the wing hair.

Considering an unclear result from our wing hair spacing experiment, we generated flies homozygous mutant for *Skp2*^{ex9} and heterozygous for either *Cyclin B* or *Cyclin A* null alleles to study the larval stages of these flies. We performed DNA staining of their wing imaginal discs (Figs. 2R-U). Reduction of *Cyclin B* does not have an apparent effect on ploidy of *Skp2* null cells (Fig. 2T), but the loss of one copy of *Cyclin A* leads to a dramatic increase in ploidy (Fig. 2U).

If reduced Cyclin A levels lead to increased polyploidy of *Skp2* null cells we may also expect that overexpression of Cyclin A could rescue the *Skp2* mutant phenotype. We therefore expressed wild type venus-tagged Cyclin A in the *Skp2* RNAi background. This leads to a clear rescue of the increased wing hair spacing phenotype (Fig. 2V). In contrast, overexpression of wild type venus-tagged Cyclin B has no effect on the *Skp2* RNAi wing phenotype (Fig. 2W). Overexpression of wild type venus-tagged Cyclin B3 also led to a partial rescue of the increased wing hair spacing phenotype (Fig.2X).

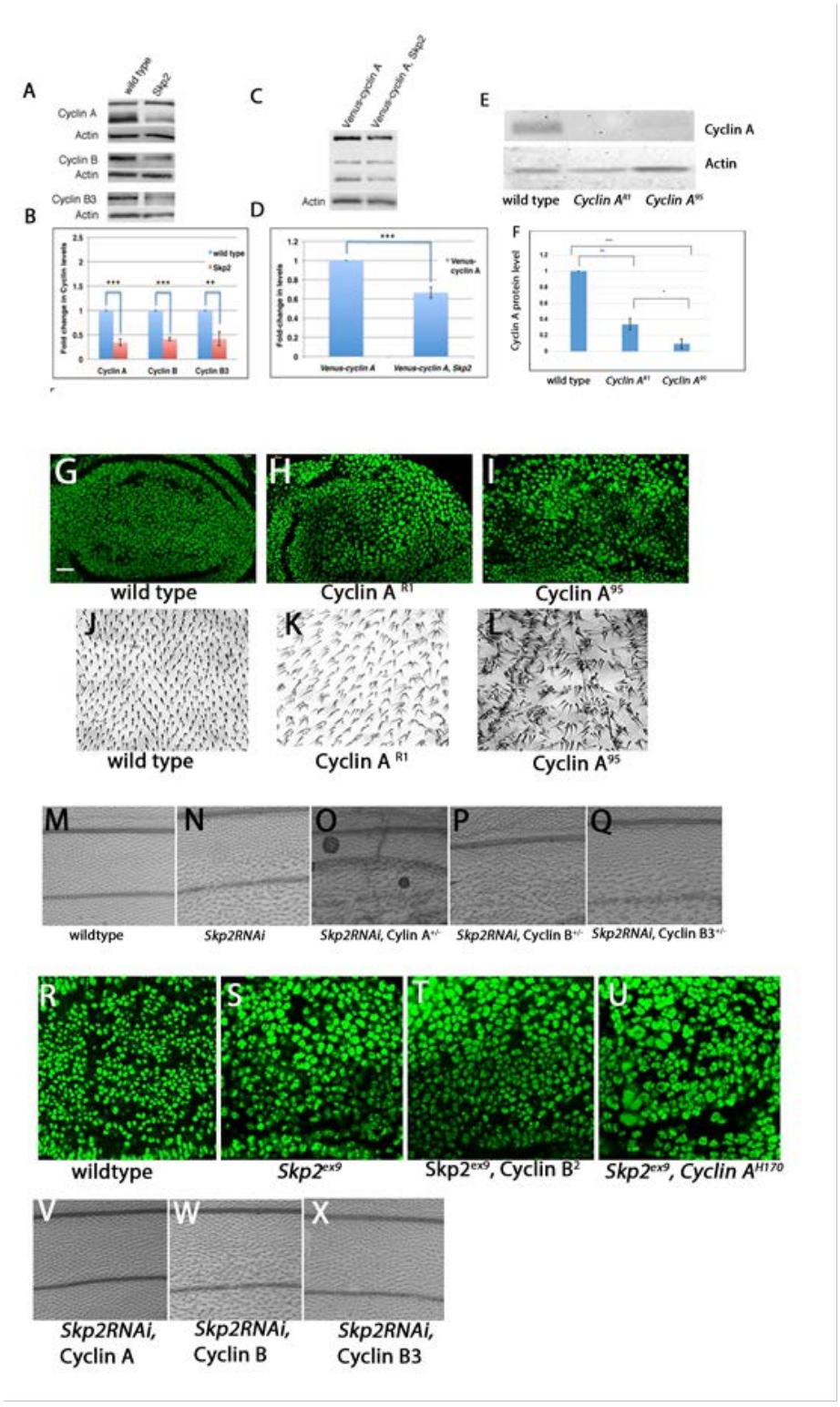

Fig. 2: Skp2 is required for maintaining mitotic cyclin levels

Fig. 2: Skp2 is required for maintaining mitotic cyclin levels. A-B) Western blot and graph on brains and imaginal discs of 3rd instar wandering larvae of the above mentioned genotypes probed for Cyclin A, Cyclin B and Cyclin B3. Actin is used as loading control. C-D) Transgenic Cyclin A lacking native promotor and UTR sequences is reduced in *Skp2*^{ex9} compared to wild type. E-F) Western blot and graph showing the knockdown efficiency of two different *Cyclin A RNAi* lines. Actin is used as loading control. G-I) DNA stained with Oligreen in 3rd instar wing imaginal discs from the genotypes shown. J-L) Representative adult wings from wild type (J), *rn-GAL4*; *Cyclin A*^{R1} (K), rn-GAL4; *Cyclin A*^{95} M-Q, V-X) Representative adult wings of the above mentioned genotypes in which *en-GAL4* is used to drive RNAi expression (R-U) DNA stained with Oligreen in 3rd instar wing imaginal discs from the genotypes shown. Scale bar in G =10 μm and applies to G-I, R-U.

2.4.3 Skp2 is required in G2 to protect Cyclin A from premature degradation

To further examine the relationship between Skp2 and Cyclin A in the wing imaginal disc we compared the effects of *Cyclin A* knockdown to that of *Skp2* null (Figs. 3A- D**)**. While *Skp2* loss results in only a modest decrease in mitotic index (Fig. 1I), knockdown of *Cyclin A* using the stronger RNAi line, *Cyclin* A^{95}, leads to a near complete absence of mitotic cells (Fig. 3C-D). This result is consistent with a critical role for Cyclin A in the entry into mitosis as has been previously described (Buendia et al., 1992; Gong et al., 2007; Jacobs et al., 2001; Lehner and O'Farrell, 1990; Stiffler et al., 1999).

If Skp2 is required for entry into mitosis through Cyclin A we might expect that reducing Cyclin A in the *Skp2* null background would result in a further decrease in mitotic index. To further test this idea we examined flies null for *Skp2* and heterozygous for *Cyclin A* (*Skp2*^{ex9}, *Cyclin* $A^{H170+/-}$). Consistent with this expectation, *Skp2*^{ex9}, *Cyclin* $A^{H170+/-}$ wing imaginal discs have a very low mitotic index (Fig. 1I), similar to the stronger *Cyclin A* knockdown (Figs. 2E-F). Interestingly, *Skp2*^{ex9}, *Cyclin* $A^{H170+/-}$ cells also show a greater predominance of G1 cells than *Skp2*^{ex9} alone, and unlike *Skp2*^{ex9} alone, a reduced frequency of G2 cells, and reduced S-phase index (Figs. 1M-P).

Collectively our results argue that the *Skp2* null phenotype is due to reduced Cyclin A. A partial reduction in Cyclin A level as seen in the *Skp2* null background causes cells to delay in G2. Polyploidy could result if some of these cells, instead of entering mitosis, revert to a G1-like state. These cells then become polyploid after the next S-phase. When Cyclin A is further reduced, as in *Cyclin* A^{95} or Skp2^{ex9},

Cyclin $A^{H170+/-}$, the G2 state is not maintained and cells instead progress through a G1-S endocycle.

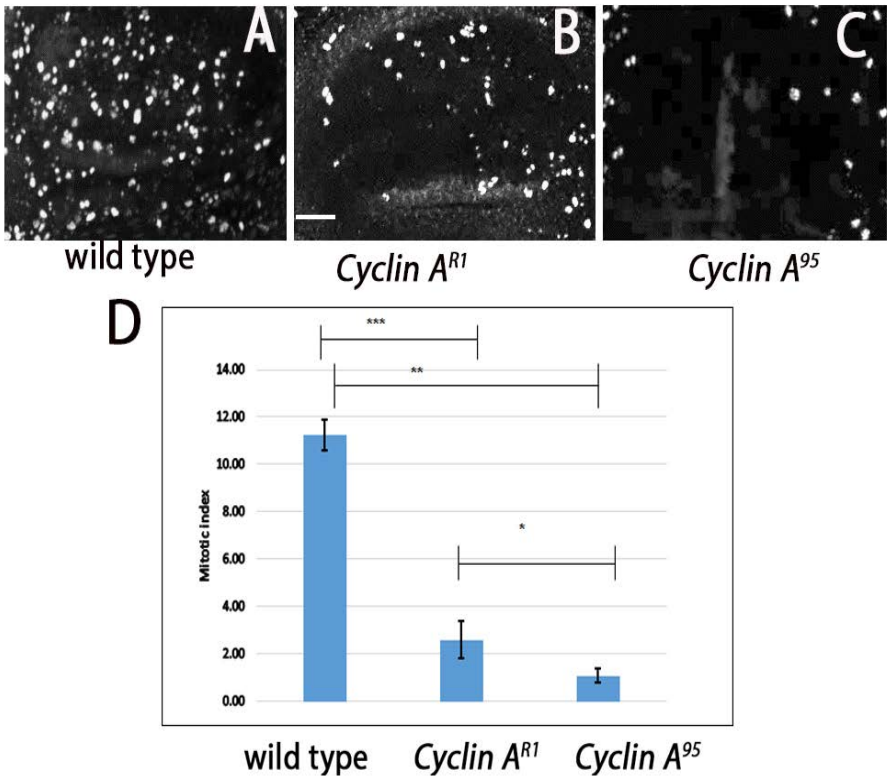

Fig. 3: Knockdown of *Cyclin A* results in less cells in mitosis

Fig. 3: Knockdown of *Cyclin A* results in less cells in mitosis A-D) Wing blade region of the imaginal discs from 3rd instar wandering larvae stained with phospho-Histone H3 to identify the mitotic cells. Representative pictures of *wild type* (A), *Cyclin* A^{R1} (B), and *Cyclin* A^{95} (C). *rn-GAL4* is used to drive the expression of the RNAi. D) M-phase index of above genotypes. Scale bar in B =20 μm and applies to A-C.

2.4.4 A complex of Skp2 and Cyclin A in larval mitotic cells

Skp2 was first identified as a Cyclin A-interacting protein (Yam et al., 1999; Zhang et al., 1995). Skp2 interacts directly with Cyclin A-CDK2 complexes via the N-terminal 90 amino acids of Skp2 (Ji et al., 2006). Given the similar phenotypes and genetic interactions between *Skp2* and *Cyclin A* we wanted to see if these proteins physically interact in *Drosophila*. We therefore, performed co-immunoprecipitations (coIPs) with HA-tagged Skp2 in *Drosophila* brains and imaginal discs. These experiments show that HA-Skp2 interacts *in vivo* with Cyclin A and Cyclin B3 but not with Cyclin B (Figs. 4A-B). We note that Skp2 interacts specifically with the higher molecular weight form of Cyclin A (61 kDA), the form that predominates in mitosis and therefore presumably the active form (Fig. 4A).We also noticed that this band migrates a little higher than 61 kDA when it interacts with Skp2. We are not sure about the kind of modification that happens to Cyclin A when it binds to Skp2, we predict that the migration of Cyclin A band might be due to some post translational modifications, possibly phosphorylation.

Surprisingly, HA-Skp2 did not detectably pull down CDK1 in these same experiments (Fig. 4A), suggesting either that Skp2 interacts with free Cyclin A that is not within CDK1 complexes, or that Skp2 bound Cyclin A is associated with another CDK. In support of this latter possibility, HA-Skp2 pulls down CDK2 in these coIPs (Fig. 4B). Cyclin A associates with both CDK1 and CDK2 in other organisms, but in Drosophila it has only been shown to interact with CDK1 (Knoblich et al., 1994; Sauer et al., 1995). However, the conclusion that Cyclin A can only interact with CDK1 came from IP experiments performed in embryos. In

embryos, Cyclin A did not CoIP with HA-Skp2 but CDK1 did (Fig. 4C). To test the possibility that Cyclin A and CDK2 can interact in larval tissues, we performed CoIP using brain and imaginal discs of 3rd instar wandering larva using MYC-tagged CDK2 and probed for Cyclin A. Our results show that CDK2 does not interact with Cyclin A (Fig. 4F). Presence of Skp2 has been shown to change Cyclin A conformation (Yam et al., 1999). To test if presence of Skp2 results in any change in Cyclin A conformation that leads to Cyclin A-CDK2 interaction, we will perform MYC-tagged CDK2 CoIP in the presence of Skp2. We are currently using CoIPs to test for the existence of Cyclin A-CDK2 complexes in the presence of HA-Skp2 in mitotic tissues of the *Drosophila* larva. The presence of CDK2 in our HA-Skp2 IPs could also represent an interaction between HA-Skp2 and Cyclin E-CDK2 complexes, a possibility that we will also address.

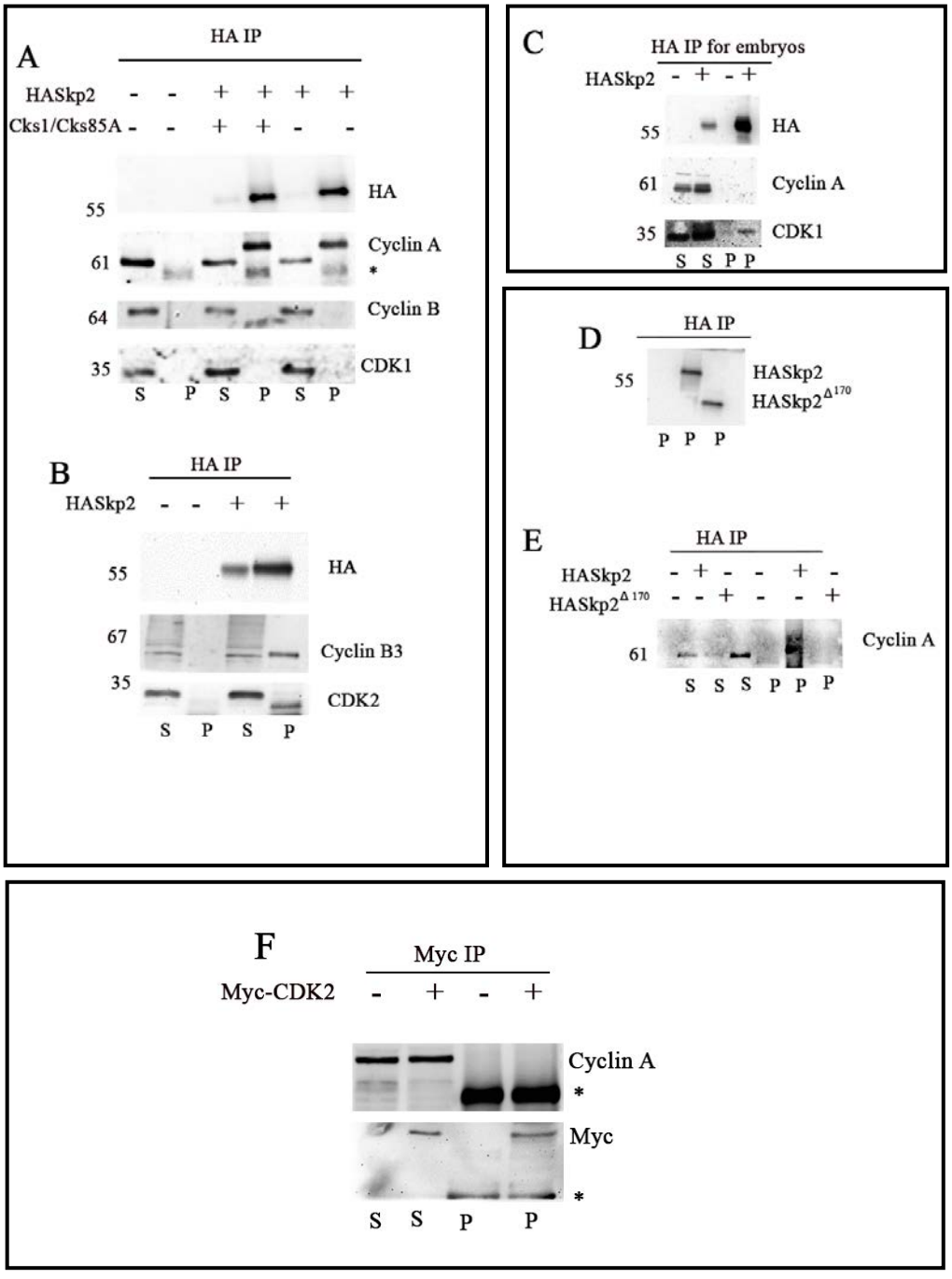

Fig. 4: Skp2 interacts with Cyclin A in larval mitotic cells

Fig. 4: Skp2 interacts with Cyclin A in larval mitotic cells. A) HA-Skp2 IP in wild type or *Cks1/Cks85A* null (indicated by a + or -). IP was performed with 3rd instar larval brains and imaginal discs. Cyclin A but not Cyclin B or Cdk1 is pulled down (S, Supernatant; P, IP pellet). B) HA-Skp2 IP in wild type 3rd instar brains/imaginal discs. C) Cyclin B3 and the lower molecular weight band of Cdk2 is pulled down. C) HA-Skp2 IP in wild type embryos. Cdk1 but not Cyclin A is pulled down. D-E) HA-IP with above mentioned genotypes in wild type 3rd instar brains and imaginal discs. (E) Cyclin A is pulled down with HA-Skp2 but not with HA-Skp2$^{\Delta 170}$. (F) Myc-CDK2 IP was performed with 3rd instar larval brains and imaginal discs. Cyclin A was not coIP'd with Myc-CDK2. * indicates antibody heavy/light chain.

2.4.5 Skp2-Cyclin A interaction is independent of Cks1/Cks85A

Skp2 could potentially interact with Cyclin A indirectly through CKS1/Cks85A since CKS1/Cks85A interacts with Skp2 and with CDKs (Cardozo and Pagano, 2004). To determine if this is the case, we repeated the HA-Skp2 IP in a background homozygous for *Cks85*^{ex15}, a null allele of *CKS1/Cks85A*. We find that Cyclin A still interacts with Skp2 in the *Cks85*^{ex15} background (Fig. 4A) indicating that the interaction is not dependent on CKS1/Cks85A. Skp2 in human appears to be able to interact directly with Cyclin A on the N-terminal domain of Skp2 (Ji et al., 2006). To determine if this domain is similarly mediating interaction with Cyclin A in *Drosophila* we generated a N-terminal truncated form of Skp2, HA-Skp2$^{\Delta 170}$ that retains the N-terminal F-box and LRR repeats required for SCF interaction and substrate recognition respectively. CoIPs with HA-Skp2$^{\Delta 170}$ reveals that it is not able to interact with Cyclin A (Fig. 4E).

To determine if Skp2 and Cyclin A directly interact, we expressed these proteins in bacteria (Figs. 5A-B) and performed a GST pull-down assay. GST-Skp2 but not GST-Skp2$^{\Delta 170}$ is able to pull down MBP-Cyclin A *in vitro* (Fig. 5C-D). An N-terminal mutated version of Skp2 (GST-SKP2^{AAAA}) has shown not to interact with Cyclin A in human. We expressed GST-Skp2^{AAAA} in bacteria and found that it pulls down MBP-Cyclin A *in vitro* (Fig. 5D). Our results show that *Drosophila* Skp2 interacts directly with Cyclin A, and this interaction depends on sequences on the N-terminus of Skp2.

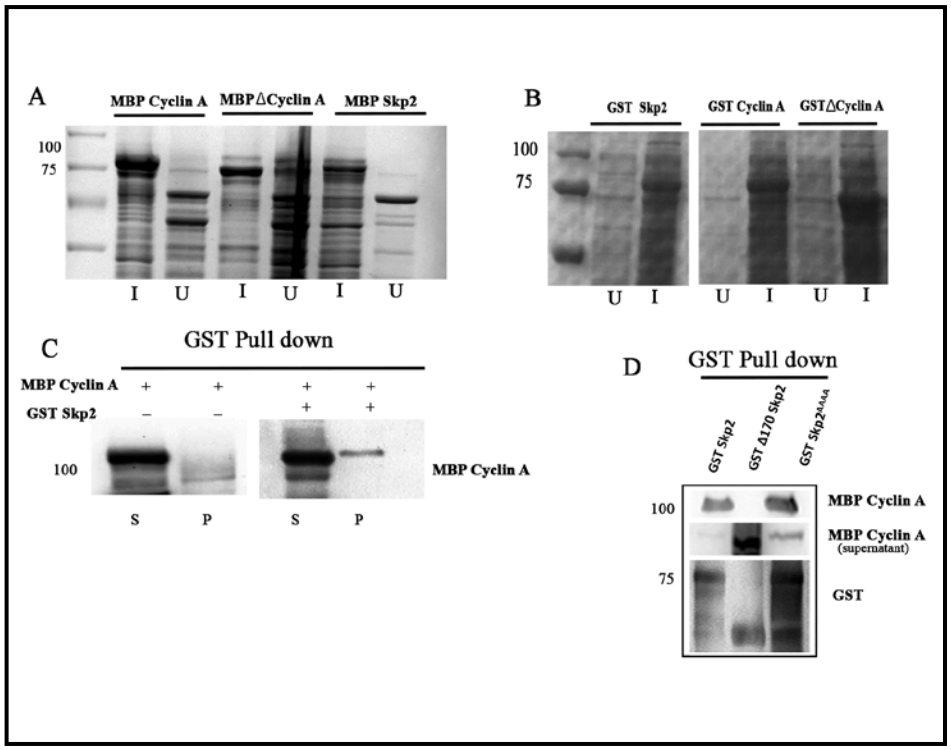

Fig. 5: Skp2 interacts directly with Cyclin A

Fig. 5: Skp2 interacts directly with Cyclin A. A-B) Coomassie staining of bacterially produced His-MBP-Cyclin A, His-MBP-Cyclin $A^{\Delta 1-53}$, His-MBP-Skp2 (A), and GST-His-Cyclin A, GST-His-Cyclin $A^{\Delta 1-53}$, GST-His-Skp2 (B), before (U) and after induction (I) with IPTG C) GST pull-down assay with GST Skp2 and His-MBP-Cyclin A. D) GST pull-down assay with indicated GST fusion proteins and His-MBP-Cyclin A. His-MBP-Cyclin A was not pulled down by GST-His-$Skp2^{\Delta 170}$ whereas GST-His-$Skp2^{AAAA}$ and GST-His-Skp2 pulled down His-MBP-Cyclin A. Indicated GST fusion proteins labelled with anti GST antibody.

2.4.6 Skp2 and Cyclin A interact in G2

We predict that Skp2 interacts with Cyclin A in G2 of the cell cycle and that this is important either for Cyclin A activity or to maintain the stability of Cyclin A, or both. Cyclin A protein accumulates in the cytoplasm and nucleus in S-phase and increases in levels until prometaphase when it is subjected to degradation via APC-CDC20/Fzy mediated ubiquitination (Sigrist et al., 1995; Su et al., 1998). We found that HA-Skp2 shows a similar localization: it is present in both cytoplasm and nucleus (Figs. 6A-A''). To determine if Skp2 also shows the same temporal accumulation as Cyclin A we co-labeled imaginal discs with phospho Histone H3 and gamma tubulin to determine mitotic phases. Skp2 seems to slowly disappear in prometaphase/metaphase (Figs. 6C- E') similar to Cyclin A. Using the tagged, constitutively expressed HA-Skp2 and Venus-Cyclin A transgenes we performed co-localization studies. The relatively uniform distribution of both Cyclin A and HA-Skp2 make it difficult to assess co-localization, though many specific sites of high Skp2 concentration correspond to sites of strong Cyclin A accumulation (Figs. 6F-F'). To better assess co-localization we employed the proximity ligation assay (PLA). In PLA, a fluorescent signal is generated only in cases where the two proteins are within 40 nm of each other. By this method we detect a specific interaction between HA-Skp2 and Venus-Cyclin A (Fig. 7A-A'). This signal is found in both nuclei and cytoplasm. Interestingly, PLA signal is enriched in a stripe of cells in the wing disc that appears to correspond to the G2 arrested cells of the Zone of non-proliferating cells (Fig. 7A',C).

