Genetic and biochemical analysis of Survivin, a chromosomal passenger protein, in chicken and human cells

Zuojun Yue

A thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

September 2007
Declaration

I hereby declare that this thesis was composed entirely by myself and that the work presented in it is my own, except where explicitly stated otherwise.

Zuojun Yue

September 30, 2007
Acknowledgements

First of all, I want express my gratitude to my Ph.D. supervisor professor Bill Earnshaw who brought me into an international and active lab. He gave me the right example of a scientist and I benefited and will continuously benefit a lot from his suggestions and supervisions. I also thank my Ph.D. committee member professor Malcolm Walkinshaw, Dr. Peter Fantes and Dr. Dietlind Gerloffe. Their constructive advice helped me very much.

I am very grateful to Dr. Sandrine Ruchaud, Dr. Ana Carvalho, Dr. Marcella Cervantes, Dr. Kumiko Samejima, Dr. Fiona Maclsaac and Dr. Jim Paulson. Their warm-hearted help was essential for my Ph.D. studies. I would offer my special thanks to Dr. Sandrine Ruchaud, whose supervision and advice helped me to finish my Ph.D. study. Stefano Cardinale’s brilliant work on movies was very important part of my project. I also thank Susana Ribeiro, Zhenjie Xu and Hiromi Ogawa for their collaborations.

I want to thank our nice colleagues: Dr. Damien Hudson, Dr. Maria Carmena, Dr. Paola Vagnarelli, Dr. Xavier Fant, Dr. Elizabeth Fairley, Dr. Chih-jui Chang, Dr. Sarah Goulding, Daniel Roth, Dr. Fan Lai, Gonzalo Fernandez-Miranda, Linda Zuurbier, Dr. Mafalda Tadeu, Nisha Broodie, Dr. Reto Gassman, Dr. Alexander Ageichik, Dr. Shinya Ohta, Tina, Thanks to the members in Margarete Heck’s lab, Eric Shrimmer’s lab, Andreas Merdes’ lab, Juri Rappsilber’s lab and Robin Allshire’s lab. I am thankful to Xuemei Li’s lab, Juri Rappsilber’s lab and John Yates’ lab for their collaborations. I am also thankful to Dr David Kelly who is always willing to help.

I would like to thank the NIH and Wellcome Trust for the founding for my study.

To my wife Junfang, my parents and my sister for their love and support.
The work presented in this thesis has led to the following publication:
Zuojun Yue, Ana Carvalho, Zhenkie Xu, Xuemei Yuan, Stefano Cardinale, 
Susana Ribeiro, Fan Lai, Ciaran Morrison, Sandrine Ruchaud and William C. 
Earnshaw. Deconstructing Survivin: genetic analysis of Survivin function by 
conditional knockout in a vertebrate cell line. (In preparing)
# Table of contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>2</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>3</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>5</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>7</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>10</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>12</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>14</td>
</tr>
</tbody>
</table>

## I. GENERAL INTRODUCTION

- THE CELL CYCLE                                                                                           | 16   |
- MITOSIS                                                                                                  | 17   |
- CYTOKINESIS                                                                                               | 18   |
- THE CENTROMERE AND THE KINETOCHORE                                                                       | 23   |
- CHROMOSOMAL PASSENGER PROTEINS                                                                          | 26   |
  - INCENP                                                                                                  | 28   |
  - Aurora B                                                                                                 | 30   |
  - Borealin/Dasra-B                                                                                        | 32   |
  - Survivin                                                                                                 | 33   |
  - TD-60                                                                                                   | 33   |
- Chromosomal passengers and kinetochore-microtubule attachment                                                    | 39   |
- Chromosomal passengers and the spindle checkpoint                                                          | 40   |
- Translocation of chromosomal passengers                                                                  | 42   |
- APOPTOSIS                                                                                                | 43   |

## II. MATERIALS AND METHODS

- INTRODUCTION OF SURVIVIN                                                                                   | 45   |
  - Discovery                                                                                            | 45   |
  - Expression of Survivin                                                                                  | 45   |
  - Structure of Survivin                                                                                     | 46   |
  - Dimerization? Or nuclear export signal? Or other functions?                                               | 48   |
  - Posttranslational Modification of Survivin                                                               | 49   |
  - Cell cycle regulation of Survivin levels                                                                | 51   |
  - Homologues and orthologues of Survivin                                                                  | 52   |
  - Survivin Isoforms                                                                                         | 57   |
  - Localization of Survivin                                                                               | 60   |
  - Survivin is also a chromosomal passenger protein and plays an important role in mitosis                 | 61   |
  - Survivin is an inhibitor of apoptosis                                                                    | 63   |
  - Survivin in development and differentiation                                                             | 65   |
  - Survivin and cancer                                                                                       | 67   |

- 2.1 CELL CULTURE                                                                                           | 70   |
- 2.2 DT40 CELL TRANSFECTION                                                                                  | 70   |
- 2.3 PCR                                                                                                   | 70   |
- 2.4 CDNA SYNTHESIS AND RT-PCR                                                                              | 72   |
- 2.5 CONSTRUCTION OF PLASMIDS                                                                                | 72   |
- 2.6 SITE-DIRECTED MUTAGENESIS                                                                               | 72   |
- 2.7 E.Coli AND TRANSFORMATION OF COMPETENT E. coli                                                         | 72   |
- 2.8 AMPLIFICATION AND EXTRACTION OF PLASMID                                                                | 73   |
- 2.9 SEQUENCING                                                                                             | 73   |
- 2.10 DNA ISOLATION AND SOUTHERN BLOTTING                                                                  | 73   |
- 2.11 SDS PAGE GEL                                                                                           | 74   |
- 2.12 IMMUNOBLOTING                                                                                         | 76   |
III. CELLULAR AND MOLECULAR ANALYSIS OF SURVIVIN FUNCTIONS .......... 83

3.1 PURPOSE OF THIS STUDY ........................................................................ 83
3.2 BACKGROUND OF SURVIVIN CONDITIONAL KNOCKOUT .................. 84
   3.3.1 Targeting of the first Survivin allele .............................................. 86
   3.3.2 Design and analysis of a new Survivin rescue system .................... 86
   3.3.3 Generating a stable conditional Survivin heterozygote .................... 87
   3.3.4 Targeting of the second Survivin allele ......................................... 89
   3.3.5 Survivin rescue construct could be fully repressed, yielding complete knockout cells ....................... 90
   3.3.6 Survivin is essential for cell survival ............................................ 93
   3.3.7 INCENP destabilization and mislocalization after Survivin repression 95
   3.3.8 Cells could initiate and traverse mitosis without Survivin .............. 98
   3.3.9 Survivin is required for mitotic spindle checkpoint after loss of tension 101
   3.3.10 Cells fail Cytokinesis without Survivin ......................................... 104
   3.3.11 Cell death in the absence of Survivin ......................................... 106
   3.3.12 GFP-tagged chicken and human Survivin can rescue the knockout 110
   3.3.13 Survivin DD72_73AA mutant still functions like wild type Survivin 112
   3.3.14 Survivin mutants reported to be pre-apoptotic ............................. 116
   3.3.15 T36 mutants have no effect on cell proliferation ............................ 118
   3.3.16 Zinc binding residues are crucial for Survivin function ............... 121
   3.3.17 The linker region between the BIR and C-terminal α-helix of Survivin is essential for its function ............. 126
   3.3.18 Predicted phosphorylation sites in Survivin are dispensable .......... 136

IV ATTEMPTS TO IDENTIFY INTERACTORS OF SURVIVIN ..................... 138

4.1 BACKGROUND .................................................................................... 138
4.2 PURPOSE OF THIS STUDY AND EXPERIMENTAL STRATEGY .............. 140
4.3 TAGGING AND PURIFYING STRATEGIES .......................................... 140
4.4 RESULTS AND DISCUSSION ............................................................... 143
   4.4.1 Stable cell lines expressing recombinant tandem-tagged Survivin in Survivin knockout cells and HeLa cells .......... 143
   4.4.2 Affinity purification and mass spectrometry of tagged Survivin .... 144
   4.4.3 Result of mass spectrometry ......................................................... 147

V DISCUSSION ........................................................................................ 152

Survivin in mitosis ................................................................................ 152
Survivin and the spindle checkpoint .................................................. 153
Survivin is required for cytokinesis .................................................... 154
Survivin is essential for cell life ......................................................... 156
Survivin and cell death ...................................................................... 156
Several Survivin structural elements are important .......................... 158
Survivin and microtubule organization .............................................. 160
Is Survivin phosphorylated? .............................................................. 160
Do other proteins interact with Survivin besides chromosomal passengers? 161

GENERAL CONCLUSIONS AND PERSPECTIVES .......................... 162

REFERENCES ....................................................................................... 164
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA</td>
<td>anti-centromere antibodies</td>
</tr>
<tr>
<td>APC</td>
<td>anaphase promoting complex</td>
</tr>
<tr>
<td>BIR</td>
<td>baculovirus IAP repeat</td>
</tr>
<tr>
<td>bp</td>
<td>base pair(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CB</td>
<td>cytoskeleton buffer</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CENP</td>
<td>centromere protein</td>
</tr>
<tr>
<td>Gg/gg</td>
<td>Chicken/Gallus gallus</td>
</tr>
<tr>
<td>CLAP</td>
<td>chymostatin, leupeptin, antipain, pepstatin A</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>C-terminus</td>
<td>carboxy-terminus</td>
</tr>
<tr>
<td>CYK-4</td>
<td>CYtoKinesis defect (C. elegans MgcRacGAP homologue)</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytidine-5'-triphosphate</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxyribonucleotide-5'-triphosphate</td>
</tr>
<tr>
<td>Drosophila</td>
<td>Drosophila melanogaster</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>ECT2</td>
<td>Rho exchange factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis-(β-aminoethyl ether)-N, N', N'-tetraacetic acid</td>
</tr>
</tbody>
</table>
EVI5  ecotropic viral integration site-5
FBS  fetal bovine serum
FITC  fluorescein isothiocyanate
GFAP  glial fibrillary acidic protein
GFP  green fluorescent protein
GST  glutathione S-transferase
HEC1  Highly expressed in cancer-1
HeLa  Henrietta Lacks
HEPES  N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HRP  horseradish peroxidase
IAP  inhibitor of apoptosis protein
kb  kilobase(s)
kDa  kilodalton
KMN  KNL-1, Mis12 and Ndc80 network
LB  Luria-Bertani medium
MgcRacGAP  Rac GTPase activating protein-1
MCAK  mitotic centromere-associated kinesin
β-ME  2-mercaptoethanol; Mercaptoethanol
MKLP  mitotic kinesin-like protein
MOPS  3-(N-Morpholino)-propanesulfonic acid
mRNA  messenger ribonucleic acid
Ndc80  yeast homologue of HEC1
NMR  nuclear magnetic resonance
N-terminus  amino-terminus
OD  optical density
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PIPES  piperazine-N, N'-bis-[2-ethanesulfonic acid]
PMSF  phenylmethylsulfonyl fluoride
PtK1  Potorous tridactylus kidney
RNA  ribonucleic acid
RNAi  RNA interference
RNase  ribonuclease
RPE cell  human retinal pigment epithelial cell
RT-PCR  reverse transcriptase or real time PCR
SBP  streptavidin binding peptide
SDS  sodium dodecyl sulphate
SF2  splicing factor 2
TAE  tris-acetate-EDTA
TAP  tandem affinity purification
TBS  Tris buffer saline
TEV  tobacco etch virus
TrAP  triple affinity purification
Tris  tris(hydroxymethyl)aminomethane
TRIzol  total RNA isolation reagent
TUNEL  terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling
Ufd1  ubiquitin binding protein 1
UV  ultraviolet
Xenopus  Xenopus laevis
ZEN-4  Zygotic epidermal ENclosure defective (C. elegans MKLP1 homologue
Abstract
The conserved chromosomal passenger protein complex, consisting of Aurora B kinase, INCENP, Survivin and Borealin/DasraB, is a key regulator of mitosis. Survivin is thought to play an important role in mediating the mitotic localization of the complex and also as an element that links cell proliferation and cell death. Here I addressed Survivin function in a null background using a conditional knockout and by proteomics studies. I successfully generated Survivin conditional knockout DT40 cell lines rescued by tetO: survivin under the regulation of tTA2 driven by the cloned Kif4 promoter. SurvivinOFF cells could be completely rescued by chicken or human Survivin fused to GFP. My experiments demonstrated that cells lacking Survivin (SurvivinoFF) could enter mitosis, and activate the spindle checkpoint in the absence of tension. However SurvivinoFF cells failed to complete cytokinesis, although initial formation of the cleavage furrow and ingress were normal. SurvivinoFF cells became multinucleated and multipolar before undergoing apoptosis during interphase. Interestingly, I found that SurvivinOFF cells were not more sensitive to etoposide, staurosporine or taxol than wild type or SurvivinON cells. Two mutants reported in the literature to be dominant negative, Survivin D55A (a mutant reported to be pro-apoptotic) and T36A/E (lacking a reportedly essential Cdk1 phosphorylation site) can rescue the knockout cells. In contrast, the C59A and C86A mutants, which destroy Zinc finger motifs in the BIR domain could not. Linker region mutants L98AV100A and L104AL106A (TS mutants) could rescue Survivin knockout at 39°C and 37°C, respectively, and target correctly in SurvivinOFF cells. By contrast, these mutants do not localize and rescue life under certain conditions at 41°C. Knockout cells stably expressing tagged Survivin were used for tandem affinity purification of Survivin and its interacting proteins. Mass spectrometry analysis of these samples identified several potential interactors. My results showed that Survivin is essential for
the completion of mitosis, but that, despite numerous published RNAi studies to the contrary, the activation of the spindle checkpoint in the absence tension does not need Survivin. The Zinc finger and linker region of Survivin are required for its function. However, our experiments failed to provide additional evidence for a role of Survivin as an essential anti-apoptotic factor.
<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Title of figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The eukaryotic cell cycle</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>The stages of mitosis</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>Different types of mitotic spindle microtubules.</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>Schematic diagram of dividing cell</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
<td>Organization of the animal kinetochore and its protein constituents</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>Localization of chromosomal passenger proteins in mitosis</td>
<td>27</td>
</tr>
<tr>
<td>7</td>
<td>Schematic representation of the chromosomal passenger complex</td>
<td>35</td>
</tr>
<tr>
<td>8</td>
<td>Chromosomal passenger complex localization and function during mitosis</td>
<td>38</td>
</tr>
<tr>
<td>9</td>
<td>Survivin structure</td>
<td>47</td>
</tr>
<tr>
<td>10</td>
<td>Schematic diagram showing the dimer interface</td>
<td>49</td>
</tr>
<tr>
<td>11</td>
<td>Amino acid sequence alignment of Survivin homologues in animals</td>
<td>57</td>
</tr>
<tr>
<td>12</td>
<td>Localization of Survivin in mitosis</td>
<td>61</td>
</tr>
<tr>
<td>13</td>
<td>Targeting constructs and strategy</td>
<td>85</td>
</tr>
<tr>
<td>14</td>
<td>Southern blot analysis of genomic DNA from wild type and heterozygous cells</td>
<td>86</td>
</tr>
<tr>
<td>15</td>
<td>Expressing rescue Survivin using the Tet-Off system before targeting of the second allele</td>
<td>88</td>
</tr>
<tr>
<td>16</td>
<td>Survivin conditional knockout cell line</td>
<td>90</td>
</tr>
<tr>
<td>17</td>
<td>Repression of the Survivin rescue</td>
<td>92</td>
</tr>
<tr>
<td>18</td>
<td>Survivin is an essential protein</td>
<td>94</td>
</tr>
<tr>
<td>19</td>
<td>INCENP level and localization are impaired in Survivin knockout cells</td>
<td>96</td>
</tr>
<tr>
<td>20</td>
<td>Levels of Ser10 phosphorylation on histone H3 are reduced in Survivin knockout cells</td>
<td>97</td>
</tr>
<tr>
<td>21</td>
<td>Mitosis without Survivin</td>
<td>99</td>
</tr>
<tr>
<td>22</td>
<td>Selected frames of representative live cell imaging performed on SurvivinON and SurvivinOFF cells</td>
<td>100</td>
</tr>
<tr>
<td>23</td>
<td>Survivin is not required for spindle checkpoint after loss of tension</td>
<td>102</td>
</tr>
<tr>
<td>24</td>
<td>Cells arrested by Taxol and nocodazole in the absence of Survivin</td>
<td>103</td>
</tr>
<tr>
<td>25</td>
<td>Cells depleted of Survivin failed Cytokinesis</td>
<td>105</td>
</tr>
<tr>
<td>26</td>
<td>Cells depleted in Survivin are not more sensitive to apoptosis</td>
<td>107</td>
</tr>
<tr>
<td>27</td>
<td>SurvivinOFF cells die in interphase after becoming tetraploid</td>
<td>109</td>
</tr>
<tr>
<td>28</td>
<td>GFP-tagged Survivin can complement the loss of Survivin</td>
<td>111</td>
</tr>
<tr>
<td>29</td>
<td>DD72,73AA mutant</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Localization of GFP-tagged DD72,73AA mutant and INCENP</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>The Survivin DD72,73AA mutation does not impair the spindle checkpoint</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>Survivin D55A mutant</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>T36 mutants have no effect on cell proliferation</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Survivin mutant T36A localizes properly</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>Survivin mutant T36E localizes properly</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>Zinc binding residue C59A mutant</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>Localization of Survivin mutant C59A-GFP and INCENP</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>Zinc binding residue C86A mutant</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>Expression of Survivin linker region mutants in KO</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>Linker regions are essential for Survivin function.</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>Localization of Survivin mutant L98AV100A and INCENP at 39°C</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>Localization of Survivin mutant L104AL106A and INCENP at 37°C</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>Localization of Survivin mutant L98AV100A and INCENP at 41°C</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>Localization of Survivin mutant L104AL106A and INCENP at 41°C</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>The L98AV100A mutation is temperature sensitive (TS).</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>Affinity purification</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>Expression constructs of tagged human Survivin</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>Localization of tagged human Survivin in HeLa cells</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>Purification from HeLa expressing TrAP tagged human Survivin</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>Tagged human Survivin expression and purification from KO1</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>Primary result of pull-down using TrAP tagged P32</td>
<td></td>
</tr>
</tbody>
</table>
### List of tables

