Tumour suppressor role of s-phase kinase associated protein 1-cullin-F box-S-phase kinase associated protein 2 (SCFSkp2)

Biju Vasavan
University of Windsor

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation
https://scholar.uwindsor.ca/etd/5675

This online database contains the full-text of PhD dissertations and Masters' theses of University of Windsor students from 1954 forward. These documents are made available for personal study and research purposes only, in accordance with the Canadian Copyright Act and the Creative Commons license—CC BY-NC-ND (Attribution, Non-Commercial, No Derivative Works). Under this license, works must always be attributed to the copyright holder (original author), cannot be used for any commercial purposes, and may not be altered. Any other use would require the permission of the copyright holder. Students may inquire about withdrawing their dissertation and/or thesis from this database. For additional inquiries, please contact the repository administrator via email (scholarship@uwindsor.ca) or by telephone at 519-253-3000ext. 3208.
Tumour suppressor role of S-phase kinase associated protein 1-Cullin-F box- S-phase kinase associated protein 2 (SCF^{Skp2})

By

Biju Vasavan

A Dissertation
Submitted to the Faculty of Graduate Studies through the Department of Biological Sciences in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada
2015

© 2015 Biju Vasavan
Tumour suppressor role of S-phase kinase associated protein 1-Cullin-F box- S-phase kinase associated protein 2 (SCF^{Skp2})

by

Biju Vasavan

APPROVED BY:

______________________________
Bruce Reed, External Examiner
Department of Biological Sciences, University of Waterloo

______________________________
Panayiotis Otis Vacratsis
Department of Chemistry & Biochemistry

______________________________
Lisa Porter
Department of Biological Sciences

______________________________
John Hudson
Department of Biological Sciences

______________________________
Andrew Swan, Advisor
Department of Biological Sciences

October 5th, 2015
DECLARATION OF CO-AUTHORSHIP/PREVIOUS PUBLICATIONS

I. Co-Authorship Declaration

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

“This thesis incorporates the outcome of research undertaken in the supervision of Dr. Andrew Swan. In all cases, experimental designs, data analysis and interpretation, were performed by the authors. Chapter two was in collaboration with Mohammad Ghorbani and Emona Kraja. Chapter three and chapter four were in collaboration with Nilanjana Das.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

II. Declaration of Previous Publication

This thesis includes one original paper that has been previously published/ submitted for publication in peer-reviewed journals, as follows:

<table>
<thead>
<tr>
<th>Thesis Chapter</th>
<th>Publication title/full citation</th>
<th>Publication status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 3</td>
<td>G2/M Role of SCF$^{Skp2}$</td>
<td>To be Submitted</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>Role of Skp2 in maintaining genomic stability</td>
<td>To be Submitted</td>
</tr>
</tbody>
</table>

I certify that I have obtained a written permission from the copyright owner(s) to include the above-published material(s) in my thesis. I certify that the above material describes work completed during my registration as graduate student at the University of
Windsor. I declare that, to the best of my knowledge, my thesis does not infringe upon anyone’s copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.
ABSTRACT

Spontaneous duplications of the genome give rise to polyploid cells and polyploidy can lead to aneuploidy. Aneuploidy is a well-documented phenomenon that could cause cancer. Therefore, a thorough knowledge of how cells maintain diploidy can help us prevent cancer. E3 ubiquitin ligases regulate the cellular levels of a wide variety of oncoproteins. The multi-protein complex, SCF (Skp1-Cullins-F-box) is an E3 ligase that is responsible for ubiquitination-mediated degradation of a large number of proteins involved in cell cycle regulation, transcription and tumor suppression. S-phase kinase-associated protein-2 (Skp2) is the receptor of the SCF complex that promotes the degradation of specific target proteins. The SCF^{Skp2} targets a wide variety of proteins including tumor suppressor protein p27. Cdc kinase subunit 1 (Cks1) is a small Cyclin – dependent kinase (CDK) interacting protein that is essential for Skp2 mediated degradation of p27. We have generated null alleles of Skp2 and Cks1 (Cks85A) in fruit flies and found that loss of these genes results in polyploidy and abnormal mitosis. These genes are also essential for normal growth of the fruit fly. Skp2 was first identified as a protein that associates with Cyclin A/ Cdk2. We provide the evidence for the first time that Skp2 is required in G2 to protect Cyclin A from premature degradation. Furthermore, our data for the first time suggest a mitotic role of Skp2. We show that Skp2 is required for maintaining the levels of mitotic cyclins and is also required for mitotic entry. Skp2 and Cks85A mutant imaginal discs undergo extensive apoptosis. We provide the first evidence of a stress response pathway that is activated upon the loss of Skp2. Our results suggest that loss of Skp2 results in polyploidy related abnormal mitosis that triggers the spindle assembly checkpoint and the DNA damage response. We also provide evidence of JNK pathway activation and autophagy in the Skp2 null background. Taken together, our finding suggests a novel Skp2 –Cyclin A interaction that plays an important role in maintaining diploidy and thus, genome stability.
Dedicated to my family for endless love and support
ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge my supervisor Dr. Andrew Swan, for his valuable guidance and support during my PhD. Without his constant support and encouragement I would not have been able to complete this degree. I would like to thank him for all the valuable discussions we had during the course of my thesis.

I would also like to thank my committee members Dr. Lisa Porter, Dr. John Hudson and Dr. Panayiotis Otis Vacratsis for their time and insightful comments.

My gratitude is extended to former and fellow Lab members of Swan lab: Emona Kraja, Mohammad Ghorbani, Rajdeep Dhaliwal, Osama Batiha, Mohammad Bourouh, Nilanjana Das and Zhihao Guo for all their assistance and encouragement.

A very special thanks goes to all my friends from Biology and Biochemistry departments for always being there for me. Finally I would like to thank my family for all their love and support throughout the years.
**TABLE OF CONTENTS**

DECLARATION OF CO-AUTHORSHIP/PREVIOUS PUBLICATIONS………iii

ABSTRACT...........................................................................................................v

DEDICATION ........................................................................................................vi

ACKNOWLEDGEMENTS....................................................................................vii

LIST OF FIGURES ..............................................................................................x

LIST OF ABBREVIATIONS ...................................................................................xi

I. **CHAPTER 1: INTRODUCTION**.................................................................1
   Ubiquitin proteasome pathway..............................................................2
   Skp2.............................................................................................................4
   Skp2 as an oncoprotein............................................................................6
   Maintenance of genetic integrity............................................................8
   Spindle Assembly checkpoints..............................................................11
   DNA damage checkpoints.......................................................................15
   Apoptosis.................................................................................................19
   Autophagy...............................................................................................22
   *Drosophila* as a model organism.........................................................24
   References...............................................................................................27

II. **CHAPTER 2** ............................................................................................38
   Cks85A and Skp2 interact to maintain diploidy and promote growth
   in *Drosophila*
   Introduction..............................................................................................39
   Materials and methods...........................................................................41
   Results.......................................................................................................44
Discussion...........................................................................................................68
References.............................................................................................................75

III. **CHAPTER 3** ..............................................................................................................78
G2/M Role of SCF$^{Skp2}$
Introduction.............................................................................................................79
Materials and methods..........................................................................................81
Results......................................................................................................................86
Discussion..............................................................................................................107
References..........................................................................................................111

IV. **CHAPTER 4** .............................................................................................................117
Role of Skp2 in maintaining genomic stability
Introduction...........................................................................................................118
Materials and methods........................................................................................120
Results......................................................................................................................122
Discussion..............................................................................................................151
References..........................................................................................................156

V. **CHAPTER 5** .............................................................................................................159
Conclusion.............................................................................................................160

**VITA AUCTORIS**......................................................................................................165
LIST OF FIGURES

CHAPTER 1
Fig. 1. Ubiquitin proteasome pathway ................................................................. 3
Fig. 2. SCF^{Skp2} E3 ligase complex ................................................................. 5
Fig. 3. Regulation of DNA replication origin licensing during G1 and S-G2 phases of the cell cycle ............................................................... 10
Fig. 4. Events in Mitosis ...................................................................................... 12
Fig. 5. Spindle assembly checkpoint ................................................................. 13
Fig. 6. DNA damage checkpoints in mammals ................................................ 18
Fig. 7. Apoptotic pathway in mammals and fruit flies ...................................... 21
Fig. 8. GAL4/UAS system .................................................................................. 25

CHAPTER 2
Fig. 1. Cks85A and Skp2 are required for growth and endoreplication .......... 45
Fig. 2. Loss of Cks85A and Skp2 results in polyploidy in brains ..................... 48
Fig. 3. Cks85A and Skp2 are required to maintain diploidy in mitotically active but not differentiated cells .............................................................. 51
Fig. 4. Polyploidy and apoptosis in Cks85A and Skp2 mutant wing imaginal discs ...................................................................................... 53
Fig. 5. Cks85A and Cks30A have distinct and antagonistic activities .............. 57
Fig. 6. Identification of the Drosophila Skp2 ................................................... 60
Fig. 7. Known SCF^{Skp2} targets are not affected in Drosophila Cks85A or Skp2 mutants .............................................................................. 66

CHAPTER 3
Fig. 1. Skp2 is required for entry into mitosis .................................................... 88
Fig. 2. Skp2 is required for maintaining mitotic Cyclin levels ......................... 91
Fig. 3. The mitotic phenotype of Skp2^{ex9} resembles Cyclin ARNAi ................. 95
Fig. 4. Skp2 interacts directly with Cyclin A .................................................... 98
Fig. 5. Skp2 and Cyclin A interact in G2 ......................................................... 102
Fig. 6. Skp2 and Cyclin A are antagonist to Fzr ............................................. 105

CHAPTER 4
Fig. 1. Polyploid Skp2 null cells undergo SAC mediated mitotic delay .......... 123
Fig. 2. Prometaphase/metaphase delayed cells are polyploid ......................... 125
Fig. 3. SAC is active in pro-metaphase/metaphase delayed Skp2 mutant cells ..... 129
Fig. 4. Skp2 cells undergo apoptosis to reduce genomic instability .............. 132
Fig. 5. Apoptotic regulators in Skp2 ............................................................... 134
Fig. 6. Skp2 null cells signal a DNA damage response .................................. 137
Fig. 7. DChk1 activates checkpoint response in Skp2 null cells ...................... 141
Fig. 8. JNK pathway is active in Skp2 null cells ............................................ 144
Fig. 9. Skp2 mutants undergo autophagy ...................................................... 147

CHAPTER 5
Fig. 1. Role of Skp2 in maintaining genomic stability .................................... 161
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEL</td>
<td>After egg lay</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase Promoting Complex</td>
</tr>
<tr>
<td>Asl</td>
<td>Asterless</td>
</tr>
<tr>
<td>Atg</td>
<td>Autophagy-related genes</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans retinoic acid</td>
</tr>
<tr>
<td>Bid</td>
<td>BH3 interacting-domain</td>
</tr>
<tr>
<td>Brca1</td>
<td>Breast cancer 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Bub1</td>
<td>Budding uninhibited by benzimidazole-1</td>
</tr>
<tr>
<td>BubR1</td>
<td>Bub-1 related kinase</td>
</tr>
<tr>
<td>CAND1</td>
<td>Cullin-associated and neddylation–dissociated</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>Cdt1</td>
<td>Chromatin licensing and DNA replication factor 1</td>
</tr>
<tr>
<td>Chk1</td>
<td>Checkpoint kinase 1</td>
</tr>
<tr>
<td>Chk2</td>
<td>Checkpoint Kinase 2</td>
</tr>
<tr>
<td>Cks</td>
<td>Cyclin-dependent kinase subunit</td>
</tr>
<tr>
<td>CMA</td>
<td>Chaperone-mediated autophagy</td>
</tr>
<tr>
<td>CPC</td>
<td>Chromosomal passenger complex</td>
</tr>
<tr>
<td>CpdA</td>
<td>Compound A</td>
</tr>
<tr>
<td>CRL4</td>
<td>Cullin-RING E3 ubiquitin ligase 4</td>
</tr>
<tr>
<td>Cul1</td>
<td>Cullin1</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>Dap</td>
<td>Dacapo</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>DFF40</td>
<td>DNA fragmentation factor 40</td>
</tr>
<tr>
<td>DIABIO</td>
<td>Direct IAP binding protein with low pI</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------</td>
<td>------------</td>
</tr>
<tr>
<td>DIAPs</td>
<td>Drosophila inhibitors of apoptosis</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signaling complex</td>
</tr>
<tr>
<td>Dmnk</td>
<td>Drosophila maternal nuclear kinase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dronc</td>
<td><em>Drosophila melanogaster</em> NEDD2-like caspase</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand breaks</td>
</tr>
<tr>
<td>Dup</td>
<td>Double parked</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EDU</td>
<td>5-Ethynyl-2′-deoxyuridine</td>
</tr>
<tr>
<td>ER-Stress</td>
<td>Endoplasmic reticulum stress</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent in situ hybridization</td>
</tr>
<tr>
<td>Fzr</td>
<td>Fizzy related</td>
</tr>
<tr>
<td>Fucci</td>
<td>Fluorescence ubiquitin based cell cycle indicator</td>
</tr>
<tr>
<td>GAPDH</td>
<td>D-glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5′-triphosphate</td>
</tr>
<tr>
<td>Hid</td>
<td>Head involution defective</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitors of apoptosis</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LRRs</td>
<td>Lucine rich repeats</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>Mad1</td>
<td>Mitotic arrest deficient 1</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose-binding protein</td>
</tr>
<tr>
<td>MCC</td>
<td>Mitotic checkpoint complex</td>
</tr>
<tr>
<td>Mcm</td>
<td>Minichromosome maintenance</td>
</tr>
<tr>
<td>Mps1</td>
<td>Monopolar spindle-1</td>
</tr>
<tr>
<td>Ncd</td>
<td>Non-claret disjunctional</td>
</tr>
<tr>
<td>ORC</td>
<td>Origin recognition complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PHH3</td>
<td>Phospho-Histone H3</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
</tr>
<tr>
<td>pre-RC</td>
<td>Pre-replication complex</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication Protein A</td>
</tr>
<tr>
<td>Rpr</td>
<td>Reaper</td>
</tr>
<tr>
<td>Rux</td>
<td>Roughex</td>
</tr>
<tr>
<td>SAC</td>
<td>Spindle assembly checkpoint</td>
</tr>
<tr>
<td>Skp1</td>
<td>S-phase kinase associated protein 1</td>
</tr>
<tr>
<td>Skp2</td>
<td>S-phase kinase associated protein 2</td>
</tr>
<tr>
<td>Smac</td>
<td>Second mitochondria-derived activator of caspases</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>UAS</td>
<td>Upstream activation sequence</td>
</tr>
<tr>
<td>Zw</td>
<td>Zwilch</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION
The ubiquitin proteasome system

Tim Hunt and his colleagues, in 1983 discovered regulated proteolysis of certain proteins called ‘cyclins’ in sea urchin embryos (Evans et al., 1983). They were called ‘cyclins’ as their proteolysis correlated with cell cycle. In 1991, it was shown that cyclin B, a key cell cycle regulator was targeted for proteolysis by ubiquitylation (Glotzer et al., 1991). Ubiquitylation involves the covalent attachment of Ubiquitins to proteins that are targeted for destruction by the 26S proteasome (Calvi et al., 2003). Ubiquitin is a 76-residue highly conserved protein found in all eukaryotic cells. A protein is ubiquitinated in 3 steps as shown in Fig. 1: The first step in ubiquitylation is an energy consuming process. Each Ubiquitin is activated by the E1 enzymes and then transferred to the E2 enzymes at the expense of 1 ATP. E2 enzymes then transfer activated Ubiquitin directly to the substrate that is bound to an E3 Ubiquitin ligase (Ciechanover, 1998; Pickart, 2001).

SCF (Skp1-Cullin1-F-box protein) and APC/C (Anaphase Promoting Complex/Cyclosome) are the most well characterized E3 enzymes (Hegde, 2010). APC/C promotes anaphase by degrading Securin and helps in metaphase-anaphase transition by degrading mitotic cyclins (Cohen-Fix et al., 1996; Sudakin et al., 1995). The SCF complex helps in the G1-S transition by ubiquitinating Cyclin E and p27. The SCF (Skp1-cullin1-F-box protein) as the name suggests consists of a scaffold protein Cullin1 with the adaptor protein Skp1 attached to its N-terminal and ROC1 RING finger protein attached to its C-terminal (Bornstein et al., 2003). Various F-box proteins bind to Skp1 by their F-box motif (~40 residues) (Bai et al., 1996).

Assembly of the SCF-complex starts with interaction of Skp1 and the F-box protein to form the Skp1-F-box protein complex. Cul1, before its interaction with Skp1-F-box protein complex, is found tightly bound to CAND1 (Cullin-Associated and Neddylation-Dissociated1) (Oshikawa et al., 2003). The Skp1-F-box protein reversibly dissociates the Cul1-CAND1 complex and forms the Skp1-cullin1-F-box protein complex (Bornstein et al., 2006).
**Fig. 1. Ubiquitin proteasome pathway.** After Ubiquitin is activated by E1 enzyme, it is passed from E1 to E2 enzymes. E3 catalyzes the transfer of Ubiquitin from E2 to the substrate. Lys48 and Lys11-linked polyubiquitinylated substrates are then destroyed by 26S proteasome complex.
Ligation of Nedd8, a Ubiquitin–like molecule to Cul1 stabilizes Skp1-cullin1-F-box protein complex and prevents it from binding with the CAND1 (Petroski and Deshaies, 2005). F-box proteins recruit and bring the substrate closer to the SCF complex thereby enhancing its overall efficiency (Schulman et al., 2000). Furthermore, the interaction between Skp1 and the F-box protein may provide an optimal position for the substrates that need to be ubiquitinated. Almost 70 F-box proteins have been identified in humans (Frescas and Pagano, 2008; Jin et al., 2004). Based on the domain that interacts with the substrate, they have been classified into three categories (FBXWs, FBXLs and FBXOs). FBXWs contains WD40 repeats. β-TRCP and FBXW7 are the most studied proteins belonging to this category. FBXLs have LRRs (Lucine rich repeats) as their substrate recognition sequence. The best-characterized protein under this category is Skp2. Proteins containing various other substrate recognizing domains have been pooled under FBXOs.

**Skp2**

In 1995, Beach and colleagues discovered that the Cyclin A-CDK2 complex in transformed human fibroblasts includes two novel proteins of mass 19 and 45kD. They named these proteins p19\(^{Skp1}\) (S-phase kinase associated protein 1) and p45\(^{Skp2}\) (S-phase kinase associated protein 2) respectively. Skp1 and Skp2 along with Roc1/Rbx1 and Cul1 form the SCF\(^{Skp2}\) complex (Lisztwan et al., 1998; Michel and Xiong, 1998). The level of Skp2 protein is cell cycle regulated although Skp2 mRNA persists through out the cell cycle. Skp2 protein starts accumulating at the G1-S phase transition and is only degraded as the cell completes M phase and therefore, absent in G0 and G1. APC\(^{Cdh1}\) degrades Skp2 in G0/G1 phase of the cell cycle (Bashir et al., 2004; Wei et al., 2004). There is hardly any change observed in the protein levels of Skp1, ROC1/RBX1 and Cul1 during cell cycle (Ohta et al., 1999).
Fig. 2. SCF\textsuperscript{Skp2} E3 ligase complex. (A) A schematic representation of SCF\textsuperscript{Skp2}. (B) Model of SCF\textsuperscript{Skp2} derived from the crystal structure. Used with permission: Nature publication, License Number: 3713700362797 (Zheng et al., 2002).
Skp2 is the substrate recognition/recruiting component of SCF\textsuperscript{Skp2} E3 ligase complex (Fig. 2A) (Schulman et al., 2000). Crystal structure reveals that the Skp1-Skp2 complex looks like a sickle (Fig. 2B) (Zheng et al., 2002). The LRR domain of Skp2 resembles the blade while the rest of Skp2 along with Skp1 make up the handle (Schulman et al., 2000). It is not clearly known how substrates bind to the LRR domain of Skp2. Two other proteins U2A and RNAse inhibitor, use the concave side of their LRR domain for protein–protein interactions (Kobe and Deisenhofer, 1995). Therefore, it is believed that substrates might be binding to Skp2 in a similar manner.