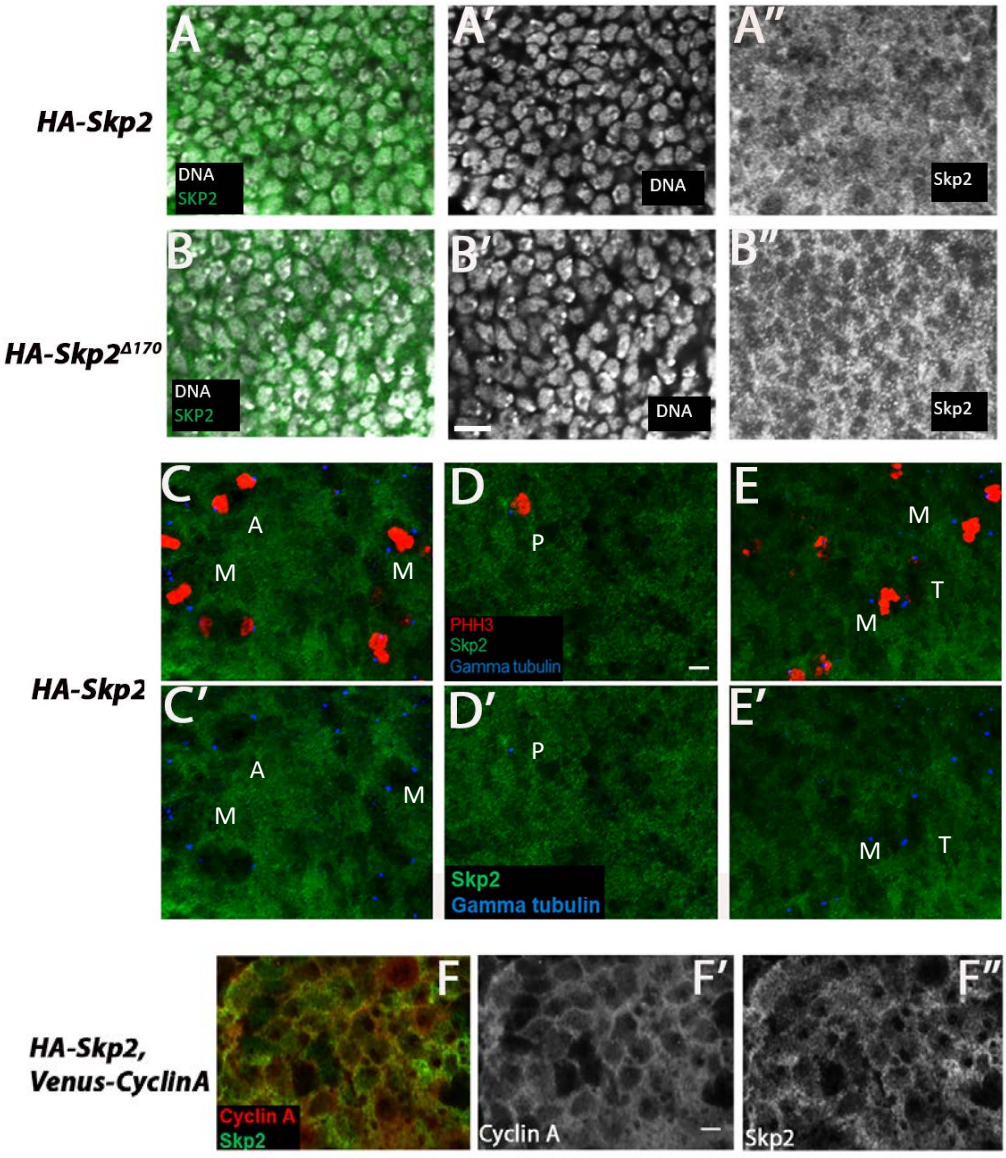

Fig. 6: Localization of Skp2 in larval mitotic cells

Fig. 6: Localization of Skp2 in larval mitotic cells. A-A'') Expression of UAS-HA-Skp2 in the blade region of wing imaginal disc of 3rd instar wandering larvae showing nuclear and cytoplasmic localization of Skp2. C-E') Localization of Skp2 in different phases of mitosis. P refers to prophase, M refers to metaphase, A refers to anaphase and T refers to telophase. F-F') Skp2 and Cyclin A protein show nuclear and cytoplasmic colocalization. Scale bar in D =5 µm, applies to C-E', F-F''. Scale bar in B' =10 µm, applies to A-B''.

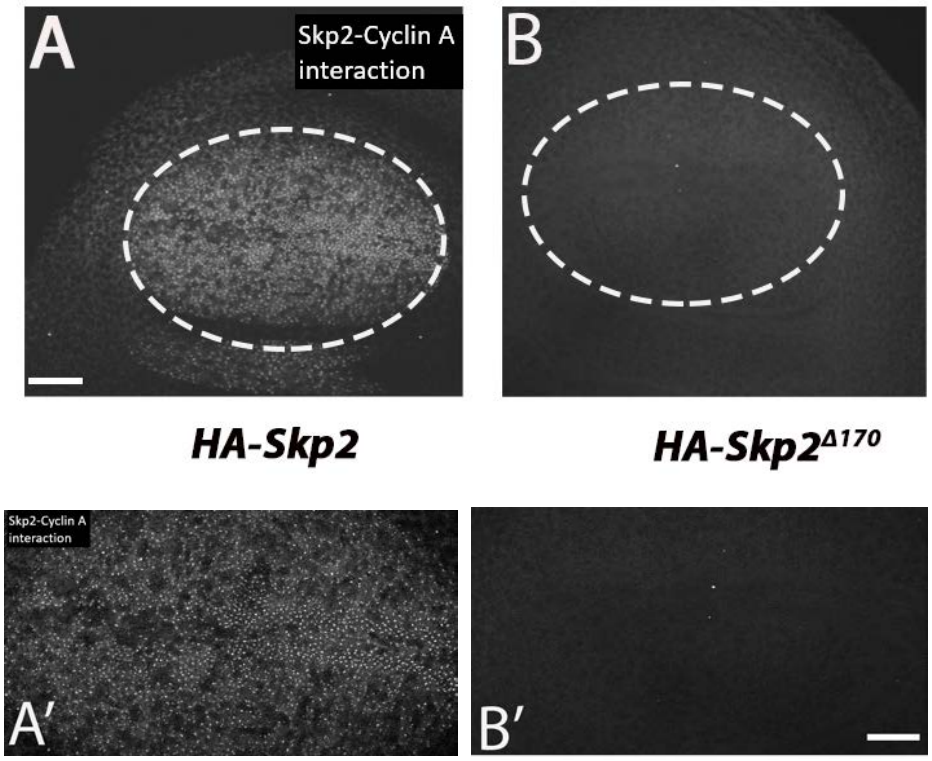

Fig. 7: Skp2 and Cyclin A interacts in G2

Fig. 7: Skp2 and Cyclin A interacts in G2. A-B') Wing imaginal disc of 3rd instar wandering larvae of the mentioned genotypes. The proximity ligation assay (PLA) signals (white dots) show Skp2 and Cyclin A interaction. C) Cartoon showing the region of G2 arrested cells in the wing imaginal disc. Scale bar in A =10 µm, applies to A-B'.

2.4.7 N- terminus of Skp2 is important for maintaining diploidy

We predicted that *Skp2* mutants that lack the ability to interact with Cyclin A would be non-functional. We tested this by expressing *HA-Skp2$^{\Delta 170}$* in *Skp2* null background. While full length *HA-Skp2* rescues *Skp2^{ex9}*, this truncated form completely fails to rescue (data not shown)**.** This is despite the fact that the truncated protein retains its F-box and its C-terminal LRR domains, and is therefore predicted to still be able to interact with the SCF complex, and with substrates such as p27.

Polyploidy in *Skp2* null wing imaginal disc cells can be fully rescued by overexpressing *HA-Skp2*. To address if the N-terminal Skp2 is responsible of this rescue, we expressed *HA-Skp2$^{\Delta 170}$* in *Skp2* null background. The results show that the ploidy observed in *Skp2 null* is not rescued by *HA-Skp2$^{\Delta 170}$* whereas full length *Skp2* completely rescued the phenotype (Fig. 8C-D). This result indicates that Skp2-Cyclin A interaction is important for genome stability.

Could HA-Skp2$^{\Delta 170}$ failed to interact with Cyclin A due to mis localization? We considered this because the N-terminus of Skp2 might have a putative NLS and deleting it might localize Skp2 in the cytoplasm. Our staining shows that the N-terminal deleted Skp2 is localized in the nucleus as well as the cytoplasm like full length Skp2 (Fig. 6A-B''). Interestingly, the accumulation of HA-Skp2$^{\Delta 170}$ was persistent in mitotic cells (Fig. 8B-B''). We observed strong often punctate accumulation of HA-Skp2$^{\Delta 170}$ in metaphase and anaphase cells whereas full length Skp2 slowly disappears during prometaphase and metaphase (Fig. 8A-B''). Persistence of HA-Skp2$^{\Delta 170}$ signal longer than full length Skp2 might indicate that the N terminal of Skp2 is needed for its degradation. Indeed we found one potential

D box, RLSLGSTGD (amino acid sequences 52-61) in the N terminus of *Drosophila* Skp2 that is similar to the D box 2 in human Skp2 (RKHLQEIPD, sequences 84-93). However, mutated D box 2 in human Skp2 was efficiently degraded like wild type Skp2 (Bashir et al., 2004). Despite the persistence of Skp2 in anaphase cells, it does not cause any lethality because ubiquitous expression of the transgene *HA-Skp2$^{\Delta 170}$* in flies is not lethal (data not shown). Our CoIP and GST pull down experiments showed that the sequences on the N terminus of Skp2 is necessary for Cyclin A interaction (Fig. 4D-E, 5E). Because there is no interaction between the N-terminal deleted Skp2 and Cyclin A, we predict that in our Proximity Ligation assay we will not observe any signal in the cells that are expressing HA-Skp2$^{\Delta 170}$ and Venus-Cyclin A. As anticipated, we observed strong PLA signal in wing disc that were expressing HA-Skp2 and Venus-Cyclin A but not in the wing disc that were expressing HA-Skp2$^{\Delta 170}$and Venus-Cyclin A (Fig. 7A-B').

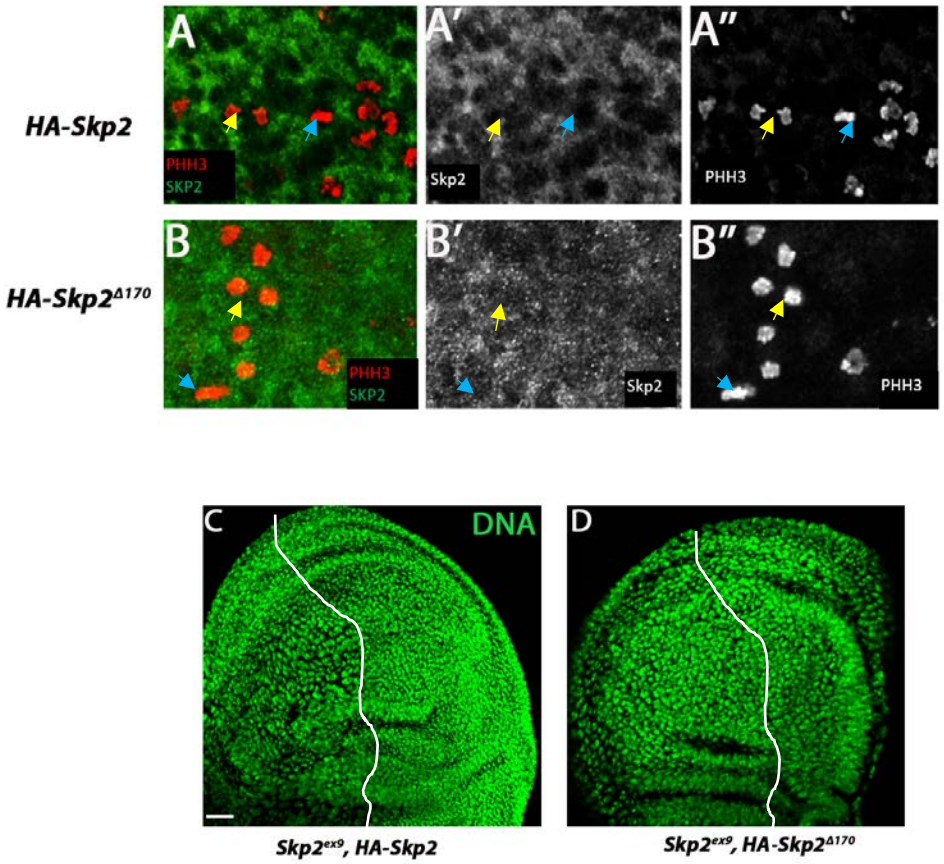

Fig. 8: Skp2-Cyclin A interaction is important to maintain diploidy

Fig. 8: Skp2-Cyclin A interaction is important to maintain diploidy. A-A'') Blade region of wing imaginal disc of 3rd instar wandering larvae showing mitotic localization of *HA-Skp2* and *HA-Skp2$^{\Delta 170}$*. A-A'') Localization of full length Skp2 in metaphase and anaphase. No HA-Skp2 localization in metaphase (blue arrowheads in (A-A'') or anaphase cells (yellow arrowheads in A-A''). B-B'') Localization of HA-Skp2$^{\Delta 170}$ in metaphase and anaphase. The blue arrowheads in (B-B'') show strong Skp2 protein in metaphase and the yellow arrowheads in (B-B'') also show Skp2 protein in anaphase. C-D) Rescue of ploidy in full length *HA-Skp2* and *HA-Skp2$^{\Delta 170}$* expressed region. These transgenes are expressed with *en-GAL4* driver in *Skp2* null background. In (F) full length *HA-Skp2*, ploidy is rescued completely whereas in (G) *HA-Skp2$^{\Delta 170}$* the ploidy did not get rescued. *en-GAL4* is expressed on the right side of the white line. Scale bar in A =10 μm, applies to A-B''. Scale bar in C =10 μm, applies to C-D.

2.5 Discussion

Skp2 and its partner, Cks1/Cks85A have a well characterized function in targeting the CKI, p27/Dap for destruction (Carrano et al., 1999; Sutterlüty et al., 1999; Tsvetkov et al., 1999). This critical function explained to a large degree the oncogenic properties of Skp2: its overexpression leads to a decrease in p27/Dap levels resulting in a failure to arrest cells in G1 (Chiarle et al., 2002; Fagan-Solis et al., 2014; Lim et al., 2002). Conversely, the loss of *Skp2* results in reduced overall growth, apparently due to elevated p27/Dap levels and thus inhibition of cell division. The *Drosophila* Skp2 and Cks1/Cks85A proteins appears to share with vertebrates this important role in regulating p27/Dap levels (Dui et al., 2013). In addition to this oncogenic activity, Skp2 might have tumour suppressive functions. Mice and *Drosophila* null for *Skp2* displayed polyploidy, a phenotype that is frequently associated with tumour formation (Ghorbani et al., 2011; Nakayama et al., 2000; Nakayama et al., 2004). In this paper, we investigated the cause of this polyploidy. We showed evidence that polyploidy in *Skp2* null *Drosophila* is a consequence of failure of cells to enter mitosis. Cells that failed to enter mitosis revert to a G1-like state and thus were able to undergo replication at the next S-phase (Davoli and de Lange, 2011; Hassel et al., 2014). Our results clearly showed that the N-terminus of Skp2 is responsible for maintaining the genome stability as *HA-Skp2*$^{\Delta 170}$ (lacking the N- terminal of Skp2) did not rescue the ploidy of Skp2 whereas full length Skp2 did. We are currently in the process of expressing *HA-Skp2*$^{\Delta 171\text{-}499}$ (lacking the LRR domains) in flies. We would predict that this version of Skp2 will retain its ability to bind and protect Cyclin A, and thus would rescue the polyploidy

phenotype of a *Skp2* null mutant. We will also make smaller deletions of the N-terminus of Skp2 to identify the interaction domain of Cyclin A.

The G2 role of Skp2 appeared to be distinct from its activity in G1-S role. Loss of *Skp2* resulted in both a decrease in S-phase and mitotic indexes. The former, we expected based on the established role for Skp2 in p27/Dap destruction (Carrano et al., 1999; Dui et al., 2013; Sutterlüty et al., 1999; Tsvetkov et al., 1999). The latter, we proposed is due to a partial failure of these cells to enter mitosis. Importantly, the effect on mitosis (but not on S-phase) is dramatically enhanced by loss of a single copy of *Cyclin A.* This is a key result in that it confirms that Skp2 is indeed required for entry into mitosis and equally important it points towards a critical interaction between Skp2 and Cyclin A.

The CoIP experiments showed that the N-terminus of Skp2 interacts physically with the active phosphorylated form of Cyclin A, which in *Drosophila* functions primarily if not exclusively in mitosis (Sigrist et al., 1995; Sigrist and Lehner, 1997). The active form of Cyclin A might result from auto phosphorylation of Cyclin A, as fly Cyclin A has five auto phosphorylation sites (Ramachandran et al., 2007). Results from our PLA experiment showed that Skp2 and Cyclin A appear to form a complex specifically in G2 of the cell cycle and both proteins disappear early in mitosis. In the absence of *Skp2*, Cyclin A levels are reduced and we showed that the effect on these levels is posttranslational. In addition to this, we also found that the mitotic specific active form of Cyclin B3 also interacts with Skp2. Cyclin B3 has not been paid much attention in cell cycle regulation but it will be interesting to tease apart the function of this specific interaction.

Another interesting observation that emerges from our research is the finding that in mitotic tissues (brain and associated mitotic discs) Cyclin A interacts with Skp2 in a complex that does not involve Cdk1. On the other hand we find that the active form Cdk2 is able to interact with Skp2 (O'Connor et al., 1993; Pagano et al., 1993). It is possible that Skp2 only interacts with a complex of Cyclin A-Cdk2 in the mitotic brain tissues. However, our Cyclin A-Cdk2, CoIP experiments did not show such interaction. Another possibility might be that Cyclin A-CDK2 interacts only in the presence of Skp2 overexpression. We are currently testing on this possibility. It is also possible that Cdk2 exists within a distinct Skp2 complex from that containing Cyclin A. In *Drosophila*, the only known partner of Cdk2 is Cyclin E. Skp2 targets Cyclin E for destruction in mammalian cells and therefore, the interaction between Skp2 and Cdk2 may reflect the targeting of Cyclin E for ubiquitination.

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CHAPTER 3

INTERACTIONS AMONGST SKP2, CYCLIN A AND APC-CDH1/FZR

3.1 Summary

S phase kinase associated protein Skp2 is the F box protein and substrate recognition component of the E3 ubiquitin ligase, the SCF complex. Skp2 is a potential oncogene and its critical target is Cyclin Dependent kinase inhibitor p27/Dap. Overexpression of Skp2 results in down regulation of p27/Dap and is observed in a variety of cancer types. Skp2 protects Cyclin A during G1-S phase transition as well as during mitotic entry. Loss of Skp2 results in loss of Cyclin A and other mitotic cyclins that results in impaired mitotic entry. This forces the mitotic cycle to enter an endoreplication cycle. The exact mechanism by which loss of Skp2 results in loss of Cyclin A and other mitotic cyclins is still unknown. Our paper tries to address this question? Here we show that *Skp2* and *Cyclin A* are antagonistic with *CDH1/Fzr*, which is the substrate recognition component and activator of another E3 ubiquitin ligase, the Anaphase Promoting Complex, APC. Our studies show that Skp2 does not affect the protein stability or localization of CDH1/Fzr nor does it compete with CDH1/Fzr to bind on Cyclin A. We also tested the idea that p27/Dap overexpression might indirectly lead to Cyclin A downregulation in *Skp2* null background. Our results show that the loss of Cyclin A leading to inability to enter mitosis in *Skp2* null cells is not due to p27/Dap overexpression.

3.2 Introduction

A cell cycle is the controlled regulation of a transition from one cell phase to another cell phase involving the strategic cooperation of a community of key regulatory proteins. Programmed protein degradation by ubiquitination is a critical pathway for cell cycle regulation. Two well-characterized and studied E3 ubiquitin ligases are the Skp1 Cullin F box (SCF) complex and Anaphase Promoting Complex (APC) (Nakayama and Nakayama, 2006; Vodermaier, 2004). The Anaphase Promoting Complex, or Cyclosome, is a 1.5 MDa protein complex with one or two copies of 19 different subunits (Pines, 2011). The subunits are divided into two distinct functional sub-complexes: the substrate recruitment sub-complex and the catalytic sub- complex. The substrate recruitment sub-complex is composed of Tetratricopeptide repeats (TPR) containing proteins and binds to the substrates. The catalytic complex interacts with the E2 ubiquitin ligase. Two WD repeat containing co-activators, CDC20/Fzy and CDH1/FZR, act as the substrate recognition subunit of the APC complex (Pines, 2011; Schwab et al., 1997; Schwab et al., 2001; Sigrist and Lehner, 1997; Visintin et al., 1997).

APC activity is regulated by Cyclin-CDK-mediated phosphorylation throughout the cell cycle to ensure proper cell cycle progression (Kraft et al., 2003). Unscheduled APC activation can trigger premature cyclin degradation and sister chromatid separation without proper kinetochore microtubule attachment. This can lead to an unequal division of nuclear material, and ultimately genome instability. The APC core complex and its activators are regulated by phosphorylation, which

can either activate or inactivate the APC co-activators. The Cyclin B-CDK1 mediated phosphorylation dependent activation of the APC activator CDC20/Fzy has been well studied (Kraft et al., 2003; Kramer et al., 2000). However, a recent study showed that phosphorylation can also inactivate CDC20/Fzy (Hein and Nilsson, 2016). According to the recent study, Cyclin A-CDK2 inactivates CDC20/Fzy during interphase; and in G2 just before entry into mitosis, Cyclin B-CDK1 activates CDC20/Fzy, which then binds to the APC core complex (Hein and Nilsson, 2016). Although APC-CDC20/Fzy is active during prophase of mitosis, it cannot degrade key mitotic substrates such as Securin/Pim and mitotic cyclins such as Cyclin B and Cyclin B3; this is due to the inhibitory binding of spindle assembly checkpoint (SAC) proteins. Spindle assembly checkpoint (SAC) proteins bind to APC-CDC20/Fzy and prevent its substrate degradation until the proper kinetochore microtubule attachment happens during metaphase (Lara-Gonzalez et al., 2011; Sacristan and Kops, 2015). Interestingly, Cyclin A is degraded by CDC20/Fzy despite the activation of SAC. Several studies have examined the ubiquitination of Cyclin A by CDC20/Fzy. Some studies have suggested that Cks1/Cks85A or Cks2/Cks30A binds to Cyclin A-CDK1 and recruits Cyclin A to the active CDC20/Fzy. This results in the degradation of Cyclin A during prometaphase (Wolthuis et al., 2008). Other studies have suggested that Cyclin A competes with SAC proteins to bind to CDC20/Fzy so that Cyclin A can be degraded (Di Fiore and Pines, 2010). After the SAC is satisfied, the active APC-CDC20/Fzy degrades Securin/Pim and the mitotic cyclins, Cyclin B and Cyclin B3, causing sister chromatid separation and the metaphase to anaphase transition (den Elzen and Pines,

2001; Geley et al., 2001; Hagting et al., 2002). Degradation of mitotic cyclins then result in low CDK1 activity, which activates CDH1/Fzr, the other APC activator. Unlike CDC20/Fzy, dephosphorylation activates CDH1/Fzr during late M phase. CDH1/Fzr stays active until late G1 phase as long as CDK activity is low and continues to promote the degradations of the mitotic cyclins, Cyclin A, Cyclin B, and Cyclin B3 (Kraft et al., 2003; Kramer et al., 2000).

CDH1/Fzr was first identified in *Drosophila* by Sigrist and Lehner in 1997, when they used *CDC20/Fzy* cDNA of *Xenopus* to clone a homologous gene from *Drosophila.* They named the gene *fizzy-related* (*Fzr*) (Sigrist and Lehner, 1997). Later, it was shown that the already-identified *Drosophila* gene *rap* (retina aberrant in pattern) encodes the CDH1/Fzr protein (Karpilow et al., 1989; Pimentel and Venkatesh, 2005). A maternally-derived *CDH1/Fzr* transcript disappears during the onset of syncytial embryonic cycles; zygotic appearance of CDH1/Fzr was observed during the 13^{th} embryonic divisions. In embryos, CDH1/Fzr is expressed in salivary gland placodes and tissues that are post mitotic (Reber et al., 2006). A high level of CDH1/Fzr was observed in mitosis 16 just before terminal mitosis, when G1 phase is introduced in cellularized embryos (Reber et al., 2006). During G1, mitotic cyclins are ubiquitinated by CDH1/Fzr (Sigrist and Lehner, 1997). In cellularized embryos, overexpression of CDH1/Fzr down-regulates mitotic cyclins and in CDH1/Fzr mutant embryos, the G1 phase is not established and cells enter S phase. (Reber et al., 2006; Sigrist and Lehner, 1997).

In *Drosophila*, Cyclin A is a mitotic cyclin. *Cyclin A* mutant embryos do not enter mitosis 16, despite the presence of Cyclin B and Cyclin B3 in those embryos

(Jacobs et al., 2001; Jacobs et al., 1998; Reber et al., 2006). Even the overexpression of Cyclin B and Cyclin B3 does not restore mitosis 16 in *Cyclin A* mutant embryos. (Reber et al., 2006). However, mitosis 16 is fully restored in *Cyclin A* and *CDH1/Fzr* double mutant embryos (Reber et al., 2006). These results point towards the critical role that Cyclin A has in mitotic entry, which is to inhibit CDH1/Fzr. Interestingly, Cyclin A can also inhibit CDH1/Fzr during the S and G2 phases of the cell cycle (Reber et al., 2006; Sigrist and Lehner, 1997; Sørensen et al., 2001). In mammals, the phosphorylation-dependent inhibition of CDH1/Fzr by Cyclin A is well established, but in *Drosophila*, the inhibition of CDH1/Fzr is not phosphorylation-dependent (Reber et al., 2006; Sørensen et al., 2001). The mechanism by which Cyclin A regulates CDH1/Fzr in *Drosophila* has not yet been addressed.