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title of table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Core proteins required for central spindle assembly</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>Chromosomal passenger proteins in various species</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>Aliases and homologues of Survivin</td>
<td>54</td>
</tr>
<tr>
<td>4</td>
<td>Cloning and generation of mutants</td>
<td>71</td>
</tr>
<tr>
<td>5</td>
<td>Recipe for lower gel</td>
<td>75</td>
</tr>
<tr>
<td>6</td>
<td>Recipe for upper gel</td>
<td>76</td>
</tr>
<tr>
<td>7</td>
<td>Primary antibodies</td>
<td>78</td>
</tr>
<tr>
<td>8</td>
<td>Secondary antibodies</td>
<td>79</td>
</tr>
<tr>
<td>9</td>
<td>Mass spectrometry result of pull-down from KO1 expressing tagged human Survivin</td>
<td>148</td>
</tr>
<tr>
<td>10</td>
<td>Mass spectrometry result of pull-down from HeLa expressing tagged human Survivin</td>
<td>149</td>
</tr>
</tbody>
</table>
General Introduction
I. General Introduction

The cell cycle

Multicellular organisms develop from a single cell. Development and repair of tissues need cell growth and division. In eukaryotes, the process by which cells coordinate their growth with DNA replication and cell division is termed the cell cycle (for details see the book by Pollard and Earnshaw, 2002). The cell cycle can be subdivided into four distinct phases according to events that coordinate with DNA replication and the gain of cell mass (Figure 1). These four phases are G1, S, G2 and M. In order to faithfully pass genetic material from one generation to next, cells depend on a series of cell cycle checkpoints to ensure all events of each phase are correctly completed before cells go to the next phase. G1 phase is a gap phase between mitosis and the S phase. Cells have to pass the restriction point and G1 DNA damage checkpoint before they can enter S phase. Some cells can exit the cell cycle during G1 into a specialized state called G0 and no longer divide. In S phase (synthesis phase), the chromosomal DNA is replicated. The subsequent gap between S and the mitotic phase is termed G2. Unduplicated or damaged DNA is detected in this phase. Following G2, another main event is the segregation of chromosomes and formation of two daughter cells, termed mitosis or M phase. In the following section, I will give a more detailed introduction to mitosis.
Figure 1. The eukaryotic cell cycle. The arrows indicate the relative lengths of each of the four phases of a standard cell cycle in cultured cells. The checkpoints are indicated in red text. Adapted from (MacIsaac, 2007).

Mitosis

The eukaryotic mitosis can be subdivided into six phases: prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis (Figure 2). This was first described by Flemming (1888. For details, see the Cell biology book by (Pollard and Earnshaw, 2002). During the G2/M transition, morphological and physiological changes are dramatic and accompanied by activation of protein kinases (recently reviewed in (Malumbres and Barbacid, 2005). In prophase, condensed chromosomes appear and the nucleolus disassembles. Asters are formed and surrounded by short microtubules. At the beginning of prometaphase, the nuclear envelope disassembles, and bipolar spindles that attach kinetochores start to assemble. Metaphase is characterized by the presence of bi-oriented chromosomes aligned midway between the two spindle poles. When all the kinetochores are correctly attached, the spindle assembly checkpoint is silenced, permitting the full
activation of the APC (anaphase promoting complex) (For details, see spindle checkpoint section) and the onset of anaphase. During anaphase, sister chromatids separate from each other and move toward the opposite spindle poles. The most obvious change during telophase is the reformation of nuclear envelope (Pollard and Earnshaw, 2002). The final stage of M-phase is cytokinesis, which I will describe in more detail.

Cytokinesis

Following mitosis, cytokinesis is the process that divides the mother cell into two new cells, each endowed with a complete set of chromosomes and cytoplasmic organelles. Just before completion of cytokinesis a thin intercellular bridge called the midbody forms.

The molecular mechanisms regulating cytokinesis are largely unknown. The main theory is that the cleavage furrow and ingression signal are transferred from the asters (Rappaport, 1961; Rappaport, 1996) to the mid region of the cell. Several experiments support this theory. Experiments in fertilized sand dollar eggs distorted into a donut-like shape showed that
cleavages occur between the asters of adjacent spindles (Rappaport, 1961). Furrow induction between the asters of two independent spindles has also been reported (Eckley et al., 1997; Rieder et al., 1997). The initiation of furrow formation was disrupted when hydrostatic pressure induced disassembly of astral microtubule arrays (Salmon and Wolniak, 1990).

There is also other data in disagreement with this theory. Spermatocytes of the Drosophila mutant asterless (asl) fail to form normal asters, but form a morphologically normal anaphase and telophase central spindle that has full ability to stimulate cytokinesis (Bonaccorsi et al., 1998). Furthermore, asters alone or truncated central spindles alone are sufficient for induction of cell cleavage. Micromanipulation experiments in grasshopper spermatocytes showed after removal of both asters and chromosomes, the residual spindle microtubules could still self-assemble into organized bundles and promote furrowing (Alsop and Zhang, 2003). These data all revealed that microtubules and their associated proteins are essential for furrow induction. In contrast, Caenorhabditis elegans spd-1 mutant embryos lack a central spindle but can nevertheless successfully complete the first embryonic divisions. But cytokinesis fails in subsequent cell divisions (Verbrugghe and White, 2004). Drug-induced monopolar spindles of mammalian PtK1 (Potorous tridactylus kidney) cells initiate and complete cytokinesis (Canman et al., 2003). But in all these mutants, microtubules were observed (Bonaccorsi et al., 1998; Canman et al., 2003; Verbrugghe and White, 2004). Another hypothesis that tries to reconcile these conflicting studies suggests that cytokinesis signals can be delivered by two kinds of microtubules (astral and spindle microtubules) (D'Avino et al., 2005; Inoue et al., 2004).

However there are many questions to answer, for example, the exact roles of the microtubule associated proteins (MAPs) and their interactions, the pathway regulating cytokinesis progress.
In the following sections I will introduce the contractile ring and central spindle. The daughter cells are normally separated by an actin-myosin contractile ring situated under the plasma membrane (Figure 4). The contractile ring creates the cleavage furrow. During furrow ingression, the cell membrane is pulled inwards to divide the cell into two. Myosin is thought to slide actin filaments against each other to draw the contractile ring tighter (Glotzer, 1997). Besides actin and myosin, contractile ring components include formin (Chang et al., 1997; Swan et al., 1998), profilin (Romero et al., 2004), cofilin (Gunsalus et al., 1995; Ono et al., 2003) and anillin (Somma et al., 2002; Straight et al., 2005). Furthermore, the contractile ring assembly and constriction are regulated by RhoA GTPase, which activates actin nucleation and myosin, the engine of cytokinesis (Guo and Kemphues, 1996; Motegi et al., 1997; Straight et al., 2003). During ingression, the contractile ring interacts with the central spindle (Giansanti et al., 1998).

The mitotic spindle is critical for sister chromatid separation, cleavage furrow positioning, and cleavage induction. Before introducing the central spindle, I will give a simple introduction to microtubules (Figure 3). The less dynamic minus ends of microtubules are located at the spindle poles, while
the more dynamic plus ends extend away from the poles. According to microtubule morphology and localization, microtubules are subdivided into three classes. The astral microtubules extend from the spindle poles towards the cell cortex. This helps to position the mitotic spindle. The kinetochore microtubule minus ends are at the spindle poles and their plus ends attach to the kinetochores. Kinetochore microtubules form morphologically distinct bundles called K fibers. The K fibers play an important role to maintain attachment of chromosomes to the spindle as well as alignment and segregation of chromosomes (Figure 3), recently reviewed in (Kline-Smith and Walczak, 2004). During anaphase and telophase, spindle microtubules become bundled to form the central spindle, which is essential for completion of cytokinesis (Figure 4). (Reviewed by (Glotzer, 2003). Core components required for central spindle assembly include microtubule associated proteins (MAPs) PRC1 and KIF (Jiang et al., 1998; Mollinari et al., 2002; Verbrugghe and White, 2004; Verni et al., 2004). The centralspindlin complex consisting of MgcRacGAP and MKPL1 is also essential (Matuliene and Kuriyama, 2002; Mishima et al., 2002; Somers and Saint, 2003). Binding of microtubules to MAPs or the centralspindlin complex is regulated by Cdk1/cyclin B (Mishima et al., 2004; Mollinari et al., 2002). Another important group of proteins that concentrate on the central spindle are the chromosomal passenger proteins (Vagnarelli and Earnshaw, 2004). Localization of centralspindlin depends on the chromosomal passenger complex (CPC)(Schumacher et al., 1998b; Severson et al., 2000). In addition to the above-mentioned proteins involved in assembling the central spindle, there are many other mitotic spindle associated proteins such as NuSAP (Raemaekers et al., 2003), orbit/Clasp1 (Inoue et al., 2000), BRCA2 (Daniels et al., 2004), and others (Sauer et al., 2005). Some of proteins required for central spindle assembly are listed in Table 1
Table 1. Core proteins required for central spindle assembly and for cytokinesis. (Adapted from (Glotzer, 2005).

The final step of cell division is the abscission of daughter cells. Actomyosins are important motors for the separation of cell mass, but it is not sufficient to fully execute cytokinesis. The targeting of membrane vesicles directed by Syntaxins is also essential to complete abscission (Burgess, Deitcher et al. 1997; Conner and Wessel 1999; Jantsch-Plunger and Glotzer,
1999). When the actomyosin contractile ring comes into close proximity to the central spindle, it concentrates regulatory proteins at the midbody. Cell and proteomics studies have identified a large number of proteins involved in the organization of the midbody (Skop et al., 2004), but mechanism of resolution of the midbody by severing of the narrow cytoplasmic bridge is still unclear. However, in some tissues the intercellular bridge remains open as canals, which are important for signal or nutrient exchange. For example, Drosophila nurse cells transfer their cytoplasmic contents to the developing eggs through these kinds of bridges (Pollard and Earnshaw, 2002).

Variations in cytokinesis are widespread normal biological phenomea. Drosophila embryos exhibit at least four variations of cytokinesis: syncytial divisions (no cleavage), cellularization, asymmetric cytokinesis, and incomplete cytokinesis. In the early nuclear divisions of the 1-13 stage of Drosophila embryo development, cytokinesis does not occur. The nuclei replicate in a syncytium till early stage 14 and start their migration outward from stage 8. After completion of the syncytial divisions, plasma membrane starts to form synchronously between all of the peripheral nuclei (Foe and Alberts, 1983). This process is called cellularization. Asymmetric cytokinesis happens in stem cells and germ cells, and leads to different fates of the daughter cells. Incomplete cytokinesis has been documented in several cell types such as Drosophila oocyte development, mammalian megakaryocytes, cardiac myocytes and vascular smooth muscle cell development (Glotzer, 2001; Ravid et al., 2002).

The centromere and the kinetochore

The centromere is a specialized chromosome region for microtubule attachment and chromosome segregation in mitosis. Human centromere sequences are made up of large tandem arrays of repetitive α-satellite DNA
(Tyler-Smith and Floridia, 2000), which is essential for de novo formation of human artificial chromosomes (reviewed by (Masumoto et al., 2004). In addition to the DNA component, the centromere also contains nearly 100 proteins: CENP-A, CENP-B, CENP-C (Earnshaw and Rothfield, 1985) and other CENPs. One common epigenetic mark that confers centromeric identity in most organisms studied so far is the replacement of histone H3 with CENP-A. CENP-A is a histone H3 variant, which incorporates into the nucleosome of inner kinetochores for the whole cell cycle (Sullivan et al., 1994; Warburton et al., 1997). Analysis of central core chromatin fibers from humans and Drosophila revealed that CENP-A nucleosomes are interspersed with histone H3-containing nucleosomes (Carroll and Straight, 2006; Sullivan and Karpen, 2004). In three dimensions, it is thought that the CENP-A containing nucleosomes face towards the kinetochore, whereas the histone H3 containing nucleosomes are on the inner side of the centromere where the sister chromatids are joined (Blower et al., 2002). In addition to the incorporation of CENP-A at the centromere, the formation of heterochromatin through histone methylation may also have a role in centromere formation (For review, see (Vos et al., 2006).

The centromere maintains sister-chromatid cohesion and attachment of chromosomes to the spindle during mitosis, and acts as the platform for the kinetochore to form (Craig et al., 1999; Pluta et al., 1995). It is also the site monitoring the attachment of chromosomes to microtubules before the onset of anaphase. The inner centromere is composed of constitutive heterochromatin and some crucial proteins. The chromosomal passengers are targeted in the inner centromere from prophase to the onset of anaphase.