**Skp2 as an oncoprotein**

Skp2 is upregulated in transformed cells (Zhang et al., 1995). The exact reason for the upregulation of Skp2 in cancer cells is not clearly understood. In some cancer tissues Skp2 upregulation has been observed due to gene amplification (Yokoi et al., 2002; Zhu et al., 2004). *In-vitro*, cell culture and animal model studies have shown that Skp2 targets p27 and promotes G1-S transition (Carrano et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999; Nakayama et al., 2000). Nakayama and his group observed that the *Skp2* null mice and *p27* null mice exhibit opposite phenotypes. *Skp2* nulls are smaller while *p27* null mice are larger than their control littermates. Hepatocytes of *Skp2* null mice are polyploid and contain multiple centrosomes. They generated *Skp2, P27* double mutant mice and observed that their body size is similar to *p27* null mice. Furthermore, polyploidy and centrosome multiplication observed in *Skp2* null mice was rescued in *Skp2, P27* double mutant mice. Taken together, these results suggest that p27 is the primary target of Skp2 and that the polyploidy in the Skp2 mutants is due to elevated p27 (Nakayama et al., 2004). Skp2 requires the aid of Cks1 to ubiquitylate p27 (Ganoth et al., 2001), (Spruck et al., 2001). Ganoth et al. showed that *in vitro* reconstituted SCF\textsuperscript{Skp2} from purified components cannot ubiquitylate p27 in the absence of Cks1. Spruck and his colleagues observed p27 accumulation in Cks1 null mice. Furthermore, they showed that cell extracts derived form Cks1 null MEFs could not ubiquitylate p27 (Spruck et al., 2001). Crystal structure of Skp1-Skp2-Cks1-p27 revealed that a major part of p27 binds
to Cks1 (Hao et al., 2005). It also showed that the pThr187 residue of p27 which is essential for its Skp2 mediated degradation, actually binds to the anion pocket of Cks1.

Skp2 targets a wide variety of proteins that are involved in important cellular processes like cell cycle regulation, apoptosis, DNA replication and cell survival. These targets include proteins such as E2F1 (Marti et al., 1999), Myc (Kim et al., 2003; von der Lehr et al., 2003), p21 (Bornstein et al., 2003; Yu et al., 1998), Cyclin D (Yu et al., 1998), Cyclin E (Yeh et al., 2001), Foxo1 (Huang et al., 2005), Cdt1 (Li et al., 2003), Orc1 (Mendez et al., 2002), Brca2 (Moro et al., 2006).

Inhibition of SCF$^{\text{Skp2}}$ E3 ligase is being considered as a promising strategy as a preventive measure or treatment for various cancers. Lin and colleagues have shown that Skp2 plays an important role in cellular senescence associated with tumorigenic conditions (Lin et al., 2010). They showed that loss of $\text{Skp2}$ alone does not lead to permanent exit of cells from cell cycle, a process known as senescence but loss of tumor suppressor ARF or PTEN in $\text{Skp2}$ null background leads to cellular senescence. They confirmed this observation both in vitro and in vivo. Furthermore, they showed that upregulation as well as synergic effect of cell cycle inhibitors p21 and p27 along with ATF4, an endoplasmic reticulum stress protein, caused senescence. Application of a small molecule, CpdA (compound A) on multiple myeloma cells causes autophagy and cell cycle arrest (Chen et al., 2008). The mode of action of CpdA is yet to be discovered. However, Skp2 does not interact with Skp1 in the presence of CpdA, therefore it is thought that CpdA prevents the interaction between Skp1 and Skp2. SMIP0004 is another small molecule that stabilizes p27 in prostrate cancer by downregulating Skp2 by unknown mechanism (Rico-Bautista et al., 2010). G1 cell cycle arrest is induced by increased degradation of Skp2 when ATRA (all-trans retinoic acid) is administered to breast cancer cell lines T-47D and MCF-7 (Dow et al., 2001). These data provide the strong evidence that inhibition of SCF$^{\text{Skp2}}$ E3 ligase can be used prevent or treat cancer to a certain extent.

Apart from E3 ligase dependent roles, Skp2 also has an E3 ligase independent role. Skp2 helps in cellular migration and invasion by regulating transcription of RhoA GTPase, a major regulator of actin dynamics (Chan et al., 2010). Myc recruits Skp2 to the RhoA promoter and subsequently Skp2 recruits Miz1 and p300 to form a complex
that regulates RhoA transcription. This activity does not require the E3 ligase activity of Skp2 dynamics (Chan et al., 2010).

Skp2 might have a tumor suppressor role apart from its well-established oncogenic role. While oncogenes lead to abnormal cell proliferation due to altered gene expression or unrestrained activity of proteins, tumor suppressors exert an opposite effect by inhibiting the cell cycle and thus, tumor growth. Skp2 null mice have polyploid hepatocytes with multiple centrosomes. This suggests that Skp2 is essential for maintaining diploidy in this tissue. Hepatocytes in Skp2 null mice become polyploid due to endoreduplication, a process in which cells enter repeated S-phase without entering mitosis (Minamishima et al., 2002), (Nakayama et al., 2004). Loss of Skp2 in HeLa cells results in re-replication due to accumulation of Cdt1, a protein essential for DNA replication (Nishitani et al., 2006). Polyploid cells can become aneuploid due to loss or gain of chromosomes and eventually multiple mutations in this background can lead to cancer (reviewed in Storchova et al., 2004). Thus, the above data suggest that Skp2 plays a critical role in maintaining genomic integrity. While targeting Skp2 may be useful to control its oncogenic functions; it will be important to better understand its tumour suppressor functions.

Maintenance of genome integrity

Eukaryotic cells employ various surveillance mechanisms to safeguard genome integrity (reviewed in Su, 2011). These surveillance machineries check that 1) replication occurs only once per cell cycle, 2) chromosomes successfully duplicate before segregation of chromosomes (DNA replication checkpoint), 3) segregated chromosomes are equally distributed among daughter cells (Spindle Assembly Checkpoint or SAC) and 4) cell cycle progression is stopped until DNA damages is fixed or that cell ceath is triggered if the damage is irreparable (DNA damage checkpoint).

DNA replication in eukaryotic cells is restricted to once during each cell cycle (Bell and Dutta, 2002). Pre-Replication Complex (pre-RC) formation also referred to as the licensing of the origin of replication takes place when the Origin Recognition Complex (ORC) recruits key components like Mem2-7 complex, Cdt1 and Cdc6. Licensing takes place in early G1 when APC/C activity is high and CDK activity is low
but origins are fired when CDK activity is high and APC/C activity is low. In early G1 APC/C targets the Cdt1 inhibitor Geminin for degradation by the 26S proteasome complex. This results in the accumulation of Cdt1, which further helps in the recruitment of MCM complexes to the origins that are already bound to Cdc6. During G1/S transition, Skp2 mediated degradation of CKIs increases CDK activity, which leads to the inhibition of APC/C activity. Accumulation of Emi1, a pseudo-substrate of APC/C, further inhibits its activity. APC/C activity is also suppressed due to degradation of UbcH10 and UbcH5, the two E2 enzymes required for its activity. After the origin is fired, multiple mechanisms are employed by the cell to prevent the assembly of pre-replication complexes until the cell cycle is completed (reviewed in Nishitani and Lygerou, 2002). In mammals Cdt1 is ubiquitinated and targeted for proteolysis by SCF^{Skp2}, phosphorylated by CDKs so that they cannot bind to DNA or sequestered by Geminin. Drosophila Cdt1 is called Dup (double parked). Dup/Cdt1 is targeted for degradation by Cul4, SCF^{Skp2} in S phase and is sequestered by Geminin in S, G2 and early M phase (reviewed in Su, 2011). Nuclear localization of Cdc6 is negatively regulated by CDK activity. Overexpression of Cdt1/Dup and knockdown of geminin causes re-replication. A general view how pre-RC is regulated is presented in Fig. 3.
Fig. 3. Regulation of DNA replication origin licensing during G1 and S-G2 phases of the cell cycle. Due to high concentration of CKIs such as p21 and p27 as well as active APC/C which ubiquitinates cyclins, CDK activity is maintained at a minimum level during G1. Geminin, a negative regulator of Cdt1/Dup is also ubiquitinated by APC/C. The low CDK activity during G1 favors the assembly of ORC along with Cdc6; Cdt1/Dup and MCM helicase complex at the origins of replication and licensing is accomplished. When CDK activity is high during S phase, origins fire. Re-replication in S and G2 phases is prevented by multiple mechanisms. Cdc6 is phosphorylated and exported out of the nucleus. Cdt1/Dup is Ubiquitinated and marked for degradation by SCF<sup>Skp2</sup> and DDB1-Cul4. APC/C is inhibited due to high CDK activity, which promotes the accumulation of Geminin that sequesters remaining Cdt1/Dup. ORC, is negatively regulated by Cyclin A-Cdk2 and MCM complexes cannot be recruited to the origin of replication in the absence Cdt1/Dup.
Spindle Assembly checkpoint

After successful DNA replication, error-free chromosome segregation is ensured by the SAC (reviewed in Lara-Gonzalez et al., 2012). Genetic screening in *Saccharomyces cerevisiae* (budding yeast) for mutants that would not arrest when subjected to microtubule destabilization, led to the discovery of SAC and its core components; Mad1, Mad2, Mad3 (*Mitotic Arrest Deficient*), Bub1 (*Budding Uninhibited by Benzimidazole-1*), Bub3 and the Mps1 (*MonoPolar Spindle-1*). The SAC is conserved from yeast to humans with the only exception of Mad3 proteins. Higher eukaryotes do not have a Mad3 homologue; instead they have BubR1 (*Bub-1 Related Kinase*), which shares homology with both Mad3 and Bub1. Chromosome segregation occurs during mitosis. Key events in mitosis are classified into 5 phases as described in Fig. 4. Error-free segregation is achieved when the sister chromatids are pulled to opposite ends of the cell before cytokinesis. The SAC monitors the attachment of the mitotic spindle to the kinetochores of sister chromatids. The SAC gets activated when the kinetochores are unoccupied. “Bi-orientation” is achieved when the kinetochores of sister chromatids attach to the microtubules originating from opposite poles of the cell (Lara-Gonzalez et al., 2012).

Studies in *Drosophila* and yeasts have shown that the onset of anaphase is achieved through the degradation of Securin and inhibition of CDK activity (Oliveira et al., 2010; Shirayama et al., 1999). Securin prevents anaphase by inhibiting Separase from cleaving the cohesin complex that binds the sister chromatids (Hornig et al., 2002). The E3 Ubiquitin ligase APC/C<sup>Cdc20</sup> promotes anaphase by Ubiquitin-mediated degradation of Securin and the CDK activator Cyclin B. In the presence of unoccupied kinetochores, the SAC inhibits APC/C<sup>Cdc20</sup> activity by sequestering Cdc20 with the aid of mitotic checkpoint complex (MCC) as shown in fig. 5. The MCC complex initially identified in HeLa cells, consists of Cdc20, Mad2, BubR1 and Bub3 (reviewed in Lara-Gonzalez et al., 2012).
**Fig. 4. Events in Mitosis.** Prophase - chromosomes condense and centrosomes duplicate. At the end of prophase, the nuclear envelope breaks down denoting the entry of the cell into pro-metaphase. Pro-metaphase - centrosomes align at opposite ends of the cells and microtubules originating from these attach kinetochores. SAC proteins recognize unattached kinetochores and inhibit APC^{Cdc20}, thus preventing premature segregation of chromosomes until all the kinetochores are occupied by microtubules. Metaphase - Sister chromatid pairs after bipolar attachment align at midzone. Anaphase – Sister chromatids are pulled to opposite poles of the dividing cell. Telophase - nuclear envelope starts forming around chromosome sets that have assembled on opposite ends while cytokinesis is in progress.
Fig. 5. Spindle assembly checkpoint. In a stepwise process, SAC proteins are recruited to the kinetochore by Kinetochore Null protein1 (KNL1) and Nuclear Division Cycle 80 (NDC80). A Bub3-Bub1 complex is initially recruited followed by Bub3-BubR1 complex with the aid of the kinase activity of Monopolar spindle protein1 (MPS1) that is bound to NDC80. Bub1 further recruits a hetero-tetramer complex of Mad1-Mad2. The Kinetochore bound Mad1-Mad2 complex converts O-Mad2 to C-Mad2. Mitotic Checkpoint Complex (MCC) is assembled when C-Mad2 bound Cell Division Control protein 20 (CDC20) binds with the BubR1-Bub3 complex. The MCC inhibits APC/C^{cdcl0}, preventing Ubiquitin-mediated degradation of securin and the CDK activator Cyclin B.
Although the exact mechanism by which SAC proteins gets assembled on the kinetochore remains elusive, it has been established that the process occurs in an orderly manner (Lara-Gonzalez et al., 2012). The Bub proteins (Bub1, Bub3 and BubR1) are recruited by KNL1, which is a core kinetochore protein. Bub1 further recruits Mad1 and Mad2. Mad2 exists in a closed confirmation (C-Mad2) when bound to Mad1 or Cdc20 and in an unbound situation it exists in an open confirmation (O-Mad2). The kinetochore bound Mad1-C-Mad2 converts O-Mad2 to C-Mad2. This conversion is essential to establish active SAC as BubR1 can only bind to C-Mad2 to generate the MCC complex.

Initial findings suggested that anaphase progression was inhibited by sequestration of Cdc20 by Mad2 alone but now it has been established that BubR1 and Bub3 are also essential for this process (Lara-Gonzalez et al., 2011). After bipolar attachment is established, the Dynein microtubule motor strips off Mad1-C-Mad2 complex from kinetochores; this prevents formation of new MCC and by an unknown mechanism APC/C activity breaks down the existing MCC (Lara-Gonzalez et al., 2012).

Studies in fruit flies have expanded our knowledge regarding the SAC (Basto et al., 2000; Basu et al., 1999; Basu et al., 1998; Buffin et al., 2007; Buffin et al., 2005). Unlike mouse and human Mad2, the Drosophila Mad2 is not required for chromosome segregation during normal mitosis (Dobles et al., 2000; Michel et al., 2001). In flies, Mad2 is only required when normal mitotic progression is obstructed due reasons such as abnormality in chromosome alignment or chromosome capture by spindle microtubules.

The loss of SAC genes like bubR1, bub3, rod and zw10 results in aneuploidy and lethality, while flies mutant for Mad2 have small number of cells that are polyploid but they are viable and fertile (Buffin et al., 2007). Based on the above observation Buffin and colleagues suggested that the sole function of Drosophila Mad2 was binding and inhibiting cdc20 while the other SAC proteins might have SAC-independent functions. Indeed, Rahmani and colleagues have shown that BubR1 also has a role in kinetochore-microtubule linkage, which requires the kinase activity of BubR1. In the BubR1-KD flies that lack a functional kinase domain, spindle microtubules do not efficiently bind to kinetochores resulting in unstable metaphase alignment of chromosomes (Rahmani et al., 2009).
Cells that are unable to satisfy SAC are delayed in mitosis; but they ultimately enter G1 as polyploid (terraploid) cells by a process known as mitotic slippage (reviewed in Rieder and Maiato, 2004). In yeast cells, mitotic slippage can occur through the inhibition of Cyclin B/Cdk1 by a CKI, Sic1. In Drosophila, Rux, a functional homolog of Sic1, has been implicated in mitotic slippage. Studies on vertebrate cell lines have shown that Cyclin B is gradually degraded, leading cells to pass into a G1-like state without having completed mitosis (Brito and Rieder, 2006).

The SAC, apart from monitoring faithful chromosome segregation, also plays an important role in the recently described phenomenon of centrosome clustering (Basto et al., 2008). Centrosomes consist of a pair of centrioles enclosed in pericentriolar material. They are responsible for organizing mitotic spindles in animal cells (Kellogg et al., 1994). Centrosomes normally duplicate only once per cell cycle but multiple centrosomes (centrosome amplification) are frequently observed in cancer cells (reviewed in D'Assoro et al., 2002). Multiple centrosomes can cluster together to avoid the formation of a multipolar spindle that can in turn lead to chromosome missegregation, chromosome breaks and other mitotic errors resulting in aneuploidy (Brinkley, 2001). A large number of proteins including microtubule-binding complex NCD80, Chromosomal Passenger Complex (CPC) and microtubule-based motors have been implicated in centrosome clustering (reviewed in Kramer et al., 2011). Different studies have shown that centrosome amplification can trigger a Mad2 dependent SAC delay (Basto et al., 2008) (Kwon et al., 2008; Yang et al., 2008). A SAC mediated delay in mitosis is observed in flies with multiple centrosomes. Though SAC is not essential for normal survival of the flies; flies with multiple centrosomes require the SAC for survival (Basto et al., 2008). Although the exact mechanism of centrosome clustering is not clearly understood, SAC protein Mad2 and motor protein Non-Claret Disjunctional (Ncd) has been shown to play an important role in flies (Kramer et al., 2011).

**DNA damage checkpoint**

Living cells are subjected to both endogenous and exogenous elements that result in DNA damage (reviewed in Podhorecka et al., 2010). Normal metabolic processes result in the accumulation of reactive oxygen species (ROS) inside cells. Nearly 90% of
single strand breaks introduced by these endogenously generated ROS are repaired while the remaining 10% are converted to double strand breaks (DSB). Ionizing radiation represents exogenous factor leading to DSB. UV rays are also reported to cause DSB. Since a single unrepaired DSB can activate programmed cell death, an efficient repair mechanism is essential for cell survival. Mammalian cells respond to DSB by phosphorylation of H2A-histone variant called H2Ax. ATM, ATR or DNA-PK can phosphorylate H2Ax (Stiff et al., 2004; Ward and Chen, 2001). Sensor proteins such as 9-1-1 complex (RAD9, RAD1, HUS1), MRN complex (MRE11-RAD50-NBS1) and RAD17 detect DNA damage and recruit downstream kinases ATM and ATR which in turn phosphorylate Chk2 and Chk1 respectively (Song, 2005). In fruit flies H2Ax is referred to as H2Av (Leach et al., 2000). After phosphorylation, H2Av is called YH2av.

The DNA damage checkpoints arrest cell cycle so that the damaged DNA can be repaired (reviewed in Song, 2005). ATR-Chk1 signaling is activated when single stranded DNA damage is generated due to UV exposure or due to chemicals that impair DNA replication such as hydroxyurea (Abraham, 2001). The Replication Protein A (RPA) rapidly coats the ssDNA and further recruits ATRIP and ATR (Zou and Elledge, 2003). ATR then phosphorylates and activates Chk1 (Niida et al., 2007). The Chk1 mediated phosphorylation of Cdc25A results in its degradation while Cdc25C is exported from the nucleus with the help of 14-3-3s (Falck et al., 2002; Peng et al., 1997). Cdc25A degradation results in the G1/S cell cycle arrest while the nuclear export of Cdc25C leads to G2 arrest.

When double stranded breaks are introduced by genotoxins and ionizing radiation, the ATM-Chk2 pathway is activated (Matsuoka et al., 2000). Some of the downstream targets of Chk2 include p53, and its negative regulator MDM2, Cdc25A and Cdc25C (Lavin and Kozlov, 2007) (Blasina et al., 1999; Chehab et al., 2000; Chen et al., 2005; Matsuoka et al., 1998). The cells with DNA damage are prevented from entering S phase by the G1/S checkpoint. This is achieved by p53-mediated upregulation of p21, which is a CDK inhibitor and the degradation of Cdc25A, a phosphatase that activates CDK by removing inhibitory phosphate. Replication origins cannot fire when CDK activity is low.

The G2/M checkpoint inhibits cells from entering mitosis. CDK1 activity is required for successful entry and exit of cells from mitosis. Chk1/Chk2 mediated
phosphorylation of Cdc25C inhibits its phosphatase activity and creates a binding site for 14-3-3s. Binding of Cdc25C with 14-3-3s results in its exclusion from the nucleus (Falck et al., 2002). Mitotic cells with DNA damage either get arrested in metaphase (Smits et al., 2000) or undergo cell death in mitosis termed ‘mitotic catastrophe’ (Andreassen et al., 2001). Mammalian DDR is summarized in Fig. 6.
Fig. 6. DNA damage checkpoints in mammals. Genotoxic radiation such as UV rays induces single stranded DNA breaks while the outcome of ionizing radiation is DSB. UV induced G1 arrest occurs through both p53 dependent and independent pathways while during DBS arrest occurs primarily through p53 dependent upregulation of p21. This is achieved by stabilization of p53 by targeting p53 inhibitor MDM2 for degradation. In p53 independent pathway, Chk1/Chk2 targets Cdc25A and Cdc25C phosphatases. Phosphorylation of Cdc25A by either Chk1/Chk2 results in its degradation. Since inhibitory phosphorylation cannot be removed from Cdk2/Cyclin A/E in the absence of Cdc25A, cell arrest in G1 phase of the cell cycle. In a similar manner nuclear export of Cdc25C by 14-3-3 proteins results in G2 arrest.