As discussed in previous papers, the E3 ubiquitin ligase SCF-Skp2 is a well-known oncogene. The most critical and well-studied target of Skp2 is p27/Dap (Carrano et al., 1999; Dui et al., 2013; Ganoth et al., 2001; Hara et al., 2001; Nakayama et al., 2004; Sutterlüty et al., 1999; Tsvetkov et al., 1999). Our work with Skp2 showed that it is essential for maintaining mitotic cyclin levels (Das et al., 2016). We also observed that the low mitotic cyclin level in *Skp2* null cells was due to a premature degradation of Cyclin A. Our studies showed that Skp2 and Cyclin A can interact in G2 of the cell cycle (Das et al., 2016). This suggests that Skp2 has a role in preventing the degradation of Cyclin A before mitotic entry; protection from Skp2 can enable Cyclin A to inhibit CDH1/Fzr. This paper tests the idea that Skp2 protects Cyclin A from CDH1/Fzr, then attempts to determine the mechanism by which CDH1/Fzr is inhibited by Skp2 and Cyclin A. Our results, discussed in this

paper, show that *Skp2* and *Cyclin A* are antagonistic with *CDH1/Fzr*. Our study shows that Skp2 does not directly target CDH1/Fzr for degradation, nor does it cause any change to CDH1/Fzr localization. We tested the idea that Skp2 competes with CDH1/Fzr so that it can bind to Cyclin A. Our results showed that Skp2 might not compete with CDH1/Fzr to bind to Cyclin A.

3.3 Materials and methods

3.3.1 Drosophila strains and genetics

All flies were maintained at 25°C unless stated otherwise. The following stocks were obtained from Bloomington Drosophila Stock Centre: *Cyclin* A^{H170} (9096), *daughterless-GAL4, patched-GAL4, rotund-GAL4* and *engrailed-GAL4*. The following stock was obtained from Vienna Drosophila Resource Centre: *Cyclin* $A^{v103595}$ *(Cyclin* A^{95}*).* *Fzr*^{e4} is a gift from Tadmiri Venkatesh, The City College of New York. *UAS-HA-Skp2*, *Skp2*^{ex9}, *yw*, and *Skp2*^{KK2101487} were described previously (Ghorbani et al., 2011). *UAS-MycDap*^{51} is a gift from Renjie Jiao, Chinese Academy of Sciences. *UASt-Fzr* is a gift from Christian Lehner, University of Zurich. The following stocks were made in Swan lab *UASp-Venus- Cyclin* $A^{\Delta 1\text{-}53}$, *UASp-Venus-Fzr*^{40} (Rajdeep Dhaliwal, unpublished) and *UASp-HA-Skp2*$^{\Delta 170}$ (Nilanjana Das, unpublished).

3.3.2 Generation of His-MBP-Cyclin A, GST-His-Cyclin A and His-MBP-Cyclin $A^{\Delta 1\text{-}53}$*, GST-His-Skp2 and His-MBP-Skp2 plasmids*

For the generation of *His-MBP-Cyclin A, GST-His-Skp2, His-MBP-Skp2* plasmid and *His-MBP-Cyclin* $A^{\Delta 1\text{-}53}$ the steps that were followed is described in (Das et al., 2016). For the generation of *His-MBP-Cyclin* $A^{\Delta 1\text{-}53}$, pENTRY*Cyclin* $A^{\Delta 1\text{-}53}$ (Rajdeep Dhaliwal unpublished) was used. The destination vector used to generate the *His-MBP-Cyclin A, His- MBP- Cyclin* $A^{\Delta 1\text{-}53}$ and *His- MBP-Skp2* constructs is pDEST-His-MBP (Plasmid 11085 from Addgene). The destination vector used to

generate *GST-His-Skp2* and *GST-His-Cyclin A* is pDEST 565 (Plasmid 11520 from Addgene).

3.3.3 Generation of Flag-Fzr plasmid

For the generation of *Flag-Fzr* plasmid, *pTiger-GFP-Fzr* plasmid (Rajdeep Dhaliwal, unpublished) was used. The *Fzr* cDNA was taken out from the donor plasmid by flanking the sites with two fast digest restriction enzymes BamH1 and Xba1 and ligated into a mammalian expression vector, pcDNA3. The pcDNA3 vector was a gift from Elizabeth Fidalgo, and Lisa Porter, University of Windsor.

3.3.4 His-MBP-Cyclin A, GST-His-Cyclin A and His-MBP-Cyclin $A^{\Delta 1-53}$, His-MBP-Skp2, GST-His-Skp2, and Flag- Fzr protein expression and purification

The steps involving the expression and purification of His-MBP-Cyclin A, GST-His-Skp2 proteins were described in (Das et al., 2016). Expression and purification of GST-His-Cyclin A and His-MBP-*Cyclin* $A^{\Delta 1-53}$, His-MBP-Skp2 expression and purification were performed according to the protocol explained in our paper (Das et al., 2016). All these above mentioned plasmids except pcDNA3Fzr were expressed in BL21-CodonPlus (DE3) RIL bacterial cells (a gift from Norah Franklin, University of Windsor).

pcDNA3Fzr was expressed in Human Embryonic Kidney 293 (HEK-293) cell line, a gift from Elizabeth Fidalgo, and Lisa Porter, University of Windsor.

3.3.5 Human Embryonic Kidney 293 (HEK293) cell culture

HEK 293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) (Thermo-Fisher) and

1% Penicillin-Streptomycin (Thermo-Fisher). The HEK 293 cells were cultured in a special 10 cm cell culture plate with fresh media containing the above mentioned media supplemented with FBS and antibiotics. The cells were raised in a 37ºC incubator with 5% CO2 until it reached 80% confluency.

3.3.6 Transfection of pcDNA3Fzr and protein expression

10 µg of the plasmid, *pcDNA3Fzr* was incubated for 10 minutes at room temperature with 500 µl of DMEM media and 30 µg of transfection agent branched polyethylenime (PEI) from Sigma. This plasmid mixture was then added dropwise on to the HEK 293 cells and left in the 37°C incubator with 5% CO2 for 20 hours for transfection.

The following day the cells were collected from the plate and centrifuged at 800 x g for 5 minutes at 4°C. The pellet containing the cells was lysed using a lysis media containing 20 mM Tris (pH 7.5), 100 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 5µg/mL of each of leupeptin and aprotinin and incubated in this media for 30 minutes on ice. Following the incubation the cells were centrifuged at 10,000 x g for 15 minutes at 4° C. The supernatant was aliquoted into 1.5ml eppendorf tubes and stored in -80°C for future use.

3.3.7 GST pull-down experiments

For the GST-His-Skp2 pull-down experiments 500µl of His-MBP-Cyclin A and 500µl of His-MBP-*Cyclin* $A^{\Delta 1\text{-}53}$were pre-incubated at 30˚C for 30 minutes followed by incubation on ice for 5 minutes. 1ml of GST-His-Skp2 or 1ml of 1x PBS for controls was added to the His-MBP-Cyclin A lysate and incubated on ice for 15

minutes followed by incubation at 30˚C for 15 minutes. The lysates were then added to 100µl of prewashed glutathione sepharose beads (Thermo Fisher Scientific) and incubated for 2 hours at 4˚C. After 2 hours, the lysate was centrifuged at 2000 rpm for 1 minute at 4˚C. The supernatant was collected and the pellet was washed three times with wash buffer (50mM Tris (8), 150mM Nacl, 0.05% Tween) and two times with wash buffer supplemented with protease inhibitor cocktail tablets (Roche Diagnostics) in 4˚C. The supernatant and the pellet were boiled with 5x and 2x sample buffer respectively for 10 minutes and then centrifuged at 13,200 rpm for 10 minutes. Samples were then loaded on SDS PAGE gel for further detection and analysis.

For the GST-His-Cyclin A pull-down experiments the above mentioned protocol was followed. As a negative control GST protein was used.

3.3.8 Competition assay

For the competition assay, the relative amount of His-MBP-Skp2 and GST protein were determined by comparing them with equalized amounts of GST-His-Cyclin A in Coomassie Blue stained gels. For this experiment, GST-His-Cyclin A and GST were pre- incubated with Flag-Fzr at 30˚C for 30 minutes, followed by incubation on ice for 5 minutes. Increasing amounts of His-MBP-Skp2 were added to the GST-His-Cyclin A and Flag-Fzr lysates and incubated on ice for 15 minutes followed by incubation at 30˚C for 15 minutes. The lysates were then added to 100µl of prewashed glutathione sepharose beads (Thermo Fisher Scientific) and incubated for 2 hours at 4˚C. After 2 hours, the lysate was centrifuged at 2000 rpm for 1 minute at 4˚C. The supernatant was collected and the pellet was washed three times with

wash buffer (50mM Tris (8), 150mM NaCl, 0.05% Tween) and two times with wash buffer supplemented with protease inhibitor cocktail tablets (Roche Diagnostics) at 4˚C. The supernatant and the pellet were boiled with 5x and 2x sample buffer respectively for 10 minutes and then centrifuged at 13,200 rpm for 10 minutes. Samples were then loaded on SDS PAGE gel for further detection and analysis.

3.3.9 Western Blotting

Western blotting of the samples was done according to the protocol described in (Das et al., 2016). The samples were probed using the following antibodies: mouse anti-Cyclin A antibody (A12) at 1/10, from Developmental Studies Hybridoma Bank, rabbit anti-Fzr antibody at 1/500, a gift from Christian Lehner, University of Zurich and mouse anti-Flag antibody, a gift from Lisa Porter, University of Windsor. Chemiluminescence imaging was performed using an Alpha Innotech FluorChemTM HD2 imager.

3.3.10 Cytology and Immunostaining

The steps used for cytology and immunostaining were described in (Das et al., 2016). The primary antibodies used were rat anti-HA antibody (Roche) at 1/100, rabbit anti-phospho histone H3 antibody (Santa Cruz) at 1/1000, rat anti-alpha tubulin antibody (Millipore) at 1/500, mouse anti-gamma tubulin antibody at 1/500, rabbit anti-GFP antibody (Abcam) at 1/10000, mouse anti-HA antibody (Abcam) at 1/1000 and rabbit anti-Fzr antibody at 1/50, a gift from Christian Lehner, University of Zurich. The following antibodies were from Developmental Studies Hybridoma Bank. They were mouse anti-Cyclin B antibody (F2F4) at 1/20, mouse anti-Dacapo antibody (NP1) at 1/5, mouse anti-Myc antibody (9E 10) at 1/15.

EDU labelling was performed for detection of S phase cells as in (Ghorbani et al., 2011).

3.3.11 Confocal microscopy

Immunostained imaginal discs were imaged using an Olympus FluoView FV1000 laser scanning confocal microscope. Images were analyzed in Olympus Fluoview software version 1.5. The images were modified (brightness and contrast) and compiled through Adobe Photoshop 2014.

3.3.12 Image J analysis of M phase and S phase indexes

The M phase and S phase analysis were done as described in (Das et al., 2016).

3.4 Results

3.4.1 Overexpression of CDH1/Fzr causes polyploidy and downregulation of mitotic cyclins in larval mitotic cells

Our previous studies of the larval mitotic tissues of *Skp2* and *Cyclin A* mutants led to our interest in *CDH1/Fzr*. Previous studies that have characterized the cell cycle role of CDH1/Fzr in *Drosophila* have been primarily conducted on cellularized embryos. (Grosskortenhaus and Sprenger, 2002; Sigrist and Lehner, 1997). To stay consistent with our previous work, we wanted to confirm the phenotypes resulting from overexpression of CDH1/Fzr in the larval and adult stages. We used two independent transgenic lines, *UAS-Fzr* (Sigrist and Lehner, 1997) and *UAS-Venus-Fzr*^{40}, (Rajdeep Dhaliwal, unpublished) and expressed the transgenes using the *GAL4-UAS* system. We tested these transgenes using 3 different *GAL4* drivers: *engrailed-GAL4* (*en-GAL4*), *patched-GAL4* (*ptc-GAL4*) and *rotund-GAL4* (*rn-GAL4*). All *GAL4* drivers were induced in different parts of the wing, as discussed later. The flies were raised at two different temperatures, 25°C and 29°C, to test if the phenotypes vary in different temperatures. In flies, the expression of a transgene by the *GAL4-UAS* system can vary at different temperatures. Generally, higher temperatures increase the expression of a transgene. For ease of use, *UAS-Fzr* will be referred to as *UAS-Fzr*^{1}, and *UAS-Venus Fzr*^{40} will be referred to as *UAS-Fzr*^{40}.

We overexpressed CDH1/Fzr in the adult wing using the transgene *UAS-*

Fzr^{40}, under the control of *en-GAL4*. *en-GAL4* is expressed in the posterior half of the adult wing. The anterior half of the wing serves as the control. Each hair in the adult wing arise from a cell, and the distance between two hairs is constant in the wild type adult wing and reflects the size of the cells. If cells become bigger, the distance between two hairs increases (Dui et al., 2013). We observed an increase in wing hair spacing (WHS) in the posterior half of the wing (Fig. 1A, B). The distance between the wing hairs increased as the temperature increased from 25°C to 29°C (Fig. 1A, B). The expression of *UAS-Fzr*^{1} with *en-GAL4* led to embryonic lethality at both 25°C and 29°C likely as a result of higher expression from this transgene.

In the adult wing, *ptc-GAL4* is expressed in the region between the anterior and posterior boundary of the wing, defined by the two longitudinal veins L3 and L4. (St Pierre et al., 2002). When we overexpressed *UAS-Fzr*^{40} in the adult wing using the *ptc-GAL4* driver, we observed an increase in WHS at both temperatures (Fig. 1C, D).

In the adult wing *rn-GAL4* driven expression is observed in the entire wing. The expression of *UAS-Fzr*^{40} with the *rn-GAL4* driver also showed an increase in WHS (Fig. 1E, F). The expression of *UAS-Fzr*^{1} with the *rn-GAL4* driver led either to no wing formation or to a rudimentary wing. Our WHS results confirm that the transgene *UAS-Fzr*^{40} is weaker than the transgene *UAS-Fzr*^{1}; the phenotypes are also temperature dependent.

Looking at wing hair spacing is an indirect way of looking at ploidy. Hence, after the study on adult wing, we wanted to test the larval wing imaginal disc to study the ploidy. *UAS-Fzr40* and *UAS-Fzr*^{1} were expressed with the *rn-GAL4 driver*.

We stained the wing imaginal discs with the DNA dye Oligreen. *UAS-Fzr*^{40} showed very little change in nuclear size compared to the wild type (Fig. 1G, H). However, when we overexpressed the *UAS-Fzr*^{1} transgene, we observed that the size of the nucleus had significantly increased compared to the wild type, indicating polyploidy (Fig.1I, J). These results support previous observation that overexpression of CDH1/Fzr causes polyploidy (Grosskortenhaus and Sprenger, 2002; Sigrist and Lehner, 1997).

CDH1/Fzr mutant cellularized embryos showed reaccumulation of mitotic cyclins after mitosis 16 where as wild type cells entered G1 with no accumulation of mitotic cyclins (Sigrist and Lehner, 1997). Overexpression of CDH1/Fzr in cellularized embryos also resulted in mitotic cyclin degradation (Grosskortenhaus and Sprenger, 2002; Sigrist and Lehner, 1997). To find whether the overexpression of CDH1/Fzr also causes a reduction of mitotic cyclins in larval mitotic tissues, we expressed *CDH1/Fzr* using the *en-GAL4* driver and looked in the wing imaginal disc of the 3rd instar wandering larvae. We observed low Cyclin B level in the posterior half of the wing imaginal disc compared to the control anterior half (Fig. 1K-K''). This result confirms the embryo results that showed that mitotic cyclins were reduced when CDH1/Fzr is overexpressed (Grosskortenhaus and Sprenger, 2002; Sigrist and Lehner, 1997).

Cdc25/String is another G2/M protein that is a substrate of the CDH1/Fzr in flies and mammals. To test whether overexpression of CDH1/Fzr causes down-regulation of the Cdc25/String protein level, we used a protein trap for Cdc25/String. We did not observe any change to the Cdc25/String protein level (Fig. 1K'''). The

reason might be that perhaps in CDH1/Fzr overexpressing cells the substrate specificity is different in embryos than in larval mitotic cells. Overexpressing CDH1/Fzr and studying the level of Cdc25/String protein in the embryos and then comparing it in larval tissues will give us the ability to better understand the result.

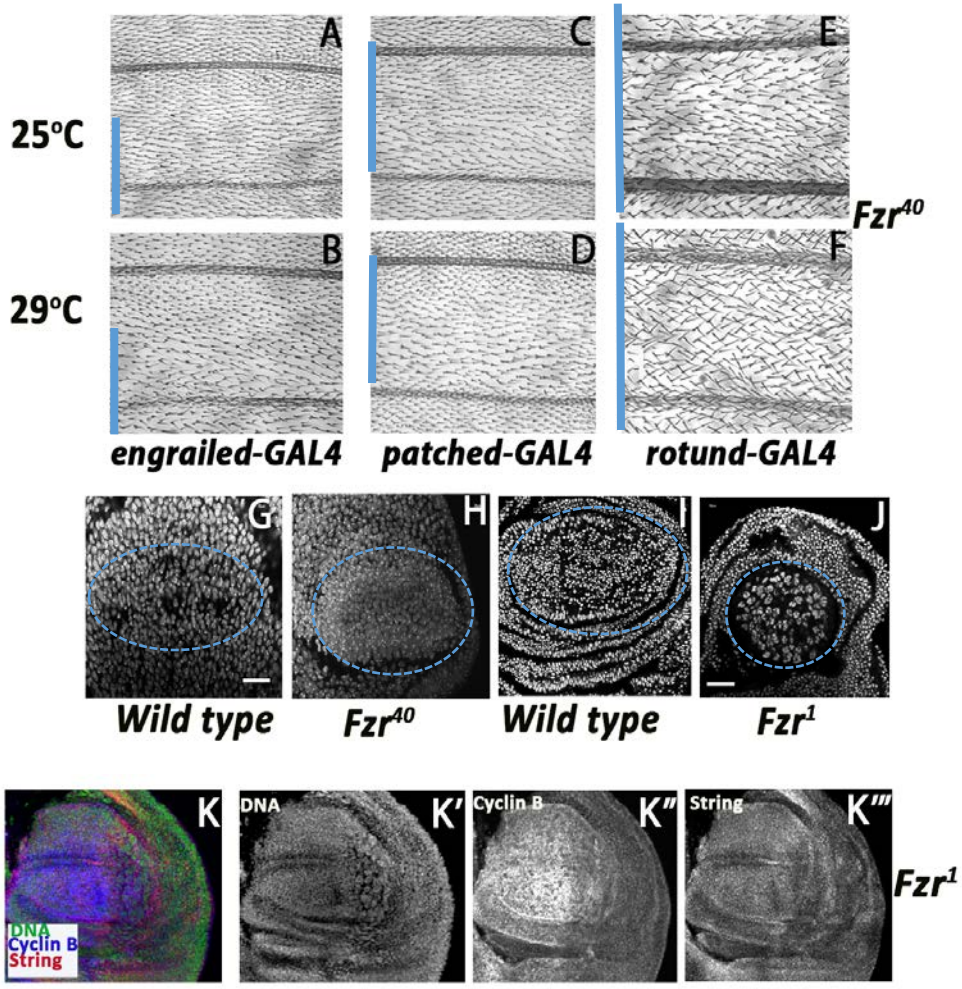

Fig. 1: Overexpression of CDH1/Fzr causes polyploidy and lower mitotic Cyclin B level.

Fig. 1: Overexpression of CDH1/Fzr causes polyploidy and lower mitotic Cyclin B level. A-F) Representative wings expressing *UAS-FZR*^{40} using different *GAL4* drivers (A-B), *engrailed-GAL4* (C-D), *patched-GAL4* and (E-F) *rotund-GAL4* at different temperatures. (A, C, E) flies were raised in 25°C and (B, D, F) flies were raised in 29°C. (A-F) Blue bars indicate the region of transgene expression on the wings. G-J) DNA stained with Oligreen in 3rd instar wing imaginal discs from the genotypes shown. Blue dashed line indicate the region of transgene expression. J) Nuclei appear larger in *UAS-FZR*^{1}, indicative of polyploidy. K-K’’’) *engrailed-GAL4* driven expression of *UAS-FZR*^{1} in 3rd instar wing imaginal discs. The discs are stained with Oligreen, Cyclin B, or CDC25/String. K’’) overall protein level of Cyclin B is reduced in CDH1/Fzr overexpressing cells compared to control cells. K’’’) CDC25/String expression shows no difference on both sides of the white dashed line. Scale bar in G =10 µm and applies to G-H. Scale bar in J =20 µm and applies to I-K’’’.

3.4.2 CDH1/Fzr overexpression phenocopies Skp2 null and Cyclin A knockdown

We expect that overexpression of CDH1/Fzr will result in a lower mitotic index, as observed in *Skp2* null and *Cyclin A* knockdown cells. We used phospho-Histone H3 to immunostain the cells of the wing imaginal disc of the 3rd instar wandering larva. Consistent with our expectation, we observed a strong reduction of the mitotic index in CDH1/Fzr overexpressing cells (Fig.. 2A-C). This result also confirms the previous observation in embryos (Sigrist and Lehner, 1997) The results so far indicate a correlation between *Skp2* null and *CDH1/Fzr* overexpression. We did not see any change to the S phase index in CDH1/Fzr overexpressing cells compared to the wild-type cells (Fig. 2D-F). This is in contrast with the previous observation in *Skp2* null cells where we observed a reduction of the S phase index (Das et al., 2016).

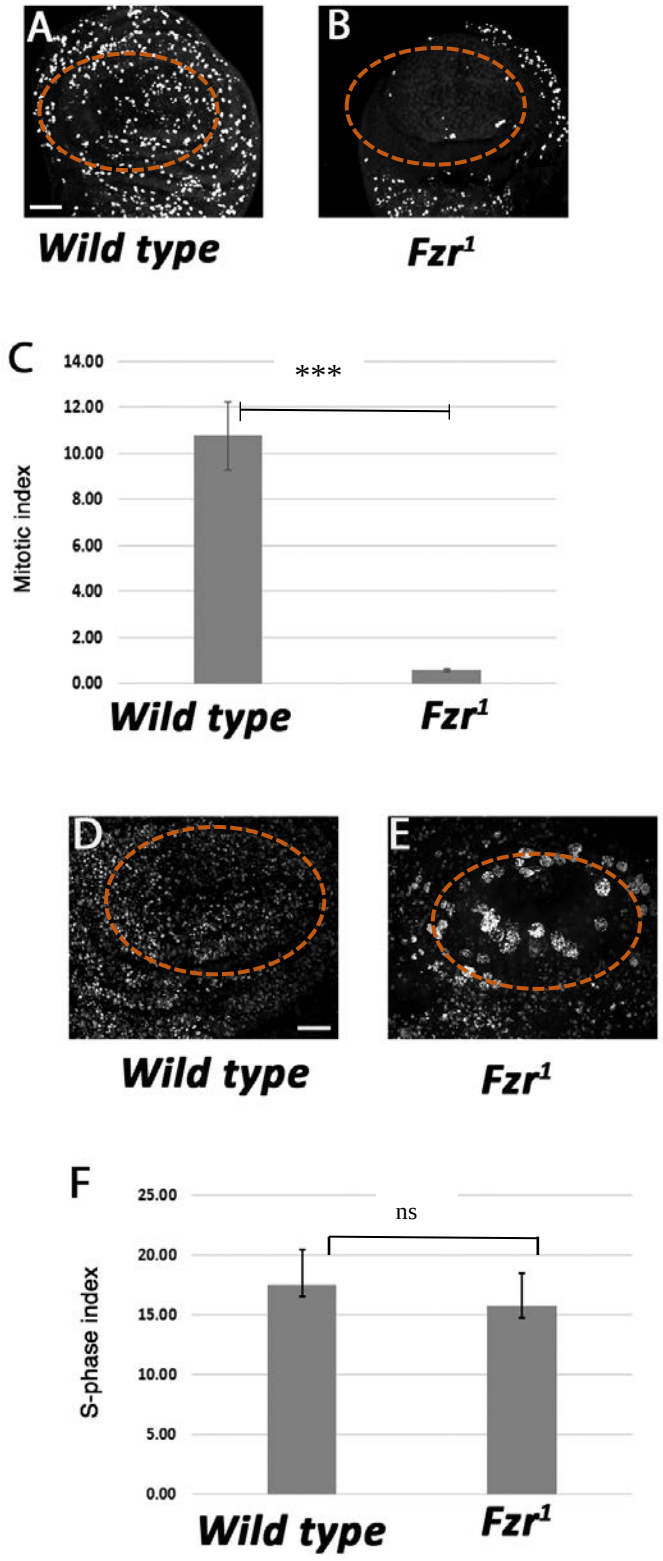

Fig. 2: Overexpression of CDH1/Fzr results in lower mitotic index in wing imaginal discs

Fig. 2: Overexpression of CDH1/Fzr results in lower mitotic index in wing imaginal discs. A-B) Representative *wild type* (A), *UAS-Fzr*^{1} (B) wing imaginal discs from 3rd instar wandering larvae immunostained with phospho-Histone H3 antibodies. C) M phase index of above mentioned genotypes. D-E) Representative *wild type* (D) and *UAS-FZR*^{1} (E) wing imaginal discs from 3rd instar wandering larvae stained with EDU (I hour incubation). F) S phase index of above mentioned genotypes. Scale bar in A =20 μm, applies to A-B and D-E. The orange dotted lines in B and E show the region of transgene expression.