The kinetochore is embedded in the surface of centromeric heterochromatin during mitosis. Kinetochores were first observed by electron microscopy (Stubblefield and Brinkley, 1966). The innermost part of the
kinetochores, called the **inner plate** contains histone H3 variant, CENP-A. In *C. elegans*, CENP-A and CENP-C direct the targeting of KNL-1 and KNL-3 to the inner kinetochore (Cheeseman et al., 2004; Desai et al., 2003). Vertebrate and Drosophila kinetochore proteins reported to localize in the **outer plate** of the kinetochore include AF15q14, Polo-like kinase (PLK), ROD, ZW10, ZWINT-1, the microtubule motor dynein, the kinesin motor CENP-E, the spindle checkpoint proteins (MPS1, BUBR1, MAD1 and MAD2) and the non-motor microtubule-associated proteins CLASPs, all depend upon CENP-A-dependent pathway for targeting (For review see Maiato and Sunkel, 2004). Proteins of the outer plate required for microtubule binding are the NDC80/Hec complex, KBP-1 to KBP-5, the MIS12 complex, CENP-F and BUB1. Localization of all of these requires KNL-1 (Cheeseman et al., 2006; Cheeseman et al., 2004; Desai et al., 2003). The KNL-1, Mis12 complex and Ndc80 complex make up a conserved (KMN) network throughout eukaryotes, which is essential for viability and kinetochore-microtubule interactions in multiple organisms (reviewed Kline-Smith, 2005). The KMN protein network is directly involved in the interaction of the kinetochore and microtubules through the Ndc80/Nuf2 subunits of the Ndc80 complex and KNL-1 (Cheeseman et al., 2006; DeLuca et al., 2006). The affinity of the Ndc80 complex for microtubules is regulated by Aurora B in vitro (Cheeseman et al., 2006). The outer plate is separated from the inner plate by an interzone where the tension receptor 3F3/2 localizes. Besides these three kinetochore layers, a fourth layer, the **fibrous corona** is seen outward of the outer plate when microtubules are absent, and where maybe the microtubule motors are recruited during mitosis (Pollard and Earnshaw, 2002). The organization of the kinetochore and its protein constituents is shown in figure 5.
Figure 5. **Organization of the animal kinetochore and its protein constituents**, according to their localization. Adapted from (Maiato et al., 2004).

**Chromosomal Passenger proteins**

Chromosomal passenger proteins display a distinctive pattern of localization throughout mitosis, first described by Cooke et al (Cooke et al., 1987). These proteins first start accumulating in the nucleus in G2. During prophase and metaphase the passengers converge at the inner centromeres of mitotic chromosomes. Subsequently the passengers associate with microtubules of the central spindle at anaphase and then concentrate at the midbody during cytokinesis (Figure 6) (For reviews see Ruchaud et al., 2007; Vagnarelli and Earnshaw, 2004). The conserved chromosomal passenger protein complex is a key regulator of mitosis. To date, five bona fide chromosomal passenger proteins have been described in vertebrates: Aurora B kinase, INCENP,
Survivin, Borealin/DasraB, and TD-60 (Adams et al., 2000; Gassmann et al., 2004; Kang et al., 2001; Sampath et al., 2004); for review, see (Vagnarelli and Earnshaw, 2004).

Figure 6. Localization of chromosomal passenger proteins in mitosis. 
Indirect immunofluorescence (upper panels) and schematic representation (lower panels) of Aurora-B localization (green) in HeLa cells during the main phases of mitosis together with kinetochores (stained with anti-centromere autoantibodies, pink), α-tubulin (red) and DNA (blue). Adapted from (Ruchaud et al., 2007).

The first four form a complex; and depletion of any one of its members by RNA interference affects the proper localization of the other subunits (Adams et al., 2000; Carvalho et al., 2003; Gassmann et al., 2004; Honda et al., 2003). Studies have shown that Aurora B, the enzymatic core of the complex, requires other proteins to guide its spatio-temporal action (Adams et al., 2001a; Carvalho et al., 2003; Vader et al., 2006b). Borealin promotes Survivin binding to INCENP, which stabilizes the passenger complex. Interestingly, a chimeric protein of Survivin fused to INCENP can target the chromosomal passenger complex to centromeres and the midbody in the absence of both Borealin and the centromere-targeting domain of INCENP (Vader et al., 2006a). Chromosomal passenger proteins are essential for
chromatin modification, correction of kinetochore attachment errors and completion of cytokinesis (Carmena and Earnshaw, 2003; Vagnarelli and Earnshaw, 2004).

INCENP
INCENP (inner centromere protein) was first identified in a monoclonal antibody screen for novel components of the mitotic chromosome scaffold. Two isoforms were identified (155 KDa and 135 kDa) (Cooke et al., 1987). Since then, homologues have been identified in all eukaryotes from budding yeast to human (Table 2). INCENP is essential in mice (Cutts et al., 1999), yeast (Kim et al., 1999), worms (Kaitna et al., 2000), flies (Adams et al., 2001b) and humans (Honda et al., 2003). Depletion of INCENP or expression of dominant-negative mutants leads to failure of chromosome congression and cytokinesis (Ainsztein et al., 1998; Mackay et al., 1998). INCENP phosphorylation by Aurora B (Sessa et al., 2005) and Cdk1 (Goto et al., 2006) regulates its function. In budding yeast, INCENP dephosphorylation by cdc14 is necessary and sufficient for its transfer to the spindle midzone at anaphase (Pereira and Schiebel, 2003).

The most recognizable feather of INCENP is a predicted central coiled-coil region (Mackay et al., 1993). Little is known about the function of this conserved portion of the protein. Another highly conserved motif close to the C-terminus is a unique identifier of INCENP members. This was termed the IN-box (Adams et al., 2000). INCENP binds Aurora B via the IN-box and partially activates Aurora B, which then phosphorylates INCENP at a highly conserved TSS motif near it C-terminus. This phosphorylation of INCENP results in a feedback loop leading to further full activation of the Aurora B kinase (Bishop and Schumacher, 2002; Honda et al., 2003; Ruchaud et al., 2007; Sessa et al., 2005). Another two chromosomal passengers, Survivin and Borealin, associate with the N-terminus of INCENP (Figure 7). In yeast, Sli15 (INCENP homologue in yeast) makes of a complex with Bir1 (Survivin
homologue in yeast), which is required for linking microtubules to kinetochores (Sandall et al., 2006). Their interaction is necessary for targeting the chromosomal passenger complex. This will be explained in more detail in the CPC section below. In chicken, INCENP residues 135–270 are required for binding heterochromatin protein-1 (HP1), which is important for heterochromatin formation (Ainsztein et al., 1998; Minc et al., 1999). The chromosomal passenger complex appears to regulate the dynamic behaviour of HP1 (Fischle et al., 2005; Hirota et al., 2005; Mateescu et al., 2004; Terada, 2006). The role of the interaction between INCENP and HP1 is still unknown. In addition to binding Aurora B, Survivin, and HP1, yeast two-hybrid and in vitro binding data demonstrated that INCENP binds directly to β-tubulin via a conserved domain encompassing residues 48–85 of the human protein (Wheatley et al., 2001b). INCENP1-405, a truncated molecule lacking the microtubule association region interfered with both prometaphase chromosome alignment and the completion of cytokinesis (Mackay et al., 1998). Near the N-terminus of INCENP, two motifs required for function were revealed by truncation mutants: The motif comprising residues 32-44 is necessary for targeting INCENP to the centromere and midbody, and the motif comprising residues 52-62 is required for targeting INCENP to the midbody (Ainsztein et al., 1998; Mackay and Earnshaw, 1993).
### Aurora B

Aurora B is a member of the vertebrate Ser/Thr protein kinase family. Another two related members are Aurora A and C (Carmena and Earnshaw, 2003). Aurora B and C are chromosomal passengers. Aurora C kinases are highly expressed in testis and some cancer cell lines (Kimura et al., 1999; Yan et al., 2005a; Yan et al., 2005b). In yeast there is one Ser/Thr protein kinase homologue Ipl1, which is thought to have functional similarities primarily to Aurora B. Drosophila has two Ser/Thr protein kinase homologues: Aurora A and B (Adams et al., 2001a). Drosophila Aurora A, which localizes to centrosomes from the time of centrosome duplication to the end of mitosis, is the original aurora kinase found by Glover (Glover et al., 1995). Its homologues in other organisms are listed in Table 2. In this thesis, I will concentrate on the Aurora B kinase.

Aurora B is the enzymatic core of the chromosomal passenger complex. Aurora B activity is required for Histone H3 phosphorylation at Serine 10 during mitosis (Adams et al., 2001b; Hsu et al., 2000; Murnion et al., 2001).

---

**Table 2 Chromosomal passenger proteins in various species**

<table>
<thead>
<tr>
<th>Organism</th>
<th>INCENP</th>
<th>Aurora B kinase</th>
<th>Survivin</th>
<th>Borealin</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. cerevisiae</td>
<td>Sli15</td>
<td>Ipl1</td>
<td>Bir1</td>
<td></td>
</tr>
<tr>
<td>S. pombe</td>
<td>Pic1</td>
<td>Ark1</td>
<td>Bir1/cut17</td>
<td></td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>Incenp</td>
<td>lal</td>
<td>Deterin</td>
<td>Borealin/Australin</td>
</tr>
<tr>
<td>C. elegans</td>
<td>ICP-1</td>
<td>AIR-2</td>
<td>BIR1</td>
<td>CSC-1?</td>
</tr>
<tr>
<td>X. laevis</td>
<td>xINCENP</td>
<td>xAurora B</td>
<td>xSurvivin</td>
<td>Dasra-A, Dasra-B</td>
</tr>
<tr>
<td>G. gallus</td>
<td>INCENP-1</td>
<td>Aurora B</td>
<td>Survivin</td>
<td>Borealin-1, Borealin-2</td>
</tr>
<tr>
<td>M. musculus</td>
<td>INCENP-A</td>
<td>AIM-1</td>
<td>Survivin</td>
<td>Borealin-1, Borealin-2</td>
</tr>
<tr>
<td>H. sapiens</td>
<td>INCENP</td>
<td>Aurora B</td>
<td>Survivin</td>
<td>Borealin</td>
</tr>
</tbody>
</table>

Adapted from (Ruchaud et al., 2007).
The phosphorylation of Histone H3 may be involved in regulating HP1 targeting to chromosomes (Ruchaud et al., 2007). Phosphorylation of CENP-A, the centromere histone-H3 variant that is the core organizer of kinetochore (Kunitoku et al., 2003; Zeitlin et al., 2001), by Aurora B at Serine-7 is reported to be required for Aurora B targeting to the centromere and for chromosome congression. Several other kinetochore proteins, Ndc-80/Hecl (Cheeseman et al., 2006; DeLuca et al., 2006) and Dam1 complex (Cheeseman et al., 2001; Kang et al., 2001) have been shown to be phosphorylated by Aurora B in vitro. These proteins regulate kinetochore microtubule attachments. Another important Aurora B substrate on kinetochore is the mitotic centromere associated kinesin (MCAK), which depolymerizes microtubules (Ohi et al., 2003). The destabilization of microtubules is also negatively regulated by the phosphorylation of MCAK in an Aurora B-dependent pathway. Depletion of MCAK could rescue microtubule stabilization in CPC-deleted egg extracts (Gadea and Ruderman, 2006; Sampath et al., 2004), whereas over-expression of MACK mimicking phosphorylation or non-phosphorylatable mutants, or treating mammalian cells with the Aurora B inhibitor hesperadin resulted in monotelic and syntelic microtubule-kinetochore attachment (Andrews et al., 2004; Hauf et al., 2003). Aurora B kinase activity is also required for loading the microtubule motors CENP-E and dynein at centromeres (Murata-Hori and Wang, 2002b). Studies using kinase dead mutants, microinjection of Aurora B antibodies or small molecule inhibitors showed that the kinase activity is essential for regulation of kinetochore-microtubule attachments, chromosomal bi-orientation and spindle checkpoint in the absence of tension (Biggins and Murray, 2001; Ditchfield et al., 2003; Hauf et al., 2003).

Aurora B regulates cytokinesis by phosphorylation of the myosin II regulatory light chain (Murata-Hori et al., 2000) MKLP1 (Liu et al., 2004b) MgcRacGAP (Minoshima et al., 2003; Tatsumoto et al., 1999), vimentin (Goto et al., 2003), GFAP and desmin (Kawajiri et al., 2003). Over-
expression non-phosphorylatable vimentin or magRacGAP mutants led cells
to fail in cytokinesis (Minoshima et al., 2003; Yasui et al., 2004). Aurora B is
also believed to be involved in transfer of the cytokinesis signal from spindle
poles to the cortex (Murata-Hori et al., 2002).

The list of Aurora B substrates is still growing. Aurora B substrates also
include its partners: INCENP (Bishop and Schumacher, 2002), Survivin
(Wheatley et al., 2004), and Borealin (Gassmann et al., 2004). The
phosphorylation of INCENP is essential for full activation of Aurora B kinase.
But the role of phosphorylation on Survivin and Borealin is not clear and
remains to be explored. Aurora B spatio-temporal function is guided by its
passenger complex partners (For recent reviews see Ruchaud et al., 2007;
Vader et al., 2006b). The phosphorylation consensus in metazoans was
reported as [(R/K) 1-3X(S/T)] (Meraldi et al., 2004).

**Borealin/Dasra-B**

Borealin/Dasra-B was first identified in a screen for new components of the
mitotic chromosome scaffold, and simultaneously in a screen for novel
Xenopus chromosome-binding proteins (Gassmann et al., 2004; Sampath et
al., 2004). Borealin promotes the interaction between Survivin and INCENP
(Vader et al., 2006a). In addition to Borealin, non-human vertebrates also
have a second more distant homologue Dasra A (Table 2). Borealin/Dasra B
is important for spindle assembly. Deletion of Borealin/Dasra B resulted in
kinetochore attachment errors, multipolar spindles and cytokinesis failure
(Gassmann et al., 2004; Hanson et al., 2005; Kelly et al., 2007; Sampath et
al., 2004). Moreover, Survivin could not bind INCENP in Borealin-deleted
cells (Vader et al., 2006a). Though larvae can survive loss of borr (fly
orthologue of Borealin), Drosophila development is impaired (Hanson et al.,
2005). Borealin promotes binding of Survivin to INCENP (Vader et al.,
2006a). Borealin was also reported to bind double-stranded DNA in vitro,
suggesting that Borealin might be the subunit within the CPC directly binding to DNA and thus localizing the CPC to the centromere (Klein et al., 2006).

Survivin
Survivin is a cell cycle-regulated protein, whose expression peaks in G2/mitosis (Lens et al., 2006b; Li et al., 1998). As one member of the chromosomal passenger complex, Survivin plays an important role to mediate the mitotic localization of the chromosomal passenger complex (Carvalho et al., 2003; Klein et al., 2006; Knauer et al., 2006a; Vader et al., 2006a). Survivin has been reported to contribute to Aurora B activity in Xenopus laevis and fission yeast (Bolton et al., 2002; Petersen and Hagan, 2003). Survivin and its yeast homolog Bir-1 are required for spindle assembly checkpoint function (Bolton et al., 2002; Petersen and Hagan, 2003). However, the exact role of Survivin in mitosis still needs more dissection. Survivin is also thought as a possible element that links cell proliferation and cell death (Li et al., 1998; Wheatley and McNeish, 2005). Survivin was originally classified as a member of the inhibitor of apoptosis protein family (IAP) (Ambrosini et al., 1998; Crook et al., 1993). It was also found to be overexpressed in a wide variety of tumours (Ambrosini et al., 1998; Li, 2003). Studies have shown that cells over-expressing Survivin are resistant to a variety of apoptotic stimuli, whereas loss of Survivin expression or function can cause spontaneous apoptosis or sensitize cancer cells to apoptotic stimuli (Beltrami et al., 2004; Carvalho et al., 2003; Jiang et al., 2001; Li et al., 1998; Mahotka et al., 1999; Mirza et al., 2002; Song et al., 2004; Temme et al., 2003). However, Survivin homologues in S.pombe (Rajagopalan and Balasubramanian, 1999), C. elegans (Fraser et al., 1999), Drosophila (Jones et al., 2000) and Xenopus (Bolton et al., 2002) did not show anti-apoptotic function. Hence, the role of Survivin in apoptosis is still controversial and unclear.