DNA damage caused by ionizing radiation leads to G2 and metaphase arrest, followed by apoptosis in Drosophila larval wing imaginal discs and brains (reviewed in Song, 2005). Unlike mammals, Decapo (Drosophila p27) mediated G1 arrest has not been documented in Drosophila after IR exposure. In humans and flies, ATM, MRE11 and RAD50 play an evolutionary conserved role in protecting telomeres. The flies mutant for atm, mre11 and rad50 die as pupae (Bi et al., 2004; Ciapponi et al., 2004). The Drosophila Chk2 was initially discovered in ovaries and was named Dmnk (Drosophila
Maternal Nuclear Kinase) (Oishi et al., 1998). Dmnk, unlike Grapes, the Chk1 equivalent in *Drosophila*, can suppress the rough eye phenotype caused by *Drosophila* p53 overexpression (Peters et al., 2002). Since loss of either p53 or Chk2 in fruit flies or mice does not cause any visible phenotype, it suggests that they are not required for normal development in these model systems. The studies done on wing imaginal discs suggest that Grapes plays the major role in IR induced G2 arrest while a minor defect in cell cycle arrest is observed in the *Dmnk* mutants (Brodsky et al., 2004). Additionally, G2 arrest is completely abolished in *grapes, dmnk* double mutants. This suggests that both Dmnk and Grapes are required for complete G2 arrest.

**Apoptosis**

Apoptosis (Greek: “Shedding of leaves”) is a form of programmed cell death that is essential for normal development and tissue homeostasis (reviewed in Shi, 2001). Misregulation of apoptotic genes results in human malignancies like cancer (down-regulation of apoptosis) and neurodegenerative disorders like Alzheimer’s disease (up-regulation of apoptosis). The “intrinsic” pathway of apoptosis goes through mitochondria while the “extrinsic” pathway is initiated by cell surface receptors. Apoptosis can be triggered due to reasons such as activation or overexpression pro-apoptotic genes, sequestration or down-regulation of anti-apoptotic genes in response to developmental cues or in response to cellular injury or stress such as ER-stress, reactive oxygen species or DNA damage.

The process of apoptosis is evolutionarily well conserved (Riedl and Shi, 2004). In mammals, when the cells receive apoptotic stimulus, Cytochrome c (Cyt c) is released from the mitochondria into the cytoplasm. Cyt c binds with APAF1 in the cytoplasm and forms an active complex called “apoptosome”, which activates procaspase-9. Caspases are the key components of apoptotic machinery. “Caspases” are so called because this family of proteins are Cysteine proteases (“c”) and they cleave after aspartic acid (“aspase”) (Alnemri et al., 1996). The active Caspase-9 then cleaves and actives Caspase-3. The active Caspase-3 breaks down a large number of proteins including the nuclear lamin and the downstream Caspases-2 and 6. DNA Fragmentation Factor 40 (DFF40) is a nuclease, which is kept inactive by DFF45. When apoptosis is initiated, cleaved Caspase-
3 degrades DFF45 and DFF40 is released from its inhibition. The DNA fragments generated by free DFF40 are considered as one of the hallmarks of apoptosis. Proteins called the inhibitors of apoptosis (IAPs) bind to Caspases and keep them inactive in the absence of apoptotic stimuli. Viral proteins can also bind and inhibit caspases, such as Baculoviral protein p35. When apoptosis is initiated, mitochondria release anti-IAPs along with Cyt c that block the activity of IAPs on caspases (Riedl and Shi, 2004).

Apoptosis can also be initiated when molecules called “Death Ligands” bind to their receptors the “death receptors” to activate the extrinsic pathway (Riedl and Shi, 2004). For example FasL is a ligand that binds to its receptor called Fas. The cytosolic region of the death ligand-receptor complex further recruits other proteins including Caspase-8 and generates the DISC (death-inducing signaling complex), which functions like the apoptosome of the intrinsic pathway. The DISC cleaves and activates caspase-8, which then activates caspase-3. Cleavage of BID by caspase-8 can generate a crosstalk between extrinsic and intrinsic pathway, as cleaved BID can aid in the release of pro-apoptotic proteins from mitochondria.

The molecular mechanism of apoptosis is conserved in fruit flies and humans (Fig. 7). As in humans, Drosophila caspases are kept inactive by IAPs called DIAPs (/Drosophila Inhibitors of Aoptosis/) (Riedl and Shi, 2004). During apoptotic stimuli, pro-apoptotic proteins called Hid (Had involution defective) Grim and Rpr (Reaper) block the fruit Drosophila DIAPs. The Drosophila Dapaf1/Dark/HAc-1 (functional homolog of mammalian APAF1) activates and cleaves Dronc (functional homolog of mammalian capase-9) that in turn cleaves Drice (functional homolog of mammalian capase-3).
**Fig. 7. Apoptotic pathway in mammals and fruit flies.** Homologues of caspases and their regulators are marked by same colour. During apoptotic stimuli, pro-apoptotic proteins, Hid Grim and Rpr block the fruit *Drosophila* DIAPs. In mammals BID and BIM transmits the signals to mitochondria to release anti-IAPs. Anti-IAPs SMAC/DIABLO block mammalian IAPs. In mammals Cytochrome c released from mitochondria activates APAF1 while apoptosis mediated by Cytochrome c from mitochondria has not yet been documented in *Drosophila*. Mammalian BCL2 protein inhibits the release of Cytochrome c and IAPs. The *Drosophila* Apaf1 (functional homolog of mammalian APAF1) activates and cleaves Dronc (functional homolog of mammalian capase-9), which in turn cleaves Drice (functional homolog of mammalian capase-3). Figure modified with permission: Nature publication, License Number: 3713691118649 (Riedl and Shi, 2004).
Dronc differs from mammalian caspases, as it prefers to cleave after Glu residue instead of Asp. Drice is inhibited by Baculoviral protein p35 (Hay et al., 1994).

In fruit flies, ATM regulates apoptosis and telomere stabilization while ATR (MEI-41) enforces G2 arrest and DSB repair after DNA damage (Jaklevic and Su, 2004) (LaRocque et al., 2007) (Bi et al., 2004) (Song et al., 2004). DNA damage caused by ionizing radiation in flies, induces both p53-dependent and independent apoptosis (McNamee and Brodsky, 2009). When p53 is overexpressed in the Drosophila eye, they become rough and their size reduces due to excessive apoptosis (Peters et al., 2002). This p53 phenotype is rescued by overexpression of dominant negative Chk2 (DN-Chk2). Peters and colleagues also showed that Chk2 could phosphorylate and stabilize p53 indirectly as well as transcriptionally regulate p53 when flies are subjected to ionizing radiation. Furthermore, their study showed that Grp does not play a role in p53 regulation (Peters et al., 2002). Once activated, p53 regulates the transcription of pro-apoptotic anti-IAPs Hid, Rpr and Skl (Brodsky et al., 2004). Apoptosis mediated by Cytochrome c from mitochondria has not yet been documented in Drosophila (Kornbluth and White, 2005); but it has been shown that Reaper can induce Cytochrome c release from mitochondria when it is ectopically expressed in cell free extracts of frog eggs (Thress et al., 1999). A similar observation was made when Grim is ectopically expressed in mouse 3T3 fibroblasts (Claveria et al., 2004).

**Autophagy**

De Duve coined the term ‘autophagy’, which means ‘self eating’ in Greek (reviewed in Mulakkal et al., 2014). It is a process by which the lysosomal system degrades and recycles organelles and long-lived cellular proteins. There are different routes by which organelles and macromolecules are delivered to lysosomes in the higher eukaryotes (Ogier-Denis and Codogno, 2003). 1) Cell surface macromolecules can be delivered to lysosomes by endocytosis. (Westbrook et al., 2007). 2) Lysosomal invagination can engulf parts of the cytoplasm by a process called the microautophagy. 3) In chaperone-mediated autophagy (CMA), cytosolic proteins with specific sequence motifs can be selectively directed to lysosomal lumen with the aid of certain chaperones. Macroautophagy is the major and the most well studied, nonselective lysosomal pathway.
Generally for simplicity, macroautophagy is often just referred to as “autophagy”. The first step in autophagy is the formation of a double-membraned “C” shaped structure of unknown origin called phagophore/isolation membrane. Phagophores then sequester cytoplasmic contents and form an autophagosome. The autophagosomes further fuse with lysosomes to generate structures called the autolysosomes (Mulakkal et al., 2014).

Much of our knowledge about regulation of the autophagy comes from the studies done in Saccharomyces cerevisiae. The core machinery of autophagy consists of two “Ubiquitin-like” conjugation systems. Atg12-Atg5 conjugation system is assembled in a similar manner to the process of ubiquitination. Atg7 activates Atg12 like an E1 enzyme and transfers it to Atg10, which behaves like an E2 enzyme. Atg10 further transfers Atg12 to Atg5. When an autophagy signal is initiated, Atg16 binds to Atg5 of Atg12-Atg5 conjugation system and together they evenly bind to phagophores. As the phagophore elongates, by an unknown mechanism, Atg12-atg5 conjugation system binds more towards the convex side and detaches from it after the formation of the autophagosome. Atg8 –phosphatidylethanolamine (PE) conjugation system is generated in four steps. Atg4 cleaves the last arginine from the c-terminal end of Atg8 to expose the glycine, which could be used by Atg7 to activate it. Atg8 is then passed on to E2 enzyme Atg3 that further passes it to phospholipid PE. In Mammals, Atg8 is known as microtubule-associated protein 1 Light Chain 3 (LC3). Atg5 recruits LC3 to the phagophores and remains there until the completion of autophagy.

Two kinases Atg1 and Vps34/PI3k are also essential for autophagy. Atg1 is believed to control the formation of autophagosome and Vps34/PI3k controls the size of phagophores and also helps in recruiting proteins that needs to be degraded (Mulakkal et al., 2014).

In flies, developmentally regulated autophagy induced by ecdysone is observed in the polyploid tissue of fat body and salivary gland of 3rd instar wandering larvae (Rusten et al., 2004). The diploid cells in these larvae do not undergo autophagy as break down of polyploid tissues helps to nourish diploid tissues likes brain and imaginal discs that end up being a part of adult Drosophila (O'Farrell et al., 2013). A similar observation is made in the midgut of of larvae, where polyploid cells are eliminated by autophagy to feed the diploid cells which eventually became a part of the adult Drosophila (Denton et al.,
Polyploid, but not diploid cells undergo autophagy due to inhibition of TOR mediated growth signaling (Rusten et al., 2004).

Autophagy is known to promote survival of apoptosis-defective tumor cells (Degenhardt et al., 2006). Paradoxically, loss-of-function of autophagic genes is also observed in tumors (Karantza-Wadsworth et al., 2007; Mathew et al., 2007). Although a lot of work has been done to understand autophagy, very little is known in which context they promote cell survival or death.

**Drosophila as a model organism**

The *Drosophila* life cycle consists of four stages. Each female is capable of laying nearly 100 eggs per day. Once the egg is fertilized, it takes about a day for the embryo to hatch as a larva. The larvae feed vigorously for nearly five days and undergo three molts before they turn into pupae. In approximately 4 days, pupae metamorphose into adult flies. The adult flies exhibit complex behavior like feeding, courtship, grooming and learning. *Drosophila* has nearly 14,000 genes and approximately 75% of the genes associated with human diseases have a familiar match in flies. In most cases, the genes are so well conserved that human genes can be used to restore the function in flies. Even though flies and humans have many differences, they still have a high degree of conserved physiology and biology that make *Drosophila* a valuable tool as a model organism (Pandey and Nichols, 2011).

There are several advantages of using *Drosophila* as a model organism. Though most strains are genetically modified, there are minimal safety and ethical issues related to *Drosophila* research (Pandey and Nichols, 2011). *Drosophila* has a short generation time of 10 days at 25 °C and females produce a large number of progeny. Therefore, a large of these can be maintained in lab. Furthermore, they have a simple diet and small size. Controlled expression of transgenes can be achieved in flies using GAL4/UAS system (Figure 8).
**Figure 8. GAL4/UAS system.** Gene of interest is expressed *in vivo* in *Drosophila melanogaster* using GAL4/UAS system. Adult flies carrying GAL4 drivers are crossed to flies that have transgenes under the control of upstream activating sequence (UAS) to produce offsprings that have both entities. Since actin-Gal4 is used in this illustration, the offspring will express the transgene ubiquitously. Tissue specific Gal4 (for example, eye) can be used to drive the gene of interest in specific tissue so that other regions of the *Drosophila* remain unaffected.
This system consists of a GAL4 transcription factor that binds and activates the GAL4 response element, called the upstream activating factor (UAS) to which the gene of interest is attached. A major advantage of this system is that it helps in the study of lethal gene by tissue specific expression; for example, in tissues like eyes and wings that are not essential for survival in flies (Duffy, 2002). Due to availability of a large number of mutants and special balancer chromosomes that have visible markers associated with them, genetic manipulation in flies is relatively easy compared to other model organisms. The function of a gene can be studied very easily in flies due low degree of redundancy in the *Drosophila* genome.

Even though *Drosophila* is an excellent model to study human diseases; it has its own limitations (Gonzalez, 2013). Flies lack mammalian tissues like bones and cartilage. Research related to mammalian organs like liver and pancreas cannot be performed in flies. Metastatic processes like intravasation and extravasation cannot be studied in flies, as they don’t have veins and arteries. Due to lack of adaptive immune system, flies cannot be used for studies related to adaptive immune defenses.

Even though fruit flies and humans have their differences, decades of ongoing research have shown that major pathways related to growth, death and signaling are well conserved. Furthermore, due to a wealth of available resources, remains one of the most tractable model organisms.

This thesis aims to address the following objectives:

- Characterization of *Skp2* mutants
- To elucidate the G2/M role of *Skp2*
- To elucidate the function of *Skp2* in maintaining genome stability

The thesis aims to decipher the tumour suppressor role of Skp2. The data obtained during this research will help in understanding the role of Skp2 in cell cycle and will also provide data that could have potential implication in cancer therapeutics.
REFERENCES


Michel, J.J., Xiong, Y., 1998. Human CUL-1, but not other cullin family members, selectively interacts with SKP1 to form a complex with SKP2 and cyclin A. Cell Growth & Differentiation 435-449.

Michel, L.S., Liberal, V., Chatterjee, A., Kirchweger, R., Pasche, B., Gerald, W., Dobles, M., Sorger, P.K., Murty, V.V., Benezra, R., 2001. MAD2 haplo-insufficiency


Oshikawa, K., Matsumoto, M., Yada, M., Kamura, T., Hatakeyama, S., Nakayama, K.I., 2003. Preferential interaction of TIP120A with Cul1 that is not modified by NEDD8 and not associated with Skp1. Biochemical and Biophysical Research Communications 303, 1209-1216.


CHAPTER 2
Cks85A and Skp2 interact to maintain diploidy and promote growth in Drosophila

Mohammad Ghorbani, Biju Vasavan, Emona Kraja, Andrew Swan
INTRODUCTION

The small Cdk subunit, Cks or suc1 was first identified on the basis of a genetic and physical interaction with the Cyclin dependent kinase (Cdk) in fission yeast and budding yeast (Pines, 1996). In addition to a completely conserved Cdk interacting region, all Cks proteins have an anion binding domain that appears to allow interaction with phosphoepitopes. All metazoans studied to date have two Cks proteins (Cks1 and Cks2 in mammals) that appear to have both distinct and redundant functions. Xenopus Xe-p9, mammalian Cks2 and Drosophila Cks30A are necessary for meiosis, and are necessary for both entry into and exit from meiosis (Patra and Dunphy, 1998, Spruck et al., 2003 and Swan et al., 2005) (Pearson et al., 2005). The anaphase role appears to involve the recruitment of cyclin–Cdk complexes to the Anaphase Promoting Complex (APC) to promote either or both APC phosphorylation by the Cdk or cyclin ubiquitination by the APC (Patra and Dunphy, 1998) (Wolthuis et al., 2008). In vitro studies in the murine model indicate that in addition to this meiotic role, Cks2 functions redundantly with Cks1 in the entry into mitosis. Surprisingly, this appears to reflect a role in transcription — specifically of mitotic cyclins and Cdk1 (Martinsson-Ahlzen et al., 2008).

Studies of mammalian Cks1 revealed yet another novel role for Cks proteins, as part of the SCF^{Skp2} ubiquitin ligase. SCF^{Skp2} is important for the ubiquitination and proteasome-mediated destruction of a number of proteins, many of which have key roles in S-phase regulation (Nakayama and Nakayama, 2006). Cks1 interacts with Skp2 and is required for the recognition of a critical SCF^{Skp2} substrate, the Cdk inhibitor p27 (Ganoth et al., 2001 and Spruck et al., 2001). Cks1 knockout in the mouse results in reduced growth and elevated p27 levels (Spruck et al., 2001), and these phenotypes are also observed upon Skp2 knockout (Nakayama et al., 2000).

While in vitro studies and the shared phenotypes suggest a close functional relationship between Cks1 and Skp2, it remains unclear to what degree Cks1 is necessary for the targeting of SCF^{Skp2} substrates other than p27. It is also not known to what degree Cks1 functions independent of SCF^{Skp2} and to what extent it is redundant with its close homologue, Cks2.

Here we present the characterization of the Drosophila Cks85A gene. We find that Cks85A is required for growth and for the maintenance of diploidy. Cks85A is the
Drosophila $Cks1$ orthologue, and interacts with Skp2 as part of the $SCF^{Skp2}$ complex. Using genetic and biochemical approaches, we determine the relationship in vivo between Cks85A and Skp2, and between Cks85A and the other Drosophila Cks protein, Cks30A.
MATERIALS AND METHODS

Fly strains and genetics

Cks85A and Cks30A null mutants as well as transgenic UAS-Flag-Cks85A and UAS-Flag-Cks30A were described previously (Swan et al., 2005). UAS-HA-Skp2 was made by PCR cloning the coding sequence of Skp2 (CG9772) from the cDNA clone RE15215 into a pUASp vector containing two copies of the HA tag in frame at the 5 prime end. This Skp2 transgene can rescue the lethality of Skp2^{ex9} (AS, unpublished). The deficiency, Df(3R)BSC197 was used to create hemizygous Cks85A individuals in some experiments and this gave identical phenotypes to Cks85A^{ex15} homozygotes. Similarly, Df(3R)Exel6140 was used to create Skp2 hemizygous individuals. Skp2^{ex9}/Df(3R)Exel6140 flies had identical phenotypes to Skp2^{ex9} homozygous flies (data not shown). Cks85A^{ex15} clones in the female germline were generated using the dominant female sterile FLP/FRT-ovoD method, using stocks obtained from Bloomington stock center. UAS-RNAi lines, Cks85A^{KK101277} and Cks85A^{GD23702}, Skp2^{GD15636} and Skp2^{KK2101487} were obtained from VDRC (Austria). An isogenic yw stock serves as a control in many experiments. All other stocks were obtained from the Bloomington stock center.

Generation of Skp2^{ex9}

To generate a null allele of Skp2 we screened by PCR for imprecise excisions of P{EPgy2}CG1103^{EY00567}, a viable P-element insertion approximately 300 bp upstream of the Skp2 open reading frame. We identified a single excision line, Skp2^{ex9} that disrupts the Skp2 gene and causes late larval lethality. Sequencing revealed a deletion that results in loss of putative promoter sequences and 62% of the coding sequence including the essential F-box sequence and most of the first LRR repeat. Therefore Skp2^{ex9} is a null allele. The lethality of Skp2^{ex9} could be fully rescued by the expression of UAS-HA-Skp2 transgene under control of the ubiquitous da-Gal4 driver, indicating that the lethal phenotype is due to the loss of Skp2 activity.
Growth measurements

To obtain synchronized larvae for determining growth, larvae that hatched within a one hour period were collected and allowed to continue development for 4 more days for a total 5 days after egg lay (AEL). Larval size was estimated by an area measurement performed on brightfield images taken using an Olympus Fluoview 1000, using the analysis software included in the program. A minimum of 12 and average of 16 larvae were measured for each genotype. Representative examples are shown in Figs. 1A-C. Whole salivary glands in Fig. 1 were taken from feeding 3rd instar larvae (4 days AEL for wild type and 5 days AEL for Cks85A and Skp2). The salivary gland nuclei in Figs. 1J-L are from wandering 3rd instar larvae (5 days AEL for wild type and 6 days AEL for Cks85A and Skp2).

Cytology, immunostaining

Imaginal discs and brains were taken from wandering 3rd instar larvae staged as described above, fixed in 3.7% formaldehyde in PBS plus 0.2% Tween. For antibody staining, blocking was performed for 1 h in PBST plus 1% BSA. For Edu labeling of S-phase cells, salivary glands were taken from feeding 3rd instar larvae, fixed as above after 1 hour incubation in Schneider's medium plus Edu (Invitrogen). DNA was detected with Oligreen or Propidium Iodide. Apoptosis was detected by incubation in Acridine Orange at 10 µM followed by live imaging, or by antibody staining with rabbit α-Cleaved Caspase 3 (Cell Signaling) at 1/250. Colchicine treatment and preparation of brain mitotic chromosomes were performed as in Gatti et al. (1994). Mitotic indexes were determined as the percent of total cells in mitosis, and were derived from counting 600 to 800 cells per genotype and treatment. Polyploidy index was determined as the percent of mitotic cells that were polyploid — combining colchicine treated and untreated cells.