3.4.3 Skp2 knockdown phenotype may be rescued by weak allele of CDH1/Fzr

To date, our results suggest that *Skp2* is important for maintaining Cyclin A levels, and that reduced Cyclin A levels lead to an endoreplication cycle in which mitosis is bypassed. The overexpression of CDH1/Fzr results in polyploidy and premature degradation of mitotic cyclins. Our results support the idea that there is an antagonistic relationship between *Skp2* and *CDH1/Fzr*. If this the case then we predict that loss of *CDH1/Fzr* in the *Skp2* null background, will rescue the loss of *Skp2* phenotype. We tested this and our results showed that a partial loss of function mutation in *CDH1/Fzr* (*fzr^{e4}*) can suppress the wing hair spacing (WHS) phenotype in *Skp2 RNAi* wings (Figs. 3C, D). An antagonistic relationship between *Cyclin A* and *CDH1/Fzr* was observed before in embryos (Sigrist and Lehner, 1997). To confirm if this is the case in larval imaginal discs, we tested this. Indeed, *fzr^{e4}* *partially* suppresses the increased WHS phenotype of *Cyclin A* RNAi (Figs. 3A, B).

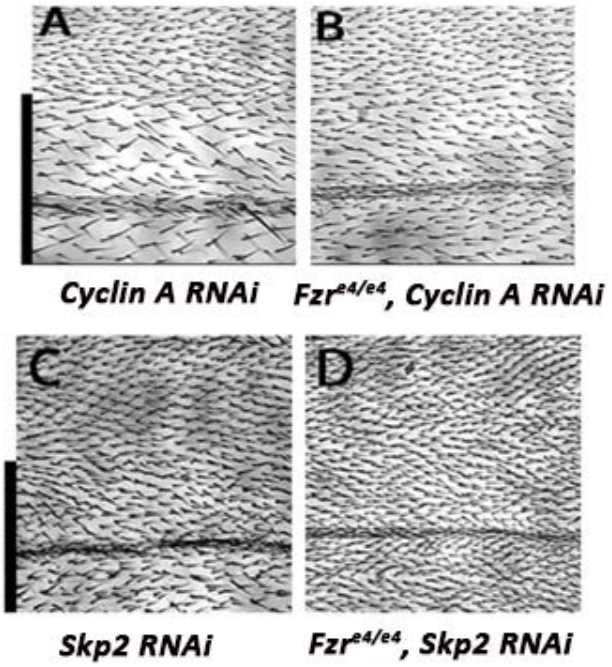

Fig. 3: *Skp2* and *Cyclin A* are antagonistic to *CDH1/Fzr*.

Fig. 3: *Skp2* and *Cyclin A* are antagonistic to *CDH1/Fzr*. A-D) *en-GAL4* driven expression of *Cyclin A RNAi* (*Cyclin* A^{R1}) (A) or *Skp2 RNAi* alone (C) or in a $Fzr^{e4/e4}$ background (B, D). All panels show a region of the adult wing at the border between engrailed expressing (indicated by black bar) and non-expressing cells. *Cyclin A* knockdown results in increased wing hair spacing. This is partially rescued by *CDH1/Fzr* hypomorph. *Skp2* knockdown results in increased wing hair spacing. This is completely rescued by *CDH1/Fzr* hypomorph.

3.4.4 Investigation of possible mechanisms of CDH1/Fzr antagonism

To investigate the possible mechanism of CDH1/Fzr antagonism, we predicted that Skp2 as an E3 ubiquitin ligase might cause instability of CDH1/Fzr protein. To test this idea, we examined CDH1/Fzr protein levels in the mitotic discs and brains of *Skp2*-null 3rd instar wandering larvae by western blotting method. We predicted that if Skp2 targets CDH1/Fzr for destruction then loss of Skp2 will result in an increase in CDH1/Fzr protein level and overexpression of Skp2 will result in a decrease in CDH1/Fzr protein level. Our results showed that there was no change in the CDH1/Fzr protein level in *Skp2*-null cells compared to wild type cells, although we observed a slight increase in the CDH1/Fzr protein level in Skp2 overexpressing cells (Fig.. 4A). To further validate this result, we looked at CDH1/Fzr protein in Skp2 overexpressing cells through immunofluorescence, but we did not see any change in the overall level of CDH1/Fzr in Skp2-overexpressing cells. Our results argue that CDH1/Fzr is not a substrate of Skp2 (Fig. 4B - B’’’).

The activity of a protein might also be regulated by a change in localization. To test whether loss of *Skp2* results in a change in the localization of endogenous CDH1/Fzr, we compared localization of CDH1/Fzr in wild-type mitotic cells with *Skp2* null cells. CDH1/Fzr is cytoplasmic during interphase (Fig. 4D-D’’) which confirms the previous observation by the Lehner lab (Jacobs et al., 2002). Our results show no change in CDH1/Fzr localization in *Skp2* null cells nor in cells that are overexpressing Skp2 (Fig. 4D-F’’). These results indicate that the regulation of CDH1/Fzr by Skp2 might not be through protein stability or a change in localization.

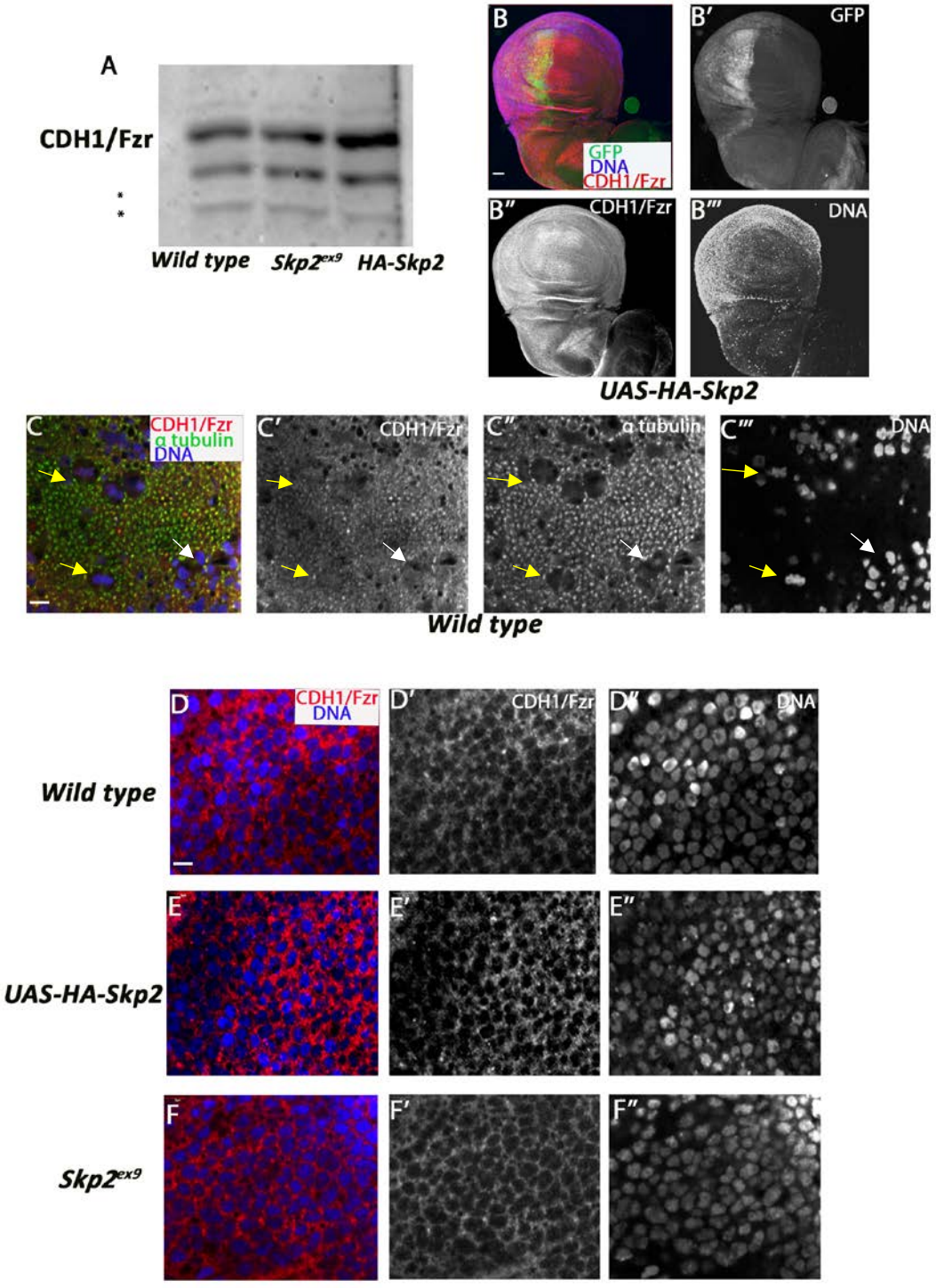

Fig. 4: Skp2 does not regulate protein level or localization of CDH1/Fzr

Fig. 4: Skp2 does not regulate protein level or localization of CDH1/Fzr. A) Western blot shows CDHI/Fzr level does not change in *Skp2*$^{ex9.}$ Asterisks denote the non-specific bands and is used as loading control. B-B’’’) *en-GAL4* driven expression of *UAS-GFP* and *UAS-HA-Skp2* in the wing imaginal disc of 3rd instar wandering larvae. Total protein level of CDH1/Fzr remains unchanged in HA-Skp2 expressing area (GFP). C-C’’’) Localization of CDH1/Fzr in different phases of mitosis. The yellow arrowheads in C’) show presence of CDH1/Fzr in metaphase and the white arrowheads in C’) show presence of CDH1/Fzr in anaphase. D-F’’) CDH1/Fzr localization does not change in the interphase cells of *wild type*, *Skp2*^{ex9} and *HA-Skp2* expressing wing imaginal disc cells. Scale bar in B =10 µm, applies to B-B’’’. Scale bar in C =5 µm, applies to C-C’’’ and D- F’’.

3.4.5 Skp2 does not interact with Cyclin A through first 53 amino acids of Cyclin A

Active CDH1/Fzr ubiquitinates Cyclin A for degradation during late M and G1 phases. The first 53 amino acids on the N-terminus of Cyclin A is required for identification of Cyclin A by CDH1/Fzr in flies (Jacobs et al., 2001; Sigrist and Lehner, 1997). We predicted that Skp2 might interact with Cyclin A through the first 53 amino acids on the N-terminus of Cyclin A and protect it from CDH1/Fzr mediated ubiquitination. In mammals, the N-terminus of Cyclin A is also needed for the interaction with Skp2 (Ji et al., 2006).

To test this model, we made transgenic flies that did not have the first 53 amino acids on the N-terminus of Cyclin A (Rajdeep Dhaliwal, unpublished). We expressed the stable Cyclin A transgene (*Cyclin* $A^{\Delta 1\text{-}53}$) along with HA-Skp2 in the wing pouch of the wing imaginal disc using rn-GAL4 (Fig. 5A-C'''). To test if Skp2 and Cyclin $A^{\Delta 1\text{-}53}$ interact, we looked at their colocalization. As anticipated, we observed a strong accumulation of Cyclin $A^{\Delta 1\text{-}53}$ in prophase and metaphase cells (Fig.. 5C-C'). We were unable to assess colocalization due to insufficient resolution and relatively uniform accumulation of Cyclin A. We also observed a high degree of abnormality in mitotic chromosomes with the expression of *Cyclin* $A^{\Delta 1\text{-}53}$ in the wing blade.

To better study the interaction of Skp2 and Cyclin $A^{\Delta 1\text{-}53}$, we tried to perform an in vivo CoIP experiment. However, when we expressed Cyclin $A^{\Delta 1\text{-}53}$ with the ubiquitous driver *daughterless-GAL4*, they were embryonic lethal. To bypass this problem we made Cyclin $A^{\Delta 1\text{-}53}$ construct (*His-MBP-Cyclin* $A^{\Delta 1\text{-}53}$) and expressed it in bacterial cells (Fig. 5D). We then performed a GST pull-down experiment using

GST-Skp2 and either His-MBP-Cyclin A or His-MBP-Cyclin $A^{\Delta 1\text{-}53}$. The amount of His-MBP-Cyclin $A^{\Delta 1\text{-}53}$ was equalized with His-MBP-Cyclin A. This was done by running different dilutions of His-MBP-Cyclin $A^{\Delta 1\text{-}53}$ and fixed amount of His-MBP-Cyclin A on a gel (Fig. 5F). We observed a stable interaction between GST-Skp2 and His-MBP-Cyclin $A^{\Delta 1\text{-}53}$ (Fig. 5G). Our *in vitro* experiment showed that Skp2 can interact with Cyclin $A^{\Delta 1\text{-}53}$, indicating that the interaction domain of Skp2 and Cyclin A might not involve the 1-53 amino acids of Cyclin A as in mammals. A verification with an *in vivo* result will better confirm our observation.

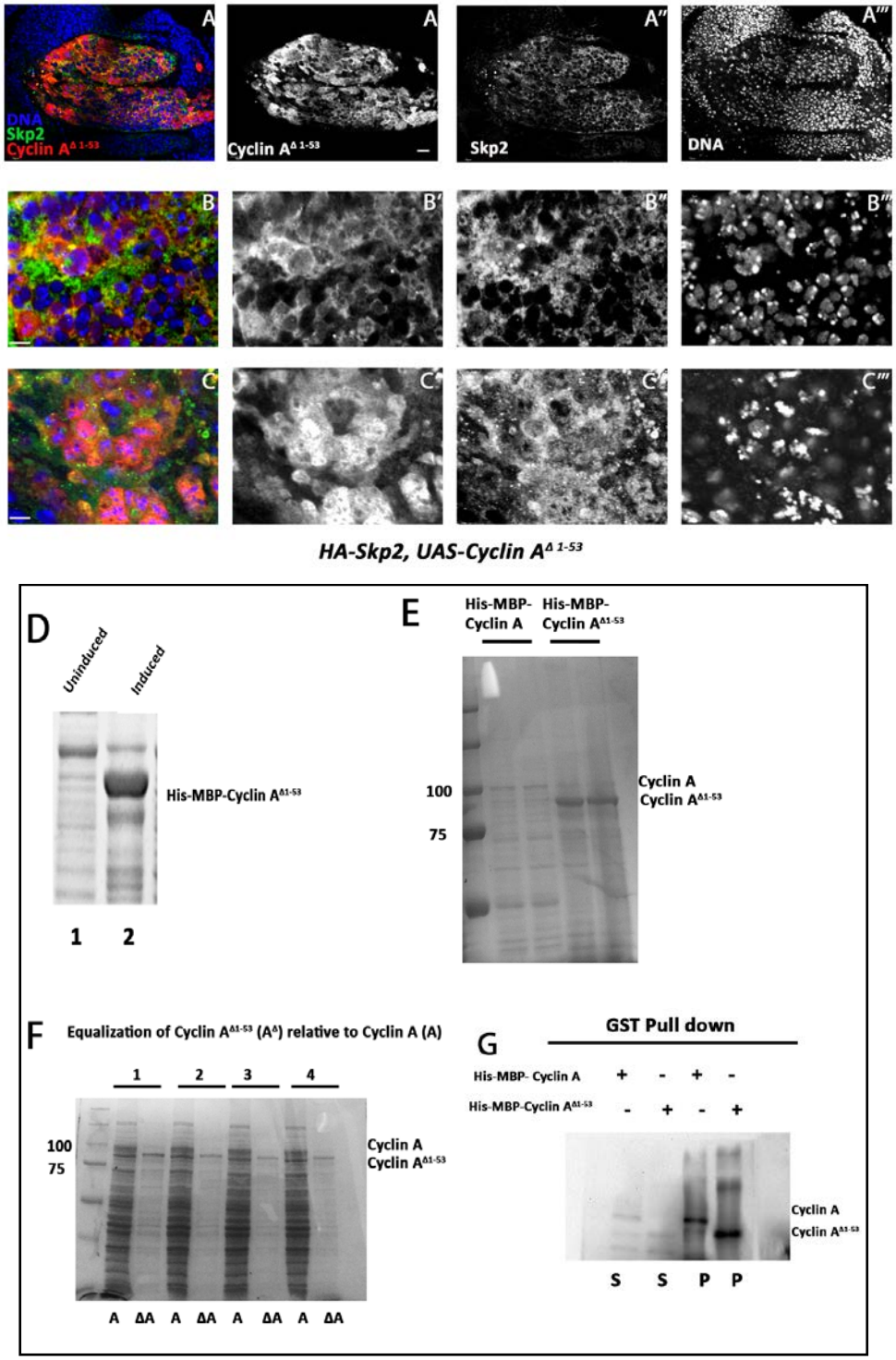

Fig. 5: The first 53 amino acid of the N-terminus of Cyclin A does not have Skp2 interaction domain.

Fig. 5: The first 53 amino acid of the N-terminus of Cyclin A does not have Skp2 interaction domain. A-A’’’) Wing imaginal blade of 3rd instar wandering larvae showing transgenic expression of *Skp2* and *Cyclin* $A^{\Delta 1\text{-}53}$. Scale bar in A - 5 µm, applies to A-A’’’. Scale bar in B - 10 µm, applies to B-C’’’. D) Expression of His-MBP-Cyclin $A^{\Delta 1\text{-}53}$ before and after IPTG induction. Lane 1 is the uninduced lane and lane 2 shows the expression of His-MBP-Cyclin $A^{\Delta 1\text{-}53}$. E) Coomassie staining of bacterially produced His-MBP-Cyclin A and His-MBP-Cyclin $A^{\Delta 1\text{-}53}$. F) Coomassie staining of 4 different sets of dilutions (indicated by 1-4) of His-MBP-Cyclin $A^{\Delta 1\text{-}53}$ with fixed amount of His-MBP-Cyclin A. G) GST pull-down assay with GST Skp2 and His-MBP-Cyclin A and His-MBP-Cyclin $A^{\Delta 1\text{-}53}$. GST Skp2 pulled down His-MBP-Cyclin A and His-MBP-Cyclin $A^{\Delta 1\text{-}53}$.

3.4.6 Skp2 does not compete with CDH1/Fzr to bind to Cyclin A

In mammals, the RXL motif on the WD 40 domain of CDH1/Fzr interacts with Cyclin A (Sørensen et al., 2001). In *Drosophila*, CDH1/Fzr also has an RXL motif on its WD 40 domain. However, there is no clear understanding of the way in which *Drosophila* CDH1/Fzr interacts with Cyclin A and whether or not the hydrophobic patch on Cyclin A is necessary for this interaction. We predicted that Skp2 might compete with CDH1/Fzr for binding to Cyclin A. To test this, we did a competition assay. We first incubated GST-tagged Cyclin and Flag-tagged Fzr. Amount of His-MBP-Skp2 or GST was equalized with fixed amount of GST-Cyclin A by densitometry analysis. We then added a 1x or 5x higher amount of MBP-Skp2, relative to GST-Cyclin A, to fixed amounts of GST-Cyclin A and Flag-Fzr. Our results show that the binding between Flag-Fzr and GST-Cyclin A was not diminished by increasing amounts of MBP-Skp2 (Fig.. 6B). Our result argues that Skp2 does not compete with CDH1/Fzr to bind Cyclin A.

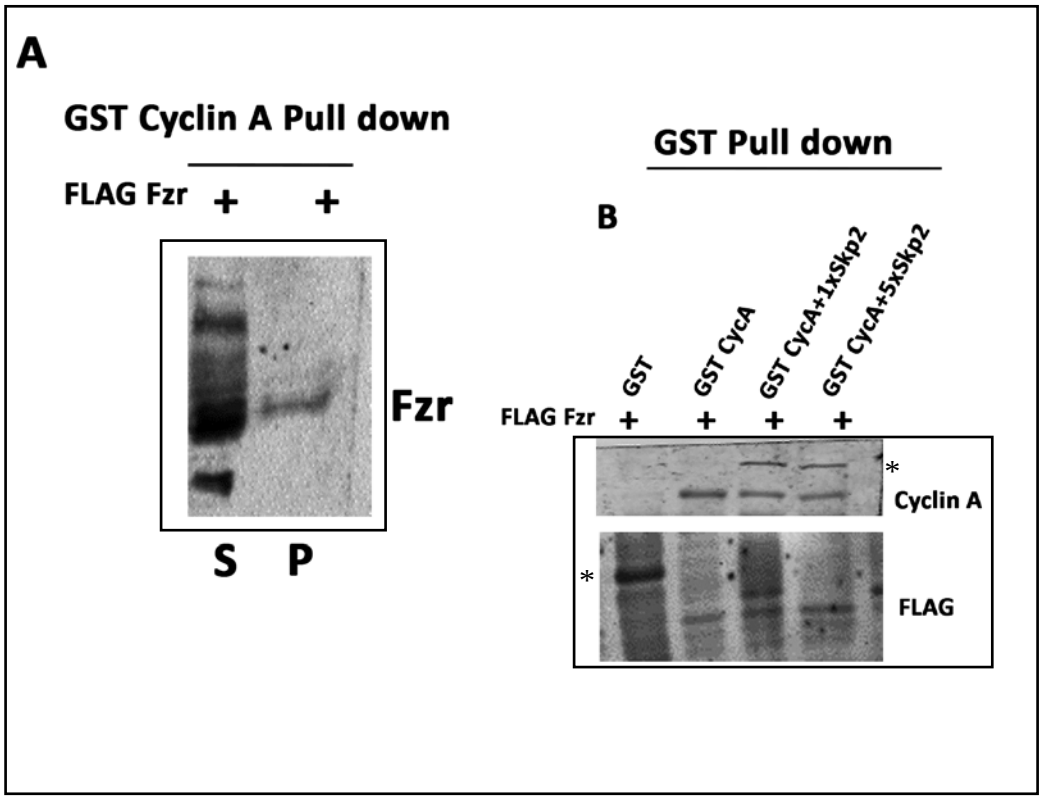

Fig. 6: Skp2 does not compete with CDH1/Fzr to bind to Cyclin A.

Fig. 6: Skp2 does not compete with CDH1/Fzr to bind to Cyclin A. A-B) GST Cyclin A pull down assay with Flag-Fzr. A) GST-Cyclin A pulled down Flag Fzr. Flag Fzr was probed with Fzr antibody. B) GST pull down assay shows no change in Flag Fzr pull down even when increasing amounts of His-MBP-Skp2 (1x or 5x), relative to GST-Cyclin A were added.

3.4.7 Overexpression of p27/Dap does not have an effect on mitotic entry

p27/Dap, a cyclin-dependent kinase inhibitor, is a critical target of Skp2 during the G1/S transition of the cell cycle (Carrano et al., 1999; Dui et al., 2013; Nakayama et al., 2004; Sutterlüty et al., 1999; Tsvetkov et al., 1999). Loss of *Skp2* results in upregulation of p27/Dap. Upregulation of p27/Dap can cause inhibition of CDK activity which can cause failure of mitotic entry. If this is the case then overexpression of p27/Dap phenotype should be similar to loss of *Skp2* phenotype. To test this idea, we looked at overexpressed p27/Dap expression in adult wings using the transgene *UAS-MycDap*^{51}. Similar to WHS observed in *Skp2* RNAi we observed increased WHS in p27/Dap overexpressing adult wings compared to wild type wings. (Fig. 7A', B'). We also observed an overall reduction of wing margins, presumably due to cell loss, something not seen with *Skp2* loss (Fig. 7A, B). Our observation confirmed a previous observation that was made by Jiao lab (Dui et al., 2013). Next we wanted to look at the localization of p27/Dap in mitotic cells. Our results show a strong nuclear accumulation of p27/Dap in interphase cells (Fig. 7C-C'''). We also observed accumulation of p27/Dap in prophase and metaphase cells (Fig. 7D-D'''). Similar localization was observed for endogenous p27/Dap (Biju Vasavan, unpublished). This indicates a possible role for p27/Dap in mitotic entry.

However, unlike *Skp2* null, overexpressing p27/Dap in *Cyclin A*$^{H170+/-}$ background did not show any change in mitotic entry (Fig.. 8A-E), indicating that failure to mitotic entry in *Skp2* null is not due to p27/Dap overexpression.