TD-60
Another protein, TD-60 (telophase disk 60 kDa) was reported as a
chromosomal passenger, as it shows a typical localization although it is not a member of the core complex (Andreassen et al., 1991; Gassmann et al., 2004). It was originally described as a putative guanosine nucleotide exchange factor (GEF) and is mislocalized when other components of the complex are perturbed (Mollinari et al., 2003) (For reviews, please see Ruchaud et al., 2007; Vagnarelli and Earnshaw, 2004).

**Function of the chromosomal passenger complex**

Chromosomal passenger proteins form a complex to carry out their function. An earlier study has shown that Ipl1, the homolog of Aurora B in budding yeast, interacts with Sli15 (the homologue of INCENP in budding yeast) (Kim et al., 1999). In Xenopus, the interaction between their orthologues XINCENP and Aurora B was confirmed using co-immunoprecipitation from egg extracts, GST-pulldown and a dominant-negative mutation in INCENP (Adams et al., 2000). The same complex was also reported in *C. elegans* (Kaitna et al., 2000). Further studies showed that Survivin was also a subunit of this complex. Survivin, INCENP and Aurora B interaction was established by yeast two hybrid assay, sucrose gradient centrifugation and immunoprecipitation (Bolton et al., 2002; Wheatley et al., 2001a) in human cell extracts. Others reported the same complex formation in budding yeast (Cheeseman et al., 2002). Borealin was also identified as a new member of the chromosomal passenger complex. Immunoprecipitation, yeast two hybrid assay and sucrose gradient centrifugation verified its physical interaction with Survivin, INCENP and Aurora (Gassmann et al., 2004; Klein et al., 2006; Sampath et al., 2004). In the chromosomal passenger complex, Aurora B binds to the C-terminal region of INCENP, while Survivin and Borealin bind to the N-terminal region of INCENP (Figure 7 (Bolton et al., 2002; Klein et al., 2006). Borealin and Survivin interact in vitro and in vivo (Gassmann et al., 2004). Localization of the chromosomal passengers depends on the interactions between the members of the complex.
Figure 7. Chromosomal passenger complex. A. Schematic representation of the chromosomal passenger complex. Survivin and Borealin bind to the N-terminus of INCENP, while Aurora B binds to the IN-box of INCENP C-terminus. Adapted from (Ruchaud et al., 2007). B. Overall structure of the core CPC formed by Survivin, Borealin10-109, and INCENP1-58. C. Conserved residues in Survivin take part in the interaction with Borealin (purple circles above the sequence) and INCENP (yellow circles above the sequence). Adapted from (Jeyaprakash AA et al. 2007).

Depletion of any one subunit impairs localization of the other members (Adams et al., 2001b; Carvalho et al., 2003; Gassmann et al., 2004; Honda et al., 2003; Kaitna et al., 2000; Lens and Medema, 2003; Lens et al., 2006a)), which suggests that they target as a complex. Borealin promotes Survivin binding to INCENP, which stabilizes the passenger complex (Vader et al., 2006a). Interestingly, a chimeric protein of Survivin fused to INCENP can target the chromosomal passenger complex to centromeres and the midbody in the absence of both Borealin and the centromere-targeting domain of INCENP (Vader et al., 2006a). These data suggest that Survivin mediates
the targeting of the whole complex.

Chromosomal passenger proteins are essential for chromatin modification and chromosome structure (Carmena and Earnshaw, 2003; Vagnarelli and Earnshaw, 2004). Serine 10 of Histone H3 is phosphorylated during mitosis by Ipl1 in yeast (Hsu et al., 2000), and by Aurora-B in metazoans (Adams et al., 2001b; Crosio et al., 2002; Ditchfield et al., 2003; Giet and Glover, 2001; Hauf et al., 2003; Honda et al., 2003; Hsu et al., 2000; Murnion et al., 2001; Speliotes et al., 2000). The chromosomal passenger complex was also reported to regulate the loading of HP1 and the condensin complex onto the chromosomes. The chromosomal targeting of condensin I was decreased when Aurora B was deleted (Lipp et al., 2007; Takemoto et al., 2007). The interactions of the passenger proteins with condensin point to a role for the chromosomal passenger complex in chromosome structure, but reports on the role of chromosomal passenger complex in chromosome structure are controversial and unclear (For detailed review, see Ruchaud et al., 2007).

The proper localization of the protector of centromeric cohesion, Shugosin/MEI-S322 depends on the chromosomal passenger complex. Shugosin/MEI-S322 (Shugosin is the human and yeast homologue of Drosophila MEI-S322) functions as a protector of centromeric cohesion before the onset of anaphase, both in meiosis and mitosis of animal cells (reviewed in (Watanabe and Kitajima, 2005). Shugosin is also required for yeast meiosis. Its interaction with CPC members was verified by GST-pulldown and TAP assay (Resnick et al., 2006; Vanoosthuyse et al., 2007).

**Chromosomal passenger proteins and microtubule assembly**

Chromosomal passengers are also involved in microtubule dynamics and organization. INCENP was shown to interact directly with beta-tubulin using the yeast two-hybrid and in vitro binding assays (Wheatley et al., 2001b). However, chromatin-induced microtubule stabilization and spindle formation
also require other subunits of the CPC. In Xenopus egg extracts, xINCENP and xDasra A depletion caused failure in spindle formation. Microtubule nucleation from sperm centrosomes was impaired in xINCENP depleted extracts (Sampath et al., 2004). When Aurora B kinase was inhibited by ZM44739, spindle assembly was blocked (Gadea and Ruderman, 2005). The destabilization of microtubules is negatively regulated by the phosphorylation of stathmin and MCAK in an Aurora B-dependent pathway. Depletion of MCAK rescued microtubule stabilization in CPC-depleted egg extracts (Gadea and Ruderman, 2006; Sampath et al., 2004). In addition to roles for INCENP, Borealin and Aurora B in microtubule assembly, the chromosomal passenger protein Survivin may play a role in microtubule stability. In RPE and COS-7 cells, Survivin was reported to modulate spindle and interphase microtubule organization (Rosa, Canovas et al. 2006; Giodini et al., 2002). In vitro experiments showed that Survivin co-sedimented with paclitaxel-stabilized microtubules (Li et al., 1998). The chromosomal passengers may stabilize the spindle by inhibiting destroyers such as stathmin and MCAK.
Figure 8. **Chromosomal passenger complex localization and function during mitosis.** Schematic representation of the chromosomal passenger complex (CPC) localization (green) correlated with its multiple functions (grey boxes) and principal targets (red boxes) during the different phases of mitosis. 1. In prophase, the CPC is found on chromosome arms where it is involved in mitotic chromosome structure. 2. In prometaphase and metaphase, CPC is at inner centromeres and required for the formation of a bipolar spindle and kinetochore-microtubule attachment. 3. In anaphase, the CPC translocates to the spindle midzone and appears at the cortex; it is involved in the formation of the central spindle. 4. In telophase, the CPC concentrates at the cleavage furrow and, subsequently, at the midbody, where it is required for completion of cytokinesis. Chromosomes, blue; tubulin, red; nuclear envelope, grey. (Adapted from Ruchaud *et al.* 2007)
Chromosomal passengers and kinetochore-microtubule attachment

Microtubule attachment to the kinetochore requires several important groups of proteins (Discussed in the centromere and kinetochore section), including the chromosomal passengers. It has been reported that Ipl1 promotes the turnover of kinetochore microtubule interactions that do not generate tension (Tanaka et al., 2002), and that Ipl1 activity is required to detach microtubules from tension-defective kinetochores (Dewar et al., 2004; Pinsky et al., 2003) and activates the checkpoint in response to tension defects by creating unattached kinetochores (Pinsky, 2006 #183). These observations are similar to studies in mammalian cell culture, where Aurora B selectively disassembles kinetochore microtubules that are attached syntelically (Lampson et al., 2004). Furthermore, impairing the function of Aurora B stabilizes kinetochore–microtubule attachments (Lampson and Kapoor, 2005; Pinsky et al., 2003). It is broadly accepted that the CPC is involved in detecting the aberrant kinetochore-microtubule attachments (Kotwaliwale and Biggins, 2006). In budding yeast, a complex of the chromosomal passenger proteins Bir1 and Sli15 (Survivin and INCENP) was shown to link centromeres to microtubules (Sandall et al., 2006). This is consistent with former studies showing that INCENP directly binds to beta-tubulin (Wheatley et al., 2001b). Destabilizing the interaction between the KMN network (KNL-1, Mis12 and Hec1/Ndc80) and microtubules depends on CPC kinase activity (Cheeseman et al., 2006; DeLuca et al., 2006). This is supposed to correct kinetochore-microtubule attachment errors. The CPC might directly be involved both in the interaction between kinetochores and microtubules and in destabilization of aberrant attachments. But there is still disagreement on this point.
Chromosomal passengers and the spindle checkpoint.

In mitosis, chromosomes must be equally and accurately segregated into two daughter cells. There is a biochemical pathway to ensure the accuracy of this conserved mechanism, termed the spindle assembly checkpoint. This checkpoint monitors whether all chromosomes have bipolar attachments and are properly aligned before the onset of anaphase. The spindle checkpoint is activated when there is either a lack of kinetochore–microtubule attachments or lack of the tension generated across the sister kinetochores by the pulling forces of the spindle (reviewed in May and Hardwick, 2006; Pinsky and Biggins, 2005; Zhou et al., 2002). If errors have occurred, cells arrest in metaphase (Lens and Medema, 2003; May and Hardwick, 2006; Nicklas, 1997). If the spindle assembly checkpoint is inactivated, unequal segregation of chromosomes can occur, possibly leading to cell death or disease. Down’s syndrome is one result of this kind of error.

The spindle checkpoint network includes Mad1, Mad2, BubR1 (Mad3 in yeast), Bub1, Bub3 and Mps1 (Hoyt et al., 1991; Li and Murray, 1991; Li and Benezra, 1996; Taylor et al., 1998; Weiss and Winey, 1996). These proteins localize to unattached kinetochores, while Bub1 and BubR1/Mad3 also localize to kinetochores lacking tension. The anaphase promoting complex/cyclosome (APC/C) activity is under the regulation of its auxiliary subunits Cdc20 and Cdh1 (Peters, 2002). BubR1 and Mad2 bind to Cdc20. This inhibits APC/C activity (Fang, 2002; Sudakin et al., 2001; Tang et al., 2001). Once stable attachment is established, the checkpoint proteins are released from the kinetochores (Skoufias et al., 2001; Taylor et al., 2001; Waters et al., 1998) and activate the APC/C. The APC/C is a multiprotein E3 ubiquitin ligase that targets the anaphase inhibitor securin, a key regulator of anaphase onset and a substrate for the APC/C (Castro et al., 2005). Securin destruction leads to release of active separase, which in turn destroys sister
chromatid cohesion and thus allows chromatids to be pulled to opposite poles.

Studies have revealed that Aurora B/Ipl1 and Survivin are also involved in the spindle checkpoint. Loss-of-function mutations of Ipl1 lead to syntelic attachments (Biggins and Murray, 2001; Chan and Botstein, 1993; Cheeseman et al., 2002; Tanaka et al., 2002). When a temperature-sensitive mutant of Ipl1 was grown at the non-permissive temperature, cells failed in chromosome segregation and proceeded through the cell cycle without activation of the spindle checkpoint (Biggins and Murray, 2001; Biggins et al., 1999). Similar phenotypes were observed in tissue culture studies. In mammalian cells, Aurora B is required for correction of chromosome mis-orientation (Ditchfield et al., 2003; Kallio et al., 2002; Lampson et al., 2004; Vagnarelli and Earnshaw, 2004). In cells treated with ZM447439 or hesparadin, inhibiting Aurora B, the spindle checkpoint was not activated in the absence of tension (Ditchfield et al., 2003; Hauf et al., 2003; Kallio et al., 2002). Carvalho et al. and Lens et al. have reported that another chromosomal passenger protein, Survivin is required to activate the spindle checkpoint in the absence of tension (Carvalho et al., 2003; Lens et al., 2003). However, a study showed Bir1-Sli15 (Survivin and INCENP in yeast) complex has no role in the kinetochore-microtubule attachment but regulate the dynamics of septin {Thomas, 2007 #271}.

The mechanism by which Aurora B and Survivin activate the spindle checkpoint in response to the absence of tension is not very clear. Studies showed that Bub1·Mad3 and Aurora B cooperate to maintain BubR1-mediated inhibition of APC/CCdc20 (King et al., 2007; Morrow et al., 2005). Mitotic arrest of Bub1-deficient cells is dependent on Aurora B, and vice versa. Assembly of checkpoint network proteins MPS1, MAD1 and MAD2 onto the kinetochore requires HEC1/Ndc80 (Martin-Lluesma et al., 2002; McCleland et al., 2003), which is negatively regulated by Aurora B.
When phosphorylation of Mad3p at two sites is prevented by site-specific mutations, the checkpoint in response to reduced kinetochore tension is abolished (King et al., 2007), similar to ipl1/Aurora B defective cells. Syntelic attachment of chromosomes was also observed in survivin depleted cells (Lens et al., 2003). In these cells BubR1 or Mad2 was not present at the kinetochores in the presence of Taxol, which stabilizes microtubules and disrupts spindle tension (Carvalho et al., 2003; Wheatley and McNeish, 2005). Another checkpoint marker, the 3F3/2 phosphoepitope, is lost prematurely from mitotic kinetochores when PtK1 cells were injected with anti-survivin antibody (Kallio et al., 2001). The chromosomal passenger complex appears to detect the tension and directly or indirectly regulate the recruitment of the spindle checkpoint network proteins to centromeres. Though many reports argue that passengers are involved in the spindle assembly checkpoint, the exact role of the CPC in the spindle checkpoint remains to be determined.

**Translocation of chromosomal passengers**

Chromosomal passenger complex targeting and relocation depends on its components, but is also regulated by several other important proteins. MKLP2, a kinesin superfamily member, is essential for the CPC relocation from the centromere to the central spindle in HeLa cells (Ulrike Gruneberg, 2004). Similar results were obtained in Drosophila. Mutants of subito (the Drosophila MKLP2 homologue) failed to localize Aurora B and polo kinase to the midzone, and were also defective in spindle assembly and chromosome segregation (Cesario et al., 2006). INCENP and Survivin localization is dependant on cohesin (Morrison et al., 2003; Sonoda et al., 2001) and loss of INCENP from the inactive centromere of a dicentric chromosome correlates with loss of sister chromatid cohesion (Vagnarelli and Earnshaw, 2001). Other important proteins regulate chromosomal passenger translocation by phosphorylation and dephosphorylation. In Drosophila and in
vertebrate cells, release of Aurora-B and INCENP onto the spindle midzone was blocked by non-degradable Cyclin-B (Murata-Hori et al., 2002; Parry et al., 2003). The same phenotype has not been reported in yeast, but the dephosphorylation of INCENP by Cdc14 is important for the transfer of INCENP from the centromere to the central spindle in budding yeast. The chromosomal passenger complex is under control of a strict spatio-temporal network regulating its proper function.