Flow cytometry

10 to 20 imaginal discs were dissected out of wandering 3rd instar larvae and dissociated in Schneider's media containing Trypsin and Propidium Iodide. Following dissociation, formaldehyde was added to 4% and cells were fixed for 20 min. Cells were pelleted at low speed and resuspended in PBST. Following one more wash, cells were
subjected to flow cytometry using a Becton Dickinson Flow Cytometer. For each experiment, wild type discs and mutant discs were analyzed in parallel. Each experimental condition was measured 3 times with over 50,000 cells per run and the combined results are shown in Fig. 4B. The individual plots shown are representative examples.

**Coimmunoprecipitations and westerns**

Coimmunoprecipitations (CoIPs) were performed on 0 to 4 hour embryos collected from female flies expressing *UAS-HA-Skp2* and either *UAS-Flag-Cks30A* or *UAS-Flag-Cks85A* under the control of the maternal *nos-Gal4* driver. IPs were performed using α-FlagM2-Agarose beads (Sigma) or α-HA-Agarose beads (Roche) as in Swan et al. (2005). Western blots were probed with rat α-HA at 1/1000 (Roche), mouse α-FlagM5 (Sigma) at 1/100, rabbit α-PSTAIR (Santa Cruz) at 1/1000, rabbit α-Cdk2 (Knoblich et al., 1994) at 1/1000, rabbit α-SkpA (Murphy, 2003) at 1/100, mouse α-Actin (Developmental Studies Hybridoma Bank) at 1/20, rabbit anti-Dap at 1/1000 (Lane et al., 1996) rabbit α-Dup (Whittaker et al., 2000) at 1/500 or rabbit α-Dup (from Michael Botchan) at 1/500.
RESULTS

Drosophila Cks85A is necessary for growth and the maintenance of diploidy

We previously reported the generation of null alleles of the two Drosophila Cks genes, Cks30A and Cks85A (Swan et al., 2005). Cks30A is essential for the activity of the Anaphase Promoting Complex (APC) in female meiosis but is dispensable in somatic cells (Pearson et al., 2005, Swan et al., 2005S and Swan and Schupbach, 2007). In contrast, Cks85A is not required for female meiosis but is essential for viability (Swan et al., 2005). Flies homozygous or hemizygous for the null allele, Cks85A^{ex15}, are lethal and this lethality is rescued by the ubiquitous expression of a UAS-Flag-Cks85A transgene using the da-Gal4 driver (Swan et al., 2005). Cks85A^{ex15} larvae develop slower than wild type (Fig. 1A,B) and following an extended 3rd instar they die after pupariation, prior to the differentiation of adult structures (Fig. 1D).

Drosophila larval growth occurs largely in the absence of cell division. Late in embryogenesis most cells exit the cell cycle and eventually enter into an endocycle in which cells undergo regular rounds of S-phase without mitosis, increasing in size as they increase in ploidy. To assay growth in endoreplicating tissues we focused on the salivary glands. Cells that make up the salivary gland start endoreplicating late in embryogenesis and by the cessation of growth at the wandering 3rd instar stage eventually reach a ploidy of up to 2048\([C \text{ (Lee and Orr-Weaver, 2003)}\]. Salivary glands are significantly smaller in Cks85A^{ex15} relative to wild type (Fig. 1G,H). Reduced overall size can be partially attributed to fewer cells per salivary gland (Fig. 1J). However, the major contributor to reduced overall growth appears to be a failure in the endoreplication cycles: the frequency of cells in S-phase in feeding 3rd instar Cks85A^{ex15} larvae is significantly lower than that of wild type controls (Figs. 1G,H,J). Correspondingly, the ploidy observed in wandering 3rd instar larvae (after termination of the endoreplication program) is reduced in Cks85A^{ex15} compared to wild type (Fig. 1K,L).
<table>
<thead>
<tr>
<th></th>
<th>Cells per salivary gland</th>
<th>S-phase cells per salivary gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>125±6</td>
<td>17±6</td>
</tr>
<tr>
<td>Cks85A</td>
<td>111±10</td>
<td>4±2*</td>
</tr>
<tr>
<td>Skp2</td>
<td>105±6*</td>
<td>1±1*</td>
</tr>
</tbody>
</table>

N. wild type
K. Cks85A
L. Skp2
M. Cks85A-RNAi
O. Skp2-RNAi
Fig. 1: *Cks85A* and *Skp2* are required for growth and endoreplication. A–C) Representative wild type (A), *Cks85A*\textsuperscript{ex15/ex15} (B), and *Skp2*\textsuperscript{ex9/ex9} (C) larvae at 5 days After Egg Lay (AEL). At this time point, wild type are at the wandering 3rd instar stage while *Cks85A* and *Skp2* null larvae are at feeding 3rd instar. *Cks85A* and *Skp2* null larvae respectively are 66% and 67% the size of wild type larvae at this time-point (see Methods). D–F) Terminal phenotype of D) *Cks85A*\textsuperscript{ex15/ex15}, E) *Skp2*\textsuperscript{ex9/ex9}, F) UAS-HASkp2/UAS-HASkp2; da-Gal4,*Cks85A*\textsuperscript{ex15}/da-Gal4,*Cks85A*\textsuperscript{ex15}. *Cks85A* and *Skp2* null mutants arrest shortly after pupariation (D,E). Overexpression of *Skp2* partially rescues *Cks85A*\textsuperscript{ex15} lethality (F). G–I) Salivary glands taken from feeding 3rd instar larvae labeled with Oligreen (green) and Edu (magenta and in grayscale in G′–H′) following a 1 hour Edu incorporation. *Cks85A*\textsuperscript{ex15}/Df(3R)BSC197 (H) and *Skp2*\textsuperscript{ex9/Df(3R)Exel6140} (I) salivary glands are significantly smaller and have less nuclei undergoing DNA replication compared to controls (G). J) Quantification of cell number per salivary gland indicates a slight reduction in overall number, and this is statistically significant for Skp2. The number of cells in S-phase is significantly less in *Cks85A* and *Skp2* mutants compared to controls. (* — p < .05). K–M) Nuclei from salivary glands taken from wandering 3rd instar larvae that have completed endoreplication cycles. Wild type nuclei (K) reach significantly higher ploidy than *Cks85A*\textsuperscript{ex15/ex15} (L) or *Skp2*\textsuperscript{ex9/ex9} (M). N–P) Salivary glands from wandering 3rd instar *ptc-Gal4/+* control (N) *ptc-Gal4/UAS-Cks85AGD23702* (O) and *ptc-Gal4/UAS-Skp2GD15636* (P), labeled with Oligreen to detect DNA. Knockdown of *Cks85A* or *Skp2* results in reduced salivary gland growth. Scale bar in G = 100 µm applies to G–I. Scale bar in K = 5 µm and applies to J–L. Scale bar in N = 100 µm and applies to M–O.
To independently establish the effect of Cks85A loss, we determined the phenotype resulting from RNAi-mediated knockdown. We obtained two UAS-RNAi lines from a genome-wide RNAi library (Dietzl et al., 2007), and crossed these to the ptc-Gal4 driver to induce knockdown in salivary glands. One of these lines, UAS-RNAi-Cks85A\textsuperscript{GD23702} produced a reduction in salivary gland size and nuclear size relative to controls (Figs. 1N,O), similar to the phenotype observed in Cks85A\textsuperscript{ex15}. Results presented below (Fig. 4) further support the conclusion that UAS-RNAi-Cks85A\textsuperscript{GD23702} affects a partial loss of Cks85A activity.

While most cells in Drosophila cease mitotic divisions late in embryogenesis and enter into an endoreplication cycle, cells of the larval brain continue to undergo canonical mitotic divisions throughout larval development. Upon examination of whole mount brains from Cks85A\textsuperscript{ex15} flies labeled for DNA, we first noticed that the regular patterning of cells, particularly apparent in the optic lobes, was disrupted (Figs. 2A,B). Further, Cks85A\textsuperscript{ex15} brains appear smaller than those of controls at equivalent times after egg laying (Figs. 2A,B), suggesting that Cks85A is important for growth in mitotically dividing cells as well as in endoreplicating cells. Strikingly, despite reduced overall growth, it appeared that some cells within brains from Cks85A\textsuperscript{ex15} appear larger than in wild type brains (arrow in Fig. 2B). To confirm that these large nuclei correspond to polyploid cells we prepared squashes of untreated and colchicine treated larval brains to directly observe mitotic chromosomes.
<table>
<thead>
<tr>
<th></th>
<th>Mitotic Index no Colchicine</th>
<th>Mitotic Index with Colchicine</th>
<th>Polyploidy Index**</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>1.0±.5</td>
<td>2.5±1.3</td>
<td>0</td>
</tr>
<tr>
<td>Cks85A</td>
<td>1.5±.4</td>
<td>2.8±.3</td>
<td>5.7</td>
</tr>
<tr>
<td>Skp2</td>
<td>1.1±.5</td>
<td>2.8±.8</td>
<td>11.1</td>
</tr>
</tbody>
</table>
Fig. 2: Loss of Cks85A and Skp2 results in polyploidy in brains. A–C) Central brain and optic lobes of 3rd instar larvae labeled with Oligreen to detect DNA. A) yw control brain/optic lobe. B) Cks85A$^{ex15/ex15}$ and (C) Skp2$^{ex9/ex9}$ brains are smaller and have larger cells. D–G) Mitotic spreads from 3rd instar brains labeled with Oligreen. Mitotic Indexes (MI) are indicated for untreated and colchicine treated brains. D) yw control. E) Cks85A$^{ex15/ex15}$ and (G) Skp2$^{ex9/ex9}$ cells with tetraploid DNA content. F) Highly polyploid cell from Cks85A$^{ex15/ex15}$. H) Mitotic and polyploidy indexes (see Methods) from untreated and from Colchicine treated brains. The total number of polyploid cells counted was very low and as a result the standard deviations are very high and the difference between Cks85A and Skp2 are not statistically significant. Scale bar in A = 20 µm and applies to A–C. Scale bar in D = 10 µm and applies to D–G.
In wild type, the full chromosome complement (two major autosomes, the sex chromosome pair and the dot-like 4th chromosomes) can be clearly observed (Fig. 2D) in approximately 1% of squashed nuclei. This frequency goes up to 2.5% upon colchicine-induced activation of the spindle assembly checkpoint (Fig. 2H). Similar frequencies of mitotic cells are observed in Cks85A<sup>ex15</sup>, indicating that mutant cells are able to enter into mitosis and arrest in response to SAC activation (Fig. 2H). A small percentage, (approximately 6% of these cells) have an exact doubling of chromosome number (Figs. 2E,H). Even more rarely, (less than 1% of mitotic cells), higher ploidy is observed (Fig. 2F).

The imaginal discs, like the brain, are composed of cells that continue to divide throughout larval development. The eye imaginal disc undergoes a wave of differentiation during the 3rd instar, marked by the passing of a morphogenetic furrow (MF) (Fig. 3A). Cells enter into G1 arrest in the MF and a subset of these cells divides once more just posterior to the MF before undergoing terminal differentiation. Eye imaginal discs taken from Cks85A<sup>ex15</sup> wandering 3rd instar larvae contain enlarged, apparently polyploid nuclei specifically anterior to the MF (Fig. 3B). Cells that exit the cell cycle posterior to the MF appear similar to those in controls. Importantly, the expression of UAS-Flag-Cks85A under control of the ubiquitous da-Gal4 driver rescues this phenotype (Fig. 3C), indicating that polyploidy is specifically due to the loss of Cks85A.

To directly assess DNA content of cells in imaginal discs we performed flow cytometry on cells dissociated from wing imaginal discs of wild type and Cks85A<sup>ex15</sup> larvae. We noticed that the degree of polyploidy in whole-mount preparations of brains and eye imaginal discs appeared to be temperature dependent (see Figs. 3B,H). We therefore performed flow cytometry on wing imaginal discs from flies raised at 18 °C, 25 °C and 29 °C to verify this observation. In all cases newly wandering 3rd instar larvae were taken to ensure that developmental stage was constant across different genotypes and rearing temperatures. Examination of DNA content in cells from wild type wing imaginal discs revealed a distribution over two peaks corresponding to 2n (G1 cells) and 4n DNA content (G2/M) (Figs. 4A,B). This distribution did not change across the 3 different rearing temperatures (Fig. 4B). In Cks85A there is a distinct 8n peak in addition to 2n and 4n. This tetraploid population is more prominent at higher temperatures.
Fig. 3: Cks85A and Skp2 are required to maintain diploidy in mitotically active but not differentiated cells. Oligreen labeled eye imaginal discs from wandering 3rd instar larvae. All images are arranged with Morphogenic Furrow (MF) in the middle, differentiating cells posterior to the furrow at the left and asynchronously dividing cells anterior to the MF on the right. A) Wild type eyes have equally sized nuclei on both sides of the MF. B) In Cks85A<sup>ex15/ex15</sup> eye imaginal discs differentiating cells posterior to the MF appear normal but cells anterior to the MF have larger nuclei. C) UAS-Flag-Cks85A; Cks85A<sup>ex15</sup>/da-Gal4,Cks85A<sup>ex15</sup> eye disc. Expression of Flag-Cks85A rescues the polyploidy of Cks85A<sup>ex15</sup>. D) Skp2<sup>ex9/ex9</sup> eye imaginal discs display pronounced polyploidy anterior to the MF. E) UAS-HA-Skp2; da-Gal4,Skp2<sup>ex9</sup>/Skp2<sup>ex9</sup> eye imaginal disc. Transgenic expression of HA-Skp2 rescues the polyploidy of Skp2<sup>ex9</sup>. F) Skp2<sup>ex9/+</sup>, Cks85A<sup>ex15/ex15</sup> eye imaginal discs from wandering a 3rd instar larva. Loss of one copy of Skp2 enhances the Cks85A null phenotype (compare to B). G) Skp2<sup>ex9</sup>, Cks85A<sup>ex15</sup> double homozygous larvae display a phenotype similar to that observed in Skp2<sup>ex9</sup> alone (compare to D). H) Cks85A<sup>ex15</sup> raised at 29 °C displays a strong polyploidy posterior to the MF. This polyploidy is rescued by overexpression of HA-Skp2. Scale bar in A = 5 µm and applies to all panels.
Fig. 4: Polyploidy and apoptosis in Cks85A and Skp2 mutant wing imaginal discs.

A,B) Flow cytometry on cells from 3rd instar wing imaginal discs reveals a distinct 8 C peak in Cks85A<sup>ex15/ex15</sup> that increases with temperature, and in Skp2<sup>ex9/ex9</sup>, independent of temperature. A sub-G1 peak indicative of cell death is also apparent in Cks85A and Skp2 mutants but not in wild type. Values in (B) are percentages averaged from 3 experiments. C–E) Acridine Orange staining of wild type (C), Cks85A<sup>ex15/ex15</sup> (D), and Skp2<sup>ex9/ex9</sup> (E) wing imaginal discs. Extensive cell death is observed in Cks85A and to a greater extent, in Skp2 mutants. F–H) Cleaved-Caspase 3 labeling (green) of wild type (F), Cks85A<sup>ex15/ex15</sup> (G) and en-Gal4/UAS-p35; Cks85A<sup>ex15/ex15</sup> (H). Extensive apoptosis is present in Cks85A mutants and is suppressed by the posterior-specific expression of the apoptosis inhibitor p35 (detected by co expressed Beta-Galactosidase-red in H). I–K) Adult wings from wild type (I), en-gal4/UAS-RNAi-Cks85A<sup>GD23702</sup>; Cks85A<sup>ex15/+</sup> raised at 29 °C (J), and en-gal4/UAS-RNAi-Skp2<sup>GD15636</sup> (K). En-gal4 mediated knockdown of Cks85A and Skp2 leads to increased wing hair spacing. En-gal4 is expected to produce knockdown in the entire posterior domain (indicated by bar in J,K) but curiously, wing hair spacing is only weakly affected near the A/P border in both Cks85A and Skp2 knockdown. Scale bar in C = 50 μm and applies to C–H.
A small but reproducible peak corresponding to 16n cells also appears at 29 °C (Figs. 4A). Therefore Cks85A is essential for the maintenance of diploidy in Drosophila in a temperature-dependent manner. We note that Cks85A<sup>ex15</sup> is a null allele and that the temperature sensitivity is therefore not allele specific but must reflect a greater requirement for Cks85A activity at elevated temperatures.

Flow cytometry on Cks85A mutant wing imaginal discs identified a population of cells with less than 2n (sub G1) DNA content (Figs. 4A,B), possibly indicative of dying cells. To directly assay for cell death we incubated wing imaginal discs with Acridine Orange, a dye that labels DNA in dying cells but that cannot enter viable cells. Acridine orange is largely excluded from cells in wild type wing imaginal discs (Fig. 4C) but labels many nuclei in Cks85A<sup>ex15</sup>, indicating a high incidence of cell death in these mutants (Fig. 4D). Immunolabeling for cleaved Caspase 3 reveals elevated caspase activation in Cks85A<sup>ex15</sup> discs (Figs. 4F,G) and this can be partially suppressed by the expression of baculovirus apoptosis inhibitor p35 (Fig. 4H). Therefore, cells that have lost Cks85A undergo apoptosis, possibly as a response to genomic instability.

Two findings argue that the polyploidy observed in Cks85A<sup>ex15</sup> animals is due specifically to the loss of Cks85A: first, mutant phenotypes are the same in Cks85A<sup>ex15</sup> homozygous and Cks85A<sup>ex15</sup> over a small deficiency (data not shown), and second, these phenotypes can be rescued by the expression of a Cks85A cDNA transgene (Fig. 3). To further verify the requirement for Cks85A in maintaining diploidy, we determined the effect of RNAi against Cks85A. We used en-Gal4 in combination with the UAS-RNAi line Cks85A<sup>GD2370</sup> to specifically knock down Cks85A in the posterior half of the wing. To maximize any possible knockdown phenotype, flies were raised at 29 °C (based on the finding that Cks85A null phenotypes are more pronounced at higher temperatures and that Gal4/UAS mediated expression is typically temperature dependent). To further maximize any possible effect of knockdown we removed one endogenous copy of Cks85A. Under these conditions (and only if both conditions were applied), the knockdown of Cks85A resulted in an increase in wing hair spacing in the posterior part of the wing (Figs. 4I,J and data not shown). Each cell of the pupal wing generates a single wing hair and as such, increased spacing between the wing hairs can be indicative of polyploidy and consequent increased cell size. We conclude from our analysis of null mutants and from RNAi-
mediated knockdown, that Cks85A is necessary for the maintenance of diploidy in Drosophila.

**Cks85A and Cks30A have non-overlapping roles in cell cycle control**

The two Drosophila Cks proteins, Cks30A and Cks85A, share 61% identity in their predicted amino acid sequences, with Cks85A having an additional 21 amino acids at the C-terminus (Swan et al., 2005). This close sequence similarity prompted us to ask if the two proteins have overlapping or redundant functions, as has been observed for the mammalian paralogues (Donovan and Reed, 2003 and Martinsson-Ahlzen et al., 2008). To determine if Cks30A has a role in the maintenance of diploidy we examined eye imaginal discs from animals null for Cks30A. Cks30A KO homozygous flies (Swan et al., 2005) are viable and eye imaginal discs taken from late 3rd instar larvae display no detectable polyploidy (Fig. 5A — compare to Fig. 3A). While Cks30A is not required on its own it is possible that it plays a partially redundant role with Cks85A. To test this possibility we created double mutants to see if loss of both Cks genes results in a more severe polyploidy than loss of Cks85A alone. While Cks85Aex15 eyes display clear polyploidy (Fig. 5B and see also (Fig. 3B), Cks85A, Cks30A double mutants have no detectable polyploidy at this stage of development (Fig. 5C). Therefore, rather than enhancing the Cks85A null phenotype, Cks30A KO appears to suppress it. We also tested if the overexpression of Cks30A can rescue the polyploidy resulting from Cks85A loss. da-Gal4, UAS-Flag-Cks30A fails to rescue the polyploidy of Cks85Aex15 eye imaginal discs (Fig. 5D). We conclude that Cks30A cannot functionally replace and in fact may functionally antagonize Cks85A.

It is interesting that Cks85A has an apparently essential role in the maintenance of diploidy, but that null animals complete embryogenesis and survive to pupariation (Fig. 1D). The results presented above demonstrate that the apparent non-essentiality of Cks85A early in development cannot be due to earlier redundancy with Cks30A. An alternative possibility is that Cks85A mRNA or protein is maternally deposited in the egg and is sufficient to rescue any early requirement. To test this possibility we generated germline + zygotic null Cks85A mutants using the FRT/ovoD germline clone method.
Fig. 5: *Cks85A* and *Cks30A* have distinct and antagonistic activities. Eye imaginal discs taken from wandering 3rd instar larvae labeled for DNA and displayed with MF in the center and anterior on the right. A) *Cks30A*<sup>KO/KO</sup> eyes have no detectable polyploidy. B) *Cks85A*<sup>ex15/ex15</sup> displays polyploidy anterior to the MF. C) *Cks30A*KO/KO suppresses the polyploidy of *Cks85A*<sup>ex15/ex15</sup>. D) da-Gal4 driven expression of UAS-Flag-*Cks30A* does not suppress the polyploidy of *Cks85A*<sup>ex15/ex15</sup>. E) *Cks85A*<sup>ex15/ex15</sup> zygotic null animals display polyploidy in late 3rd instar imaginal discs (as in B). F) Loss of maternal *Cks85A* does not enhance this phenotype. Scale bar in A = 5 μm and applies to all panels.
We first noted that Cks85A heterozygous flies from germline homozygous females are viable and appear phenotypically normal (data not shown), indicating that maternal Cks85A is not essential. Furthermore, Cks85A maternal/zygotic homozygous null larvae survive to the larval/pupal transition and display the same onset and degree of polyploidy as maternally rescued larvae raised in parallel (Figs. 5E,F). We conclude that Cks85A is required for the maintenance of diploidy, specifically late in larval development, and has no essential role in this respect in the embryo or early larva.