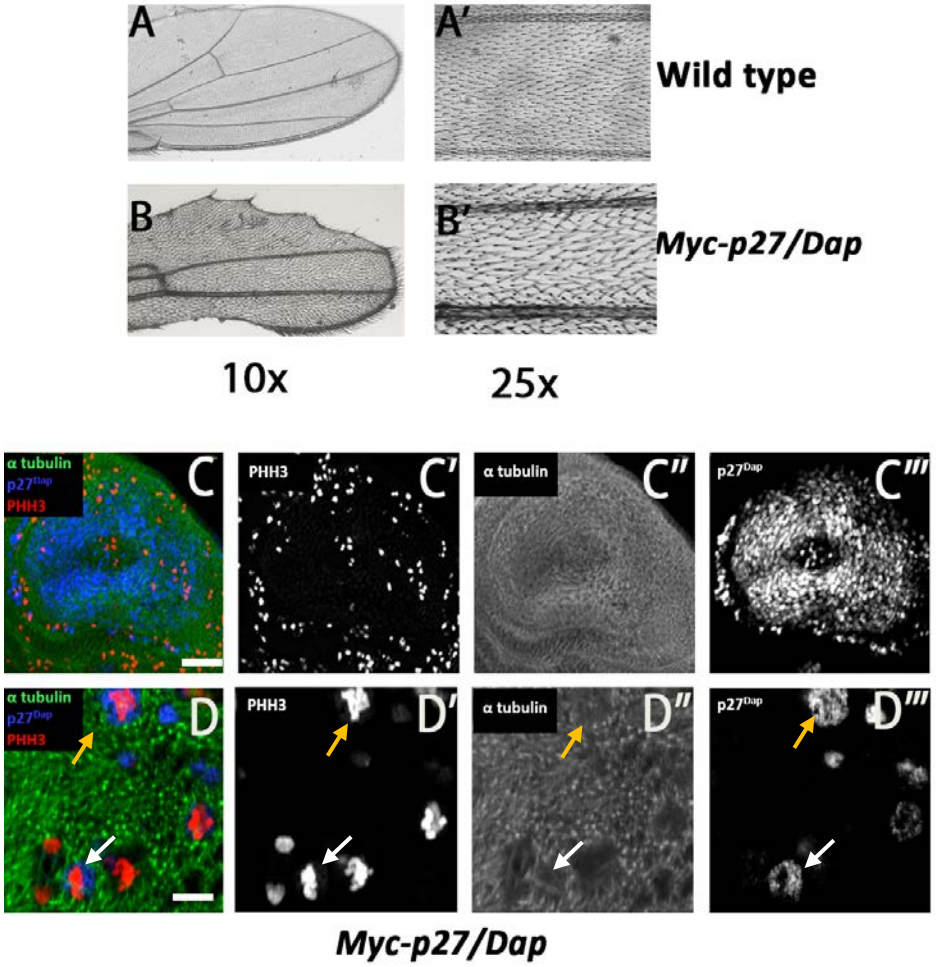

Fig. 7: Transgenic p27/Dap accumulates in interphase and mitotic cells of wing imaginal disc

Fig. 7: Transgenic p27/Dap accumulates in interphase and mitotic cells of wing imaginal disc A-B') Representative wings from *wild type* (A-A') *rotund-GAL4*; *UAS-Myc-p27/Dap* (B-B') Increased WHS and loss of wing margin is observed in p27/Dap overexpression compared to wild type. C-D''') *rotund-GAL4* driven accumulation of *UAS-Myc-p27/Dap* in the wing blade of 3rd instar wandering larvae shows accumulation of p27/Dap in interphase and mitotic cells. C-C''') Strong nuclear accumulation of p27/Dap is observed. D-D''') Localization of p27/Dap in different phases of mitosis. The yellow arrowhead shows p27/Dap protein accumulation in prophase and the white arrowhead shows p27/Dap protein in metaphase. Scale bar in B =10 μm, applies to C-C'''. Scale bar in D =5 μm, applies to D-D'''.

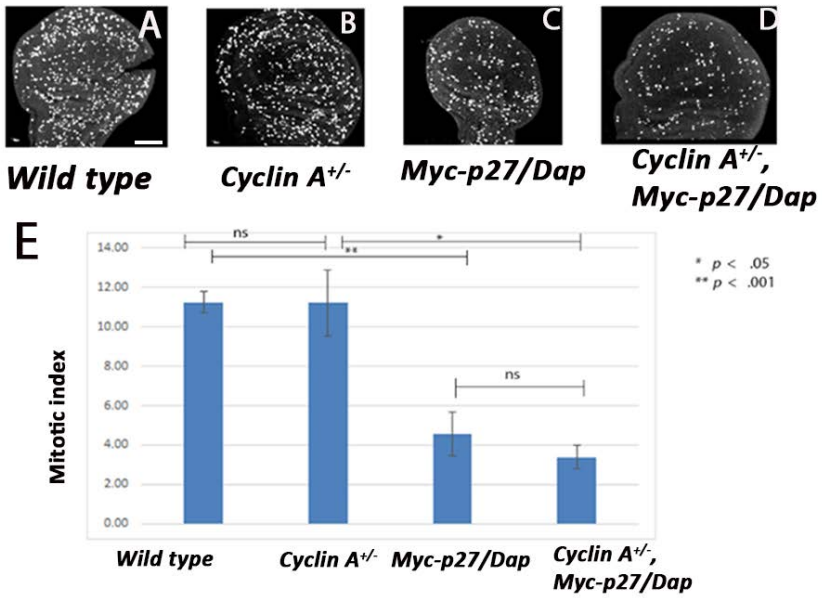

Fig. 8: Overexpression of p27/Dap results in lower mitotic index

Fig. 8: Overexpression of p27/Dap results in lower mitotic index A-D) Representative *wild type* (A), *Cyclin* $A^{H170+/-}$ (B), *UAS-Myc-p27/Dap*, and *Cyclin* $A^{H170+/-}$, *UAS-Myc-p27/Dap* wing imaginal discs from 3rd instar wandering larvae immunostained with phospho-Histone H3 antibodies. E) M phase index of above mentioned genotypes. Scale bar in C =20 µm, applies to A-D.

The N-terminus of Skp2 interacts with Cyclin A, and the C-terminus LRR domain is needed for p27/Dap degradation (Das et al., 2016; Ji et al., 2006). We predicted that HA-Skp2$^{\Delta 170}$ which cannot interact with Cyclin A can still degrade p27/Dap. We compared endogenous p27/Dap expression in *wild-type* and *Skp2* null cells. We observed a strong accumulation of endogenous p27/Dap in metaphase of *Skp2* null cells, compared to wild type cells (Fig. 9A-B'''). If HA-Skp2$^{\Delta 170}$ is still able to target p27/Dap we predict that there would be less p27/Dap accumulation in *HA-Skp2$^{\Delta 170}$* cells in *Skp2* null background than in *Skp2* null cells alone. Our results showed a strong accumulation of p27/Dap in the nucleoplasm and on the chromatin of *Skp2* null metaphase cells (Fig. 9C-C''). However, in *HA-Skp2$^{\Delta 170}$* metaphase cells there was a reduction of p27/Dap accumulation in the nucleoplasm (Fig. 9E-F'). We also studied p27/Dap accumulation in *HA-Skp2* as above, but did not observe any reduction in p27/Dap accumulation (Fig. 9D-D'). This result was surprising, as *HA-Skp2* has a C-terminal LRR tail and has been shown to rescue the lethality of *Skp2* null. However, *HA-Skp2$^{\Delta 170}$* may be more stable as discussed in (Das et al., 2016), and might have resulted in an increased Skp2 expression compared to wild type *HA-Skp2*. Our results argue that HA-Skp2$^{\Delta 170}$ is still able to target p27/Dap for destruction. Its failure to rescue a *Skp2* null mutant argues therefore that Skp2 has a G2/M role that is independent of its ability to target p27/Dap.

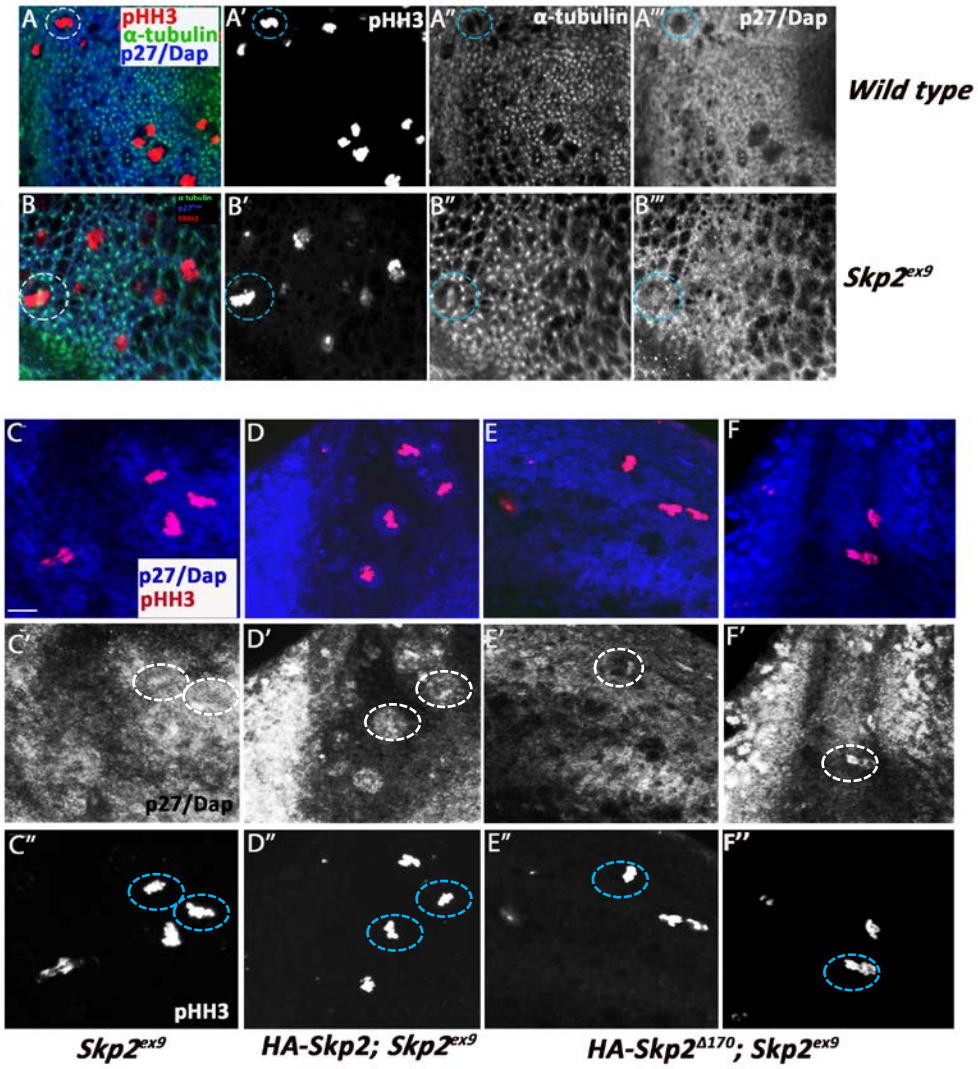

Fig. 9: Overexpression of p27/Dap does not cause failure to mitotic entry in *Skp2* null cells

Fig. 9: Overexpression of p27/Dap does not cause failure to mitotic entry in *Skp2* null cells. A-B’’’) Endogenous p27/Dap expression in *wild type* and *Skp2* null wing imaginal disc cells. Wing imaginal disc of third instar wild type larva immunostained with anti–Dap antibodies. Blue dotted circle shows the cells that are in metaphase. C-F’’) Representative *Skp2* null (C-C’’), *HA-Skp2, Skp2* null (D-D’’) and *HA-Skp2$^{\Delta 170}$, Skp2* null (E-E’’, F-F’’) wing imaginal discs from 3rd instar wandering larvae immunostained with anti-Dap antibodies. Strong p27/Dap expression is observed in the chromatin and nucleoplasm in *Skp2* null metaphase cells (C, C’’) and *HA-Skp2, Skp2* null metaphase cells (D, D’’) compared to p27/Dap expression in *HA-Skp2$^{\Delta 170}$, Skp2* null metaphase cells (E, E’’ and F, F’’). The white and blue dotted circles mark the metaphase cells. Scale bar in C= 5 µm, applies to A-F’’.

3.5 Discussion

Our previous work with Skp2 revealed an interesting mitotic role of Skp2 plays in protecting Cyclin A from degradation. That led to our focus on CDH1/Fzr. Our rescue experiment on *Skp2 RNAi* and *Cyclin A RNAi* adult wings showed a genetic antagonism between *Skp2*, *Cyclin A*, and *CDH1/Fzr*.

We tried to study the mechanism that Skp2 uses in protecting Cyclin A in G2 by inhibiting CDH1/Fzr. We proposed that Skp2 binds to Cyclin A through the first 53 amino acids of Cyclin A, and this prevents CDH1/Fzr from identifying Cyclin A. To test this model, we sought to first identify the sequence on Cyclin A needed for Skp2 interaction. Our results shows that the first 53 amino acids on the N-terminus of Cyclin A are not needed for Skp2 interaction unlike the situation in CDH1/Fzr. This suggests that Skp2 interacts with Cyclin A through some other domain. Our future work will focus on identifying Cyclin A–Skp2 interaction domain. We have made two deletion constructs of Cyclin A: an N-terminal Cyclin A construct (Cyclin $A^{\Delta\ 171\text{-}491}$) and a C-terminal Cyclin A construct (Cyclin $A^{\Delta 1\text{-}170}$). We predict that Skp2 probably interacts with the N-terminus of Cyclin A as the sequences on the N–terminus are not conserved in Cyclin B.

In our quest to understand the mechanism of Skp2 mediated inhibition of CDH1/Fzr, we proposed that SKP2 and CDH1/Fzr competes to bind to Cyclin A. However the results of our competition assay showed that Skp2 does not compete with CDH1/Fzr to bind to Cyclin A. There might be several reasons why we did not see any change in CDH1/Fzr interaction with Cyclin A. Firstly, the assay was done with lysates not with purified protein. Other proteins in the lysate might have

interfered with result. Secondly, when we performed the experiment we incubated Cyclin A with CDH1/Fzr and then added increasing amounts of Skp2. If we incubate Cyclin A with Skp2 first and then add CDH1/Fzr we might see a different outcome. There is also a possibility that Cyclin A-Skp2 interaction might change the conformation of Cyclin A which causes its stability through means other than CDH1/Fzr interaction. Degradation of Cyclin A in flies and in mammals is very complex and different than any other cyclins. Apart from the D box or the KEN box (not present in mammals) there are lysine residues near the D box or far away from the D box which plays an important role on Cyclin A stability. If that is the case then Skp2 and Cyclin A can both bind with CDH1/Fzr and still Skp2 can protect Cyclin A. There is also a possibility for the involvement of accessory proteins in protecting Cyclin A stability in G2.

Skp2$^{\Delta 170}$ cannot bind with Cyclin A but appears to bind to p27/Dap, and also cannot rescue the polyploidy (Das et al., 2016) argues that polyploidy arising from loss of *Skp2* is different from polyploidy arising from overexpression of Skp2. Moreover, we showed that p27/Dap overexpression does not cause the mitotic failure that we observe in *Skp2* null nor does the degradation of p27/Dap in *HA-Skp2*$^{\Delta 170}$ rescue the polyploidy phenotype of *Skp2* null.

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CHAPTER 4

MITOTIC ENTRY IN *SKP2* NULL CELLS TRIGGERS SPINDLE ASSEMBLY CHECKPOINT AND APOPTOSIS

4.1 Summary

S phase kinase associated protein Skp2 is the substrate recognition component of the E3 ubiquitin ligase SCF complex. Loss of Skp2 results in tetraploidy, a critical first step that can lead to cancer. Skp2 functions with Cyclin A during mitotic entry and loss of *Skp2* results in impaired mitotic entry. Instead *Skp2* null cells start endoreplication. Loss of *Skp2* also results in cell death. To understand the fate of tetraploid cells in *Skp2* mutant has interested us particularly we wanted to study if Skp2 has any role in determining the fate of these cells. Our study shows that the endoreplicating *Skp2* null cells sometimes enter mitosis but they delay in prometaphase due to Spindle assembly checkpoint activation. We also observed DNA damage in these cells that led to either Chk1 mediated G1/S arrest or Chk2 and p53 dependent apoptosis. Our results show that if polyploid cells enter mitosis they activate these checkpoints that led to cell cycle arrest or cell death. However, if the polyploid cells skip mitosis they avoid DNA damage and evade these checkpoints and continue to endoreplicate.

4.2 Introduction

A common theme in all cancers is evasion of checkpoints. A large number of checkpoints that operate in normal cells have to detect problems that could lead to cancer and trigger pathways to take action to fix the problem while at the same time delaying cell division; and if the problems are too great, checkpoints induce permanent cell arrest or apoptosis. The best known of these checkpoints are those responding to DNA damage or improper chromosome attachments in mitosis. In the DNA damage response (DDR), exposed single stranded or double stranded DNA act as signals to activate key sensor kinases, ATM and ATR. Signaling through these kinases can lead to, amongst other outcomes, stabilization and activation of the p53 tumour suppressor. p53 in turn promotes cellular senescence or apoptosis. Failure of this checkpoint leads to chromosomal instability, aneuploidy and cancer (Kastan and Bartek, 2004).

By delaying anaphase in the presence of incorrect kinetochore attachments the Spindle Assembly Checkpoint (SAC) plays a key role in inhibiting aneuploidy (Burgess et al., 2007; Burgess et al., 2014), and a weakened SAC is frequently implicated in cancer (Malmanche et al., 2006). While the SAC has a protective role it does not produce a permanent cell cycle arrest. *In vitro* experiments using microtubule poisons to activate the SAC have shown that SAC arrest can eventually be overcome – a process called mitotic slippage – and that this can lead to further aneuploidy. *In vitro* experiments point to a critical role for p53 in driving these cells that escape SAC arrest into senescence (Davoli et al., 2010).

Polyploidy, and in particular, tetraploidy – an exact doubling of DNA content, is a critical first step in many cancers. Tetraploidy can arise via many different paths including re-replication, failed cytokinesis or failure to enter mitosis. While the causes of tetraploidy are clear it is not clear how this leads to tumourigenesis and nor is it well understood how cells respond to tetraploidy. The existence of tetraploidy checkpoints has been postulated but these are not well understood (Davoli et al., 2010; Ganem et al., 2007).

Skp2 is the substrate recognition component of the SCF-Skp2 ubiquitin ligase, a major regulator of the cell cycle and an important oncogene (Nakayama and Nakayama, 2006). The best-characterized function of Skp2 is to target the CDK inhibitor, p27/Dap (Carrano et al., 1999; Sutterlüty et al., 1999; Tsvetkov et al., 1999). It does this in cooperation with the small CDK-interacting protein, Cks1/Cks85A (Ganoth et al., 2001; Spruck et al., 2001). In doing so Skp2 promotes entry into S-phase. This role accounts for a large part of the oncogenic activity of Skp2. Less well characterized, Skp2 has potential tumour suppressive functions as loss of *Skp2* in diverse organisms from *Drosophila* to mice, results in tetraploidy and further polyploidy (Ghorbani et al., 2011; Nakayama et al., 2000; Nakayama and Nakayama, 2006).

We previously found that Skp2 cooperates with Cyclin A to promote mitotic entry in *Drosophila*. We found that the loss of *Skp2* leads to a bypass of mitosis resulting in tetraploidy or further polyploidy (Das et al., 2016). Here we study the consequences of polyploidy in *Drosophila Skp2* mutants. We show that polyploid *Skp2* null cells undergo SAC-mediated mitotic arrest as well as DNA damage

checkpoints leading to a Chk1-dependent cell cycle arrest or Chk2 and p53-dependent apoptosis. We show that the JNK pathway also contributes to the polyploidy checkpoint response in these cells. We provide evidence that it is mitosis that triggers these checkpoints and that polyploid cells that avoid mitosis also avoid these checkpoints.

4.3 Materials and methods

4.3.1 Drosophila strains and genetics

All flies were maintained at 25°C. The following stocks were obtained from Bloomington *Drosophila* Stock Centre: *UASp-GFP-mCherry Atg8a* (37749), *Chk2RNAi* (35152), Cyclin A^{H170} (Bl9096), *p53* (Bl23283), puc^{lacZ} (109029), JNK^{DN} (Bl9311), *BUBR1* (Bl10526), *GFP-LC3* (Bl8730), *UAS-p35* (Bl5072). Following Stocks were obtained from Vienna *Drosophila* RNAi center (VDRC): $Chk1RNAi^{v12860}$, $HidRNAi^{v8269}$, $Reaper\ RNAi^{v12045}$, $Cyclin\ A^{v103595}$ $(Cyclin\ A^{95})$, *MAD2p* (gift from Roger Karess), *MAD1-GFP* (gift from Roger Karess, Paris Diderot University), $Cyclin\ A^{5940R-1}$ $(Cyclin\ A^{R1})$, and *GST-D1* (gift from Helen McNeill, University of Toronto), *Cks85A*, *Skp2*, (Ghorbani et al., 2011), *Venus-Cyclin A 21C* (R.Dhaliwal, unpublished).

4.3.2 Cytology and immunostaining

Immunostaining and BrdU labeling of wing imaginal discs and brains obtained from wandering 3rd instar larvae was performed as described in (Ghorbani et al., 2011). BrdU incorporation was done for 30 min. Cleaved Caspase staining was performed according to (McNamee and Brodsky, 2009) with slight modification. Briefly, wing imaginal discs of third instar wandering larvae were dissected in 1x PBS and fixed in 4% paraformaldehyde for 30 min at room temperature (RT). Samples were rinsed 3 times in 1x PBS and extracted with 1 ml PBST (1x PBS+0.3% triton X-100) for 30 min at RT. Samples were then blocked in blocking solution (1xPBS+0.3% triton X-100+1% BSA) for 1 hr at RT. Samples were then incubated with primary antibody in blocking solution overnight at 4°C. After

incubation with primary antibodies, the samples were rinsed 3 times with 1 ml PBST followed by three washes for 20 min each in 1 ml PBST. Samples were then incubated with secondary antibody at RT for 4 hr. Following secondary incubation, the samples were rinsed 3 times with 1 ml PBST followed by three washes for 20 min each in 1 ml PBST. After the washes, samples were stored in 80% glycerol in 1x PBS at 4°C until slides were prepared. 80% glycerol in 1x PBS was used as mounting medium for all slide preparations. The above-mentioned protocol was used for all antibodies except the following that required some modification such as p-JNK. For ASL and γH2AV antibodies, tissues were extracted, blocked and incubated in both primary and secondary antibodies containing 1x PBS+0.1% triton X-100. β-galactosidase, anti-phospho-Histone H3, anti-alpha Tubulin and anti-gamma Tubulin antibodies required extraction in 1x PBS+0.2% tween 20+0.05% Triton-X-100. The extraction step was omitted for all other antibodies. The following primary antibodies were used: Rabbit anti-cleaved Caspase 3 1/100 (Cell Signaling Technology), rabbit anti-phospho-Histone 3 antibody 1/1000 (Santa Cruz), rat anti-alpha Tubulin antibody 1/500 (Milipore), mouse anti-gamma Tubulin antibody 1/500 (Sigma), rabbit anti-GFP 1/10000 (Abcam). Rabbit anti-γH2AV 1/500 (gift from Kim McKim, Rutgers University), rabbit anti-p-JNK1/500, rabbit anti-Asl 1/500 (gift from Jordan Raff, University of Oxford), mouse anti-β-galactosidase 1/500.

4.3.3 Western Blotting

Immunoblotting was performed using standard techniques as described in (Das et al., 2016). The following antibodies were used: mouse anti-Cyclin A (A12) at 1/10, mouse anti-Cyclin B antibody (F2F4) at 1/20, mouse anti-Actin at 1/250 (all obtained from Developmental Studies Hybridoma Bank), rabbit anti-Cyclin B3 at 1/2000 (a gift from Christian Lehner, University of Zurich), rabbit anti-GFP at 1/10000 (Torrey Pines Biolabs). Densitometry analysis was performed in Alpha Innotech FluorChemTM HD2 imager.

4.3.4 Confocal microscopy and image analysis

Images were captured on Olympus FluoView FV1000 laser scanning confocal microscope. In general pictures were taken in Z-stacks. The step-size was set to optimum and all layers obtained were merged and used for analysis. They were analyzed in Olympus Fluoview software version 1.5. Adobe Photoshop used for brightness/Contrast adjustment.

4.4 Results

4.4.1 Polyploid Skp2 mutant cells undergo SAC-mediated mitotic delay

We previously found that Skp2 is required for the entry into mitosis, and that it physically associates with Cyclin A and protects Cyclin A from premature destruction (Das et al., 2016). In keeping with a requirement for entry into mitosis, we had found that *Skp2* null cells have a lower mitotic index than controls. To determine if those cells progress normally through mitosis, we examined *Skp2* imaginal disc cells using phospho-Histone H3, γ-Tubulin and α-Tubulin antibodies. With these antibodies we can readily detect the major phases of mitosis in wild type cells (Figs. 1A-E). We noticed that compared to wild type cells in mitosis, *Skp2* null cells in mitosis appear larger, and the chromatin appears more dispersed, rarely showing the compact arrangement seen in wild type metaphase cells (Fig. 1F–J). We classified cells with dispersed chromatin on a fully formed mitotic spindle as being in anaphase based on absence of BUBR1 staining in those cells. In wild type cells BUBR1 is present in prometaphase and disappears in anaphase. We also noted that such cells are particularly common in the *Skp2* mutants (Fig. 1G). We rarely see a normal anaphase in which 2 equal and compact DNA masses are seen separating towards opposite poles. (Fig. 1I). *Drosophila Skp2* functions with the CDK-interacting protein, Cks1/Cks85A (Dui et al., 2013; Ghorbani et al., 2011). Consistent with a close functional relationship, *Cks1/Cks85A* null mutants show the same mitotic phenotype (Fig. 1K, L).