**Chromosomal passengers and cytokinesis**

Chromosomal passenger proteins are essential for completion of cytokinesis. Depletion or interference with the function of any member of the passenger complex in different organisms leads to failure of cytokinesis with consequent multinucleation (Carvalho et al., 2003; Gassmann et al., 2004; Honda et al., 2003; Lens et al., 2003; Schumacher et al., 1998a; Tatsuka et al., 1998; Terada et al., 1998). The phosphorylation and localization of several proteins involved in cytokinesis depends on the chromosomal passenger complex. Myosin II regulatory light chain (Murata-Hori and Wang, 2002a), MKLP1 (Guse et al., 2005; Liu et al., 2004b), MgcRac1GAP (Minoshima et al., 2003; Tatsumoto et al., 1999) and vimentin (Yasui et al., 2004) are Aurora B substrates. Non-phosphorylated mutants of these proteins led to failure in cytokinesis. Though there are more and more proteins discovered to be essential for cytokinesis, the regulation of this process remains elusive. Further studies are needed to answer whether the chromosomal passenger complex is the scissors, or the hands controlling the scissors.

**Apoptosis**

Keeping a balance between cell division and cell death is an important feature of the maintenance of cell and tissue homeostasis. In the cell cycle section, I have discussed the checkpoints that maintain the fidelity of the genetic material. If cell cycle errors cannot be corrected, checkpoint
signalling may activate the pathway of programmed cell death. On the other hand, checkpoint defects may result in gene mutation, chromosome damage or aneuploidy. Programmed cell death is a process whose mechanism is evolutionarily conserved in virtually all cells of multicellular organisms and occurs in response to genetic or environmental cues. Death of chord and adjacent cartilage of metamorphic toads was first reported by Vogt et al in 1842. Lockshin (1965) first proposed the term 'programmed cell death' to describe a phenomenon whereby some cells were destined to die during tadpole and insect metamorphosis, as if driven by a cell-intrinsic program (Lockshin and Williams, 1965). Programmed cell death plays an essential role in embryonic development, maintenance of tissue homeostasis, establishment of immune self-tolerance, defence against pathogens and regulation of cell viability by hormones and growth factors (Pollard and Earnshaw, 2002).

Apoptosis, one of the major types of programmed cell death, is characterized by a series of distinct morphological and biochemical changes that include nuclear and cytoplasmic condensation, DNA fragmentation, and membrane blebbing. The word apoptosis was first coined by Kerr et al (Kerr, 1971). It is extremely important in many physiological processes. As many as 85% of neurons undergo programmed cell death during vertebrate nervous system development (Cowan et al., 1984) and up to 95% of immature T cells die by apoptosis without leaving the thymus (Krammer, 2000). Insufficient apoptosis can cause cancer or autoimmunity, while excessive cell death leads to acute and chronic degenerative diseases, immunodeficiency, and infertility (Danial and Korsmeyer, 2004). The molecular identities of two genes that are key to C. elegans cell death, namely ced-3 and ced-9, and their homology to vertebrate counterparts, namely the caspase family and the Bcl-2 family, have propelled apoptosis into one of the mainstreams of biological research (Degterev et al., 2003; Yuan et al., 1993).
Introduction of Survivin

Discovery

Survivin was discovered by Ambrosini in 1997 when she was doing a hybridization screen of a human genomic library using a cDNA probe to effector cell protease receptor-1 (EPR-1). The coding strand sequence of Survivin is complementary to EPR-1, and its open reading orientation is opposite to EPR-1 (Ambrosini et al., 1997). The relationship between these two genes is not clear. In a case of natural antisense regulation, the induction of EPR-1 transcript resulted in a downregulation of survivin expression, with an increase in apoptosis and an increase in the sensitivity to anticancer agents (Ambrosini et al., 1998; Yamamoto et al., 2002). Survivin is a small acidic protein (pI 5.1) with molecular weight around 16.5 kDa. Its gene has been localized to band 17q25 in human chromosome 17, spanning about 15 kb (Ambrosini et al., 1997). Survivin is conserved from yeast to humans. Its homologues have been identified in several different organisms (Table 3). This will be further discussed in the Survivin homologues section.

Expression of Survivin

Survivin is present during fetal development; but is undetectable in terminally differentiated cells and quiescent cells (Ambrosini et al., 1997). Survivin is abundantly expressed in proliferating cells and rapidly down-regulated by cell cycle arrest in G1 phase, with a peak level in the G2/M phase in a cell-cycle-regulated manner (Li et al., 1998; Wheatley and McNeish, 2005). Some studies have shown that Survivin is also detected in G1 interphase cells (Fukuda and Pelus 2001; Temme et al., 2003). However, Survivin is prominently expressed in transformed cell lines and in most common cancer cells (Ambrosini et al., 1997) as will be discussed in a later section: Survivin and cancer. Some scientists even use Survivin as a tumor biomarker (Duffy et al., 2007; Reed, 2001).
Structure of Survivin

The human survivin gene locus spans around 15 kb and includes 4 exons (Mahotka et al., 1999). The cDNA of human survivin is 426 bp and encodes a polypeptide of 142 amino acids. The chicken survivin gene locus is smaller, with just 750 bp, due to much shorter introns. However, as for the human protein the chicken Survivin consists of 142 amino acids. The Survivin N-terminus contains a single baculovirus-IAP (inhibitor of apoptosis protein) repeat domain (residues 18-88, also called a BIR domain) (Figure 9A). Therefore Survivin is also called BIRC5 or IAP4. BIR proteins belong to a large family and will be discussed in the IAP section. The N-terminus of human Survivin consists of three helices (aa14-21, 31-41, 68-80) and a three-stranded antiparallel β-sheet (aa43-45, 55-58, 61-64). Four conserved Zinc finger residues (Cys57, Cys60, His77 and Cys84) lie inside the BIR domain and are important for stabilizing the globular BIR domain structure (Chantalat et al., 2000; Verdecia et al., 2000). Expression of a Survivin with C84A BIR mutation in HeLa cells resulted in a progressive increase in caspase-3 activity and apoptosis (Li et al., 1998). The C84A mutant could not restore the spindle checkpoint when endogenous Survivin was knocked down by RNAi (Lens et al., 2006a). Another Survivin mutant in the BIR domain, DD70, 71AA was reported to abolish the direct interaction of Survivin with Aurora B. This mutant was not able to localize properly in mitosis; and its over-expression disrupted cytokinesis, resulting in multinucleation (Cao et al., 2006). In contrast to other IAPs, Survivin lacks a C-terminal ring finger (Ambrosini et al., 1997). The C-terminus of Survivin is a continuous α-helix (residues 100-142) and forms a hydrophobic patch, which is a potential site for protein-protein interaction (Figure 9B and C) (Li 1998; Chantalat L 2000) (Verdecia et al., 2000).
Figure 9. Structure of Survivin. A. The primary amino acid sequence of human Survivin and its secondary structure. B. Crystal structure of Survivin. The zinc atoms are shown as blue spheres. (Adapted from (Chantalat et al., 2000). C. Surface features of human survivin. Survivin solvent accessible surface colored to approximately reflect the underlying electrostatic potential, where blue is positive, red is negative and white is neutral (Adapted from Verdecia et al., 2000).
Both the N-terminus (Survivin 1-106) and the C-terminus of Survivin are required for its localization (Skoufias et al., 2000). Between the BIR domain and C-terminal \( \alpha \)-helix lies a linker region (residues 89-102). X-ray structural studies on human and murine Survivin crystals have demonstrated that it is a bow-tie-shaped homodimer (Figure 9). Its special structure suggests that it may function as an adaptor (Chantalat, Skoufias et al. 2000; Verdecia, Huang et al. 2000; (Wheatley and McNeish, 2005).

**Dimerization? Or nuclear export signal? Or other functions?**

Solution studies and crystal analysis of Survivin support the conclusion that it can form a dimer (Chantalat et al., 2000; Verdecia et al., 2000). Song et al have also reported that Survivin forms a heterodimer in vivo (Song et al., 2004). But the structural studies show that it does not dimerize by means of the C-terminal coiled-coil. Chantalat and colleagues found that three segments are involved in the monomer-monomer interaction: the N-terminal region, the linker region, and the N-terminus of the \( \alpha 4 \) helix (Figure 9A). The molecular interaction surface is mostly hydrophobic. In the core interaction region, four hydrogen bonds between the main chains were observed (Figure 10) (Chantalat et al., 2000). Multiangle laser measurements of molar mass revealed that the double site mutant of human Survivin P101A L102A could not dimerize (Engelsma et al., 2007). Dimerization of Survivin was also stabilized by the interaction of the two residues Glu76 and His80 with Zn\(^{2+} \) (Muchmore et al., 2000). Transient expression of Survivin E76A or H80A mutants in HeLa caused spontaneous apoptosis. Therefore Survivin appears to be able to form a dimer.
Several studies discovered that the linker region is also involved in nuclear export in a Crm1-dependent pathway. Survivin-GFP microinjected into nuclei was quantitatively exported into the cytoplasm, while the L96A L98A double-site dominant-negative mutant was not. The export of Survivin-GFP could be blocked by the Crm1 inhibitor leptomycin B (Colnaghi et al., 2006; Knauer et al., 2006a; Knauer et al., 2006b). Another report, however, claimed the dominant-negative the murine NES-deficient (L96A V98A) Survivin mutant is still able to form homo- as well as heterodimers and interacts with Aurora B (Stauber et al., 2006). To date, the studies on Survivin linker region are still controversial, though the linker region is highly conserved. This region might be involved in both dimerization and nucleo-cytoplasmic shuttling. It may be involved as well in interaction with other proteins.

Posttranslational Modification of Survivin

Modifications of Survivin after translation have been reported, including phosphorylation and ubiquitination. Survivin physically associates with the cyclin-dependent kinase p34 (CDK1) and is phosphorylated by p34-cyclin B1 on Thr34 both in vitro and in vivo. This residue is conserved from C. elegans to human (Figure 11). Using a specific antibody against Survivin phosphorylated on Thr34, O'Connor found that the level of phosphorylation of Thr34 peaked in M phase (O'Connor et al., 2000). Loss of phosphorylation...
on Thr34 resulted in dissociation of a survivin-caspase-9 complex, while forced expression of the Survivin non-phosphorylatable T34A mutant in HeLa, A594, MCF-7 and YUSEC2 melanoma cells slowed cell growth and induced greater apoptosis, based on sub-G1 population increase, chromatin condensation and caspase-9 cleavage. However the caspase inhibitor, Z-VAD-fmk, blocked these changes (Blanc-Brude et al., 2003; Liu et al., 2004a; Mesri et al., 2001; O'Connor et al., 2000; Wall et al., 2003). Similarly, addition of the CDK1 inhibitor flavopiridol, which prevents phosphorylation on Thr34, increased apoptosis induced by doxorubicin, Taxol and UV-irradiation in MCF-7 and HeLa cells (Wall et al., 2003). Controversially, another group reported that T34A, the non-phosphorylatable Survivin mutant, still functioned as well as wild type as an anti-apoptotic factor, though it caused aberrant execution of cytokinesis and failed to localize on centromeres in HeLa cells (Temme et al., 2003). The data on Survivin threonine34 mutants has yet to give a clear idea on its function in mitosis and apoptosis, even though mutations of this residue appears to have effects on these process.

In our lab, Dr Wheatley mapped an Aurora B phosphorylation site in Survivin using an in vitro kinase assay and mass spectrometry analysis. Human Survivin is specifically phosphorylated at threonine 117 by aurora-B kinase. Mutation of residue 117 from threonine to alanine (T117A) did not alter its localization in HeLa cells, but the phospho-mimic mutant threonine117 to glutamic acid (T117E) could not localize properly in mitosis. Both Survivin mutants at threonine117 lose the ability to interact with INCENP in vitro (Wheatley et al., 2004). However, FRAP experiments showed that the Survivin T117A mutant was stably associated with centromeres. Survivin T117A expression induced a prometaphase arrest in cells depleted of endogenous Survivin (Delacour-Larose et al., 2007). The function of T117 phosphorylation is still controversial. One group found that Survivin T117A was unable to rescue the phenotypes of cells depleted endogenous Survivin (Delacour-Larose et al., 2007), while in another report,
non-phosphorylatable Survivin T117A could still support cell proliferation when the endogenous wild type Survivin was knocked down (Wheatley et al., 2007). On the other hand the phospho-mimic mutant, Survivin T117E could not restore viability, nor could it complement chromosome congression and spindle checkpoint defects due to depletion of endogenous Survivin. This result suggests that once Survivin is phosphorylated at Thr117, dephosphorylation at this residue is also crucial for chromosome congression and progression into anaphase (Wheatley et al., 2007). However this site is only conserved in mammals. More data is still needed to verify the phosphorylation in vivo and to resolve the controversy over its function.

With the exception of phosphorylation at T34 and T117, no other data on phosphorylation sites on Survivin has been published to date. However there are other conserved threonines and serines in Survivin. T21 (conserved) and T127 (conserved in mammals) are predicted sites for phosphorylation by protein kinase C. Conserved T48 and T97 are predicted sites for casein kinase-2 phosphorylation. GFP-tagged Survivin T97A and T97E localize in both the cytoplasm and nuclei, but compared with the wild type Survivin it is predominantly cytoplasmic. Survivin T97A and T97E mutants have been reported to increase sensitivity to X-irradiation, but they are still able to inhibit caspase-3 activity (Colnaghi et al., 2006). The reason for this controversial result is not clear. T48 and T88 are also predicted sites for CDK1, and S81 is a predicted site for protein kinase A. However, in all cases, these predicted sites need experimental verification (http://kinasephos.mbc.nctu.edu.tw/) or (http://bioinformatics.lcd-ustc.org/PPSP/).

Cell cycle regulation of Survivin levels

The amount of Survivin is cell-cycled regulated. After mitosis, the Survivin levels decrease (Li et al., 1998). Hence, many studies focused on Survivin ubiquitination for its degradation (Wang et al., 2004). Survivin accumulates
when cells are treated with proteasome inhibitors (Zhao et al., 2000). Ubiquitination of Survivin has been reported in vitro, and Smac/DIABLO can accelerate Survivin clearance (McNeish et al., 2005). However one report demonstrates that the ubiquitination of Survivin is involved in the regulation of mitosis by governing the dynamics of Survivin. In mitosis, Survivin is ubiquitinated by both Lys48 and Lys63 ubiquitin ligases. Association of Survivin with centromeres requires the Lys63 ubiquitination under the mediation of the ubiquitin binding protein Ufd1 that interacts with Survivin (Vong et al., 2005). hFAM, a de-ubiquitinating enzyme that co-immunoprecipitates with Survivin, mediates chromosome alignment and segregation by controlling both the dynamic association of Survivin with centromeres and the proper targeting of Survivin and Aurora B to centromeres. Lys63 de-ubiquitination, controlled by hFAM, is essential for the dissociation of Survivin from centromeres after metaphase (Vong et al., 2005). Therefore the dynamic association of chromosomal passengers with centromeres is mediated by ubiquination and de-ubiquination, and sequentially regulates chromosome alignment and segregation. Moreover the ubiquitination of Survivin by the Lys63 ubiquitin ligase is not involved in Survivin degradation (Vong et al., 2005). There are 16 lysines residues in human Survivin, of which nine (K23, K62, K78, K79, K90, K91, K110, K112, and K115) are highly conserved. Currently, it is unknown which lysines of Survivin are ubiquitinated, and it is also not clear whether the elimination of Survivin in a ubiquitin-proteasome-dependent manner is critical or not.

Homologues and orthologues of Survivin

Survivin orthologue in yeast

Survivin orthologues in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* were discovered by genome searches for BIR-containing proteins, both were given the name Bir1 (Rajagopalan and Balasubramanian, 1999; Uren et al., 1998). The different names given to yeast Survivin can be
confusing. People use different aliases for Survivin. In this manuscript, I use Bir1 to refer to both *S. pombe* and *S. cerevisiae* Survivin. Their aliases and other genetic information are listed in Table 3.