**Cks85A physically associates with SCF\textsuperscript{Skp2} and cyclin-Cdk complexes**

The requirement for Cks85A in growth suggested that Cks85A could be the orthologue of the mouse Cks1 gene. Cks1 is not essential for viability in mice but it is required for growth (Spruck et al., 2001). Cks1 physically associates with the F-box protein Skp2 and is necessary for the SCF\textsuperscript{Skp2} dependent destruction of the Cdk inhibitor, p27 (Ganoth et al., 2001 and Spruck et al., 2001). The mouse Skp2, like Cks1 is required for growth and interestingly, it is also required for the maintenance of diploidy in some cell types (Nakayama et al., 2000). These similarities lead us to examine the possibility that Cks85A is a component of the Drosophila SCF\textsuperscript{Skp2} ubiquitin ligase complex. We therefore assayed for direct physical association of Cks85A and Skp2 in Drosophila. The closest homologue to Skp2 in the Drosophila genome, CG9772, encodes a predicted protein with 23% identity and 41% similarity to the human Skp2 (Fig. 6A,B). If comparison is limited to the sequence starting at amino acid 94 of the human Skp2, composed mainly of the F-box and 8 LRR domains, the human Skp2 and CG9772 are 33% identical and 59% similar. We conclude from this sequence conservation and from the biochemical and genetic evidence presented below that CG9772 encodes the Drosophila Skp2 gene.

To test for physical interaction between Skp2 and Cks85A we generated a transgene consisting of the HA-epitope fused to Skp2 cDNA and under control of Gal4. UAS-HA-Skp2 was co-expressed with UAS-Flag-Cks85A in Drosophila embryos using the nos-Gal4 driver. We then performed immunoprecipitations (IPs) with either anti-Flag or anti-HA antibody beads. Flag-Cks85A and HA-Skp2 were able to coIP each other, indicating that the two proteins interact in vivo (Fig. 6C-lanes 3,6).
Fig. 6: Identification of the Drosophila Skp2. A) Sequence alignment between human Skp2 and Drosophila CG9772-PA. Blue line indicates the F-box domain and the black lines indicate the 8 LRR domains on the human Skp2. B) Genomic organization around CG9772 (Skp2), located at cytological position 82A on chromosome 3R. Also shown are the molecular breakpoints of Skp2\textsuperscript{ex9} and the original P-element insertion, P(EP)EY00567 from which this excision line was generated. P(EP)EY00567 is homozygous viable and shows no detectable polyploidy or growth impairment (data not shown). C) Coimmunoprecipitations from embryos with nos-Gal4 driven expression of UAS-HA-Skp2 and either UAS-Flag-Cks85A (lane 3 and 6) or UAS-Flag-Cks30A (lane 2 and 5). Western blotting for HA (Skp2), Flag (Cks30A or Cks85A), Cdk1, Cdk2 and SkpA. Lane 2: Flag-Cks30A associates with Cdk1 but not SkpA or Skp2. Lane 3: Flag-Cks85A associates with Skp2, Cdk1 and SkpA. Lane 5: HA-Skp2 associates with Cdk1, SkpA but not with Cks30A. Lane 6: HA-Skp2 associates with Cdk1, SkpA and Cks85A. Control IPs in lanes 1 and 3, from embryos not expressing transgenes, reveal that the anti-HA and anti-Flag beads do not bind non-specifically to any of the proteins tested.
Further, Flag-Cks85A and HA-Skp2 could coIP the SCF component, SkpA (Fig. 6C-lanes 3,6). Therefore, Cks85A associates with SCF$_{\text{Skp2}}$ in Drosophila.

To determine if the other Drosophila Cks, Cks30A also physically interacts with SCF$_{\text{Skp2}}$ we co-expressed Flag-Cks30A with HA-Skp2 and then IPd either Flag-Cks30A or HA-Skp2. We find that Cks30A, in contrast to Cks85A, does not interact with Skp2 or SkpA (Fig. 6C-lanes 2, 5). This inability of Flag-Cks30A to interact with SCF$_{\text{Skp2}}$ may explain its inability to functionally compensate for the loss of Cks85A (Fig. 5 and Swan et al., 2005).

Cks was first identified as a subunit of the Cyclin dependent kinases (reviewed in Pines (1996), and we previously showed that Drosophila Cks85A shares this conserved ability to interact with Cdk2 as well as Cdk1 and its cyclin partners A, B and B3 (Swan et al., 2005) (Fig. 6). HA-Skp2 also associates with Cdk1 and Cdk2 (Fig. 6-lanes 5,6). Therefore Cdk1 and Cdk2 can associate with SCF$_{\text{Skp2}}$, possibly with Cks85A forming the link between the Cdk and SCF complexes as predicted from in vitro studies of their mammalian counterparts (Hao et al., 2005).

**Skp2 is necessary for growth and maintenance of diploidy in Drosophila**

Our results support a role for Cks85A in the SCF$_{\text{Skp2}}$ ubiquitin ligase. To test this possibility we sought to compare the Cks85A null phenotype to the Skp2 null phenotype. There are no known Skp2 mutants in Drosophila. Therefore we generated a null allele of Skp2 by imprecise excision of a P-element inserted upstream of the gene (Fig. 6B). Skp2$^{ex9}$ removes 62% of the predicted coding sequence, including the essential F-box and 1st LRR domain, in addition to 300 bp upstream of the transcription start site; and is therefore a null allele. Skp2$^{ex9}$ has the same lethal phase as Cks85A$^{ex15}$: homozygous or hemizygous individuals die at the larval/pupal transition with no sign of differentiation of adult structures (Fig. 1E). Viability of Skp2$^{ex9}$ is restored and normal-appearing adults are produced upon the expression of UAS-HA-Skp2 under da-Gal4 control (data not shown), demonstrating that lethality is due specifically to loss of Skp2 function.

Larval growth is reduced in Skp2$^{ex9}$ (Fig. 1C), and as in the Cks85A null mutant, this corresponds to reduced DNA replication and final ploidy of endoreplicating cells (Fig. 1I,J,M). ptc-Gal4 mediated knockdown of Skp2 in the salivary gland using the UAS-
RNAi line, Skp2<sup>GD15636</sup> (Dietzl et al., 2007) also results in reduced salivary gland size and ploidy (Fig. 1P). A more subtle reduction in salivary gland size is observed with a second UAS-RNAi line, Skp2<sup>KK2101487</sup> (data not shown). Therefore Skp2, like Cks85A is required for growth and this at least in part reflects a role in the endoreplication cycle.

To determine if Skp2, like Cks85A, is required for the maintenance of diploidy, we examined brains and imaginal discs from Skp2 null larvae. Whole mount and chromosome squash preparations of Skp2<sup>ex9</sup> brains revealed polyploid cells at a low frequency (Figs. 2C,G). As in the Cks85A mutant, polyploidy was not associated with a failure to enter mitosis, since the mitotic index with or without colchicine treatment was not lower than seen in wild type (Fig. 2H).

We also examined imaginal discs to determine if Skp2 is required for the maintenance of diploidy in these tissues. Eye imaginal discs from Skp2<sup>ex9</sup> 3rd instar larvae contain large nuclei anterior to the morphogenetic furrow, and apparently normal nuclei posterior to the MF (Fig. 3D). This is similar to the phenotype in Cks85A null mutants in which polyploidy arises in dividing cells anterior to the MF but not in differentiated cells posterior to the MF. However, the Skp2 null phenotype is more pronounced (compare Figs. 3B,D) and is not temperature sensitive (data not shown). Polyploidy is fully rescued by da-Gal4 driven expression of HA-Skp2, indicating that it is specifically due to loss of Skp2 activity (Fig. 3E).

Finally, we examined cell ploidy in Skp2<sup>ex9</sup> wing imaginal discs by flow cytometry. This revealed a distinct peak of tetraploid cells and a smaller population of cells of greater ploidy. Comparing flies raised at 25 °C or 29 °C, the relative number of cells that are polyploid is similar between Cks85A and Skp2. However, unlike Cks85A, Skp2 is equally necessary at lower temperatures, and the incidence of polyploid cells in Skp2<sup>ex9</sup> is the same at 18 °C as it is at higher temperatures (Fig. 4B).

Flow cytometry analysis also revealed the presence of a sub-G1 cell population in Skp2<sup>ex9</sup> wing imaginal discs (Figs. 4A,B), possibly due to cell death. Acridine Orange labeling of Skp2<sup>ex9</sup> wing imaginal discs confirmed the presence of widespread cell death (Fig. 4E). The extent of apoptosis appeared significantly greater in the Skp2 null background than in the Cks85A null background (compare Figs. 4D,E).
To independently examine the requirements for Skp2 in maintaining diploidy, we determined the effect of en-Gal4 mediated RNAi knockdown of Skp2 in the developing wing. The two Skp2 RNAi lines UAS-RNAi-Skp2<sup>GD15636</sup> and UAS-RNAi-Skp2<sup>KK2101487</sup> produced an increase in wing hair spacing (Fig. 4K and data not shown). In conclusion, Cks85A and Skp2 null mutants display qualitatively similar phenotypes, though Skp2 loss results in stronger expression of these phenotypes, particularly at lower temperatures.

**Genetic evidence that the main role of Cks85A is to promote SCF<sup>Skp2</sup> activity**

The similarity between Cks85A and Skp2 loss of function phenotypes and the observed physical association in vivo supports the idea that Cks85A functions with Skp2 as part of the SCF<sup>Skp2</sup> ubiquitin ligase. On the other hand, the relatively milder phenotypes observed upon Cks85A loss may indicate that SCF<sup>Skp2</sup> has some activity in the absence of Cks85A. To test this possibility we first asked whether reducing the dose of Skp2 could enhance the Cks85A null phenotype. If Cks85A were essential for all SCF<sup>Skp2</sup> activity, reducing the amount of Skp2 should not enhance the Cks85A null phenotype. Halving Skp2 gene dose in the Cks85A<sup>ex15</sup> homozygous background leads to more pronounced polyploidy (Fig. 3F — compare to Fig. 3B). This genetic enhancement is consistent with Cks85A and Skp2 acting in the same pathway, but with Skp2 playing a more critical role.

To extend this analysis we examined flies double homozygous for Cks85A and Skp2. If Cks85A has activity outside of the SCF<sup>Skp2</sup> complex we might expect that double mutants for Cks85A and Skp2 would display a stronger phenotype than either single mutant. We found instead that Cks85A, Skp2 double homozygous individuals displayed the same lethal phase (data not shown) and degree of polyploidy as Skp2 single mutants (Fig. 3G — compare to Fig. 3D). Overall, these results are consistent with a model in which Cks85A functions as part of the SCF<sup>Skp2</sup>, but with a less critical role than Skp2 in this complex.

The relatively milder phenotype resulting from Cks85A loss can be understood if Cks85A plays an essential role in the recognition of only a subset of SCF<sup>Skp2</sup> targets as has been suggested (Hao et al., 2005). Alternatively, Cks85A could play a supporting but non-essential role in the recognition of all or most SCF<sup>Skp2</sup> targets. In this latter scenario
we predict that merely increasing the levels of Skp2 may compensate for the absence of Cks85A. To test this possibility, we overexpressed Skp2 using UAS-HA-Skp2 and the da-Gal4 driver, in a Cks85A null background. As described above, Cks85A null flies invariably arrest at pupariation with no sign of differentiation of adult structures (Fig. 1D). When we overexpressed Skp2 in the Cks85A null background, flies progressed to the pharate adult stage and died during or shortly after escaping from their pupal cases (Fig. 1F). We then asked if the overexpression of Skp2 could also rescue the polyploidy that results from Cks85A loss. When raised at 29 °C Cks85A\textsuperscript{ex15} flies display a strong polyploidy in the posterior half of the eye imaginal disc (Fig. 3H). This polyploidy appears completely rescued by the overexpression of HA-Skp2 (Fig. 3I). This surprising finding, that the requirement for Cks85A can be bypassed, to a large degree, simply by overexpressing Skp2, suggests that much of SCF\textsuperscript{Skp2} function can be performed in the absence of Cks85A.

**Loss of SCF\textsuperscript{Skp2} function results in growth impairment and polyploidy, independent of p27 and Cdt1 accumulation**

*Skp2* or *Cks1* knockout in the mouse leads to the over-accumulation of p27 (Nakayama et al., 2000 and Spruck et al., 2001), and genetic evidence suggests that in the *Skp2* knockout, high levels of p27 are at least partially responsible for the polyploidy that is observed (Nakayama et al., 2004). To directly test the possibility that Cks85A and Skp2 target p27 for destruction in Drosophila we examined levels of Dacapo (Dap), the Drosophila p27 homologue (de Nooij et al., 1996 and Lane et al., 1996) in wild type, *Skp2*\textsuperscript{ex9} and *Cks85A*\textsuperscript{ex15}. Dap levels are not affected in whole larvae (Fig. 7A), while in isolated mitotic tissues (combined brains and imaginal discs) Dap levels were actually reduced in *Cks85A*\textsuperscript{ex15} and more so in *Skp2*\textsuperscript{ex9} (Fig. 7B). Therefore, Cks85 and Skp2 loss results in polyploidy and reduced growth by a mechanism that does not involve the overaccumulation of p27/Dap.

While p27 is the only SCF\textsuperscript{Skp2} target for which Cks has been found to play an essential role, SCF\textsuperscript{Skp2} has a number of other targets that may contribute to the polyploidy observed in *Skp2* null animals.
Fig. 7: Known SCF\textsuperscript{Skp2} targets are not affected in Drosophila Cks85A or Skp2 mutants. A,B) Western blots on extracts from whole 3rd instar larvae (A), and mitotic tissues (brains and imaginal discs) from 3rd instar larvae (B) probed for p27/Dap. Dap runs as a doublet at approximately 27 kDa. Dap levels are not elevated in mutants for Cks85A or Skp2. C,D) Western blots on extracts from whole feeding 3rd instar larvae (C), and mitotic tissues from feeding 3rd instar larvae (D). Dup migrates as 105/106 kDa bands in wild type mitotic tissues. An 80 kDa band (not shown) of unknown origin is variably enriched in Cks85A and Skp2 null mutants. However, separate experiments with a myc-dup transgene fail to detect a corresponding band in wild type or Skp2 null background (data not shown). Asterisk indicates a non-specific band in Dup Westerns.
Cdt1 (Double parked or Dup in Drosophila) is a particularly attractive candidate as it is a key component of the pre-replication complex and SCF^{Skp2} has been shown to mediate in the destruction of Cdt1 in cultured mammalian cells (Li et al., 2003). To determine if Cks85A and Skp2 are necessary for the targeted destruction of Drosophila Cdt1/Dup we examined levels of Dup in whole larvae and in mitotic tissues from wild type, Cks85A and Skp2 null animals. We found no increase in Dup levels in either whole larvae (Fig. 7C), or in mitotic tissues (Fig. 7D). Therefore Cks85A and Skp2 are not required to maintain overall Dup levels.

To independently test if an overaccumulation of Dap or Dup contributes to the Cks85A null phenotype we asked if genetically lowering the overall levels of dap or dup could suppress Cks85A^{ex15}. We found no such suppression in flies heterozygous for dup^1 or in flies homozygous for the hypomorphic dup^PA77 allele (data not shown). While the failure to detect a genetic interaction cannot be given as strong evidence, it further supports the idea that SCF^{Skp2} has targets other than p27 and Cdt1 that are important for controlling cell ploidy.

DISCUSSION

Cks85A-SCF^{Skp2} maintains genomic stability in Drosophila

Cks1 and Skp2 have emerged in recent years as important oncogenes and potential therapeutic targets for cancer treatment. Both genes are overexpressed in a wide spectrum of cancers and in many cases their expression levels are predictive of outcomes (reviewed in Hershko, 2008). The increased expression of Cks1 and Skp2 in tumors has been correlated with decreases in levels of the tumor suppressor, p27, suggesting that the major oncogenic activity of Cks1 and Skp2 corresponds to their role in p27 destruction. Our characterization of the Drosophila Cks1 (Cks85A) and Skp2 leads us to conclude that Cks and Skp2 may have more complicated roles in the cell cycle and in cancer progression. First we find that Drosophila Cks85A and Skp2 control growth independent of any effect on p27 levels. Second, we find that Cks85A and Skp2 have potential tumor suppressive roles, being required for the maintenance of genomic stability.

Loss of either Cks85A or Skp2 in Drosophila results in polyploidy. Flow cytometry analysis reveals up to 35% of cells being polyploid (depending on genotype
and temperature). Of these, the vast majority appear to be tetraploid, with much lower frequency of cells with greater than 8n DNA content. A similar conclusion can be reached from chromosome squashes of larval brains. While tetraploidy may be more prevalent than higher ploidies, this conclusion has to be somewhat qualified. Based on the appearance of nuclei in whole mount preparations, the ploidy level of cells in wing imaginal discs seems much less than in eye imaginal discs or brains. While we did not quantitate the degree of polyploidy in eye imaginal discs we note that most nuclei posterior to the MF appear larger than those anterior to the MF, suggesting that the majority of these undifferentiated cells are polyploid. Also, the size of nuclei posterior to the MF varies considerably dependent on genotype and temperature. Presumably the larger nuclei represent cells of greater than tetraploid DNA content. Chromosome squashes on the other hand, could also lead to an underestimate of both the frequency and degree of polyploidy, as only cells that are in mitosis are examined for ploidy. If mutant cells bypass mitosis and undergo endoreplication cycles they will not be identified by this method.

Cks85A and Skp2 appear to be required in multiple cell types to maintain diploidy, but interestingly the requirement for these genes is limited to late in development. In the case of Cks85A this is clearly not due to maternal rescue, as demonstrated by the finding that loss of the maternal contribution of Cks85A does not result in an earlier onset or more severe polyploidy (Fig. 5). Similar experiments could not be performed with Skp2 as the centromere-proximal location of the Skp2 gene precludes the use of mitotic recombination to generate germline clones.

The distinctive phenotype observed in the eye imaginal disc also suggests a specifically late requirement for Cks85A and Skp2. During the 3rd instar, a morphogenic furrow passes from posterior to anterior of the eye imaginal disc and cells stop dividing and differentiate in its wake. Eye discs from Cks85A and Skp2 null wandering 3rd instar larvae show clear polyploidy only in mitotically active, undifferentiated cells anterior to the furrow. The simplest interpretation is that polyploidy only arises in mitotically active cells and only late in development. It is interesting that there is often a fairly abrupt transition from polyploid cells anterior to the furrow, to apparently diploid cells posterior to the furrow (e.g. Fig. 3F). Assuming that the MF is continually moving in these mutants
we might expect more of a gradient of polyploidy that extends posterior to the MF. Perhaps MF progression is also affected in these mutants, or alternatively, polyploid cells posterior to the MF are eliminated via apoptosis.

In addition to causing polyploidy, the loss of Cks85A or Skp2 results in a high frequency of apoptosis. Genomic instability often leads to the activation of checkpoint pathways that trigger apoptosis (Storchova and Pellman, 2004). Further study will be necessary to determine if apoptosis in Cks85A and Skp2 is a result of such a checkpoint dependent response to aneuploidy.

A role for Skp2 and Cks85A in maintaining diploidy appears to be conserved from Drosophila to vertebrates, but how they perform this function may not be conserved. There is, in fact, little consensus on how Cks1 and Skp2 maintain diploidy in mammalian cells. Cks1 has been implicated in a redundant role with Cks2 in promoting Cdk1 and cyclin transcription and entry into mitosis (Martinsson-Ahlzen et al., 2008). Knockdown of both Cks1 and Cks2 results in failure to enter mitosis and consequent re-replication. By a completely different mechanism, Skp2 (and by inference, Cks1) are implicated in promoting mitotic entry by regulating p27 levels (Nakayama et al., 2004). Neither model fits well with what we know so far about Drosophila Cks85A and Skp2. Contrary to predictions of both models, we do not observe a failure to enter mitosis in Skp2 or Cks85A null cells (Fig. 2). With respect to the latter model, p27 levels are not elevated upon Cks85A or Skp2 knockout in Drosophila. Furthermore, p27/Dap only binds and inhibits Cdk2, not the mitotic Cdk1 (Lane et al., 1996), and it is therefore difficult to imagine how excess p27 could disrupt mitosis in Drosophila.