We determined the frequency of prophase, prometaphase/metaphase, anaphase/telophase and cytokinesis amongst mitotic cells from wild type, *Skp2* and

Cks1/Cks85A mutants. Representative wing discs for each genotype are shown in Fig. 1M and the quantification of this analysis is shown in Fig. 1R. This mitotic profile confirms that *Skp2* and *Cks1/Cks85A* null mutants have a higher incidence of prometaphase/metaphase cells and lower incidence of anaphase than wild type (Fig. 1 M-O, R).

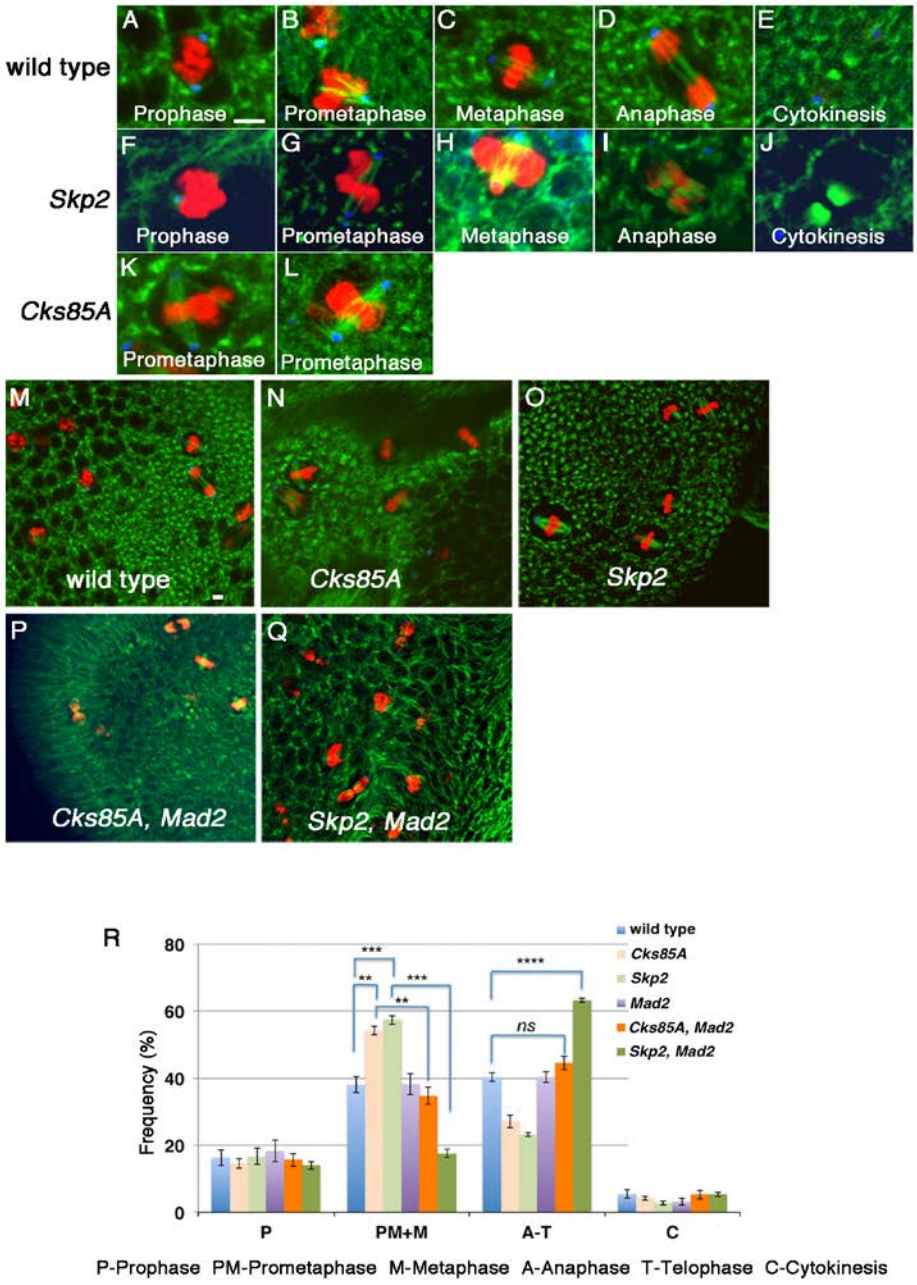

Fig. 1: Polyploid *Skp2* null cells undergo SAC mediated mitotic delay.

Fig. 1: Polyploid *Skp2* null cells undergo SAC mediated mitotic delay. A-L) Mitotic progression in the wing imaginal discs of 3rd instar wandering larvae of wild type, *Cks85A* and *Skp2* mutants was monitored by immunostaining for phospho-Histone H3 (red), microtubules (green) and the centrosomal marker γ-tubulin (blue). A-E) Representative wild type cells undergoing prophase, prometaphase, metaphase, anaphase and cytokinesis. F-J) *Skp2* cells undergoing prophase, prometaphase, anaphase and cytokinesis. K-L) *Cks85A* cells in pro-metaphase. M-O) Compared to wild type, *Cks85A* and *Skp2* show high frequency pro-metaphase/metaphase. P-Q) Loss of SAC gene *MAD2* in a *Cks85A* or *Skp2* background suppresses the prometaphase/metaphase delay. R) Graph showing relative frequencies of mitotic phases in wild type, *Cks85A*, *Skp2*, *MAD2*, *Cks85A*, *MAD2* double mutants along with *Skp2*, *MAD2* double mutants. Scale bar in A is =2 μm and applies to all panels.

In addition to increased DNA content in *Skp2* mutants, we noticed larger centrosomes. Centrosome duplication is frequently observed in *Skp2* null cells in mammals (Nakayama et al., 2000). To test if *Skp2* null cells in flies also show centrosome duplication, we probed *Skp2* null cells with centriole-specific marker, Asl. While wild type cells have single Asl focus, interestingly *Skp2* cells show two overlapping foci (Fig. 2A, B). This increase in apparent centrosome size appears to be a result of the combined effects of increased centrosome number and clustering of these centrosomes as we describe later. The correlation between increased centrosome number and increased ploidy indicates that centrosome amplification occurs in parallel with genome reduplication. This fits with our previous evidence that *Skp2* mutant cells bypass mitosis and go into S-phase with duplicated chromosomes and centrosomes (Das et al., 2016)

Polyploid cells make up only 30% of the total population in *Cks1/Cks85A* (25°C) and *Skp2* null wing imaginal discs (Ghorbani et al., 2011). It is striking, then, that almost all *Cks1/Cks85A* and particularly *Skp2* null cells in mitosis appear to have more chromatin and larger centrosomes than wild type cells (Fig. 1F-L). To more precisely compare centrosome sizes we measured the diameter of centrosomes from mitotic cells of wild type and *Skp2* null and plotted them (Fig. 2D). The graph shows almost no overlap between wild type and *Skp2* null cells, indicating that essentially all *Skp2* null that are in mitosis have larger centrosomes than wild type suggesting that almost all are polyploid. Putting together these findings it may mean that in *Skp2* mutants, the cells that become polyploid tend to delay or arrest in mitosis and argue against the idea that delay in metaphase leads to polyploidy in these cells.

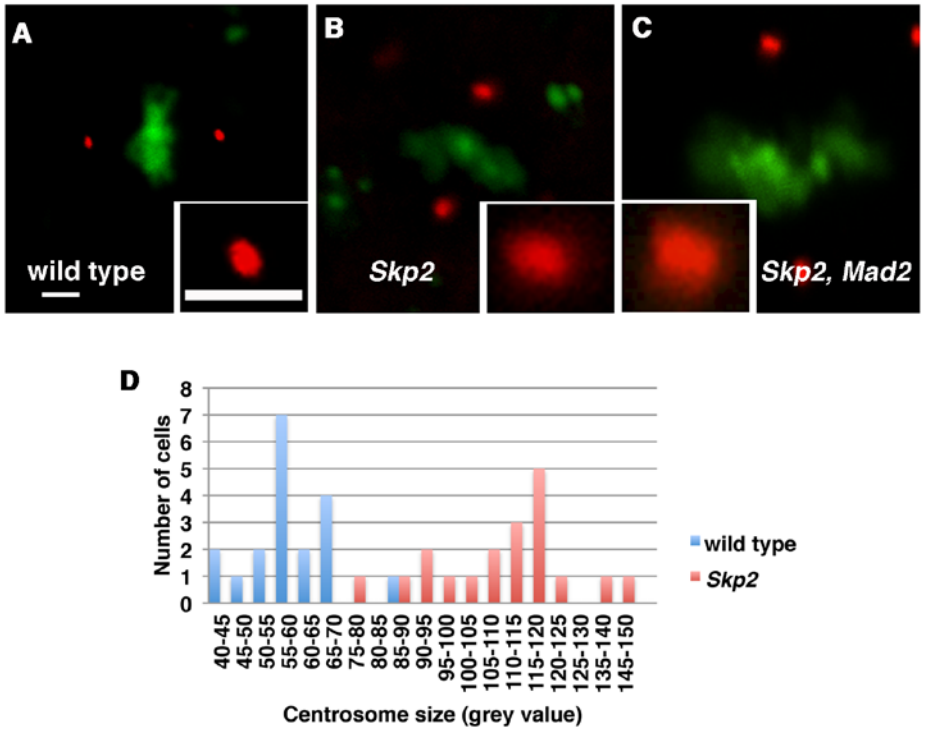

Fig. 2: Prometaphase/metaphase delayed cells are polyploid

Fig. 2: Prometaphase/metaphase delayed cells are polyploid A-C) Representative wing imaginal discs of 3rd instar wandering larvae were stained to mark DNA using Oligreen (green) and immunostained for centriole specific antibody, Asl, (red). D) The majority of *Skp2* cells delayed in pro-metaphase/metaphase have larger centrosomes based on the area. Scale bars in A - 2 μm applies to A-C.

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Knockdown of *Cyclin A* in mitotic cells also bypass mitosis and enters an endoreduplication cycle. Interestingly, the weaker knockdown of *Cyclin A* (*Cyclin* A^{R1}) more closely resembles the phenotype seen in *Skp2* mutants. We observed multiple centrosomes as well as centrosome clustering in all of the Cyclin A knockdown cells that are in prometaphase or metaphase (Fig. 3A, B’’’). Centrosome clustering is more prevalent in *Cyclin* A^{R1} (weaker knockdown) (Fig. 3C, C’) and multiple centrosomes are more prevalent in *Cyclin* A^{95} (stronger knockdown) (Fig. 3D, D’). *Cyclin* A^{R1} cells also show a partial reduction in mitotic index with consistently more cells arrested in prometaphase – metaphase than anaphase or telophase (Fig. 3E, G) (Das et al., 2016). The classification was done based on chromosomal arrangement, and microtubule positions. Due to small sample size we were not able to quantify our observation. Our findings might indicate a correlation between the phenotype that we observed in mitotic cells of *Skp2* null and *Cyclin* A^{R1}.

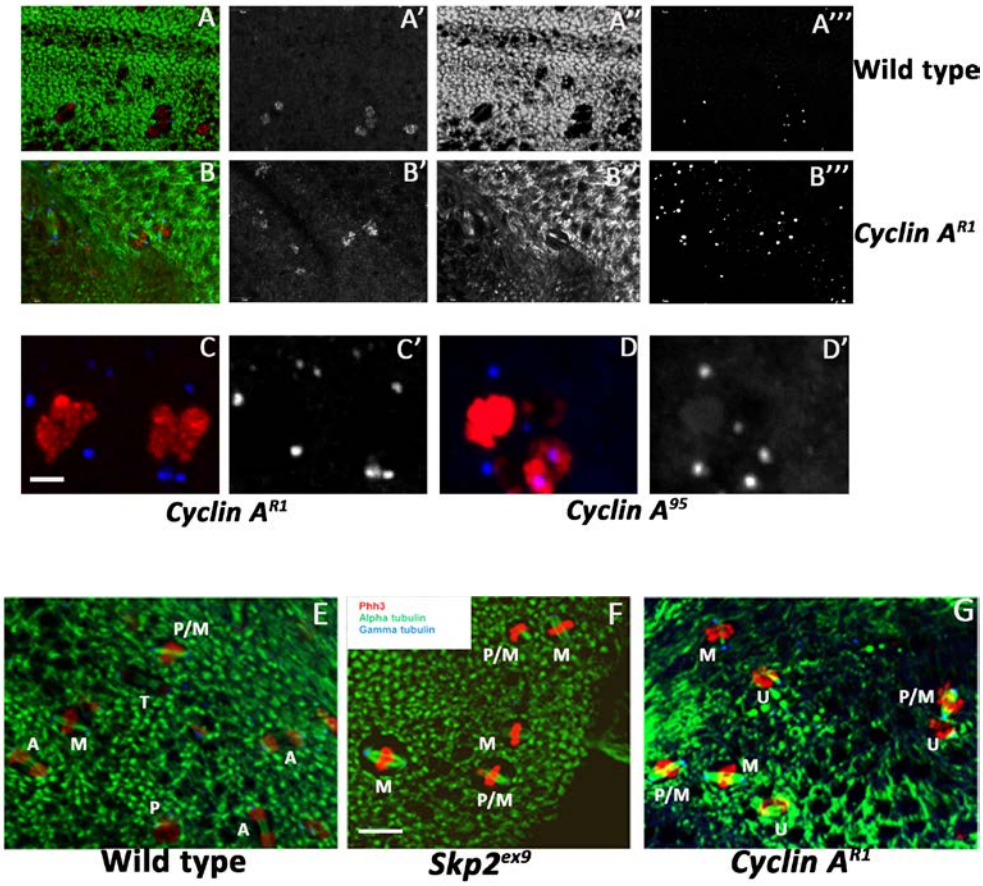

Fig. 3: Knockdown of *Cyclin A* in mitotic cells results in centrosome amplification

Fig. 3: Knockdown of *Cyclin A* in mitotic cells results in centrosome amplification A-B'') Wing imaginal discs of 3rd instar wandering larvae were stained to mark mitotic cells with phospho-Histone H3 antibodies (red), α-Tubulin antibodies (green) to identify microtubules, and γ-Tubulin antibodies (blue) to identify the centrosomes. C-D') zoomed in figure of mitotic cells showing increase in centrosome number and larger centrosome foci in *Cyclin* A^{R1} and *Cyclin* A^{95} mitotic cells. E-G) wing discs from 3rd instar wandering larvae immunostained with phospho-Histone H3 antibodies (red), α-Tubulin antibodies (green), and γ-Tubulin antibodies (blue) to identify the different phases of mitotic cells. Representative wild type (E), *Skp2*^{ex9} (F), *Cyclin* A^{R1} (G). The cell phases indicated as P for prophase, M for metaphase, P/M for prometaphase/metaphase, A for anaphase, T for telophase, and U for unclassified. Chromosomal arrangement and γ-Tubulin positions indicate a higher number of prometaphase/metaphase cells in *Skp2*^{ex9} and *Cyclin* A^{R1} than wild type. Scale bar in E=5μm, applies to A-B'''and E-G. Scale bar in C=2μm, applies to C-D'.

4.4.2 SAC activation in Skp2 null cells

The timing of this apparent delay of polyploid in *Skp2* and *Cks1/Cks85A* cells in prometaphase or metaphase suggests the possibility that they encounter a spindle assembly checkpoint (SAC)-mediated delay or arrest. To test this possibility, we looked for evidence of SAC activation in these cells by examining the distribution of the SAC proteins BUBR1 and MAD1. In wild type cells, BUBR1 appears on chromosomes in prometaphase and seems to abruptly disappear at anaphase (Figs. 3A, B). The cells that we classify as prometaphase in *Skp2* null are also BUBR1 positive, indicating that they are in prometaphase (Fig. 4C). Using BUBR1 staining as a guide we could also see that in *Skp2* null cells where chromatin appears to stretch to either spindle pole, BUBR1 staining is absent, indicating that these are undergoing an aberrant anaphase (Fig. 4E), perhaps due to escape from the SAC arrest. We also examined the localization of MAD1, using a MAD1-GFP transgene. Though MAD1 appears more diffuse, it shows a similar temporal appearance in wild type or present in prometaphase and then disappearing at anaphase (Figs. 4F, G). It also shows a similar pattern in *Skp2* mutant cells, and like BUBR1 its overall levels appear higher than is seen in wild type cells (Figs. 4H, I).

To confirm that *Skp2* and *Cks1/Cks85A* null cells undergo a SAC-mediated delay in the cell cycle we generated flies double mutant for *MAD2* and either *Skp2* or *Cks1/Cks85A*. These flies lack a functional SAC and we therefore expect them to no longer show an elevated frequency of pro-metaphase/metaphase. Indeed, *Cks1/Cks85A, MAD2* and *Skp2, MAD2* double mutants are rescued with respect to the increased metaphase and prometaphase frequency (Figs. 1P, Q, R). *MAD2* alone

has no appreciable effect on mitotic profile (Fig. 1R). While *MAD2*, *Skp2* double mutants are rescued for the prometaphase delay, mitosis appears even more dramatically disrupted than in *Skp2* alone. In the double mutants we frequently observe cells in late anaphase or telophase with chromosomes having failed to segregate (Figs. 4J, K, L). We also observe isolated chromosome fragments in these mutants that are undergoing late anaphase or telophase, indicating chromosome breakage (Fig. 4L). The degree of polyploidy is also greater (Figs. 4J, L and Fig. 5H), suggesting that the SAC-mediated cell cycle delay is important in these polyploid cells for limiting polyploidy.

As mentioned above, *Skp2* mutant cells appear to have larger/more centrioles, apparently resulting from centrosome re replication and clustering. It was previously found that some components of the SAC are required for centrosome clustering – in particular BUBR1 and MAD2 has been shown to be required for this (Kwon et al., 2008). It is interesting to note that in *Skp2, MAD2* cells in mitosis there are still only 2 centrioles even though they often appear even larger than those observed in *Skp2* (Figs. 2B,C). Therefore, centrosome clustering still seems to occur relatively normally even though the SAC is non-functional. We tried to compare *Skp2, MAD2* to *Skp2, BUBR1* mutants. However, the latter were extremely difficult to obtain due to high lethality of this mutant combination. Nonetheless, in a preliminary result, out of only a small number of mitotic cells we found evidence of multiple centrosomes in the *Skp2, BUBR1* mutant, but not in BUBR1 alone (Figs. 4M,N). While this result will need to be repeated it suggests the interesting possibility that centrosome

clustering depends not on SAC activity per se but rather on specific components of the SAC.

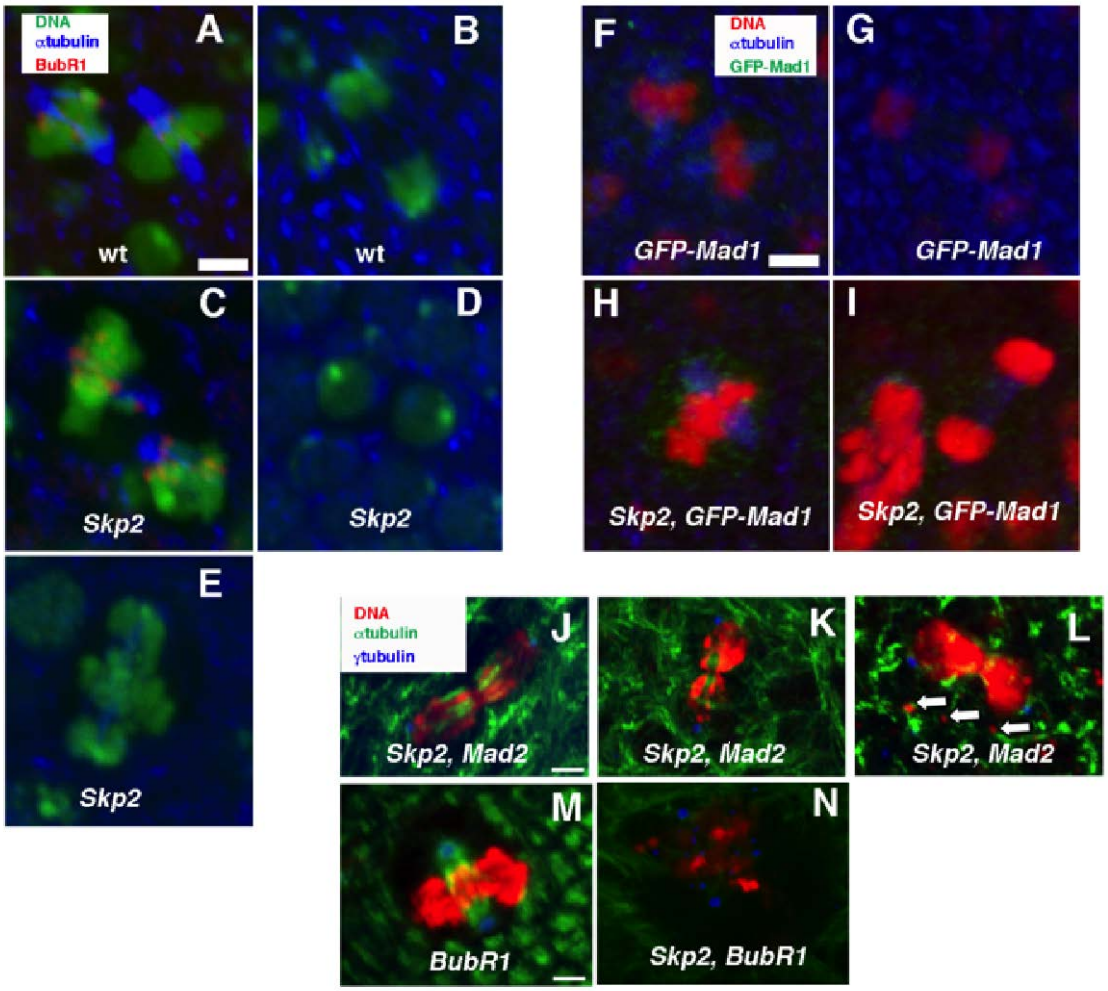

Fig. 4: SAC is active in pro-metaphase/metaphase delayed *Skp2* mutant cells.

Fig. 4: SAC is active in pro-metaphase/metaphase delayed *Skp2* mutant cells. A-E) The wing imaginal discs of 3rd instar wandering wild type and *Skp2* larva were probed with BUBR1 antibodies (red). DNA is stained with oligreen (green) and microtubules are stained with α-tubulin antibodies (blue). F-I) GFP–MAD1 can be seen in wild type pro-metaphase and disappears in anaphase likewise in *Skp2*, GFP–MAD1 accumulates in pro-metaphase and disappears in anaphase. Strong accumulation of GFP–MAD1 is seen in *Skp2*. J-N) Wing imaginal discs were immunostained for phospho-Histone H3 (red), microtubules (green) and the centrosomal marker γ-tubulin (blue). J-L). Representative images of abnormal-anaphase phenotype seen in *Skp2, MAD2* double mutants. L) Arrows showing chromosome fragments in a cell undergoing abnormal anaphase. M-N) *BUBR1, Skp2* double mutants show a scattered centrosome phenotype, while in BUBR1 alone they seem to be clustered together. Scale bar in A is - 2 μm and applies to all panels.

4.4.3 Polyploid Skp2 null cells undergo a DNA damage response

Our results suggest that *Skp2* cells undergo aberrant mitoses that may lead to genome instability, and that SAC activity helps protects *Skp2* null animals from this genomic instability. *Skp2* and *Cks1/Cks85A* null mutants undergo a high degree of apoptosis (Ghorbani et al., 2011) (Figs. 5A, B). We predict that this apoptosis is the outcome of a pathway or pathways that sense genome instability. If this is the case we expect that in the absence of SAC function, *Skp2* null cells will be subject to increasing genome instability and as a consequence, they will show elevated levels of apoptosis. Indeed we find that *Skp2, MAD2* double mutants have much greater levels of apoptosis than *Skp2* alone (Figs. 5B, D) while *MAD2* alone has no effect (Fig. 5C). This indicates that SAC arrest protects *Skp2* mutant cells from genome instability and resultant activation of a checkpoint pathway that leads to apoptosis.