Bir1 behaves as a passenger protein in mitosis and is essential in yeast. Bir1 mutants fail to localize Ark1 (The Aurora B homologue in fission yeast) to centromeres (Morishita et al., 2001); and dysfunctional Bir1 leads to failure in chromosome condensation and chromosome segregation (Morishita et al., 2001; Petersen and Hagan, 2003; Rajagopalan and Balasubramanian, 1999; Uren et al., 1999). Like Survivin in animals, Bir1 is also a component of a complex consisting of Sli15 (INCENP) and Ipl1 (Aurora B) in *S. cerevisiae* (Cheeseman et al., 2002; Thomas and Kaplan, 2007) (Sandall et al., 2006). Even though Bir1 is not essential for budding yeast vegetative growth, it is essential for meiosis (Uren et al., 1999): *S. cerevisiae* cells deleted of Bir1 although viable, suffer a chromosome-loss and metaphase-anaphase transition defect (Uren et al., 1999; Yoon and Carbon, 1999).

Bir1 in yeast is much larger than its mammalian counterparts, and has two BIR domains (Uren, Coulson et al. 1998; Uren AG1999). It was reported that yeast cells lacking Bir1 are more sensitive to apoptosis induced by oxidative stress. Consistently, overexpression of Bir1 reduces apoptosis-like cell death (Walter et al., 2006). The mechanism of cell death in yeast remains far from clear.

**Survivin homologue in *C. elegans***

*C. elegans* BIR1 was firstly identified and characterized by Fraser and colleagues, and is 37% identical to human Survivin. The worm BIR1 is highly expressed during embryogenesis, with detectable expression throughout other stages of development (Fraser et al., 1999). Embryos lacking BIR1 are unable to complete cytokinesis and become multinucleated. However,
transgenic expression of human Survivin can partially suppress the cytokinesis defect of worm embryos.

<table>
<thead>
<tr>
<th>Specie</th>
<th>Name</th>
<th>Size</th>
<th>Disruption phenotype</th>
<th>Accession/NCBI number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Galus gallus</em></td>
<td>Survivin</td>
<td>420 bp 142 aa 16.5 kDa</td>
<td>No data</td>
<td>AAG42494/Gl:1192273</td>
</tr>
<tr>
<td><em>homo sapiens</em></td>
<td>Survivin/ Birc5/ IAP4</td>
<td>420 bp 142 aa 16.5 kDa</td>
<td>Multinucleation apoptosis</td>
<td>U7828.1/NM_001168</td>
</tr>
<tr>
<td><em>Mus musculus</em></td>
<td>Survivin/ Birc5/ IAP4/ TIAP</td>
<td>420 bp 140 aa</td>
<td>Embryonic lethal; Multinucleation; Disruption in microtubule oraganization</td>
<td>AF115517.1/Gl:4588767</td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>xSurvivin/ xBir/Su1</td>
<td>480 bp 160 aa 18 kDa</td>
<td>No data</td>
<td>AF442492/Gl:25990776</td>
</tr>
<tr>
<td></td>
<td>SIX/Su2</td>
<td>471bp 157 aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>Deterin</td>
<td>459 bp 153 aa</td>
<td>apoptosis</td>
<td>NP_650608</td>
</tr>
<tr>
<td><em>Caenorhabditis elegans</em></td>
<td>Bir1</td>
<td>265 bp 155 aa 17.7 kDa</td>
<td>Embryonic lethal; Multinucleation; Defects in chromosome segregation</td>
<td>UB85911.1/Gl:2738001</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Bir1/Bir1p</td>
<td>2862 bp 954 aa 108.6 kDa</td>
<td>Defects in chromosome condensation and segregation; aneuploidy</td>
<td>AAB39312.1/Gl:1019708</td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>Bir1/Bir1p/Cut17/Pbh1</td>
<td>2991 bp 997 aa 112.5 kDa</td>
<td>Essential; Defects in chromosome condensation and segregation; “cut” phenotype</td>
<td>AB031034/CAA20432</td>
</tr>
</tbody>
</table>

Table 3. Aliases and Homologues of Survivin. Adapted from (Wheatley and McNeish, 2005).
Worm BIR1 makes up a complex with CSC-1 (the possible orthologue of Borealin in C elegans), AIR-2 (the orthologue of Aurora B in C elegans), ICP-1 (the orthologue of INCENP in C elegans) (Romano et al., 2003). Similar to vertebrate Survivin, BIR-1 localizes AIR-2 to chromosomes and the spindle midzone. In the absence of BIR-1, AIR-2 was not present on chromosomes and histone H3 phosphorylation staining was reduced (Speliotes et al., 2000). However, ectopic expression of BIR-1 failed to suppress cell death in vivo, whereas inhibition of BIR-1 expression did not increase cell death (Fraser et al., 1999). To date, there is no data to suggest that C elegans Bir1 is involved in the apoptosis pathway.

Survivin in Drosophila melanogaster

A Survivin homologue has been identified in Drosophila. Deterin has one BIR domain (Jones et al., 2000). Deterin differs from its vertebrate counterparts at both the N- and C-termini, but it is 45% identical to human Survivin. The deterin transcript is expressed throughout early stage embryos, but it becomes progressively restricted to the central nervous system and gonads in later stage embryos. Expression of deterin can prevent insect SF9 and S2 cells from apoptosis induced by the caspase-dependent apoptosis activator reaper or by cytotoxic agents. When cells are depleted of deterin by RNAi or transfected with its C-terminal domain, cell survival is strongly reduced (Jones et al., 2000). HeLa cell death induced by Survivin depletion can be partially complemented by Deterin (Jiang et al., 2001). To date, relatively little data on deterin is available. Whether or not it is involved in the cell cycle is still unknown.

Survivin in Xenopus laevis

The xSurvivin cDNA was identified from a Xenopus EST library by its high homology to human and mouse Survivin, and was then isolated from a Xenopus stage 11.5-14 cDNA library by PCR. At the same time, its cDNA was also generated independently from stage VI oocyte mRNA by another
Survivin homologues have also been identified in other organisms (http://www.ensembl.org/Homo_sapiens/geneview?gene=ENSG00000089685). The chicken homologue of the Survivin consists of 142 amino acids and is 61% identical to human Survivin. The BIR domain is well conserved (81%). The chicken Survivin gene is localized on chromosome 3, spanning 750 bp. The alignment for the amino acid sequence of animal Survivin homologues is
shown in Figure 11.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ce</td>
<td>bir-1</td>
<td>MAPC1KKSDMARTPPDDMSKHFEDRDPPDAKESQAVIATG0-VS</td>
</tr>
<tr>
<td>Xl</td>
<td>survivin</td>
<td>MYSXNRFQCVQQLQQFDKNDYDAEALADETTN-XSSXKIKSYM1</td>
</tr>
<tr>
<td>Dr</td>
<td>survivin</td>
<td>MDVSDDDDEKMYFENQEVWVEFG-VYLYVIVSLK</td>
</tr>
<tr>
<td>Gg</td>
<td>survivin</td>
<td>MHDYPRKVELNLYVLTVVAAAPRTFG-AAYPLSAKLY1</td>
</tr>
<tr>
<td>Cf</td>
<td>survivin</td>
<td>MCIAPTLPKQFFLKDHEEAKHNPLFLG-SVPWATZ911</td>
</tr>
<tr>
<td>Mm</td>
<td>survivin</td>
<td>MCAIFPHQVLKILKHAIASKNQLPLD-1A20</td>
</tr>
<tr>
<td>Rn</td>
<td>survivin</td>
<td>MCAITALPIQKNKLHDETAYKKNPLDG-SAL121</td>
</tr>
<tr>
<td>Hm</td>
<td>survivin</td>
<td>MCAIPLPPKQFRLKHEEITKNPLDLG-SAB121</td>
</tr>
</tbody>
</table>

**Figure 11. Amino acid sequence alignment of Survivin homologues in animals.** The species abbreviations are: ce, Caenorhabditis elegans; xl, Xenopus laevis; dr, Danio rerio; gg, Gallus gallus; rn, Rattus norvegicus; cf, Canis familiaris, mm, Mus musculus; and hm, Homo sapiens. The four conserved residues binding Zinc ion are labeled * in the last line. Three conserved residues (D53, D70, D71) which were reportedly pro-apoptotic are also indicated with “D” in the last line. T34 and T117 residues in human Survivin, which were previously reported to be phosphorylated, are also indicated in cyan squares, as are the predicted phosphorylation sites T21, T48, S81, T97, T127. (Adapted from Cao et al., 2006)).

**Survivin Isoforms**
In addition to Survivin (142 amino acids), four other splicing isoforms have been reported in human cells: Survivin-2α (128 amino acids), Survivin-2β (165 amino acids), Survivin-δEx3 (137 amino acids) and Survivin-3β (120 amino acids) (Mahotka et al., 1999; Noton et al., 2006). Survivin-2α and Survivin-3β are predicted to be truncated forms, while Survivin-2β and Survivin-δEx3 are the results of alternative splicing at exons 2 and 3. Survivin-2β has an additional exon (part of intron 2) between exons 2 and 3. Survivin-δEx3 lacks exon 3. Wild type Survivin, Survivin-2β and Survivin-δEx3 have been detected in renal cell carcinomas and commonly used cell lines, including HeLa and U2OS (Krieg et al., 2002; Mahotka et al., 1999). In breast, colorectal, head and neck cancer, and in lymphoma and leukemia patients, Survivin and Survivin-2β were found to be overexpressed (Knauer et al., 2007), but wild type Survivin was the predominant form detected in most malignant tissues. The levels of each of the isoforms vary among different tumour tissues (Li, 2005).

The localization of Survivin isoforms in cells has been reported in a number of studies. GFP-tagged Survivin-2β and Survivin-δEx3 are diffuse throughout the cytoplasm at all stages of the cell cycle. In contrast to wild type Survivin, Survivin-2β and Survivin-δEx3 do not localize on centromeres and do not concentrate on the midbody (Mahotka et al., 2002; Noton et al., 2006). Another group has also reported that GFP-tagged Survivin-2β localizes to mitochondria and in the microtubule organizing centre (MTOC) (Ling et al., 2007). Some also argue that Survivin-δEx3 prefers to stay in the nucleus (Mahotka et al., 2002). There is still disagreement concerning the localization of Survivin isoforms.

The function of Survivin isoforms also remains unclear. Survivin-2α, Survivin-2β and Survivin-δEx3 do not interact with chromosomal passenger proteins in vivo (Knauer et al., 2007; Noton et al., 2006). Only survivin-3β is
reported to efficiently interact with chromosomal passenger complex (CPC) proteins (Knauer et al., 2007). Functional studies have also been carried out by over-expressing these Survivin isoforms in cells. U2OS cells stably expressing Survivin-2β and Survivin-δEx3 proliferate normally. Survivin-2β and Survivin-δEx3 are not essential and cannot compensate for loss of wild type Survivin (Noton et al., 2006). However another group has reported that Survivin-2β behaves as a pro-apoptotic molecule. Forced expression of survivin-2β blocked tubulin polymerization, decreased the mitotic population, inhibited cell growth and induced mitochondrion-dependent apoptosis by releasing Smac from mitochondria and downregulating Bcl-2 in HeLa and MCF7 cells (Ling et al., 2007; Mahotka et al., 1999). Survivin-δEx3 was reported to protect HepG2 cells from apoptosis induced by methotrexate (Mahotka et al., 1999; Malcles et al., 2007). Survivin-δEx3 is reported to act as an adaptor, allowing the formation of a complex between Bcl-2 and activated caspase-3, which inhibits the activity of caspase-3 (Malcles et al., 2007). The conflicting results coming from different laboratories might be due to the presence of the endogenous Survivin in these overexpression studies, the expression level or other unknown factors. More data is needed to resolve this controversy.

Survivin isoforms have also been reported in mice and chicken, but these forms are different from the human variants. Six splice variants were identified in chicken granulosa cells (Johnson et al., 2002). Chicken Survivin-β (76 amino acids) only contains the first two exons. Chicken Survivin-δ encodes 150 amino acids, which has an insertion after exon 3 and changes reading frame. Chicken Survivin-s just has exon 1 (39 amino acids). Survivin-γ has a 129 bp insertion after exon 1, which encodes 59 amino acids (Johnson et al., 2002). Another isoform (135 amino acids) has a different exon 4. To date, there is no report on the function of these different chicken Survivin isoforms.
Localization of Survivin

Survivin has a very intriguing localization in mitotic cells, like other chromosomal passenger proteins. In prometaphase and metaphase Survivin targets to centromeres. In anaphase it transfers to the cell cortex and midzone, and it concentrates on midbodies in telophase. Survivin is degraded when daughter cells are separated (Figure 12) (Skoufias et al., 2000; Temme et al., 2003; Uren et al., 2000; Wheatley et al., 2001a). In interphase human cells, Survivin is found both in the cytoplasm and the nucleus (Chiou et al., 2003; Dohi et al., 2004; Falleni et al., 2003; Fortugno et al., 2002; Frost et al., 2002; Lo Muzio et al., 2003; Okada et al., 2004; Takai et al., 2002; Temme et al., 2003). However, GFP-tagged human Survivin is predominantly cytoplasmic (Beardmore et al., 2004; Rodriguez et al., 2006; Skoufias et al., 2000; Wheatley et al., 2001a). Survivin was also reported to exist in a mitochondria pool in tumor cells (Dohi et al., 2004). The localization of Survivin during interphase maybe due to a CRM1-mediated nucleocytoplasmic shuttling pathway (Rodriguez et al., 2006; Rodriguez et al., 2002). Two laboratories have identified the nuclear export signal (NES) in human Survivin (Colnaghi et al., 2006; Knauer et al., 2006a; Knauer et al., 2006b). Mutations of L98 to A or addition of the Crm1 inhibitor leptomycin B blocks the export of Survivin-GFP from nucleus to the cytoplasm. In mitosis, the NES and the interaction between Survivin and CRM1 are required for localization of Survivin and the chromosomal passenger complex (Knauer et al., 2006a; Knauer et al., 2006b). Whether the interaction between CRM1 and Survivin has a role in apoptosis regulation is unknown at present, but it is reported that a Survivin NES mutant failed to protect tumor cells against chemo- and radiotherapy-induced apoptosis (Colnaghi et al., 2006; Knauer et al., 2006a; Knauer et al., 2006b). It was proposed that different localized forms of Survivin might accomplish different functions (Colnaghi et al., 2006; Knauer et al., 2006b; Li et al., 2005a).
Figure 12. Localization of Survivin in mitosis. Prometaphase, metaphase, anaphase and telophase DT40 cells were stained with INCENP (red), DNA (blue) and Survivin (green). The localizations of Survivin are shown by arrows in different phases of mitosis.

Survivin is also a chromosomal passenger protein and plays an important role in mitosis

Targeting the chromosomal passenger complex

The location and translocation of chromosomal passengers depends on several proteins, and on the passengers themselves (discussed in the CPC section above). Several reports have shown that Survivin plays a critical role in directing the localization of the chromosomal passenger complex. As one member of chromosomal passenger complex, Survivin plays an important role to mediate the mitotic localization of the complex (Carvalho et al., 2003;
Klein et al., 2006; Knauer et al., 2006a; Vader et al., 2006a). A chimeric protein of Survivin fused to INCENP can target the chromosomal passenger complex to centromeres and mid-body in the absence of both Borealin and the centromere-targeting domain of INCENP. These observations suggested that Survivin might mediate the targeting of the whole complex (Vader et al., 2006a). The chromosomal passengers' dynamic association with centromeres is also mediated by ubiquination and de-ubiquination of Survivin, which sequentially regulates chromosome alignment and segregation (Vong et al., 2005). Survivin is the critical guide of the chromosomal passenger complex and an important of mitotic regulation, so the chromosomal passenger complex can function in the right place at the right time.