Skp2, but as yet not Cks1, has been implicated in a completely different pathway to maintain diploidy — preventing re-replication by promoting Cdt1 degradation (Li et al., 2003). Again our results do not support a similar model for Drosophila Skp2 (or Cks85A), as Cdt1/Dup levels are not elevated in either Skp2 or Cks85A null cells (Fig. 7). The control of re-replication in different cell types has been found to vary greatly and to involve multiple mechanisms (Arias and Walter, 2007). It is therefore possible that Drosophila Cks85A and Skp2 are indeed involved in preventing re-replication but that they target pre-replication factors other than Cdt1.
Cks85A as a co-receptor in the SCF<sup>Skp2</sup> complex

Regardless of how Cks85A and Skp2 maintain diploidy, our findings strongly support a model in which they function together in this capacity. Cks85A and Skp2 null mutants are similar, with Skp2 having the more severe phenotypes. This and the fact that double mutants appear similar to Skp2 alone (Fig. 3), argue that Cks85A has little additional function outside its role with Skp2. This possibility is more dramatically illustrated by the finding that the lethality and polyploidy of Cks85A null mutants can be at least partially overcome by over-expressing Skp2 (Fig. 3). Therefore, Cks85A has little or no essential functions outside of the SCF<sup>Skp2</sup> complex.

It is also interesting that polyploidy resulting from loss of Cks85A is relatively low at 18 °C but is similar to that of Skp2 null mutants at higher (Fig. 4). This may simply reflect a need for stronger interactions between substrate and SCF complex at higher temperatures, and if so it implies that Cks85A functions essentially to increase the efficiency of SCF<sup>Skp2</sup>-substrate interactions. This may be analogous to the role of Cks2 in mitosis (Patra and Dunphy, 1998), where it is proposed to link its associated Cdk to specific substrates, thereby increasing the efficiency of substrate phosphorylation.

While SCF<sup>Skp2</sup> has numerous putative in vivo substrates, little is known about the role of Cks1, if any, in the recognition of these. The exception is p27, for which Cks1 appears to be absolutely essential (Ganoth et al., 2001, Hao et al., 2005 and Spruck et al., 2001). Our results argue that in Drosophila and perhaps in other organisms, Cks85A plays a more general role in the recognition of substrates by SCF<sup>Skp2</sup>, and that the absolute requirement for mammalian Cks1 in p27 recognition may be the exception. To date we do not have a clear idea of what these targets are in Drosophila and it will clearly be of great interest to identify these and to determine if these are conserved.

Distinct roles for Cks85A-SCF<sup>Skp2</sup> in promoting and preventing DNA replication

The endoreplication cycle, in which cells undergo repeated rounds of DNA replication without intervening mitosis represents a unique variation on the normal mitotic cell cycle and an excellent system for examining growth control and S-phase control in the absence of mitosis. We found that Cks85A and Skp2 are necessary for growth in endoreplicating tissues and that this requirement appears to reflect a role in the
endoreplication cycle (Fig. 1). It seems contradictory that Cks85A and Skp2 are necessary for achieving polyploidy in endoreplicating cells while they are necessary for preventing polyploidy in diploid cells. This could reflect two distinct roles for Cks85A and Skp2. Cks85A and Skp2 interact with both Cdk1 and Cdk2 (Fig. 6) and it is possible that interaction with each Cdk confers different functions on the SCF$^{Skp2}$ complex. In mitotic cells, Cks85A and Skp2 may interact with the mitotic Cdk1 to prevent DNA re-replication. Loss of Cdk1 and cyclin A in Drosophila results in apparent polyploidy (Hayashi, 1996, Sigrist and Lehner, 1997 and Weigmann et al., 1997) and it is intriguing to speculate that this reflects a functional interaction with SCF$^{Skp2}$. In endoreplicating tissues, where Cdk1 is not expressed (Zielke et al., 2008), Cks85A and Skp2 may instead interact with Cdk2 to promote S phase. We note that Cks85A and Skp2 may be necessary for growth in mitotic tissues (Fig. 2) and this may also reflect a Cdk2-dependent role in S phase of the canonical cell cycle. Cks85A and Skp2 may therefore interact with Cdk2 to promote DNA replication in S-phase, and interact with Cdk1 following S phase to prevent DNA re-replication. It is not yet clear how Cdk association with SCF$^{Skp2}$ might contribute to its function. SCF$^{Skp2}$ specifically targets phospho-proteins (Nakayama and Nakayama, 2006) and therefore, association with Cdk2 may allow efficient coordination between the phosphorylation and ubiquitination of substrates. Alternatively, Cdk2 could play a non-catalytic, recruitment role. It has been proposed that Cdk2 helps to recruit associated p27 to the SCF$^{Skp2}$ (Xu et al., 2007) while Cdk1 may recruit cyclin A to the APC for ubiquitination (Wolthuis et al., 2008).

**Drosophila Cks proteins have distinct functions**

The Cks proteins are all closely related at the sequence level and share common Cdk-interacting and phospho-epitope binding domains. Cks genes from humans can replace their budding yeast counterpart (Richardson et al., 1990), illustrating the remarkable functional conservation of these proteins. In metazoans, in which there are two Cks genes, there is also evidence of functional conservation between the two homologues: in mice, ectopic expression of Cks1 can functionally rescue meiotic defects resulting from the loss of Cks2 (Donovan and Reed, 2003), while mammalian Cks1 and Cks2 appear to function redundantly to promote mitosis (Martinsson-Ahlzen et al., 2008).
In contrast, the two Drosophila Cks proteins appear to have distinct and non-overlapping functions. Cks30A is required for spindle assembly and anaphase progression in female meiosis but has no essential function in mitotic cells (Pearson et al., 2005 and Swan et al., 2005). Cks30A appears to be the functional equivalent of Xenopus Xe-p9, mouse Cks2 and C. elegans Cks1 — all of which have specific requirements in meiosis (Patra and Dunphy, 1996; Polinko and Strome, 2000; and Spruck et al., 2003). We previously showed that Cks85A cannot functionally replace Cks30A in promoting APC activity in female meiosis (Swan et al., 2005) and we now show that Cks30A cannot functionally replace Cks85A in the SCF$^{Skp2}$. In fact Cks30A appears to act antagonistically to Cks85A (Fig. 5). The failure of Cks30A to functionally replace Cks85A could easily be attributed to its inability to interact with Skp2 (Fig. 6). The apparent antagonistic behavior is more difficult to explain. Cks30A promotes the APC-dependent destruction of mitotic cyclins (Swan and Schupbach, 2007). Therefore we speculate that Cks30A antagonizes SCF$^{Skp2}$ function through its effect on associated cyclins. Again, this model hinges on the assumption that cyclin-Cdk association with SCF$^{Skp2}$ is important for its in vivo activity, a possibility that clearly needs to be tested experimentally.

In conclusion we find that the two closely related Cks genes in Drosophila are functionally distinct and that Cks85A acts mainly or perhaps exclusively as a part of the SCF$^{Skp2}$ complex. We demonstrate that SCF$^{Skp2}$ has essential roles in the maintenance of diploidy and in promoting growth. It is therefore possible that the human orthologues, Cks1 and Skp2 possess both tumor promoting and tumor suppressing functions.

**Acknowledgments**

We are grateful to Trudi Schüpbach for support in the early stages and for helpful comments throughout the course of this work. We thank Gail Barcelo for help with Skp2 cloning and Anuradha Mukhopadhyay for help with Edu labeling experiments. We also thank members of the Swan lab for helpful discussions. We thank Christian Lehner, Terry Orr-Weaver, Michael Botchan, and Terrence Murphy for generous gifts of Drosophila stocks and antibodies. Other antibodies were obtained from the Developmental Studies Hybridoma Bank. Other fly stocks were obtained from the Bloomington stock center, and
the Vienna Drosophila Resource Center. This research was funded by grants to AS from
the Cancer Research Society (grant 2008–13159) and the Canadian Cancer Society.
REFERENCES


Saccharomyces cerevisiae and Schizosaccharomyces pombe. Genes & Development 4, 1332-1344.


CHAPTER 3
G2/M Role of SCF$^{Skp2}$
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 potent proto-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; Li et al., 2004; Lim et al., 2002; Masuda et al., 2002; Nelsen et al., 2001; Shim et al., 2003; Sorbye et al., 2012; Wang et al., 2012a; Yang et al., 2014; Yokoi et al., 2004). The best characterized target of SCF<sup>Skp2</sup> is the Cyclin dependent kinase (Cdk) inhibitor, p27/Kip1 (Carrano et al., 1999; 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 (Chiarle et al., 2002; Fagan-Solis et al., 2014; Lim et al., 2002). Skp2 functions in tandem with Cdk2, which first phosphorylates p27 at T187, allowing p27 to be recognized by SCF<sup>Skp2</sup> (Tsvetkov et al., 1999). p27 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 in 2000 observed that Skp2 knockout mice are polyploid and have reduced growth indicating a possible tumour supressive 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 levels: Skp2, p27 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). Studies in flies also showed that p27 (Dacapo/Dap) is an important substrate of Skp2 (Dui et al., 2013). According to Nakayama et al. 2004, Skp2 mediated degradation of p27 is required in G2-M to activate Cdk1 (Nakayama et al., 2004). In Drosophila, overexpression of Dap has been shown to cause G1 arrest by inhibiting Cdk2/Cyclin E activity (de Nooij et al.,
Dap has not been shown to bind or inhibit Cdk1 function in flies and the role of 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 tumor suppressive role of Skp2 is essential before any clinical use of anti-Skp2 therapeutics is considered for clinical use.

The small Cdk-associated protein, Cks1 associates with Skp2 to form part of the p27 binding interface of the SCF$^{Skp2}$ complex. Cks1 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 (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 (Hao et al., 2005), but their importance for SCF$^{Skp2}$ function is not clear.

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 mediated inhibition (Ji et al., 2006). In Drosophila, Cyclin A is a mitotic cyclin and appears to only interact 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 thought to interact with mitotic cyclins and have a mitotic role apart from inhibition of p27 (Nakayama et al., 2004).

Here we show that Skp2 has a critical role in the entry into mitosis. We find that Skp2 physically associates with Cyclin A to protect it 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 results in reversion to a G1 state, leading to polyploidy. Therefore Skp2 with Cyclin A plays an important role in G2/M transition in addition to its established role at the G1/S transition.
MATERIALS AND METHODS

*Drosophila* strains and genetics

All flies were maintained at 25°C. The following stocks were obtained from *Bloomington Drosophila Stock Centre:* Cyclin A1170 (9096), Cyclin B2 (6630). The following stock was obtained from *Vienna Drosophila Resource Centre:* Cyclin A442 (Cyclin A10595). The following stock was obtained from *National Institute of Genetics (NIG-FLY):* Cyclin A5940R (Cyclin A10201). Cyclin B2L540 is a gift from Christian Lehner, University of Zurich. Fzred is a gift from Tadmiri Venkatesh, The City College of New York. UAS-HA-Skp2, Skp2ex9, yw, Cks85Aex15, Skp2GD15636, Skp2KK2101487 were described previously (Ghorbani et al., 2011) The following stocks were made in the Swan lab. UAS-Venus-Cyclin A21c, UAS-Venus-Cyclin B31a (Dhaliwal, unpublished), UAS-Venus-CyclinB3 (Swan, unpublished).

**Generation of UASp-HA-Δ170Skp2 transgenic line**

UASp-HA-Δ170Skp2 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’-AAAGCAGGCTTAACGCATGGGCTACCGTTCGTACG - 3’ and the Skp2 specific reverse primer used is 5’-GAAAGCTGTTTATTAGTCCCTGCGCGTGACGACCCCA – 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’ – ACAAGTTGTACAAAAAAGCAGGCT – 3’ and the full attB2 sequence is 5’-ACCCAGCTTTCTTGTACAAAGTGTTG – 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 10s, annealing at 64.3°C for 30 seconds, and elongation at 72°C for 25s, 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 10s, annealing at 60°C for 30 seconds, and elongation at 72°C for
55s, 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-Δ170Skp2 transgene. Several different transgenic lines were obtained. UASp-HA-Δ170Skp2 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- delta N terminal Skp2 plasmid.

**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. For the generation of MBP-His-Cyclin A plasmid, Cyclin A cDNA (LD44443) was used.

**GST-His-Skp2, GST-His-delta N Skp2 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-delta N terminal Skp2 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 for 10 times and then centrifuged at 4°C, 10000 x g for 15 minutes. 1ml aliquots of supernatant were collected in cold room and saved in -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 OD\textsubscript{600} reached 0.5. The induced culture was transferred to a 25°C shaker for 5 hrs instead of 28°C for 4 hrs.

**GST pull-downs**

For GST pull-downs 500\mu l 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 100\mu l 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) 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.

**Co-Immunoprecipitations (CoIPs) from Larvae and Embryos**

HA-Skp2 or HA-Δ170Skp2 was crossed with da-Gal4 to express the protein ubiquitously. 100 brains and imaginal discs of 3\textsuperscript{rd} 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, Barcelo, & Schüpbach, 2005). The CoIP of embryos were performed as in (Ghorbani et al., 2011).
Western Blotting

Brains and imaginal discs of 3rd instar wandering larva were dissected in 1xPBS and flash frozen in liquid nitrogen. The tissues were lysed 20 µl with 2x sample buffer, boiled for 10 minutes and then centrifuged at 13,200 rpm for 10 minutes in 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 the these antibodies 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 in Alpha Innotech FluorChemTM HD2 imager.

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. PLA detects protein–protein interaction. Briefly, 3rd instar larval wing imaginal discs were incubated with rabbit anti-GFP antibodies (Abcam) to detect Venus-Cyclin A and mouse anti-HA antibodies (Abcam) 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.

Drosophila FUCCI system

Fucci (Fluorescent ubiquitination-based cell cycle indicator) system was developed by Zielke and his colleagues to study cell cycle profile in vivo (Zielke et al., 84
Briefly, the system consists of GFP tagged E2F(1-230) and mRFP tagged Cyclin B (1-266). To study the cell cycle profile, these two tagged transgenes are expressed in the cells of interest. From late M phase to end of G1 phase E3 ubiquitin ligase APC/C is active therefore, mRFP-cyclin B will be degraded and therefore, the G1 cells will appear green due to accumulation of GFP-E2F. During S phase ubiquitin ligase CRL4\textsuperscript{Cdt2} is active which degrades GFF-E2F. Therefore, cells appear red due to accumulation of mRFP-cyclin B. Since APC/C and CRL4\textsuperscript{Cdt2} are absent from G2 to late M phase, the cells appear yellow due to accumulation of both the transgenes.

**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 1xPBST 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 Biotechnology) at 1/1000, rat anti-alpha tubulin antibody (Milipore) at 1/500, mouse anti-gamma tubulin antibody (Fenger et al.) 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. The 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).
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.

Image J analysis of M phase and S phase indexes

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-infinity 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 100 pixel area is calculated. The measurements of particles were plotted on Windows 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. 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.

RESULTS

Skp2 is required for entry into mitosis

Loss of Skp2 results in polyploidy in a number of experimental systems (Nakayama et al., 2000); (Dui et al., 2013); (Ghorbani et al., 2011) 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 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. While other S-phase cells show expression of GFP-PCNA, a marker of DNA replication, 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 is overexpressed, cells posterior to the furrow continue to incorporate EDU, suggesting that they are re-replicating in G2. 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).
**Fig. 1: Skp2 is required for entry into mitosis.** A-B) GFP-PCNA was expressed as S-phase marker in wild type wing imaginal discs or imaginal discs in which Skp2 was knocked down and probed with anti-Achaete antibody (red) to mark cells arrested in G2-phase of the cell cycle. Co-labeling of GFP-PCNA and and Achaete antibody is not seen either in wild type discs or discs in which Skp2 is knocked down. C) Eye imaginal discs of 3rd instar wandering larvae were 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. We do not see an increase in the band of S-phase cells in the dorsal compartment (Skp2 knockdown) compared to the ventral compartment (wild type). 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. Wild type FISH dots appear smaller and less intense than in Skp2.) 1E-H) Representative wild type (E), Cyclin A<sup>H170+/–</sup> (F) Skp2<sup>ex9</sup> (G) and Skp2<sup>ex9</sup>, Cyclin A<sup>H170+/–</sup> (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<sup>ex9</sup> (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 indexes of above mentioned genotypes. Scale bar in E =20 µm, applies to F-H, J-K. Scale bar in M =10 µm, applies to N-O”.
This indicates that polyploid \textit{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 \textit{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 \textit{Skp2} imaginal discs is lower compared to controls (Figs. 1E,G,I) suggesting that \textit{Skp2} null cells are specifically delayed in the entry into mitosis. The frequency of S phase also appears to be decreased in \textit{Skp2} null cells compared to controls (Figs. 1J-L). This might be due to the up-regulation of Dap in \textit{Skp2} null cells (Dui et al., 2013) since upregulation of Dap has been shown to arrest cells in G1 (Dui et al., 2013; de Nooij et al., 1996; Zielke et al., 2014).

We also employed the \textit{Drosophila} FUCCI system (Zielke et al., 2014) to distinguish cell cycle phases \textit{in vivo} (Figs. 1M-P). This reveals that the G1 population is increased in \textit{Skp2} null cells (Fig. 1P). The G2/M population in \textit{Skp2} null cells is similar to that of wild type cells (Fig. 1P). If we consider that fewer cells go into mitosis in \textit{Skp2} null, we can conclude that the G2 population is at least somewhat greater in the \textit{Skp2} mutant. We conclude that \textit{Skp2} mutants are delayed in G2 and may bypass mitosis.

**\textit{Skp2} is required to maintain mitotic Cyclin levels**

The combined effects of mitotic Cyclin accumulation and activation of Cdk-Cyclin complexes determine mitotic entry. To determine how \textit{Skp2} could affect mitotic entry we first examined the levels of mitotic cyclins in \textit{Skp2} null cells. We found that loss of \textit{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 \textit{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 \textit{Skp2} mutants. As mentioned before, loss of \textit{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
Fig. 2: **Skp2 is required for maintaining mitotic Cyclin levels.** A-B) Mitotic cyclin levels are low in Skp2

*ex9*. 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. Actin is used as loading control. G-I) DNA stained with oligreen in 3rd instar wing imaginal discs from the genotypes shown. Nuclei appear larger in Cyclin A

*R1* (H) and Cyclin A

*95* (I) indicative of polyploidy. J-L) Representative wings from wild type (J), *rn-Gal4; Cyclin A

*R1* (K), *rn-Gal4; Cyclin A

*95* (L). Wing hair spacing is increased in both Cyclin A knockdown lines compared to wild type. M-Q) Adult wings in which *en-Gal4* is used to drive RNAi expression. N) Skp2RNAi shows increased wing hair spacing, indicative of polyploidy. O) Cyclin A

*H170+/-* in Skp2RNAi background does not enhance the wing hair spacing phenotype but the wings are reduced in size and have other abnormalities similar to what we observe in Cyclin A RNAi

*95* (I). Heterozygosity for Cyclin B

*2* in a Skp2RNAi background has no effect on wing hair spacing (P) whereas Cyclin B

*L6* is shown to partially enhance the phenotype (Q). R-U) DNA stained with Oligreen in 3rd instar wing imaginal discs from the genotypes shown. Nuclei appear larger in Skp2

*ex9* (S) indicative of polyploidy. This phenotype is enhanced by Cyclin A

*H170+* (U) but not Cyclin B

*2+/* (T). V-X) Wing hair spacing observed in Skp2 knockdown phenotype is suppressed by Cyclin A overexpression (V) but not by Cyclin B overexpression (W). Cyclin B3 also rescues the phenotype partially (X). Scale bar in G =10 µm and it applies to G-I, R-U.
RNAi lines against *Cyclin A* (*Cyclin A<sup>95</sup> and *Cyclin A<sup>R1</sup>*) 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) consistent with increased ploidy. 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<sup>95</sup>* (Figs. 2F,I,L) results in a greater reduction in protein levels and a more severe wing phenotype compared to *Cyclin A<sup>R1</sup>* (Figs. 2F,H,K). In addition to the increase in wing hair spacing in *Cyclin A<sup>95</sup>* wings show a more 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<sup>R1</sup>*, 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 cyclin, Cyclin B) 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* or *Cyclin B* null mutants (Figs. 2M-Q). While reduction of *Cyclin B* dose has no effect on the *Skp2 RNAi* phenotype (Fig. 2P), the loss of one copy of *Cyclin A* leads to an increase in wing hair spacing (Fig. 2O). In addition, wing hairs appear less evenly spaced and the overall size of the posterior wing is reduced similar to the phenotype of the strong Cyclin A knockdown.