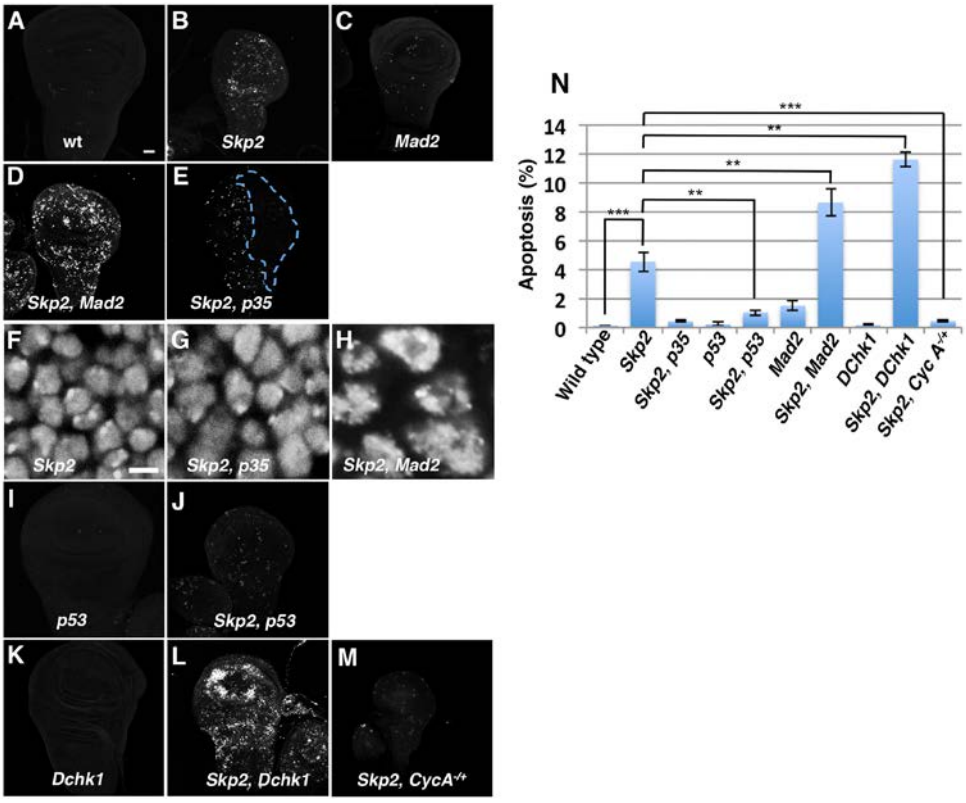

Fig. 5: *Skp2* cells undergo apoptosis to reduce genomic instability

Fig. 5: *Skp2* cells undergo apoptosis to reduce genomic instability. A-E) Wing imaginal discs of 3rd instar wandering larvae were assayed for apoptosis by probing with anti-cleaved Caspase 3 antibodies. Expression of Baculoviral protein p35 in half of *Skp2* null imaginal wing disc using *en-GAL4* driver (area within dotted line), almost completely abolishes apoptosis (E). F-H) Wing imaginal disc were stained with Oligreen to mark DNA to show the level of ploidy seen in *Skp2* alone, *Skp2, p35* and *Skp2*, *MAD2*. I-M) Wing imaginal discs of 3rd instar wandering larvae were assayed for apoptosis by probing with anti-cleaved Caspase 3 antibodies. (N) Graphical representation of regulation of apoptosis by various genes in *Skp2* null background. Scale bar =30 μm and applies to A-E, I-M and while scale bar in F-J =2 μm.

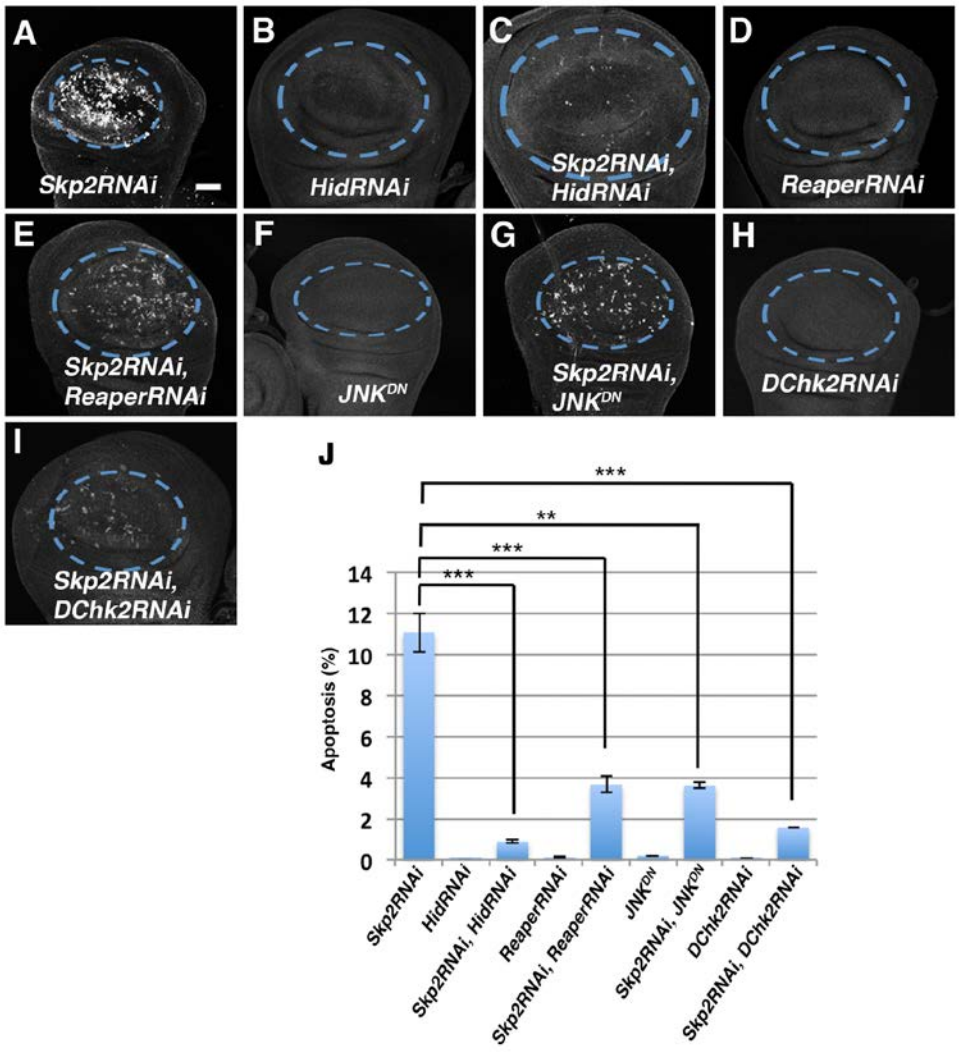

Fig. 6: Hid and reaper are both necessary for apoptosis in *Skp2*

Fig. 6: Hid and reaper are both necessary for apoptosis in *Skp2* A-I) Cleaved Caspase 3 staining of wing imaginal discs of the above mentioned genotypes. The dotted lines represent the wing blade region where the genes are knocked down. (J) Graphical representation showing percentage of apoptosis of the above mentioned genotypes. Scale bar =30 μm applies to A-I.

Aberrant mitosis can lead to chromosome breaks, which in turn can signal a DNA damage response that leads to apoptosis. To determine if such a pathway is activated in *Skp2* and in *Skp2, MAD2* cells we probed wing imaginal discs from these mutants for the presence of phosphorylated H2Av Histone (γH2Av). This variant histone is phosphorylated at sites of chromosome breaks and can thus be used as a marker of DNA breaks in *Drosophila* (Jang et al., 2003; Mehrotra et al., 2008). We find that *Skp2* mutant wing imaginal discs show an increase in number of γH2Av foci, suggesting that cells in this mutant do in fact incur DNA damage, possibly as a result of mitotic defects (Fig. 7B). We then examined *Skp2, MAD2* double mutants and found that the number of γH2Av foci is much greater than *Skp2* alone (Fig. 7C). These results indicate that *Skp2* null cells undergo apoptosis, possibly as a result of DNA damage, and that the SAC helps to protect these cells from this fate.

We previously showed that apoptosis in the *Cks1/Cks85A* null mutant is suppressed by expression of the Baculoviral p35 protein (Ghorbani et al., 2011). Similarly, almost all apoptosis in the *Skp2* null wing disc is suppressed by expression of p35 (Fig. 5E). We also find that the suppression of apoptosis results in an overall increase in cell ploidy (Fig. 5G), indicating that apoptosis serves to restrict polyploidy in the *Skp2* mutant. Similarly loss of SAC also increases ploidy (Fig. 5H), indicating that this checkpoint also helps to control ploidy in the *Skp2* mutant.

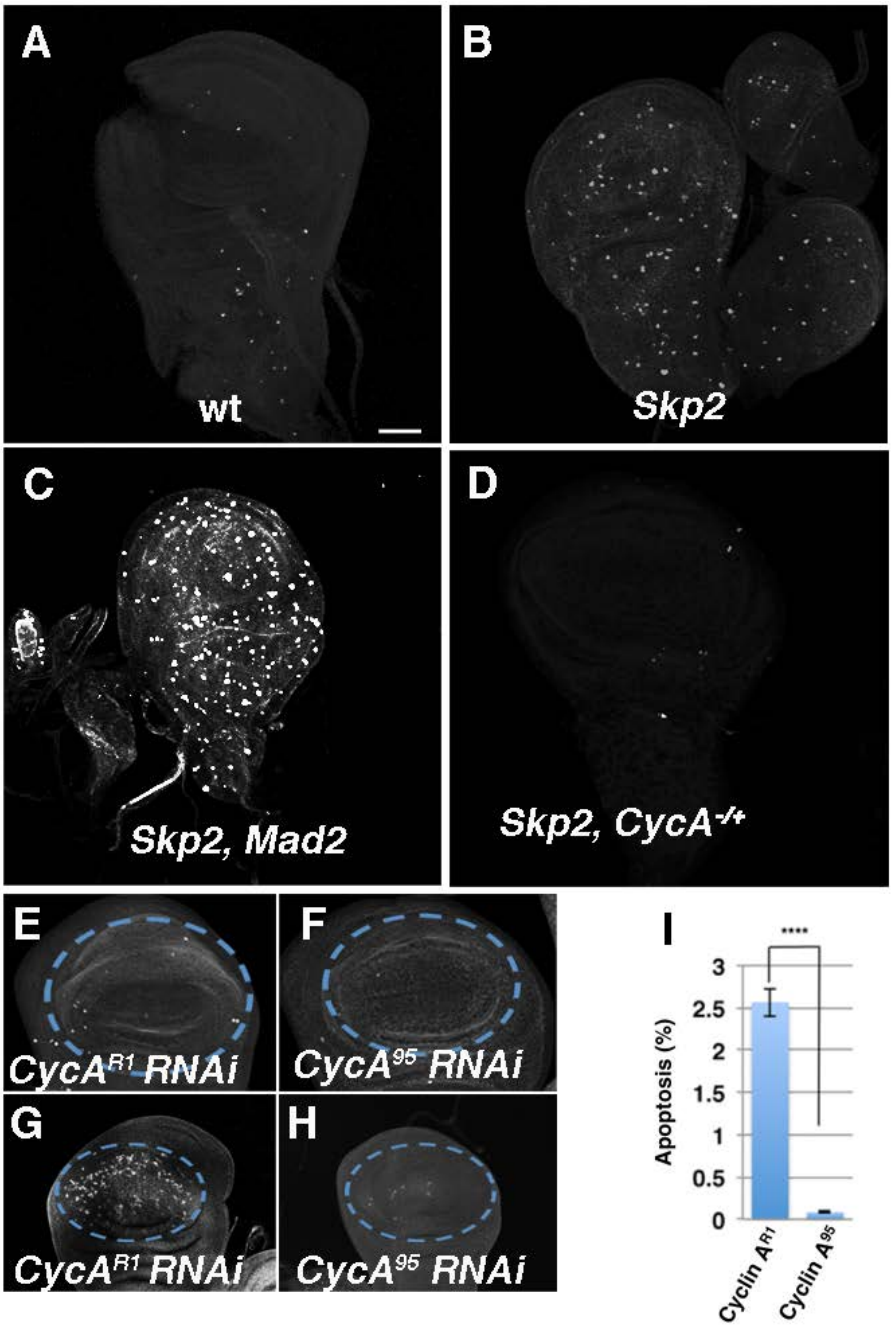

Fig. 7: *Skp2* null cells signal a DNA damage response

Fig. 7: *Skp2* null cells signal a DNA damage response. A-D) Wing imaginal discs of 3rd instar wandering larvae were assayed for γH2av by probing with anti-γH2av antibodies. E-F) γH2av staining of wing imaginal discs were done in which *rn-GAL4* was used to knock down *Cyclin A* either using *Cyclin* A^{R1} or *Cyclin* A^{95}. G-H) Cleaved Caspase 3 staining of wing imaginal discs in which *rn-GAL4* was used to knock down *Cyclin A* either using *Cyclin* A^{R1} or *Cyclin* A^{95} lines. I) Graph shows the level of apoptosis observed when *Cyclin A* is knocked down using the two different RNAi lines. Scale bar in A is=50 μm and applies to all panels.

Apoptosis in *Drosophila* is mediated through the activity of anti-IAPs Grim, Hid and Reaper (Goyal et al., 2000).To determine if these are involved in *Skp2* cell death we combined *Skp2* knockdown with RNAi against *Reaper* and *Hid*. We found that loss of *Hid* strongly reduces apoptosis in the *Skp2* background while loss of *Reaper* also significantly reduces apoptosis (Fig. 6C, E, and J). Therefore the pro-apoptotic anti-IAPs, Hid and Reaper are both necessary for apoptosis in the *Skp2* null background.

γH2Av is generated by the activity of the ATR and ATM kinases in response to double strand breaks in mammals(Stiff et al., 2004; Ward and Chen, 2001). These kinases recruit proteins involved in repair and they activate a DNA damage response that triggers apoptosis (Tanaka et al., 2007). To characterize the pathway activated in *Skp2* cells we tested for a requirement for known checkpoint genes in the *Skp2* mutants. In *Drosophila* as in mammals, a number of cell stresses including DNA damage converge on the p53 tumour suppressor. We find that loss of *p53* reduces the degree of apoptosis by approximately 70% (Fig. 5J, N) indicating that it plays a major role. On the other hand, comparing the effect of the *p53* null to that of *p35* overexpression it is clear than p53 is not responsible for all of the apoptosis in polyploid *Skp2* cells (Fig. 5N).

4.4.4 DChk1 and DChk2 function in distinct checkpoints in polyploid Skp2 null cells

The checkpoint kinases, Chk1 and Chk2 function downstream of ATM (and ATR) to transduce DNA damage signaling(Song, 2005). We found that *Chk2, Skp2* double knockdown results in a reduction in apoptosis similar to that seen in the *p53, Skp2* double mutant (Fig. 5J, N and Fig. 6I, J). It is consistent with the possibility that Chk2 plays a key role upstream of p53 in the apoptotic response of *Skp2* null cells.

In striking contrast to the *Skp2, Chk2* double knockdown which showed reduced apoptosis; in *Skp2, Chk1* double mutants, apoptosis is greatly elevated (Fig. 5L, N). Therefore, as with MAD2, Chk1 may be required for a cell cycle checkpoint that if compromised, might lead to activation of the Chk2 dependent apoptotic pathway. To identify a possible Chk1-dependent cell cycle checkpoint in *Skp2* null cells, we compared cell cycle profiles for *Skp2, Chk1* double knockdown and *Skp2* alone. If Chk1 affects a G2 arrest or G1 arrest in S*kp2* null cells, we expect to observe a higher mitotic index or S-phase index respectively in the double knockdown than in *Skp2* alone. We find that the S-phase index of *Skp2, Chk1* double knockdown is higher than *Skp2* alone (Fig. 7J). This suggests that Chk1 promotes a G1 arrest in the *Skp2* mutant background. Surprisingly, the mitotic index of *Skp2, Chk1* double knockdown is highly reduced (Fig. 7K). We also looked at the mitotic phase profile of *Skp2, Chk1* double knockdown to look for any difference compared to *Skp2* alone. The profile appears similar to *Skp2* alone with one exception – we observe a higher frequency of cytokinesis in the double (Figs. 7L, H, I).

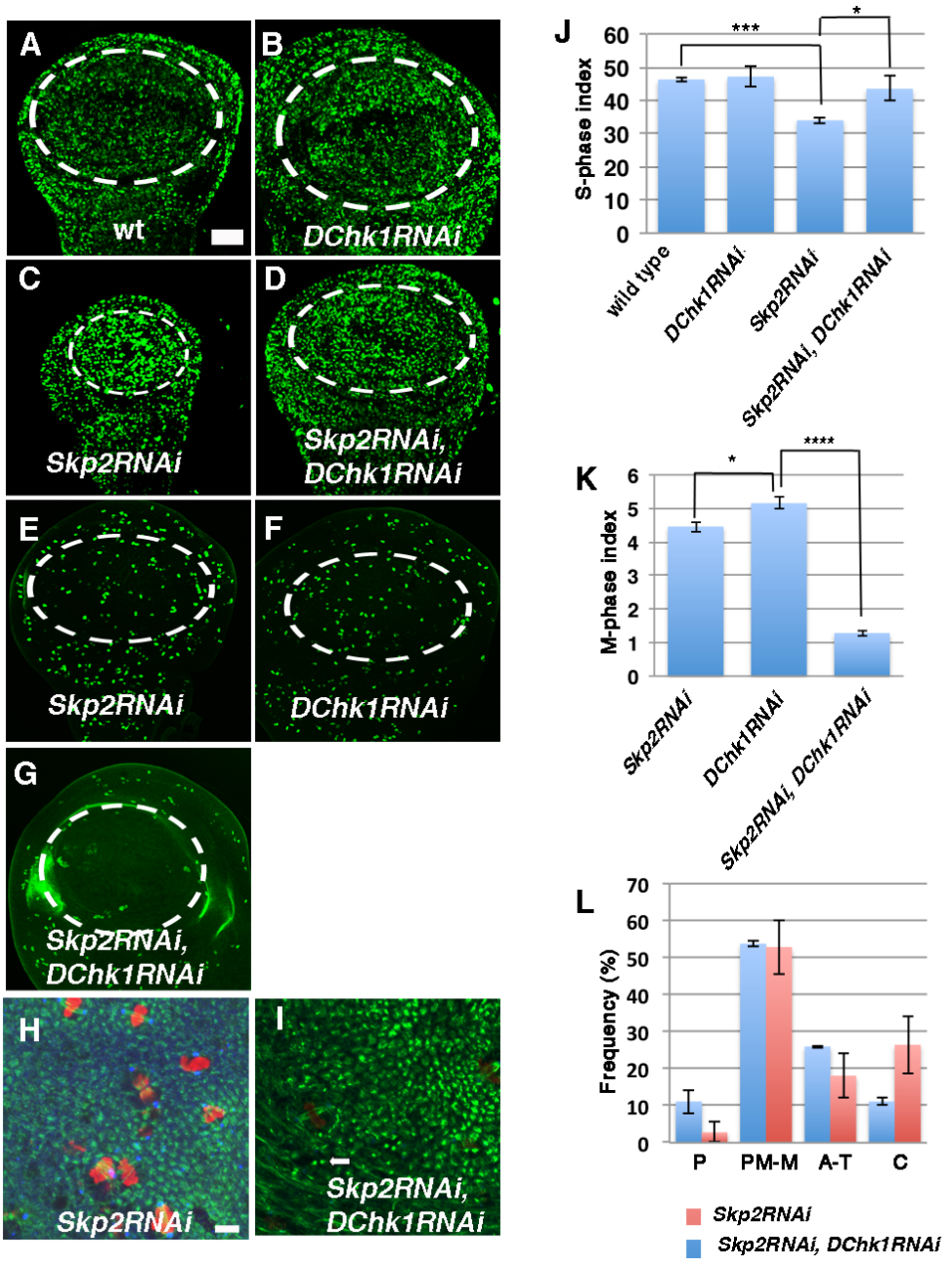

Fig. 8: DChk1 activates checkpoint response in *Skp2* null cells

Fig. 8: DChk1 activates checkpoint response in *Skp2* null cells. A-D) BrdU labeling was used to label S-phase cells of wild type, *DChk1* knockdown, *Skp2* knockdown and *Skp2*, *DChk1* double knockdown. Wing blade region (marked by dotted lines) specific knockdown was done using *rn-GAL4* driver. E-G) Mitotic frequency using anti-phospho-Histone H3 antibody was determined in *Skp2* RNAi, *DChk1* RNAi and double knock down of *Skp2*, *DChk1* H-I) Mitotic progression in the wing imaginal discs of 3rd instar wandering larvae of *Skp2* RNAi and *Skp2*, *DChk1* double knockdown was monitored by immunostaining for phospho-Histone H3 (red), microtubules (green) and the centrosomal marker γ-tubulin (blue). The arrow points to one of the cells undergoing cytokinesis. J) Graphical representation of S-phase index. K) Graphical representation of M-phase index. L) Graph showing relative frequencies of mitotic phases in mentioned genotypes. Scale bar in A=30 μm and applies to panels A-F. Scale bar in H = 2 μm and applies to panels H-I.

4.4.5 JNK pathway activation in Skp2 null cells

While *Hid* knockdown or *p35* expression largely suppresses apoptosis in the *Skp2* null background, *p53* has only a partial effect, suggesting that another pathway functions in parallel with p53 to promote apoptosis in *Skp2* null animals. The JNK pathway has been implicated as a proapoptotic pathway in mammalian and *Drosophila* cells (McEwen and Peifer, 2005; Stadheim and Kucera, 2002), so we tested for a role in *Skp2* null cells. First we looked for evidence that the JNK (Basket, in *Drosophila*) pathway is activated in *Skp2*. We probed wing discs with a phospho-JNK antibody and found that in the region of *Skp2* knockdown, phospho-JNK levels are elevated (Fig. 9A). JNK pathway activation is also be assessed by looking at transcription of a downstream target *puckered* (*puc*) (Martín-Blanco et al., 1998). Using the puc-lacZ reporter we see activation of JNK in cells where *Skp2* has been knocked down (Fig 9B). To determine if JNK activation in *Skp2* null wing discs promotes apoptosis, JNK^{DN}, which is a dominant negative form of JNK, was co-expressed along with *Skp2* RNAi. These show a reduction in apoptosis (Fig. 6G, J) indicating that this pathway is also activated in *Skp2* and serves to promote apoptosis.

The JNK pathway is activated in response to a number of different cellular stresses. One of the better-established triggers is the accumulation of free radical oxygen species (ROS). Glutathione-S-Transferase (GST), encoded by *gstD1* gene, can be used as a direct readout of ROS, and *gstD-GFP* reporter flies can be used to monitor the level of ROS (Sykiotis and Bohmann, 2008). Using *gstD-GFP* reporter flies, we find that indeed *Skp2* mutant cells accumulate reactive oxygen species (ROS) (Fig. 9C). While we do not yet know if the ROS accumulation in *Skp2* null

cells contributes to JNK pathway activation, these results nonetheless suggest that some of the apoptosis observed in the *Skp2* null mutant may be due to an ROS-triggered JNK-mediated stress response.

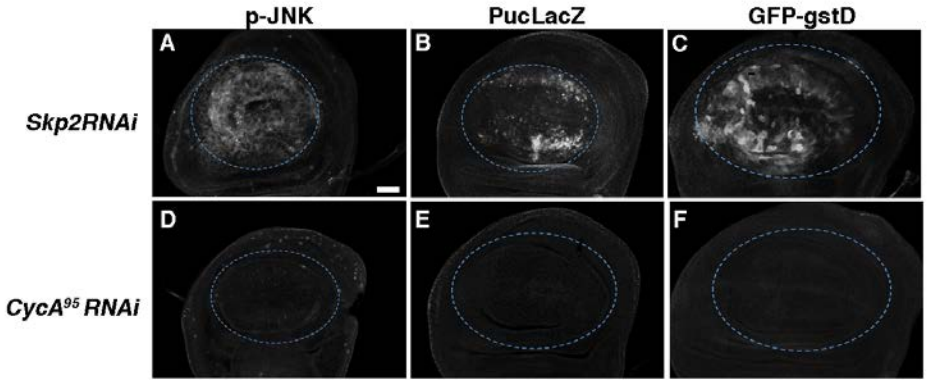

Fig. 9: JNK pathway is active in *Skp2* null cells

Fig. 9: JNK pathway is active in *Skp2* null cells. A-F). Wing imaginal discs of 3rd instar wandering larvae were immunolabeled using anti-phospho-JNK (p-JNK) antibody to monitor JNK activity, probed with anti- β-galactosidase in wing discs of puc LacZ enhancer trap lines, monitored for the activity of JNK target Puckered (Puc) or GST accumulation was monitored in *gstD-GFP* transgene expressing wing discs to see the level of ROS. A-C) *Skp2* was knocked down in wing blade region of these discs using *rn-GAL4* (marked by dotted lines). Scale bar in A =30 μm and it applies to all panels.

The JNK pathway can transcriptionally upregulate autophagic genes in *Drosophila* to protect them from oxidative stress(Wu et al., 2009). We knocked down *Skp2* in wing imaginal discs of flies that expressed GFP-tagged LC3 (homolog of human ATG8) that labels the autophagosome (Rusten et al., 2004). We observed GFP–LC3 accumulation in the region of the wing disc where *Skp2* was knocked down, confirming autopahgic activity (Fig. 10 B). Extracts of larval brain and wing disc shows the free GFP that is generated as a result of autopahgic degradation of mCherry-GFP-Atg8a. Free GFP is not observed in imaginal disc extracts from larvae expressing mCherry-GFP-Atg8a alone but can be observed when mCherry-GFP-Atg8a is expressed in *Skp2* mutant background (Fig. 10 C). We further analyzed the rate of autopahgic degradation using mCherry-GFP-Atg8a. Autophagosome bound mCherry-GFP-Atg8a is positive for both GFP and mCherry but when autophagosomes fuse with lysosomes to form autolysosomes, GFP gets quenched, rendering the reporter positive only for mCherry (Nagy et al., 2013).Therefore, mCherry positive cells are a confirmation of active autophagy. While we hardly see any mCherry puncta in wild type discs, the discs in which *Skp2* is knocked down contain many mCherry dots (Fig. 10 D, E). This clearly confirms that *Skp2* cells are undergoing autophagy.