**Deletion of Survivin leads to cell division failure**

Several methods have been used to study Survivin function. Those approaches include knockout, knockdown or inactivation by microinjection of Survivin antibodies.

When the Survivin gene is disrupted in mice, the null embryo becomes polyploid, shows impaired spindles, and dies at day E4.5. This phenotype is similar to mice in which INCENP is disrupted (Uren et al., 2000). Another report also confirmed that lack of Survivin resulted in mouse embryo death (Zwerts et al., 2007). In worms, embryos lacking Bir1, the Survivin homologue in C elegans, fail in cytokinesis and became a multinucleated single cell embryo (Fraser et al., 1999). Deletion of Survivin homologue Bir1 in yeast resulted in a similar phenotype: chromosome segregation defects and aneuploidy (Li et al., 2000).

Another widely used method to knock down Survivin levels is RNAi or antisense RNA transfection in mammalian cells. The dominant phenotype is defects in chromosome segregation, failure in cytokinesis and polyploidization (Carvalho et al., 2003; Honda et al., 2003; Li et al., 1998).
Survivin has been reported to contribute to Aurora B activity in Xenopus laevis and fission yeast (Bolton et al., 2002; Petersen and Hagan, 2003). Survivin and its yeast homologue Bir1 are required for spindle assembly checkpoint function (Lens et al., 2003; Petersen and Hagan, 2003), but the exact role of Survivin in mitosis still needs more study.

**Survivin is an inhibitor of apoptosis**

**IAP (Inhibitor of apoptosis proteins)**

Survivin plays an important role in mitosis, but Survivin is also considered a possible element that links cell proliferation and cell death (Li et al., 1998; Wheatley and McNeish, 2005). Survivin was originally classified as a member of the inhibitor of apoptosis protein family (IAP), which is a subgroup of BIR (baculovirus IAP repeat) containing proteins. To date, eight IAP members have been identified in mammals. IAPs contain one to three BIR domains (Crook et al., 1993). All BIR motifs contain a core of 65 amino acids that includes four short α-helices and a three-stranded β-sheet. Three cysteines and a histidine make up of a Zinc finger in the BIR domain (Luque et al., 2002). In addition to the BIR domain, XIAP, cIAP1, cIAP2 and ML-IAP also have a RING (really interesting new gene) motif at their C-terminus. However, Survivin lacks a carboxyl-terminal RING finger and contains only one BIR domain (residues 18-88), which resembles the Bir2 domain of XIAP (Ambrosini et al., 1997; Crook et al., 1993).

IAP family proteins were reported to inhibit apoptosis by binding caspases, the central components of the apoptotic mechanism (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001). This would be an important function. Aberrant activation of caspases is lethal to cells. Activity of IAPs is regulated by RHG (reaper, hid, and grim) proteins: Smac/DIABLO in mammalian cells. Smac/Diablo is a mitochondrial protein that is released into the cytosol in
response to some apoptotic stimuli. Smac/DIABLO can directly bind Bir2 and Bir3 domains of IAPs, removing the inhibition of caspases (Du et al., 2000; Liu et al., 2000; Verhagen et al., 2000; Wu et al., 2000). Hence, apoptosis is under strict control. For the details of the interaction between caspases, IAPs and Smac/DIABO, see the review by Shiozaki and Shi 2004; Wheatley and McNeish 2005.

**Survivin’s role in apoptosis**

As a BIR-containing protein, people have paid much attention to Survivin’s apoptotic role. Studies have shown that cells overexpressing Survivin are resistant to a variety of apoptotic stimuli, whereas loss of Survivin expression by RNAi, or forced expression of BIR mutants T34A and C84A causes spontaneous apoptosis or sensitizes cancer cells to variety of apoptotic stimuli in HeLa, U251 and MCF-7 cells (Beltrami et al., 2004; Carvalho et al., 2003; Jiang et al., 2001; Li et al., 1998; Mahotka et al., 1999; Mirza et al., 2002; Song et al., 2004; Temme et al., 2003), and leading to increased caspase-3 activity in HeLa and A594 cells (Conway et al., 2000; Li and Altieri, 1999; Olie et al., 2000). Survivin was also found to be overexpressed in a wide variety of tumours (Ambrosini et al., 1997; Li, 2003). The mechanism through which Survivin inhibits apoptosis remains unclear. Studies have shown that Survivin like other IAPs was able to bind caspase-3 and caspase-7 and inhibit apoptosis induced by a wide variety of stimuli (Li et al., 1998; Tamm et al., 1998; Xu et al., 1998). Other studies disagree with this (Banks et al., 2000; Song et al., 2003b; Verdecia et al., 2000). Another report showed that Survivin might interact with caspase-9 in an indirect way. Survivin can make a complex with hepatitis B X-interacting protein (HBXIP), and this complex inhibits the activation of procaspase-9 (Marusawa et al., 2003). However, the interaction between Survivin and caspases is still controversial (Song et al., 2003b).
Similar to other IAPs, Survivin was also reported to bind Smac/DIABLO both in vitro and in vivo (Du et al., 2000; McNeish et al., 2005; Song et al., 2003a; Song et al., 2003b; Sun et al., 2005). Survivin and Smac/DIABLO co-localized within the cytosol (Song et al., 2003b) and mitochondria during interphase. The interaction between Survivin and Smac/DIABLO requires the RHG motif of Smac and the BIR domain of Survivin (McNeish et al., 2005; Song et al., 2003b). The BIR domain site mutant, D71R and the truncated mutant Survivin-BIR abolished the interaction with Smac, leading to failure to inhibit apoptosis induced by Taxol in HeLa cells (Song et al., 2003b). Expression of another BIR mutant, D53A, that is not able to bind Smac/DIABLO, increased apoptosis induced by TRAIL, doxorubicin, and RIP3 in HeLa, A549 and H1299 cells (Song et al., 2004). Adenoviral delivery of Smac alone could induce apoptosis, but neither reduction in Survivin level by RNAi nor over-expression of Survivin had any impact on this Smac-mediated apoptosis (McNeish et al., 2005). This report suggests that survivin may not act to inhibit Smac-mediated apoptosis (McNeish et al., 2005).

However, not all IAPs have been confirmed to play a critical role in apoptosis (Salvesen and Duckett, 2002). Survivin homologues in S. pombe (Rajagopalan and Balasubramanian, 1999), C. elegans (Fraser et al., 1999), Drosophila (Jones et al., 2000) and Xenopus (Bolton et al., 2002) do not show an anti-apoptotic function. In short, the role of Survivin in apoptosis is still controversial and unclear.

**Survivin in development and differentiation**

Survivin is an essential gene. When Survivin was deleted in mice, degeneration was observed in E2.5 day embryos. By days E5.5 and E6.5, all survivin−/− embryos were grossly abnormal and showed deteriorated cell masses and giant cells (Uren et al., 2000). Survivin may also be involved in angiogenesis and cardiogenesis. In another mouse knockout study, Survivin
knockout embryos displayed hemorrhages from day 9.5 (E9.5) that were prominent and diffuse, and the embryos died before E13.5. Heart development was strikingly abnormal in these knockout embryos. Survivin-null endocardial lineage cells could not undergo a normal epithelial-mesenchymal transformation (EMT), resulting in hypoplastic endocardial cushions and in utero heart failure (Zwerts et al., 2007). Survivin knockout mice also had nerve development problems. 30% of mutant embryos had a neural tube closure defect. Thus, survivin is crucial for normal embryonic angiogenesis, cardiogenesis, and neurogenesis (Zwerts et al., 2007). Survivin is also required for normal hematopoiesis. Survivin is expressed throughout the cell cycle in normal CD34(+) hematopoietic stem and progenitor cells stimulated by growth factors. Forced expression of Survivin in primary mouse marrow cells increased granulocyte macrophage-colony-forming units (Fukuda et al., 2002; Fukuda and Pelus, 2001; Fukuda and Pelus, 2002). In Xenopus, transgenic expression of Su1 (xSurvivin) leads to an enlargement of the tadpole’s blood vessels with an increase in the number of endothelial cells (Du Pasquier et al., 2006). Survivin might regulate development via its role in mitotic progression.

Increasing data reveals that Survivin is involved in differentiation. Incomplete cytokinesis has been documented in Drosophila oocyte development, mammalian megakaryocytes, cardiac myocytes and vascular smooth muscle cell development (Glotzer, 2001; Ravid et al., 2002). Actually mammalian sperm are also produced by a pathway with incomplete cytokinesis (Pollard and Earnshaw, 2002). The underlying molecular mechanism is not clear, but Survivin appears to be involved. Bone marrow megakaryocytes are terminally differentiating cells that lack Survivin at any stage of the endomitotic cell cycle (Zhang et al., 2004). Megakaryocytes apparently skip late anaphase and cytokinesis during endomitosis though Aurora B was normally expressed and correctly localized at prophase in polyploidizing mouse bone marrow megakaryocytes (Zhang et al., 2004).
However erythroid cells follow a different maturation path, even though they and megakaryocytes arise from a common progenitor. The erythroid cells express Survivin throughout maturation, whereas Survivin protein is not detectable in the megakaryocytes (Gurbuxani et al., 2005). While over-expression of survivin in mouse bone marrow cells blocked megakaryocyte growth, maturation, and polyploidization, it had no effect on erythroid development. On the other hand, knockdown of survivin by RNAi interfered with the formation of erythroid cells but not megakaryocytes (Gurbuxani et al., 2005). In vascular smooth muscle cells, another polyploidizing cell line, similar phenotypes were observed (Nagata et al., 2005). Aurora B and INCENP are properly co-localized in the centromeres, the midzone, and the midbody during mitosis in these cells. But Survivin levels are low and the protein does not co-localize with Aurora B and INCENP. Interestingly, this polyploidization in vascular smooth muscle cells can be overridden by ectopic expression of Survivin. However, quantitative PCR analysis reveals that the mRNA level of survivin is not downregulated in these cells, suggests that Survivin is regulated at the translation or protein level (Raslova et al., 2007). Therefore, Survivin might play an important part in the regulation of development and differentiation. These reports highlight some variations of cytokinesis, which may occur in a Survivin-dependent manner.

Survivin and cancer

Survivin has been found to be over-expressed in a wide variety of tumors including lung, breast, colon, stomach, bladder, esophagus, pancreas, liver, uterus, ovaries, Hodgkin's Disease, non-Hodgkin's lymphoma, leukemias, neuroblastoma, soft-tissue sarcomas, gliomas and melanomas (for review, see Li 2003. The Survivin transcript was the fourth most frequently over-expressed transcript in cancer cells as compared with corresponding normal tissues (Velculescu et al., 1999). Some scientists regard Survivin as a potential cancer marker and a promising target for cancer therapy.
Alterations in the levels of Survivin have been observed to correlate with cell proliferation in normal cells; and its levels can be upregulated by growth factors (Fukuda et al., 2002; Fukuda and Pelus, 2001). There is no evidence to link cancer with Survivin mutants or the Survivin promoter (Xu et al., 2004; Yu et al., 2004). Also, in virally induced cancers, Survivin cannot account for the cell proliferation, because other cell-cycle dependent proteins are altered in the same way as Survivin. More and more studies are demonstrating that Survivin is not a cancer-specific anti-apoptotic protein, but a regulator of mitosis.

**DT40 cells**

DT40 cells were derived from an avian leukemia virus (ALV)-induced bursal lymphoma, and are only approximately 10 μm in diameter (Baba et al., 1985; Baba and Humphries, 1984). DT40 cells are very easy to culture, with a short generation time of around 8-10 hours. They can grow at 34°C to 43°C, allowing temperature sensitive mutants to be isolated (Winding and Berchtold 2001; Fukagawa et al., 2001). The biggest advantage of DT40 cells is that these cells have a high rate of homologous recombination, often permitting a more than 50% ratio of specific to random DNA integration event (Buerstedde and Takeda, 1991). This ratio is far higher than in most human and mouse cells (Lahti, 1999), and makes it much easier to target single or multiple genes in DT40 cells (Winding and Berchtold, 2001). The chicken genome is almost fully sequenced, which also makes the identification of chicken gene homologues easy and convenient. Many genes have been targeted in DT40 cells to date (Fukagawa and Brown, 1997; Fukagawa et al., 2001; Fukagawa et al., 2004; Hudson et al., 2003; Lahti et al., 1997; Sonoda et al., 2001).
II. Materials and methods
II. Materials and methods

2.1 Cell culture

DT40 cells were grown in suspension in RPMI 1640 medium supplemented with 10% FBS, 1% chicken serum, 1 mM glutamine and Penicillin (100 U/ml)/streptomycin (100 µg/ml) maintained in 5% CO₂ at 39 °C.

HeLa cells were maintained in RPMI 1640 (Gibco 26873) supplemented with 10% FBS, 1 mM glutamine, Penicillin (100U/ml)/streptomycin (100 µg/ml) in 5% CO₂ at 37 °C.

2.2 DT40 Cell transfection

1-2 x 10⁷ cells were centrifuged at 450 xg for 4 minutes at room temperature. After removed of medium, cells were washed once with Optimem medium (Gibco). The cells were then resuspended in 500 µl Optimem. 10-20 µg DNA (in 2-20 µl) was added in a Gene Pluser cuvette (0.4 cm) (Biorad). Cell transfection was performed by electroporation (280 volt, capacitance 950 µF) using the Gene Pulser apparatus (BioRad). After electroporation, cells were incubated in normal RPMI 1640 with 10% FBS at 39 °C. For stable cell lines, the drug used for selection was added after 24 hours.

2.3 PCR

PCR conditions (final concentration) (total volume 20 µl)
Reaction conditions are as follows: 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, pH 8.3 (25 °C), dNTP 250 µM each, Primer 250 nM, Template 5 ng and
0.2 U Taq. The procedure is: 95 °C for 2 minutes, [94 °C, 30 seconds, Tm depends on primers, 72 °C extension ] X 30-35 cycles. Primers used in my experiments are listed below in Table 4.