To determine if these phenotypes are related to increased ploidy we generated flies homozygous mutant for *Skp2<sup>ex9</sup>* and heterozygous for either *Cyclin B* or *Cyclin A* null alleles and 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-GFP tagged Cyclin A in the Skp2RNAi background. This leads to a clear rescue of the increased wing hair spacing phenotype (Fig. 2V). In contrast, overexpression of wild type GFP-tagged Cyclin B has no effect on the Skp2RNAi wing phenotype (Fig. 2W). Together, these results suggest that the polyploidy resulting from loss of Skp2 is at least in part due to reduced Cyclin A levels. Therefore Skp2 appears to be required to maintain Cyclin A levels and thereby prevent polyploidy.

**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 (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). Interestingly, the weaker knockdown of Cyclin A more closely resembles the phenotype seen in Skp2 mutants: Cyclin A\(_{R1}\) cells show a partial reduction in mitotic index (Figs. 3B-D). These findings suggest that Skp2 loss is similar to a partial loss of Cyclin A.

To further test this idea we examined flies null for Skp2 and heterozygous for Cyclin A (Skp2\(_{ex9}\), Cyclin A\(_{H170+/-}\)). 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. 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 alone, and unlike Skp2\(_{ex9}\) alone, a reduced frequency of G2 cells, and reduced S-phase index (Figs. 1M-P).
Fig. 3: The mitotic phenotype of $Skp2^{ex9}$ resembles $Cyclin ARNAi$. A-D) Wing blade region of the imaginal discs from 3$^{rd}$ instar wandering larva stained with phospho-Histone H3 to identify the mitotic cells. Representative pictures of the wild type (A), $Cyclin A^{R1}$ (B), and $Cyclin A^{95}$ (C). D) M-phase index of above genotypes.
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.

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 (Fig. 4A-B). We note that Skp2 interacts specifically with the higher molecular weight form of Cyclin A, the form that predominates in mitosis and therefore presumably the active form. 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. 4C). 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. 4D).

We are currently using CoIPs to test for the existence of Cyclin A-Cdk2 complexes 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 directly with Cyclin A.** A) HA-Skp2 IP in wild type or *Cks85A* null (indicated by a + or -). IP was performed with 3rd instar brains and imaginal discs. Cyclin A but not Cdk1 is pulled down (S, Supernatant; P, IP pellet). B-C) HA-Skp2 IP in wild type 3rd instar brains/imaginal discs. B) Cyclin B3 but not Cyclin B is pulled down. C) The lower molecular weight band of Cdk2 is pulled down. D) HA-Skp2 IP in wild type embryos. Cdk1 but not Cyclin A is pulled down. E-F) Coomassie staining of bacterially produced His-MBP-Cyclin A (E) and GST-His-Skp2 (F) before and after induction with IPTG. G) 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-Δ170Skp2 (first panel). Indicated GST fusion proteins labelled with anti GST antibody (third panel). H) HA IP with above mentioned genotypes in wild type 3rd instar brains/imaginal discs. Cyclin A is pulled down with HA-Skp2 but not with HA-Δ170 Skp2. * indicates antibody light chain, ** indicates antibody heavy chain.
**Skp2-Cyclin A interaction is independent of Cks85A**

Skp2 could potentially interact with Cyclin A indirectly through Cks85A since Cks85A interacts with Skp2 and with Cdk5. To determine if this is the case, we repeated the HA-Skp2 IP in a background homozygous for Cks85\textsuperscript{ex15}, a null allele of Cks85A. We find that Cyclin A still interacts with Skp2 in the Cks85\textsuperscript{ex15} background (Fig. 4A) indicating that the interaction is not dependent on Cks85A. Skp2 in humans 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\textsuperscript{Δ170} that retains the N-terminal F-box and LRR repeats required for SCF interaction and substrate recognition respectively. CoIPs with HA-Skp2\textsuperscript{Δ170} reveals that it is not able to interact with Cyclin A (Fig. 4H).

To determine if Skp2 and Cyclin A directly interact we expressed these proteins in bacteria (Figs. 4E-F) and performed a GST pull-down assay. GST-Skp2 but not GST-Skp2\textsuperscript{Δ170} is able to pull down MBP-Cyclin A *in vitro* (Fig. 4G). Therefore, *Drosophila* Skp2 interacts directly with Cyclin A, and this interaction depends on sequences in the N-terminus of Skp2.

**Skp2 and CyclinA-Cdk2 interact in G2**

We predict that Skp2 interacts with Cyclin A in G2 of the cell cycle and that this is important either for Cdk2-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/C\textsuperscript{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. 5A-A’’). To determine if skp2 also shows the same temporal accumulation as Cyclin A we co-labeled imaginal discs for HA-Skp2, phospho Histone H3 and gamma tubulin to determine mitotic phases. Skp2 seems to slowly disappear in prometaphase/metaphase (Figs. 5B-D’). 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. 5E-E’). 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. 5F). This signal is found in both nuclei and cytoplasm. Interestingly, PLA signal is not detected in most cells but it is enriched in a stripe of cells in the wing disc that are G2 arrested (Fig. 5D). We will confirm the specificity of this interaction by performing the PLA with HA-Skp2Δ170. We expect that this truncated form of Skp2 will not interact in the PLA assay, since it does not interact with Cyclin A by coIP or GST pull-down (Figs. 4G-H).
Fig. 5: Skp2 and Cyclin A interact in G2. A-A’’ ) *rn-Gal4* driven expression of UAS-HA-Skp2 in the wing imaginal blade of 3rd instar wandering larvae showing nuclear and cytoplasmic expression of Skp2. B-D’ ) Localization of Skp2 in different phases of mitosis. The yellow arrowheads in (B-B’) show reduced Skp2 protein in metaphase and the white arrowheads in (B-B’) shows almost no Skp2 protein in anaphase. In C-C’ ) yellow arrowhead shows Skp2 protein in prophase. The white arrowhead in (D-D’) show no Skp2 protein in telophase. E-E’ ) Skp2 and Cyclin A protein show similar nuclear and cytoplasmic colocalization. F ) Skp2 and Cyclin A proteins interact in G2 as shown by the proximity ligation assay (PLA). The PLA signals are concentrated in the G2 arrested region showing that Skp2 and Cyclin A interact in G2. G ) Cartoon showing the region of G2 arrested cells in the wing imaginal disc. Scale bar in C =5 µm, applies to B-D’, F. Scale bar in E’ =10 µm, applies to A-A’’, E- E’’.
Skp2 and Fzr may compete for binding to Cyclin A

Our results to date suggest that Skp2 is important for maintaining Cyclin A levels and that the reduced Cyclin A levels lead cells to enter an endoreplication cycle in which M-phase is bypassed. Several studies have pointed to a critical antagonism between Cyclin A and APC/C\(^{Fzr}\). APC/C\(^{Fzr}\) is active from late mitosis and throughout G1 of the cell cycle and promotes the G1 state by targeting the mitotic Cyclins for destruction. Overexpression of Fzr results in polyploidy in Drosophila, presumably due to its effect on mitotic Cyclin levels (and on Cdc25/string) (Reber et al., 2006); (Sigrist and Lehner, 1997); (Hassel et al., 2014). Meanwhile Cyclin A, by an unknown means inhibits APC/C\(^{Fzr}\) activity (Hassel et al., 2014). Consistent with this antagonistic relationship between Cyclin A and Fzr, we find that partial loss of function mutations in fzr (fzre4) can suppress the wing hair spacing phenotype in Cyclin A RNAi wings (Figs. 6A, B). We have proposed that Skp2 interacts with Cyclin A to promote mitosis, and therefore Skp2 should also show antagonism with Fzr. Indeed, fzre4 suppresses the increased wing hair spacing phenotype of Skp2RNAi (Figs. 6C,D).

What could be the molecular basis for the antagonistic relationship between Skp2 and Fzr? We are currently testing the possibility that Skp2 binding to Cyclin A protects Cyclin A from APC/C\(^{Fzr}\) mediated destruction. With this model we predict that Skp2 mutants that lack the ability to interact with Cyclin A would be non-functional. We tested this by expressing HA-Skp2\(^{A170}\) 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.
Fig. 6: Skp2 and Cyclin A are antagonistic to Fzr. A-D) en-Gal4 expression of Cyclin $A^R1$ (A) or Skp2 alone (C) or in a Fzr$^{ed/ed}$ 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 Fzr hypomorph. Skp2 knockdown results in increased wing hair spacing. This is completely rescued by Fzr hypomorph.
DISCUSSION

Skp2 and its partner, Cks1 (Cks85A in Drosophila) have a well-characterized function in targeting the CKI, p27 (Carrano et al., 1999; Sutterlüty et al., 1999; Tsvetkov et al., 1999). This critical function explains to a large degree the oncogenic properties of Skp2: its overexpression leads to a decrease in p27 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 levels and thus inhibition of cell division. The Drosophila Skp2 and Cks85A proteins appear to share with vertebrates this important role in regulating p27 levels (Dui et al., 2013). In addition to this oncogenic activity, Skp2 may have tumour suppressive functions. Mice and Drosophila null for Skp2 display 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 investigate the cause of polyploidy. We present evidence that polyploidy in Skp2 null Drosophila arises as a consequence of a failure of cells to enter mitosis. Cells that fail to enter mitosis revert to a G1-like state and thus are able to undergo replication at the next S-phase (Davoli and de Lange, 2011; Hassel et al., 2014).

This G2 role for Skp2 appears to be distinct from its activity in G1/S. Loss of Skp2 results in both a decrease in S-phase and mitotic indexes. The former, we expected based on the established role for Skp2 in p27/Dacapo destruction (Carrano et al., 1999; Sutterlüty et al., 1999; Tsvetkov et al., 1999; Dui et al., 2013). The latter, we propose 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. We found that Skp2 interacts physically with Cyclin A, which in Drosophila functions primarily if not exclusively in mitosis (Sigrist et al., 1995; Sigrist and Lehner, 1997). 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. We propose that Skp2
binding to Cyclin A during G2 protects this Cyclin from destruction by APC/C-Fzr. This is critical for a switch from APC/C-Fzr active to inactive state. In Drosophila, similar to vertebrates, Cyclin A (and Cyclin E) are able to inhibit APC/C-Fzr activity (Reber et al., 2006; Lukas et al., 1999; Sørensen et al., 2001; Knoblich et al., 1994). In yeast and mammals this occurs by phosphorylation but the exact mechanism in flies has not been established (Reber et al., 2006; Kramer et al., 2000; Zachariae and Nasmyth, 1999); (Lukas et al., 1999; Sørensen et al., 2001). Nonetheless, by protecting Cyclin A in G2, Skp2 establishes the conditions in which Cyclin A can then inhibit APC/C-Fzr activity. This model explains why Skp2 loss results in not only a decrease in Cyclin A levels but also a decrease in Cyclins B and B3 – two other targets of APC/C-Fzr.

How could Skp2 association with Cyclin A serve to protect Cyclin A from APC/C-Fzr? Cyclin destruction depends on specific motifs, the destruction box (D-box) and KEN box motifs (Jacobs et al., 2001). In most Cyclins these motifs are necessary and sufficient for APC/C-mediated destruction (den Elzen and Pines, 2001; Glotzer et al., 1991; Jacobs et al., 2001). Drosophila Cyclin A has 2 of each of these motifs though it appears that its destruction is more complex as simply deleting these putative KEN and D-box sequences is not sufficient to completely stabilize Cyclin A (Jacobs et al., 2001). Nonetheless, deletion of the first 53 amino acids of Cyclin A renders it stable (Jacobs et al., 2001). We predict that Skp2 association with Cyclin A through the Cyclin A N-terminus such that when Skp2 is bound, APC/C-Fzr cannot bind.

The model that we are proposing is similar in some respects to one that has been proposed for Skp2 in S-phase, based on in vitro studies of the mammalian Skp2 and Cyclin A. It was found that Skp2 association with Cyclin A prevents p27 binding to Cyclin A (Ji et al., 2006). It was proposed that by competing with p27 for Cyclin A binding, Skp2 can help to promote CyclinA-Cdk2 activity at the G1/S transition (Ji et al., 2006). It is theoretically possible that the Skp2-Cyclin A interaction in Drosophila serves exactly the same purpose – to protect Cyclin A from p27. It was found that the overexpression of Drosophila p27/Dap leads to an increase in spacing between adult wing hairs, a phenotype that is also seen upon loss of Skp2, and that may be due to polyploidy (Dui et al., 2013). Therefore it is possible that the Cyclin A-Skp2 interaction serves primarily to protect Cyclin A from p27. We will have to test this possibility, but in the
meantime several observations suggest that this is not likely. First, p27 in *Drosophila* has so far only been identified in complexes with Cdk2-Cyclin E, and not in complexes with Cyclin A (de Nooij et al., 1996; Lane et al., 1996) Second, *Drosophila* p27 has not been implicated in mitosis in *Drosophila* (de Nooij et al., 1996; Lane et al., 1996; Zielke et al., 2014). Third, we found that, while loss of a single copy of Cyclin A dramatically enhances the polyploidy phenotype in *Skp2* mutants, it has no effect on *p27* mutants (Nila Das, unpublished).

While *p27* does not genetically interact with *Skp2*, *fzr* does. We showed that a weak allele of *fzr* that has no wing phenotype on its own suppresses the wing hair spacing phenotype in both *Cyclin A* and *Skp2* mutants. Obviously genetic interactions can be open to interpretation, so it will be necessary to directly test our model. This can be done by performing direct competition assays using purified Skp2, Cyclin A and Fzr proteins.

If it turns out that Skp2 interaction with Cyclin A protects Cyclin A from destruction, several questions emerge. We found that Skp2 and Cyclin A have similar timing of destruction – both in early mitosis. Early in mitosis, dependent on rising Cdk activity, the APC/C^{Fzy} complex becomes active. Cyclin A is one of its earliest known targets. It is possible then that Skp2, like Cyclin A, is targeted for destruction by the APC/C^{Fzy} complex. If this is the case, how does APC/C^{Fzy} target these two proteins in mitosis while APC/C^{Fzr} is unable to target them before this point?

An interesting prediction of our model is that Skp2 plays a non-catalytic role in protecting Cyclin A. This is something that can be tested by generating a *Skp2* transgene that is mutated within its LRR domain, and therefore unable to bind to substrates. 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 *Skp2* null mutant.

Another interesting possibility that emerges from our research is the finding that Cyclin A interacts with Skp2 in a complex that does not involve Cdk1. On the other hand we find that Cdk2 is able to interact with Skp2. It is possible that Skp2 interacts specifically with a complex of Cyclin A-Cdk2. While Cyclin A-Cdk2 is a major player in vertebrate cell cycle such a complex has not been seen in *Drosophila* to date. We note that all reported studies of Cyclin-Cdk complexes in *Drosophila* have relied on embryo extracts. It is possible that in embryos Cyclin A associates preferentially with Cdk1 but
that in imaginal tissues it associates also with Cdk2. If this is the case, our results might imply an as yet undetected role for a Cdk2-Cyclin A complex in mitotic entry in *Drosophila*.

While our identification of Cdk2 in a complex with Skp2 may relate to a putative Cyclin A-Cdk2-Skp2 complex, 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.

**Acknowledgements**

We thank Christian Lehner for generous gift of *Drosophila* stocks and antibodies. We also thank Norah Franklin for the generous gift of competent cells for bacterial transformation. This research was funded by grants to AS from Seeds For Hope and Canadian Cancer Society.
REFERENCES


CHAPTER 4
Role of Skp2 in maintaining genomic stability
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., 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 (Carrano et al., 1999; Sutterluty et al., 1999; Tsvetkov et al., 1999). It does this in
cooperation with the small Cdk-interacting protein, Cks1 (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 tumour suppressive functions and loss of Skp2 in diverse organisms from Drosophila to mice, results in tetraploidy and further polyploidy (Ghorbani et al., 2011; Nakayama et al., 2000).

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 (Chapter 3 of this thesis). 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.
MATERIALS AND METHODS

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), Cyclin A\(^{5940R\_1}\) (Cyclin A\(^{R1}\)) , and gst-D1 (gift from Helen McNeill), Cks85A (Ghorbani et al., 2011), Skp2 (Ghorbani et al., 2011), Venus-Cyclin A 21C (R.Dhaliwal, unpublished).

Cytology and immunostaining

Immunostaining and BrdU labeling of wing imaginal discs and brains obtained from wandering 3\(^{rd}\) 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 further 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 44 °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 antibodies such as p-JNK while other antibodies required some minor modifications. 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. For all other antibodies, washing blocking and incubation required 1x PBS+0.2% tween 20. 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 Biotechnolgy), rat anti-alpha Tubulin antibody 1/500 (Milipore), mouse anti-gamma Tubulin antibody 1/500 (Fenger et al.), rabbit anti-GFP 1/10000 (abcam). Rabbit anti-γH2AV 1/500 (gift from Kim McKim), rabbit anti-p-JNK1/500, rabbit anti-Asl 1/500 (gift from Jordan Raff), mouse anti-β-galactosidase 1/500.

**Western Blotting**

Immunoblotting was performed using standard techniques as described in chapter 3. 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), rabbit anti-GFP at 1/10000 (Torrey Pines Biolabs). Densitometry analysis was performed in Alpha Innotech FluorChemTM HD2 imager.

**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.

**S-phase index analysis**

S-phase index analysis was performed as described in chapter 3 of this thesis.
RESULTS

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 destruction. 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, \(\gamma\)-Tubulin and \(\alpha\)-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 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 prometaphase and we note that such cells are particularly common in the Skp2 mutants (Fig. 1G). We also rarely see a normal anaphase in which 2 equal and compact DNA masses are seen separating towards opposite poles. Instead we see chromosome dispersed on the microtubules as the cells proceeds towards anaphase (Fig. 1I). Drosophila Skp2 functions with the Cdk-interacting protein, Cks85A (Ghorbani et al., 2011; Dui et al., 2013). Consistent with a close functional relationship, 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 Cks85A mutants. Representative wing discs for each genotype are shown in (Fig. 1M-O) and the quantification of this analysis is shown in (Fig. 1R). This mitotic profile confirms that Skp2 and Cks85A null mutants have a higher incidence of prometaphase/metaphase cells and lower incidence of anaphase than wild type (Fig. 1 M-O, R).

In addition to increased DNA content in Skp2 mutants, spindles appear larger and centrosomes have a greater diameter (Fig. 1G, H). We probed Skp2 cells for the centriole-specific marker, Asl and saw a similar overall increase in Asl-positive foci (Fig. 2B).
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-J) Cks85A cells in pro-metaphase. They all appear polyploid based on phospho-Histone H3. These polyploid chromosomes appear to lag at the midzone or sometimes to the poles. 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. Csk85A and Skp2 show elevated pro-metaphase and this is suppressed by loss of Mad2. Scale bars in A is =2 µm and applies to all panels. One asterisk indicates $P \leq 0.05$, two $P \leq 0.01$, three $P \leq 0.001$ and four $P \leq 0.0001$. 
Fig. 2: Prometaphase/metaphase delayed cells are polyploid A-C) Wing imaginal discs of 3rd instar wandering larvae were stained to mark DNA using Oligreen (green) and immunostained for centriole specific antibody, Asl, (red). Asl reveals that polyploid cells of Skp2 have bigger /or multiple centrosomes clustered together (two large dots), while wild type cells have smaller single dots. Skp2, Mad2 double mutants have bigger Asl-positive foci than Skp2 mutants alone. Magnified images of centrioles are shown in insets. D) The majority of Skp2 cells delayed in pro-metaphase/metaphase have bigger centrosomes based on the area. Scale bar in A is = 2 µm
While wild type cells have single Asl foci, 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 that 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 (Chapter 3 of this thesis).

We previously found that polyploid cells make up only 30% of the total population in $Cks85A$ (25°C) and $Skp2$ null wing imaginal discs. It is striking, then, that almost all $Cks85A$ 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 from wild type and $Skp2$ and plotted these sizes (Fig. 2D). This shows almost no overlap between wild type and $Skp2$ cells, indicating that essentially all $Skp2$ null that are in mitosis have larger centrosomes than wild type cells in mitosis, 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.

**SAC activation in $Skp2$ cells**

The timing of this apparent delay of polyploid $Skp2$ and $Cks85A$ 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 first 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 prophase 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. 3C). 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. 3E), 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, present in prometaphase and then disappearing at anaphase (Figs. 3F,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. 3H,I).