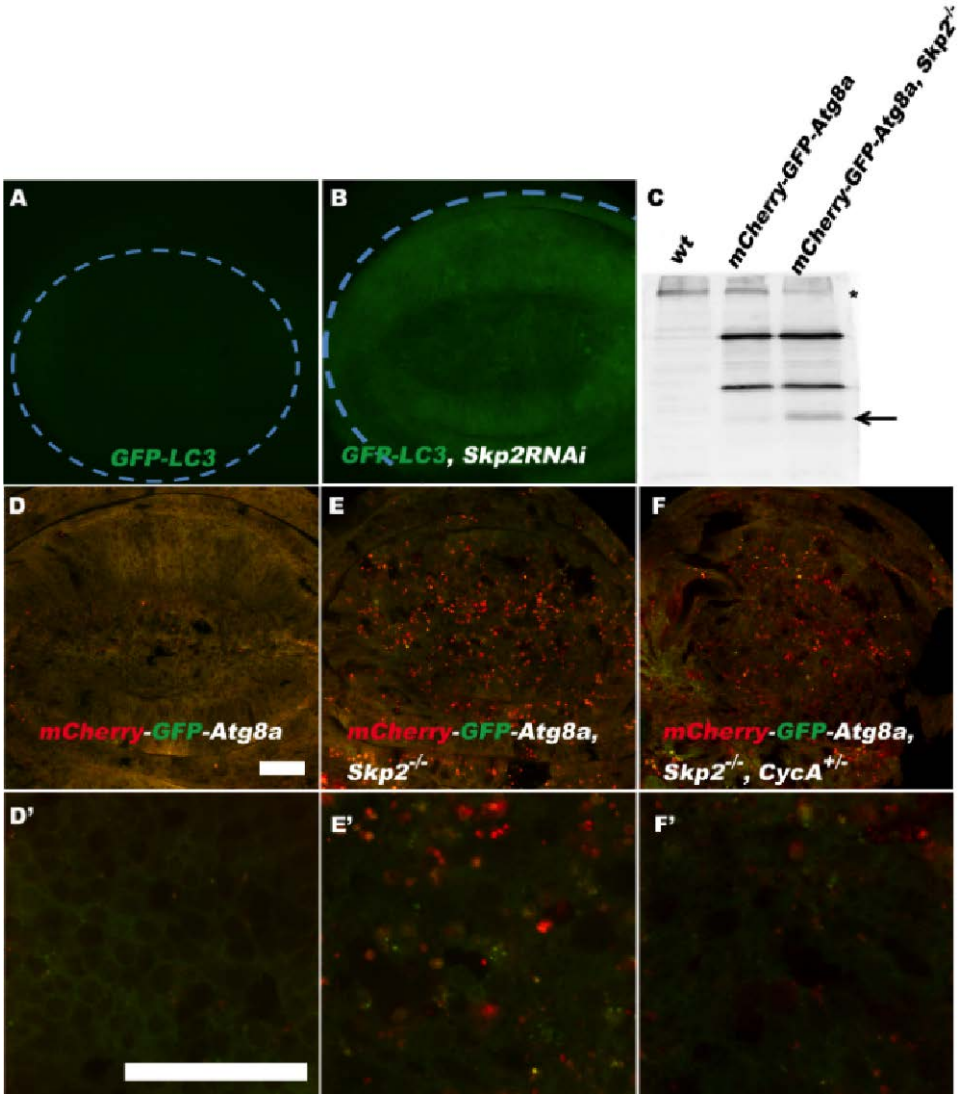

Fig. 10: *Skp2* mutants undergo autophagy

Fig. 10: *Skp2* mutants undergo autophagy. A-B) GFP-LC3 and *Skp2RNAi* was expressed in in the wing blade region using *rn-GAL4* represented by dotted line. C) Immunoblot of larval brain and wing disc extracts that was probed with anti-GFP antibody. Free GFP is not observed in imaginal disc extracts of larvae expressing mCherry-GFP-Atg8a alone but can be observed when mCherry-GFP-Atg8a is expressed in *Skp2* mutant background. Wild type imaginal disc extract serves as a control for non-specific bands. Asterisk denotes the band that is used as a loading control. D-F) mCherry and GFP expression was monitored in imaginal discs of larvae expressing either mCherry-GFP-Atg8a alone or in the background of *Skp2* or *Skp2, Cyclin* A^{H170}. D'-F') Picture taken at higher magnification of wild type (G*), Skp2* (H) and *Skp2,* $A^{H170+/-}$. Scale bars-30 μm and applies to A-F.

4.4.6 Are checkpoints due to a Skp2-specific pathway or polyploidy?

We have shown that *Skp2* null cells are subject to multiple checkpoint pathways, apparently arising from the presence of DNA damage (as shown by γH2Av accumulation) and from cell stress resulting from ROS. We were interested to understand what causes the DNA damage and cell stress. We consider the following possibilities: 1st polyploidy itself may lead to DNA replication errors or incomplete replication that can lead to the accumulation of γH2av marks. 2nd, Skp2 may have a role in protecting the genome that is distinct from its role in preventing polyploidy. 3rd aberrant mitosis in *Skp2* null cells could lead to DNA damage, possibly through chromosome breakage resulting from aberrant anaphase.

Previously we showed that *Skp2* null cells become polyploid in part as a result of failure to enter mitosis, which in turn reflects a role for Skp2 in protecting Cyclin A (Das et al., 2016). Strong knockdown of *Cyclin A* results in polyploidy but unlike *Skp2*, these cells rarely enter mitosis, instead they seem to enter an endocycle. We had found that loss of a single copy of *Cyclin A* in the *Skp2* null background converts these cells to endocycling cells (Das et al., 2016). We considered that by comparing checkpoint activation in *Cyclin A RNAi* lines and in *Skp2, Cyclin* $A^{H170/+}$ cells we could distinguish between models for how *Skp2* promotes these checkpoints. We first examined apoptosis in flies expressing the RNAi line, *Cyclin* A^{95} and found that these cells do not undergo any apoptosis (Fig. 6F, I). This is despite the fact that these cells are significantly more polyploid than *Skp2* null cells (Das et al., 2016).

We next examined *Skp2, Cyclin* $A^{H170+/-}$ cells. Like *Cyclin* A^{95} knockdown, these cells do not enter into mitosis but become considerably more polyploid than

Skp2 alone (Das et al., 2016). When we performed cleaved Caspase staining, we found that these cells do not undergo apoptosis (Fig. 4 M, N). The observation that *Skp2* null cells avoid apoptosis when heterozygous for *Cyclin A* strongly argues against, the idea that the apoptosis observed in *Skp2* is due to a specific role for *Skp2* in apoptosis. It also argues against polyploidy per se as the stimulus for apoptosis, an argument that is further supported by the fact that apoptosis is not observed when *Cyclin A* is knocked down using *Cyclin* A^{95} (Fig. 7 G, H, I)

It has been recently shown that endocycling cells are resistant to apoptosis even under conditions of DNA damage that lead to a strong apoptotic phenotype in mitotic cells (Mehrotra et al., 2008). Our results could therefore indicate that *Skp2, Cyclin* $A^{H170+/-}$ cells are protected from apoptosis despite the damage they incur. We examined γH2Av to determine if indeed these cells incur DNA damage. Strikingly, we saw very low levels of γH2Av in *Skp2, Cyclin* $A^{H170+/-}$ cells (Fig. 7D). The absence of γH2Av signal indicates that somehow *Skp2, Cyclin* $A^{H170+/-}$ cells avoid DNA damage and thus avoid induction of apoptosis. Given that the obvious difference between *Skp2* and *Skp2, Cyclin* $A^{H170+/-}$ is that *Skp2* cells undergo mitosis, we hypothesized that it is aberrant mitosis resulting from polyploidy that results in DNA damage and DDR.

Knockdown of *Cyclin A* using the *Cyclin* A^{R1} results in a weaker polyploid phenotype than the stronger *Cyclin* A^{95} (Das et al., 2016). Furthermore, these cells enter mitosis like *Skp2* null cells. If passage through mitosis that triggers apoptosis then we predict that *Cyclin* A^{R1} will show an increase of apoptosis compared to

Cyclin A^{95}. To test this we performed cleaved Caspase assay on these wing discs. Indeed, we find that these wing discs undergo apoptosis (Fig. 7G, I) and they have a slightly elevated γH2Av foci compared to *Cyclin A*^{95} knockdown (Fig. 7A, E, F).

We found that in addition to the DDR, *Skp2* null cells have an active JNK pathway, possibly as a consequence of accumulation of reactive oxygen species, and that *Skp2* null cells undergo autophagy. To determine if these pathways differ in their activation in *Skp2* cells versus endocycling cells, we looked at *Cyclin A*^{95} with the reporters for JNK pathway and reactive oxygen species (Fig. 9D, E, F). We do not see any JNK activation or upregulation of ROS in these cells. To check if endocycling cells undergo autophagy we looked for autophagy in *Skp2, Cyclin A*$^{H170+/-}$. The results suggest that this occurs in the endocycling cells but at a lower level (Fig. 10F).

4.5 Discussion

There is a clear link between polyploidy and cancer and accumulating evidence suggests that polyploidy specifically tetraploidy is an important early step in cancer progression (Storchova and Pellman, 2004). Here we investigate the consequences of the polyploidy that arises in *Skp2* null cells. We found that *Skp2* null cells are subject to a SAC arrest. The relationship between the SAC and polyploidy is complex and there are multiple causal relationships between these two phenomena. Cells that arrest due to SAC activation can exit from this arrest through a process known as mitotic slippage. Mechanistically, this occurs because of gradual depletion of mitotic cyclins that occurs despite this arrest. As a consequence of mitotic slippage, cells can bypass mitosis, re-enter a G1-like state, and become polyploid after the next S-phase. In this scenario SAC arrest leads (indirectly) to polyploidy (Ganem et al., 2007).

It is possible that at least some of the polyploidy found in *Skp2* null cells is due to mitotic slippage. However, it is likely not the only or even the major reason for polyploidy in *Skp2* null cells. 1st, we found that the SAC arrested cells observed in *Skp2* invariably were already polyploid, even though most cells in these mutants are diploid (Ghorbani et al., 2011). 2nd, the polyploidy that results from loss of *Skp2* is elevated when a single copy of *Cyclin A* is removed. However, in this genetic background the cells almost completely bypass mitosis and rarely undergo SAC-mediated arrest. Therefore, polyploidy appears to occur independent of SAC function.

It is possible that in *Skp2* null cells we see the opposite relationship between SAC and polyploidy: that polyploidy leads to SAC activation. If this is the case,

what is the specific feature of polyploidy that triggers SAC activation in these mutants? One possibility is that abnormal chromosomes in these cells fail to make proper bipolar attachments to the mitotic spindle. Our FISH experiment against a peri-centromeric region of the X-chromosome shows, that polyploid *Skp2* null cells do not have more chromosomes than wild type (Das et al., 2016). Thus these chromosomes are polytene – they remain attached as they endoreplicate. The presence of multiple centromeres may affect kinetochore assembly, possibly even allowing for multiple attachments to a single polytene chromosome. In *Skp2*, polyploid chromosomes appear to lag at the midzone or sometimes appear near the poles and this may be due to aberrant kinetochore attachments. When the SAC is eliminated in the *Skp2,MAD2* double mutant we found a much greater incidence anaphases with lagging chromosomes, and we see examples of fragmented chromosomes, consistent with the idea that in *Skp2*, chromosomes do not establish proper bipolar attachments to spindle microtubules and that this normally results in a SAC-mediated arrest.

A third possible cause of the SAC arrest in *Skp2* null cells may be the presence of supernumerary centrosomes. Supernumerary centrosomes are expected to result in the formation of multipolar spindles and a consequent SAC-mediated arrest. We showed that *Skp2* null cells have ectopic centrosomes. However, we find that the extra centrosomes appear to efficiently cluster and the majority of mitotic spindles appear to be bipolar. Therefore it is not clear if the presence of extra centrosomes seen in *Skp2* null mitotic cells can explain the SAC arrest phenotype. When centrosome clustering is disrupted, as in the *Skp2, BUBR1* double mutant, this

appears to lead to a far more severe mitotic phenotype, and likely accounts for the high degree of larval lethality of these double mutants.

We found that polyploid cells resulting from loss of *Skp2* or to a lesser degree, partial knockdown of Cyclin A, accumulate DNA damage. DNA damage results in the activation of a well-characterized DNA damage response that can in turn lead to cell cycle arrest or apoptosis. We show that the apoptotic response occurs in *Skp2* null cells and that this depends in part on the checkpoint kinase, Chk2 as well as p53. P53 in *Drosophila* promotes apoptosis in large part by transcription of pro-apoptotic genes *hid*, *grim* and *reaper*. We show that both Hid and Reaper contribute to the apoptosis that occurs in *Skp2* null cells.

Chk1 and Chk2 are thought to play somewhat overlapping roles in transducing checkpoint signals from the upstream sensor kinases, ATM and ATR. We find that loss of Chk1 has a dramatically different effect on *Skp2* mutants than does loss of Chk2. In the absence of *Chk1, Skp2* null cells undergo extensive DNA damage and apoptosis. In this respect, loss of Chk1 in this background resembles the effect of loss of the SAC. We propose that, like the SAC, Chk1 is required for a cell cycle checkpoint that arrests *Skp2* null cells. In the absence of Chk1, cells continue through aberrant cell divisions, incur further DNA damage and activate the Chk2-dependent apoptotic pathway. In many ways this double checkpoint is similar to the situation in the embryo during the midblastula transition. In late state syncytial embryos Chk1 mediates a cell cycle arrest that is necessary for cellularization and other gastrulation events. In the absence of Chk1, a Chk2-dependent checkpoint is activated that leads to centrosome inactivation (Sakurai et al., 2011). Chk1 in flies

has been implicated in G1 and G2 checkpoints. Our result suggests that Chk1 is required for G1 cell cycle arrest in the *Skp2* null background.

We also show that the JNK pathway is active and contributes to the apoptotic response in *Skp2* null cells. The JNK pathway promotes apoptosis through Hid specifically (McNamee and Brodsky, 2009). Thus, in *Skp2* mutants the Chk2/p53 and JNK pathways are likely to both rely on Hid to mediate the apoptotic response. This explains the near complete inhibition of apoptosis in *Skp2* null cells upon *Hid* knockdown. The JNK pathway is activated through a number of cell stresses. It has been shown that *Drosophila* cells exposed to IR undergo JNK dependent apoptosis, though it appears likely that it is not the DNA damage per se that triggers the JNK pathway following IR, but rather some cellular stress that occurs as a consequence of DNA damage or anneuploidy (McNamee and Brodsky, 2009). We show that one of the known signals for JNK activation, ROS, accumulates in *Skp2* null cells and therefore it is likely that this at least in part contributes to the JNK arm of the apoptotic pathway.

It is interesting that DNA damage occurs and DNA damage checkpoints are activated in *Skp2* null cells and in cells with a weak *Cyclin A* knockdown, but not in *Skp2* null cells that are also heterozygous for *Cyclin A* or upon strong knockdown of *Cyclin A*. This is despite the fact that these latter genotypes incur more polyploidy than *Skp2* null cells. The critical difference between *Skp2* null cells and these others is that *Skp2* null cells undergo mitosis. This correlation suggests that DNA damage results from progression through mitosis. There are a number of ways that passage of polyploid cells through mitosis may lead to DNA damage (Hayashi and Karlseder,

2013). In *Skp2* null cells it is rare to observe an anaphase in which all sister chromatids appear to be separating cleanly. In most cases we observe chromosomes spread over the spindle. These aberrant anaphases are much more common when the SAC is eliminated. In particular, in *Skp2, MAD2* double mutants we see many examples in which chromosomes seem to get caught in the cleavage furrow during cytokinesis. We predict that these chromosomes break at the completion of cytokinesis. Therefore, chromosome breaks resulting from failed chromosome segregation may result in activation of the DNA damage response.

Recent work from the Calvi lab has shown that polyploid cells in *Drosophila* inactivate DNA damage checkpoints and as such are incapable of undergoing apoptosis following induced DNA damage(Hassel et al., 2014; Hayashi and Karlseder, 2013; Mehrotra et al., 2008; Zhang et al., 2014). They showed that cells induced to enter an endocycle downregulate several components of the apoptotic pathway, including p53. Our results add another layer to this picture of how polyploid cells evade apoptosis. One of our major conclusions is that polyploid cells evade checkpoint-mediated cell cycle arrest or apoptosis by avoiding mitosis and thereby avoiding DNA damage.

Upregulation of autophagy has been observed in a number of diseases like cancer and neurodegeneration and in most cases it appears to have a role in protecting these cells (Hara et al., 2006; Høyer-Hansen and Jäättelä, 2008). Similarly, *Drosophila* becomes hypersensitive to H_2O_2 in the absence of important autopahgic genes such as *ATG7* and *ATG8A* (Wu et al., 2009). Autophagy has been implicated as a means to survive against oxidative stress. Wu and colleagues have shown that

JNK pathway can upregulate the transcription of autopahgic genes when *Drosophila* cells are subjected to oxidative stress(Wu et al., 2009) . *Skp2* cells show high level of ROS and it has been shown that ROS can activate JNK pathway. Therefore, the autophagy seen in *Skp2* could be a protective mechanism employed by these cells to survive the oxidative stress.

Autophagy can also be used by cells to promote cell death. For example, developmentally regulated autophagy is involved in the programmed cell death of salivary gland and midgut cells of *Drosophila*. It is possible that autophagy in the *Skp2* mutant contributes to cell death. We found that over the course of larval development, *Cyclin* A^{95} and *Skp2, Cyclin* $A^{H170+/-}$ wing imaginal discs grow larger than *Skp2* wing discs, consistent with a lack of apoptosis in the latter endocycling cells. However, at least in the case of *Cyclin* A^{95}, the size of the adult wing is greatly reduced in the region corresponding to knockdown. This suggests that these cells eventually are killed, likely during pupal stage. It will be interesting to determine if autophagy plays a role here or if the apoptotic pathway is reactivated.

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CHAPTER 5

GENERAL DISCUSSION

In the mid-1990s, Beach Lab discovered an interesting protein that associates with Cyclin A in transformed cell lines. They named this protein S-phase kinase-associated protein 2, or Skp2 (Zhang et al., 1995). Interest in Skp2 grew over the years, as it was seen to be overexpressed in a variety of cancer types and had strong correlation with the p27/Dap downregulation in those cell types, reviewed in (Frescas and Pagano, 2008). p27/Dap is an important tumor suppressor and a G1/S regulator. In 2004, it was established as a critical target of Skp2 (Nakayama et al., 2004). During the G1/S transition, Skp2 targets p27/Dap and other cyclin-dependent kinase inhibitors for degradation; this activates the Cyclin A/E-CDK2 complex that is necessary for a successful transition from G1 to S phase. In cancer cells, overexpression of Skp2 promotes an early G1 to S transition by causing premature degradation of p27/Dap. This led to precocious S phase without proper G1 arrest. Hence, the inhibition of Skp2 function was thought to be an excellent way to prevent tumorigenesis (Frescas and Pagano, 2008). While inhibition of Skp2 was thought to prevent genetic instability, it was shown in 2000 that *Skp2* knockout mice were also polyploid (Nakayama et al., 2000). They showed that *Skp2* null mice had a smaller body size than their littermates. The cells in these mice had an accumulation of multiple centrosomes, enlarged nuclei, and accumulation of Cyclin E and p27/Dap. They also observed slower than normal growth rate and a high degree of apoptosis (Nakayama et al., 2000).

When our lab started to work with *Skp2* in *Drosophila*, our findings were similar to that of Nakayama lab (Nakayama et al., 2000). We observed that *Skp2* null flies had a smaller body size than wild type flies (Ghorbani et al., 2011). The mitotic

cells in these flies had enlarged nuclei. We also observed that loss of *Skp2* in *Drosophila* resulted in a slower growth rate and a high degree of cell death. We saw that *Skp2* null flies were not viable after the third instar larval stage. This indicates that the function of *Skp2* is essential in flies unlike the case in mammals. Another interesting point that we observed in *Skp2* null flies was that the nuclei of their endoreplicating cells were smaller than those of wild-type flies, indicating that Skp2 promotes diploidy in mitotic cells where as it promotes polyploidy in endoreplicating cells. Understanding the role of Skp2 in endoreplicating cells will be included in our future work.

This initial work on mice and flies left us with a fundamental question: How does inhibiting the function of Skp2 lead to polyploidy in mitotic cells? In 2004, Nakayama lab showed that Skp2 function is needed in G2/M for the downregulation of p27/Dap (Nakayama et al., 2004). According to them, the overexpression of p27/Dap during G2 phase can cause CDK1 inhibition, which might lead to G2/M arrest in *Skp2* null cells (Nakayama et al., 2004).

This thesis is focused on finding the reason for polyploidy in *Skp2* null, discussed in our first paper (Das et al., 2016b), finding the mechanism wherein loss of *Skp2* leads to premature degradation of cyclins, discussed in our second paper (Das et al., 2016a) and lastly finding the consequences of polyploidy in mitotic cells, discussed in our third paper (Vasavan et al., 2016).

Reason for polyploidy in *Skp2* null cells

In *Skp2* null cells, Cyclin A is prematurely degraded. This results in a lower mitotic index in these cells. Further reduction of Cyclin A, not Cyclin B in *Skp2* null

cells (*Skp2*$^{-/-}$, *Cyclin A*$^{+/-}$) results in almost complete loss of cells entering mitosis. The cells in *Skp2*$^{-/-}$, *Cyclin A*$^{+/-}$ have enlarged nuclei compared to *Skp2* null alone arguing that reduction of Cyclin A in *Skp2* null results in less cells entering mitosis. We have shown that the N-terminus of Skp2 directly interacts with Cyclin A; our PLA experiments have shown that G2-arrested cells in wing imaginal disc cells have an increase in Skp2-Cyclin A interaction foci. We have also shown that the N-terminus of *Skp2* is required for its role in genome stability. Therefore, our results point to a novel role that Skp2 plays in protecting Cyclin A during G2/M of the cell cycle. In flies, Cyclin A is critical for mitotic entry. A loss of Cyclin A also causes a premature activation of APC-CDH1/Fzr. This leads to the premature degradation of Cyclin B and Cyclin B3, which in turn results in cells not entering mitosis. They enter into a G1-like state and start endoreduplicating instead.

How does Skp2 protect Cyclin A?

In our second paper, we discussed our attempt to find the mechanism wherein Skp2 protects Cyclin A (Das et al., 2016a), Even though our studies have showed that an overexpression of CDH1/Fzr is antagonistic with loss of *Skp2*, we were not able to determine the possible role of Skp2 in regards to inhibiting the premature activation of CDH1/Fzr. We showed that loss of *Skp2* or overexpression of Skp2 did not cause any change in CDH1/Fzr protein level or its localization, and that Skp2 did not compete with CDH1/Fzr for binding to Cyclin A. Our results also indicate that the role that Skp2 have in protecting Cyclin A was independent of its role in p27/Dap degradation. Finding the domain of Skp2-Cyclin A and Cyclin A-CDH1/Fzr interaction will be useful in understanding the mechanism of Cyclin A protection.

Additionally, it may also be a possibility that Skp2 interacts with Cyclin A to protect itself from CDH1/Fzr mediated degradation.

Consequences of *Skp2* loss

Our third paper discusses the consequences of polyploidy in *Skp2* mutants (Vasavan et al., 2016). Our results argue that the activation of SAC in *Skp2* mutants does not cause polyploidy, but polyploid cells enter mitosis and activate SAC. These cells then delay in prometaphase/ metaphase due to SAC activation. *Skp2* null cells undergo extensive DNA damage which might be the reason for high degree of apoptosis in them. We also observed similar rate of apoptosis in the weaker *Cyclin A* knockdown (*Cyclin* A^{R1}), which enters mitosis in similar frequency to *Skp2* null cells. In contrast, the stronger *Cyclin A* knockdown (*Cyclin* A^{95}), which skips mitosis and is more polyploid undergoes reduced rate of apoptosis. We also observed reduced rate of apoptosis in $Skp2^{ex9}$, *Cyclin* $A^{H170+/-}$. This shows that it is not the polyploidy itself that leads to apoptosis but the entry into mitosis that leads to several checkpoint activation which might lead to cell death as a survival mechanism to prevent genome instability.

Overall, our results show that the function of Skp2 is important not only during the G1/S transition but also during the G2/M transition where it protects Cyclin A from premature degradation. Maintaining Cyclin A levels during mitotic entry is important for successful mitosis and this prevents polyploidy and genome instability.

Future Directions

It was interesting to find that the N terminus of Skp2 is essential for Cyclin A interaction as well as to maintain diploidy. Future work will be focused on finding the domain and later the exact amino acid sequences needed for the interaction between Skp2 and Cyclin A. Mutating those amino acid sequences will enable us to understand the significance of this stable and conserved interaction between Skp2 and Cyclin A.

In our study we showed that Skp2 interacts with Cyclin B3, a mitotic cyclin that is conserved in all organisms but not well understood. It will be interesting to see if this interaction between Skp2 and Cyclin B3 is conserved in mammals. Exploring this interaction might help us understand the versatility and importance of G2-M role of Skp2.

It will be also interesting to find out some novel interactors of Skp2. For this we will Co-immunoprecipitate Skp2 and perform mass spectrometry analysis.

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