To confirm that Skp2 and Cks85A null cells undergo a SAC-mediated delay in the cell cycle we generated flies double mutant for Mad2 and either Skp2 or Cks85A. These flies lack a functional SAC and we therefore expect them to no longer show an elevated frequency of pro-metaphase/metaphase. Indeed, Cks85A, Mad2 and Skp2, Mad2 double mutants are rescued with respect to the increased 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 mutant we frequently observe cells in late anaphase or telophase with chromosomes having failed to segregate (Figs. 3J,K,L). We also observe isolated chromosome fragments in these mutants that are undergoing late anaphase or telophase, indicating chromosome breakage (Fig. 3L). The degree of polyploidy is also greater (Figs. 3J,L and Fig. 4H), 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 replication and clustering. It was previously found that some components of the SAC are required for centrosome clustering – in particular BubR1 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. 3M,N). While this result will need to be repeated it suggests the interesting possibility that
**Fig. 3:** 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). In wild type, BubR1 staining can be observed in pro-metaphase (A) and disappears in anaphase (B). *Skp2* cells show a similar pattern (C-D), except, BubR1 staining is stronger in *Skp2*. E) Some *Skp2* cells have their chromosomes stretched to their poles with no BubR1 foci suggesting that they are in anaphase. F-I) SAC protein Mad1 shows a similar pattern of accumulation to that observed in BubR1 staining. 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. J-K) images showing lagging chromosomes in anaphase. J) More lagging is observed in the cell with higher ploidy. 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.
centrosome clustering depends not on SAC activity per se but rather on specific components of the SAC.

**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 Cks85A null mutants undergo a high degree of apoptosis (Ghorbani et al., 2011) (Figs. 4A,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. 4B,D) while Mad2 alone has no effect (Fig. 4C). Therefore SAC arrest protects Skp2 mutant cells from genome instability and resultant activation of a checkpoint pathway that leads to apoptosis.
Fig. 4: *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. In wild type very few cells undergo apoptosis while *Skp2* mutants have a high degree of apoptosis. Cells in *Mad2* nulls undergo apoptosis, but apoptosis dramatically increases in *Skp2, Mad2* double mutants. 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 ploidy seen in *Skp2* alone, *Skp2, p35* and *Skp2, Mad2*. Suppression of apoptosis by Baculoviral protein p35 enhances the ploidy of *Skp2*. Loss of SAC protein *Mad2* further enhances the ploidy of *Skp2*. I-M) Wing imaginal discs of 3rd instar wandering larvae were assayed for apoptosis by probing with anti-cleaved Caspase 3 antibodies. No apoptosis is observed in *p53* null discs. *Skp2, p53* double mutant, partially suppress apoptosis. Apoptosis is not observed in *DChk1* alone but a high level of apoptosis is observed in *Skp2, DChk1* double mutants. Apoptosis in *Skp2, Cyclin A H170*+/− is drastically reduced compared to Skp2 alone. (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. For N, One asterisk indicates P≤ 0.05, two P≤ 0.01, three P≤ 0.001, and four P≤ 0.0001.
Fig. 5: Apoptotic regulators in Skp2 A-I) Cleaved Caspase 3 staining of wing imaginal discs in which rm-Gal4 was used to knock down Skp2 alone or the genes indicated. The dotted lines represent the region were the genes are knocked down. Knockdown of Hid, Reaper or DChk2 alone does not cause any apoptosis. Varying degree of reduction in apoptosis is observed when Hid, Reaper, or DChk2 is knocked down in Skp2RNAi background. Overexpression of dominant negative JNK suppresses apoptosis caused as a result of Skp2 knockdown. (J) Graphical representation of regulation of apoptosis by various genes in Skp2RNAi background. Knockdown of hid, reaper, DChk2 and overexpression of dominant negative form of JNK in Skp2 knockdown background partially rescues the apoptosis. Scale bar =30 µm. One asterisk indicates P ≤ 0.05, two P ≤ 0.01, three P ≤ 0.001, and four P ≤ 0.0001.
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 \textit{Skp2} and in \textit{Skp2, Mad2} cells we probed wing imaginal discs from these mutants for the presence of phosphorylated H2Av Histone (\(\gamma\)H2Av). This variant histone is phosphorylated at sites of chromosome breaks and can thus be used as a marker of DNA breaks in \textit{Drosophila} (Jang et al., 2003; Mehrotra and McKim, 2006). We find that \textit{Skp2} mutant wing imaginal discs show an increase in number of \(\gamma\)H2Av foci, suggesting that cells in this mutant do in fact incur DNA damage, possibly as a result of mitotic defects (Fig. 6B). We then examined \textit{Skp2, Mad2} double mutants and found that the number of \(\gamma\)H2Av foci is much greater than \textit{Skp2} alone (Fig. 6C). These results indicate that \textit{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 \textit{Cks85A} null mutant is suppressed by expression of the Baculoviral p35 protein (Ghorbani et al., 2011). Similarly, almost all apoptosis in the \textit{Skp2} null wing disc is suppressed by expression of p35 (Fig. 4E). We also find that the suppression of apoptosis results in an overall increase in cell ploidy (Fig. 4G), indicating that apoptosis serves to restrict polyploidy in the \textit{Skp2} mutant. Similarly loss of SAC also increases ploidy (Fig. 4H), indicating that this checkpoint also helps to control ploidy in the \textit{Skp2} mutant.
**Fig. 6: 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. Basal level of H2av phosphorylation can be detected in 3rd instar wandering wild type larval wing discs. Skp2 null wing discs show elevated levels of γH2av foci. Skp2, Mad2 double mutants have more γH2av foci than Skp2 alone. Skp2, Cyclin A<sup>H170/+</sup> wing discs show γH2av foci similar to wild type. 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<sup>R1</sup>* or *Cyclin A<sup>95</sup>* lines. γH2av foci in *Cyclin A<sup>95</sup>* wing discs look like wild type while *Cyclin A<sup>R1</sup>* has a subtle increase in the number of foci. 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<sup>R1</sup>* or *Cyclin A<sup>95</sup>* RNAi lines. Apoptosis is seen in *Cyclin A<sup>R1</sup>* while *Cyclin A<sup>95</sup>* looks like wild type. 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. For I, **** P≤ 0.0001
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. 5C,E,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 (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. 4J,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. 4N).

**DChk1 and DChk2 function in distinct checkpoints in polyploid Skp2 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 show a reduction in apoptosis similar to that seen in the p53, Skp2 double mutant (Fig. 3J,N and Fig. 4I,J), suggesting 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. 4L,N). Therefore, as with Mad2, Chk1 may be required for a cell cycle checkpoint that if compromised, leads 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 Skp2 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. 7: 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 knock down. Wing blade region specific knockdown was done using rnGal4 driver. Wing blade region is marked by dotted lines. E-G) Mitotic frequency using anti-phospho-Histone H3 antibody was determined in Skp2RNAi, DChk1 RNAi and double knock down of Skp2 RNAi, DChk1 RNAi . Mitotic frequency in Skp2 knockdown wing disc is significantly lower than DChk1 knockdown alone. Double knockdown of Skp2 and DChk1 results in drastic reduction of mitotic frequency. H-I) Mitotic progression in the wing imaginal discs of 3rd instar wandering larvae of Skp2RNAi and Skp2RNAi, DChk1 double mutants were 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. Wild type and DChk1RNAi has similar S-phase index while knockdown of Skp2 causes a reduction in S-phase index. The Loss of DChk1 in Skp2RNAi background rescues the reduced S-phase index observed in Skp2 knockdown alone. K) Graphical representation of M-phase index. Skp2RNAi has a lower mitotic index compared to DChk1RNAi alone. Double knockdown of Skp2RNAi and DChk1RNAi has significantly lower mitotic index. L) Graph showing relative frequencies of mitotic phases in Skp2 knockdown and Skp2, DChk1 double knockdown. In Skp2RNAi, DChk1 double mutants, we see an accumulation of cells in pro-metaphase /metaphase, similar to Skp2 alone but at the same time we also see an accumulation of cells in cytokinesis along with a decrease in number of cells in prophase. Scale bar in A is=30 µm and applies to panel (A-F). Scale bar in H is = 2 µm and applies to panel (H-I). In J, K One asterisk indicates P ≤ 0.05, two P ≤ 0.01, three P ≤ 0.001, and four P ≤ 0.0001.
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 phosphor-JNK antibody and found that in the region of Skp2 knockdown, phosphor-JNK levels are elevated (Fig. 8A). JNK pathway activation is also be assessed by looking at transcription of a downstream target Puckered (Puc) (Martin-Blanco et al., 1998). Using the Puc-lacZ reporter we see activation of JNK in cells where Skp2 has been knocked down (Fig 8B). 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 Skp2RNAi. These show a reduction in apoptosis (Fig. 5G,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. 8C). 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. 8: JNK pathway is active in Skp2 null cells.** A-F). Wing imaginal discs of 3rd instar wandering larvae were immunolabeled using anti- phosphor-JNK (p-JNK) antibody to monitor JNK activity, probed with anti- β-galactosidase in wing discs of PucLacZ 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 *rnGal4* (marked by dotted lines). A-B) P-JNK and β-galactosidase staining show that JNK is active in the area of the wing discs where Skp2 is knocked down. C) The high GFP expression in the wing blade region of the wing discs where Skp2 is knocked suggests oxidative stress. D-F) Cyclin A was knocked down in the region marked by dotted line using *Cyclin A<sup>95</sup> RNAi*. D-E) p-JNK and β-galactosidase staining show that JNK is not active in these regions. F) Absence of any GFP signal suggests that cells do not undergo oxidative stress when *Cyclin A* is knocked down. Scale bar in A =30 µm and it applies to all panels.
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 (human ATG8 homolog) 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 autophagic activity (Fig. 9B). Extracts of larval brain and wing disc shows the free GFP that is generated as a result of autophagic degradation of mCherry-GFP-Atg8a. Free GFP is not observed in imaginal disc extract from larvae expressing mCherry-GFP-Atg8a alone but can be observed when mCherry-GFP-Atg8a is expressed in *Skp2* mutant background (Fig. 9C). We further analyzed the rate of autophagic 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. 9D,E). This clearly confirms that *Skp2* cells are undergoing autophagy.
Fig. 9: Skp2 mutants undergo autophagy. A) GFP-LC3 was alone expressed in the wing blade region using *rn-Gal4* represented by dotted line. Overexpression of GFP-LC3 alone does not lead to its accumulation. B) Both GFP-LC3 and *Skp2RNAi* was expressed in the wing blade region using *rn-Gal4* represented by dotted line. Knockdown of Skp2 along with GFP-LC3 overexpression results in the accumulation of GFP-LC3. C) Immunoblot of larval brain and wing disc extracts that was probed with anti-GFP antibody. The arrow shows the free GFP that is generated as a result of autophagic degradation of mCherry-GFP-Atg8a. Free GFP is not observed in imaginal disc extract 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) Very few mCherry–GFP-Atg8A dots are seen in wild type discs. (E) A large number of punctate m-Cherry dots is visible in Skp2 null background while very few GFP dots are seen in these discs. This suggests that GFP has been quenched by the formation of autolysosome, confirming active autophagy in Skp2 null background. (F) A lot of m-Cherry puncta can be observed in Skp2, *Cyclin A* $^{H170/+}$ wing imaginal disc but it is comparatively less than Skp2 nulls. This suggests that these discs undergo less autophagy compared to Skp2 alone. D’-F’) Picture taken at higher magnification of wild type (G), Skp2 (H) and Skp2, *Cyclin A* $^{H170/+}$ Scale bars=30 µm
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 (Chapter 3 of this thesis). Strong knockdown of Cyclin A results in polyploidy but unlike Skp2, these cells rarely enter mitosis, instead they seem to enter an endocycle (Chapter 3 of this thesis). We had found that loss of a single copy of Cyclin A in the Skp2 null background converts these cells to endocycling cells. 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 (Chapter 3 of this thesis).

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 (Chapter 3 of this thesis). When we performed cleaved Caspase staining, we found that these cells do not undergo apoptosis (Fig. 3M,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 $A^{95}$ RNAi line.
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, \( Cyclin^{H170/+} \) cells are protected from apoptosis despite the damage they incur. We examined \( \gamma H2Av \) to determine if indeed these cells incur DNA damage. Strikingly, we saw very low levels of \( \gamma H2Av \) in \( Skp2, Cyclin^{H170/+} \) cells (Fig. 6D). The absence of \( \gamma H2Av \) signal indicates that somehow \( Skp2, Cyclin^{H170/+} \) cells avoid DNA damage and thus avoid induction of apoptosis. Given that the obvious difference between \( Skp2 \) and \( Skp2, Cyclin^{H170/+} \) 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 \( Cyclin \ A^{R1} \) RNAi line results in a weaker polyploid phenotype than the stronger \( Cyclin \ A^{95} \) (Chapter 3 of this thesis). Furthermore, these cells enter mitosis like \( Skp2 \) null cells. To check our hypothesis that aberrant mitosis resulting from polyploidy results in DNA damage and DDR we performed cleaved Caspase assay on these wing discs. Indeed, we find that these wing discs undergo apoptosis (Fig. 6G, I) and they have a slightly elevated \( \gamma H2Av \) foci compared to \( Cyclin \ A^{95} \) knockdown (Fig. 6A,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. 8D,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 \( Skp2, Cyclin^{H170/+} \). The results suggest that this occurs in the endocycling cells but at a lower level (Fig. 9F).
DISCUSSION

There is a clear link between polyploidy and cancer and accumulating evidence suggests that polyploidy 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 are 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., 2011a). 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. We previously showed by FISH against a peri-centromeric region of the X-chromosome, that polyploid Skp2 null cells do not have more chromosomes than wild type. 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 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 find a much greater incidence of 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 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 JNK pathway following IR, but rather some cellular stress that occurs as a consequence of DNA damage or aneuploidy (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; Hoyer-Hansen and Jaattela, 2008). Similarly, *Drosophila* becomes hypersensitive to H$_2$O$_2$ in the absence of important autophagic 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 autophagic 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.
Acknowledgements

We thank Roger Karess and Helen McNeill for the generous gift of *Drosophila* stocks. We also thank Christian Lehner for antibodies. This research was funded by grants to AS from Seeds For Hope and Canadian Cancer Society.
REFERENCES


CHAPTER 5
CONCLUSION
Beach and his colleagues discovered Skp2 in 1995 (Zhang et al., 1995). 20 years of research has established Skp2 as an important oncogene. Skp2 targets a wide variety of proteins including tumor suppressor protein p27. Cks1, a small Cdk-interacting protein, is essential for Skp2 mediated degradation of p27. Although a wide variety of proteins have been identified as Skp2 substrates, how these substrates are essential for Skp2 function remains unknown. To further characterize the role of Skp2 and Cks1 we generated null alleles of Skp2 and Cks1 (Cks85A) in Drosophila. We found that both Skp2 and Cks85A are essential for growth, maintenance of diploidy and also essential for normal endoreplication. Loss of these genes results in large polyploid cells in brain and imaginal discs of 3rd instar wandering larvae. Both Skp2 and Cks85A die as third instar larvae. Lethality observed in Cks85A can be partially rescued by overexpression of Skp2 suggesting Cks85A has very little function outside the SCF^Skp2 complex.

Though the loss of Skp2 has been shown to cause polyploidy in a number of experimental systems the exact cause for this is not clear. We observe low levels of mitotic cyclins in Skp2 mutants. Our immunoprecipitation study show that Skp2 forms a complex with cyclin A and the protein-protein interaction study using PLA shows that Skp2 and cyclin A specifically interact during G2. Skp2 has low mitotic index and this phenotype closely resembles the loss of cyclin A phenotype. Furthermore, we observe a dramatic effect on mitotic entry when a single copy of cyclin A is lost in Skp2 mutant background. This suggests that Cyclin A functions with Skp2 in mitotic entry. The observation that Skp2 is essential for maintaining cyclin A level suggests that Skp2 protects cyclin A from degradation. The low mitotic index of Skp2 suggests some cells fail to accumulate sufficient cyclin A and therefore, are unable to enter mitosis. As a result they undergo endoreplication and become polyploid (Fig. 9). Prometaphase/metaphase delayed cells of Skp2 mutants have more chromatin and larger centrosomes compared to wildtype, which suggests that they are polyploid. The observation that diploid cells are not delayed in prometaphase/metaphase suggests that mitotic delay and thereafter mitotic slippage is not the reason for observed polyploidy in Skp2 mutants. These polyploid prometapase/metaphase cells show enhanced accumulation of SAC proteins, BubR1 and Mad1. Taken together these results suggest that the polyploid cells that enter mitosis become delayed due to SAC
activation. Cells of Skp2, Mad2 double mutants are more polyploid and undergo extensive DNA damage compared to Skp2 alone which indicates that SAC is essential to control ploidy and genomic stability in Skp2 mutants. As mentioned in the discussion of Chapter 4, some of these SAC arrested cells might undergo mitotic slippage and could further contribute to polyploidy and thus enhance genomic instability.

We see extensive apoptosis in Skp2 mutants. Our result show that apoptosis observed in Skp2 is Chk2 dependent. We also observe both p53 dependent and independent apoptosis. P53-independent JNK mediated apoptosis has been observed in

Figure 1: Role of Skp2 in maintaining genomic stability. Refer to text for details.
Drosophila wing imaginal discs in response to chromosomal instability (Dekanty et al., 2012). We also observe JNK activation in the Skp2 mutants. Therefore, the JNK pathway might control the p53-independent apoptosis. Our results show that pro-apoptotic proteins Hid and Reaper also play an important role in apoptosis observed in Skp2 mutants. It would be interesting to know if JNK and p53 are both regulating Hid as loss of Hid almost completely abolishes the apoptosis observed in Skp2 mutants. Furthermore, we also see autophagy in Skp2 mutant. JNK pathway is known to promote autophagy. It would be interesting to determine if it is required for autophagy in Skp2 mutants. It would be interesting to know if the autophagy seen in Skp2 is a pro-survival mechanism against oxidative stress or a mechanism to eliminate damaged cells. Our results show that loss of Chk1 alone does not cause apoptosis but we do observe an enhancement of apoptosis in Skp2, Chk1 double mutants. This suggests that Chk1 might be essential for a cell cycle arrest checkpoint. Our analysis of S-phase index suggests that indeed Chk1 is essential for G1/S cell cycle arrest in Skp2 mutant cells.

Nakayama and colleagues observed that the polyploidy observed in Skp2 mutant mice can be rescued in Skp2, p27 double mutants (Nakayama et al., 2000). They further concluded that the polyploidy observed in Skp2 mutants is due to elevated p27 in G2-M, which inactivates Cdk1 causing the cells to endoreplicate (Nakayama et al., 2004). Dui and colleagues have shown that Drosophila p27/Dap is a target of Skp2 in flies (Dui et al., 2013). Drosophila p27/Dap is known to bind only Cdk2 and its overexpression leads to a G1 arrest due to inhibition of Cdk2 activity (de Nooij et al., 1996). Therefore, the polyploidy phenotype observed in Skp2 mutants might not be related to Skp2 targeting Dap. Therefore, it would be interesting to know if p27/Dap is the critical target of Skp2 in Drosophila.

A diploid cell can become polyploid due to many reasons such as cell fusion, abortive cell cycle and endoreduplication (reviewed in Storchova et al., 2004). Loss or gain of chromosomes leads to the generation of aneuploid cells. Aneuploidy is a common phenomenon observed in cancer (Ganem et al., 2007). Aneuploid cells can be formed from tetraploid intermediates that are unstable. Multiple mutational “hits” of tumour suppressor genes in an aneuploid background can result in cancer (reviewed in Storchova
et al., 2004). Mice and Drosophila models have shown that loss of Skp2 results in polyploidy. This suggests that Skp2 has a less well characterized tumour suppressor function. To further confirm this interesting tumour suppressor role, allograft transplantation experiments could be performed (Dekanty et al., 2012). The Allograft transplantation technique in Drososphila model consists of transplanting pieces of wing imaginal disc expressing p35, GFP and a UAS transgene that could potentially lead to chromosomal instability and tissue migration. Expression of p35 will prevent the tissue from undergoing apoptosis and GFP will aid in the tracking of metastatic cancer cells. Therefore, allograft transplantation of wing imaginal discs expressing p35, GFP and UAS-Skp2RNAi transgene and monitoring for metastatic cells would aid in establishing Skp2 as a tumour suppressor.
References


VITA AUCTORIS

Name: Biju Vasavan

Education:

PhD Biology (2015)
Department of Biological Sciences
University of Windsor

M.Sc Biochemistry (2009)
Department of Chemistry and Biochemistry
University of Windsor

Honors and Scholarships:

- Windsor Prostate Cancer Scholarship, University of Windsor (2014).
- Verdecchia Family Graduate Scholarship, University of Windsor (2014).
- University of Windsor Tuition scholarship (2009-2013).

Publications And Presentations:

Publication:

* Equal contribution

Poster presentation:

1. Role of SCFSkp2 in maintaining genome stability. (2nd Prize)

2. Role of SCFSkp2 in maintaining genome stability.
3. SCFSkp2 and Cks85A are essential for maintenance of diploidy in *Drosophila melanogaster*.

**Biju Vasavan**, Mohammad Ghorbani, Emona Kraja, Andrew Swan.
CANFLY XI. Canadian Drosophila Research conference. Brock university, St. Catharines, Canada (June 4-8, 2011)

**Leadership Qualities/Volunteer Work**

- **Union representative** for Department of Biology Graduate Students Association ([DBGSA](#)). University of Windsor (2010-2011).
- **Graduate representative (PhD)** for Department of Biology Graduate Students Association ([DBGSA](#)). University of Windsor (2012-2013).