<table>
<thead>
<tr>
<th>Coding sequence</th>
<th>Vector</th>
<th>Primer pair</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP tag</td>
<td>pBluescript t-KS</td>
<td>5'-acattgacagagaagaccaccg-3'</td>
<td>70 °C</td>
</tr>
<tr>
<td>Gg WT Survivin-GFP</td>
<td>EGFP-N1</td>
<td>5'-acatcgataagcccccagttgcaagc-3'</td>
<td>70 °C</td>
</tr>
<tr>
<td>Gg WT Survivin</td>
<td>SV40 EGFP-C1</td>
<td>From Ciaran Morrison (Sal I &amp; KpnI)</td>
<td></td>
</tr>
<tr>
<td>Gg Survivin-T23A-GFP</td>
<td>EGFP-N1</td>
<td>Site-directed mutagenesis on pEGFP-N1-gSvn</td>
<td>88 °C</td>
</tr>
<tr>
<td>Gg Survivin-S50A-GFP</td>
<td>EGFP-N1</td>
<td>5'-gtgcttgagcccccgcagaacgacc-3'</td>
<td>84 °C</td>
</tr>
<tr>
<td>Gg Survivin-D55A-GFP</td>
<td>EGFP-N1</td>
<td>Site-directed mutagenesis on pEGFP-N1-gSvn by Ana</td>
<td></td>
</tr>
<tr>
<td>Gg Survivin-DD72, 73 AA</td>
<td>SV40 EGFP-C1</td>
<td>Cut from Gg Survivin-DD72, 73 AA (Nhe I &amp; BamH I)</td>
<td></td>
</tr>
<tr>
<td>Gg Survivin-S80A-GFP</td>
<td>EGFP-N1</td>
<td>Site-directed mutagenesis on pEGFP-N1-gSvn</td>
<td>85 °C</td>
</tr>
<tr>
<td>Gg Survivin-C86A-GFP</td>
<td>EGFP-N1</td>
<td>Site-directed mutagenesis on pEGFP-N1-gSvn by Ana</td>
<td></td>
</tr>
<tr>
<td>Gg Survivin-C86A-GFP</td>
<td>SV40 EGFP-C1</td>
<td>Cut from Gg Survivin-C86A-GFP (Nhe I &amp; BamH I)</td>
<td></td>
</tr>
<tr>
<td>Gg Survivin-P95F-GFP</td>
<td>pcDNA3- GFP</td>
<td>Given by Steuber</td>
<td></td>
</tr>
<tr>
<td>Gg Survivin-T99A-GFP</td>
<td>EGFP-N1</td>
<td>Site-directed mutagenesis on pEGFP-N1-gSvn</td>
<td>77 °C</td>
</tr>
<tr>
<td>Gg Survivin-L98AV100A-GFP</td>
<td>EGFP-N1</td>
<td>Site-directed mutagenesis on pEGFP-N1-gSvn</td>
<td>85 °C</td>
</tr>
<tr>
<td>Gg Survivin-L104AL106A-GFP</td>
<td>EGFP-N1</td>
<td>Site-directed mutagenesis on pEGFP-N1-gSvn</td>
<td>85 °C</td>
</tr>
</tbody>
</table>

Table 4. Cloning and generation of mutants.
2.4 cDNA synthesis and RT-PCR

Total mRNAs were prepared using TRIzol reagent (Invitrogen) according to the manufacturer’s recommendations. RT-PCR was done according to the Invitrogen protocol (Invitrogen #11904-018).

2.5 Construction of plasmids

The targeting vectors for deleting the survivin gene in DT40 cells were constructed by Dr. Ciaran Morrison and Dr. Ana Carvalho. Survivin genomic DNA was screened and isolated from a λ Fix II DT40 genomic library. To disrupt the Survivin gene, targeting vectors containing a selectable marker that confers resistance to neomycin, puromycin or histidinol were constructed (tv-Neo, tv-Puro and tv-HisD). The resistance cassettes were flanked by a 5'-genomic arm situated upstream of the initiation codon of the Survivin ORF and a 3'-genomic arm situated downstream of its stop codon.

2.6 Site-directed mutagenesis

Site-directed mutagenesis of Survivin was carried out by PCR, according to the instructions in the QuikChange™ Site-Directed Mutagenesis Kit from Stratagene. Mutations were verified by restriction mapping and DNA sequencing.

2.7 E.coli and transformation of competent E. coli

Top10 E.coli strain was used for plasmid amplification. E.coli was grown at 37 °C in LB media.
100 μl aliquots of competent cells were thawed on ice and mixed with ligation reactions. The cells were incubated for 20 min on ice, heat shocked for 40 seconds at 42 °C, then chilled on ice for 1 min. 1 ml LB was then added and the cells were incubated on a shaking platform at 37 °C for 1 h. After pelleting and resuspending in 100 μl LB, cells were spread on LB agar plates containing the appropriate antibiotic.

2.8 Amplification and Extraction of plasmid

After transformation of plasmids into competent E. coli, single colonies were picked and grown in LB containing the appropriate antibiotic for 16 hours at 37 °C. Plasmid DNA was isolated from these cultures using miniprep, midiprep, or maxiprep plasmid kit (Qiagen), which is based on the alkaline lysis method (Sambrook and Russell, 2001).

2.9 Sequencing

Plasmids were sequenced using the Big Dye Terminator v3.1 Cycle sequencing kit. A mix of 2 μl of Big Dye, 3 pmol of primer and 200 ng of template DNA was prepared in a total volume of 10 μl. The cycling protocol was 96 °C for 4 minutes, 25 cycles of 94 °C for 30 seconds, 51 °C for 30 seconds and 60 °C for 4 minutes, followed by an extension at 60 °C for 10 minutes. Samples were analysed in a 3100 Genetic Analyser (Applied Biosystems) by School of Biology Sciences Sequencing Service, Ashworth Laboratories, University of Edinburgh.

2.10 DNA isolation and Southern blotting

5 x 10⁶ cells were collected by centrifugation at 400 xg for 5 minutes. After the medium was removed, cells were lysed in 1 ml lysis buffer (10 mM Tris-
HCI pH7, 100 mM EDTA, 0.5% SDS). Next, Rnase was added to a final concentration of 20 μg/ml and samples were incubated for 2 hours at 37. The samples were shaken vigorously for 1 minute, 200 μl of saturated NaCl was added and shaking was repeated. Cells were then centrifuged at 20,000 xg for 10 minutes. Supernatants were transferred to new tubes and 1 volume of isopropanol was added to precipitate the DNA. Samples were centrifuged again at 20,000 xg for 10 minutes and the DNA pellet washed in 70% ethanol. After another centrifugation, the DNA pellet was air-dried and resuspended in 70 μl of distilled water. Genomic DNA was digested with the appropriate restriction enzyme, in the presence of 10 μg/ml RNase and 0.1 mg/ml BSA, overnight at 37 °C. Digested DNA and molecular weight markers (Gibco BRL) were electrophoresed on a 0.8% agarose gel (Invitrogen) in TAE buffer (0.04 M Tris-acetate, pH 7.5, 0.001 M EDTA) containing 0.3 μg/ml ethidium bromide (EB) and photographed alongside a fluorescent ruler in order to calculate the size of bands after blotting. The gel was then depurinated in 0.25 M HCl for 20 minutes and denatured in 0.5 M NaOH, 1.5 M NaCl for 20 minutes. After the gel is neutralized in 1.5 M NaCl, 0.5 M Tris-HCl 8.0, the gel was transferred to a nylon membrane (Amersham Hybond-N) in 10 x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7 adjusted with citric acid), by upwards capillary transfer. After transfer, membrane was UV cross-linked and hybridised overnight. The probe was generated by PCR using primers GsvnC (CAGCATGGTCCCAGAGAGTTC) and GsvnD (AATGCGGAGGTGAAGAGATTC). The membrane was washed and exposed to photographic film at −80 °C.

2.11 SDS PAGE gel

4X Lower gel (resolving gel/ cast gel) buffer: 1.5 M Tris-HCl, pH 8.8 with 0.4% SDS.
4X Upper gel (stacking gel) buffer: 0.5 M Tris-HCl, pH 6.8 with 0.4% SDS
<table>
<thead>
<tr>
<th>Concentration of gel</th>
<th>5%</th>
<th>7.5%</th>
<th>10%</th>
<th>12.5%</th>
<th>15%</th>
<th>16%</th>
<th>17%</th>
<th>18%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75 mm gel/8 ml/2 mini</td>
<td>Lower buffer (ml)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>30% Acryl (ml)</td>
<td>1.3</td>
<td>2</td>
<td>2.6</td>
<td>3.3</td>
<td>4</td>
<td>4.3</td>
<td>4.5</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>H₂O (ml)</td>
<td>4.7</td>
<td>4</td>
<td>3.3</td>
<td>2.7</td>
<td>2</td>
<td>1.7</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>10% APS</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>TEMED</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>1 mm gel/12 ml/2 mini</td>
<td>Lower buffer (ml)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>30% Acryl (ml)</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>6.4</td>
<td>6.8</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>H₂O (ml)</td>
<td>7</td>
<td>6</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2.6</td>
<td>2.2</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>10% APS</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>TEMED</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>0.75 mm gel/20 ml/1 big</td>
<td>Lower buffer (ml)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>30% Acryl (ml)</td>
<td>3.3</td>
<td>5</td>
<td>6.5</td>
<td>8.3</td>
<td>10</td>
<td>10.5</td>
<td>11.3</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>H₂O (ml)</td>
<td>11.7</td>
<td>10</td>
<td>8.5</td>
<td>6.7</td>
<td>5</td>
<td>4.5</td>
<td>3.7</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>10% APS</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>TEMED</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>1.5 mm gel/40 ml/1 big</td>
<td>Lower buffer (ml)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>30% Acryl (ml)</td>
<td>6.5</td>
<td>10</td>
<td>13</td>
<td>16.5</td>
<td>20</td>
<td>21.5</td>
<td>22.5</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>H₂O (ml)</td>
<td>23.5</td>
<td>20</td>
<td>17</td>
<td>13.5</td>
<td>10</td>
<td>8.5</td>
<td>7.5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10% APS</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
<td>250</td>
</tr>
</tbody>
</table>

Table 5. Recipe for lower gel. The volume is for 2 mini gel and 1 big gel.
Table 6. Recipe for upper gel.

<table>
<thead>
<tr>
<th>Stock</th>
<th>2 mini Bio-rad 0.75 mm gels</th>
<th>2 mini Bio-rad 1 mm gels</th>
<th>1 big 1.5 mm gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper gel buffer</td>
<td>0.8 ml</td>
<td>1.2 ml</td>
<td>3.6 ml</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>0.4 ml</td>
<td>0.6 ml</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>H2O</td>
<td>2 ml</td>
<td>3 ml</td>
<td>9 ml</td>
</tr>
<tr>
<td>10%APS</td>
<td>40 µl</td>
<td>60 µl</td>
<td>180 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>3 µl</td>
<td>4.5 µl</td>
<td>13.5 µl</td>
</tr>
</tbody>
</table>

SDS-PAGE running buffer: 2.5 mM Tris, 19.2 mM glycine, 0.1% SDS, pH 8.8.

SDS-PAGE transfer buffer: 0.2 M Glycine, 25 mM Tris, 0.1% SDS, 20% Methanol.

2.12 Immunoblotting

Whole cell lysates (from 0.1-0.2 x 10⁶ cells) were sonicated and boiled for 5 minutes in Laemmli sample buffer (50 mM Tris-HCl pH 6.8, 15% sucrose, 2 mM EDTA, 3% SDS, 1.43 M β-mercaptoethanol). Samples were then subjected to SDS-PAGE gel electrophoresis for 40 minutes at 200 volts. Proteins were then blotted onto nitrocellulose membranes (Amersham Bioscience). The membrane was blocked with 10% non-fat dry milk dissolved in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) or Tris-buffered saline (TBS, 137 mM NaCl, 2.7 mM KCl, 25 mM Tris, pH 7.4) for 1 hour on a shaker at room temperature. The membrane was incubated with the primary antibodies diluted in 5% milk on a shaker for 1 hour (incubated overnight at room temperature for S tag antibodies), and washed 3 times (10 minutes each) at room temperature in PBS. Then the membrane was incubated with horseradish peroxidase-linked secondary antibodies for 1 hour at room temperature. The membrane was washed 3 times in PBS/Tween-20 (1XPBS...
0.1% Tween-20). Bound antibodies were detected using ECL enhanced chemiluminescence reagent (Amersham Bioscience).

### 2.13 Indirect immunofluorescence microscopy

Cells were gently dropped onto coverslips or microscope slides treated with poly-L-lysine and incubated at 39 °C for 15-20 minutes to allow attachment. Cells then were fixed with 4% formaldehyde in cytoskeleton buffer (CB buffer: 137 mM NaCl, 5 mM KCl, 1.1 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 2 mM MgCl₂, 2 mM EGTA, 5 mM PIPES, 5.5 mM glucose, pH 6.1) and permeabilized with 0.15% Triton X-100 in CB buffer. Coverslips were blocked by immersion in PBS, 1% BSA for 30 minutes at room temperature, or overnight at 4 °C. Cells were incubated with primary antibodies diluted in blocking solution for 30 minutes at 37 °C. The coverslips or slides were washed in blocking buffer for 15 minutes (5 minutes x 3) at room temperature. Secondary antibodies (see Table 8) were diluted in blocking solution. Coverslips or microscope slides were incubated with secondary antibody for 30 minutes at 37 °C. After washes (5 minutes x 3) the coverslips or slides were mounted using Vectashield with DAPI. Image stacks were taken using an Olympus IX-70 microscope controlled by Delta Vision SoftWorx (Applied Precision) and a 100x objective (NA 1.4). Image stacks were deconvolved, quick-projected and saved as tiff images.

### 2.14 Live cell imaging

Chicken Survivin knockout cells stably expressing H2B-RFP were pre-grown on 40 mm cover slips coated with Concanavalin A (Calbiochem Cat.No 234567). After cells attached on cover slips, they were transferred into a Nikon chamber (Bioptechs) and kept at 39°C in the presence of RPMI without
phenol red (Gibco-BRL Cat. No11835). Three-dimensional data sets were collected every 2 or 10 minutes and movie frames were processed by IMAGE Analysis 6.0 software. This live cell microscopy was performed by Stefano Cardinale.

2.15 Antibodies

Table 7. Primary antibodies. Their dilutions for Immunofluorescence and Immunoblotting were listed respectively.
<table>
<thead>
<tr>
<th>Secondary Antibodies</th>
<th>Species</th>
<th>Source</th>
<th>Dilution for Immunofluorescence</th>
<th>Dilution for Immunoblotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-rabbit, HRP conjugated</td>
<td>Donkey</td>
<td>Amersham Pharmacia, NA934</td>
<td></td>
<td>1:10000</td>
</tr>
<tr>
<td>Anti-mouse, HRP conjugated</td>
<td>Sheep</td>
<td>Promega</td>
<td></td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-rabbit, FITC conjugated</td>
<td>Goat</td>
<td>Jackson Laboratories 111-096-045</td>
<td>1:200</td>
<td></td>
</tr>
<tr>
<td>Anti-rabbit, Texas Red conjugated</td>
<td>Goat</td>
<td>Jackson Laboratories 111-076-045</td>
<td>1:200</td>
<td></td>
</tr>
<tr>
<td>Anti-mouse, FITC conjugated</td>
<td>Goat</td>
<td>Jackson Laboratories 115-096-062</td>
<td>1:200</td>
<td></td>
</tr>
<tr>
<td>Anti-mouse, Texas Red conjugated</td>
<td>Goat</td>
<td>Jackson Laboratories 115-076-062</td>
<td>1:200</td>
<td></td>
</tr>
<tr>
<td>Anti-human, Cy5 conjugated</td>
<td>Goat</td>
<td>Jackson Laboratories 109-176-127</td>
<td>1:200</td>
<td></td>
</tr>
<tr>
<td>Anti-rabbit, Cy5 conjugated</td>
<td>Goat</td>
<td>Jackson Laboratories 111-176-045</td>
<td>1:200</td>
<td></td>
</tr>
</tbody>
</table>

Table 8. Secondary antibodies. Their dilutions for Immunofluorescence and Immunoblotting were listed respectively

2.16 Flow cytometry analysis

The apoptotic status of cells was assessed using TUNEL Label and Annexin V Staining Kits according to the manufacturer’s instructions (Roche).

To assess ploidy, cells were washed in PBS and fixed for 1 hour in 70% ethanol at 4°C. Cells were washed with PBS and incubated with 40 μg/ml propidium iodide (Sigma) for 30 minutes.

All samples were analysed using a Fluorescence Activated Cell Sorter (FACSCalibur, Becton Dickinson, Mountain View, CA) and Cell Quest software.
2.17 Elutriation

I adjust steady flow rate to 40ml/s and started from 3750 rpm. 1 X 10^8 Cells were suspended in 5 ml media with 1mM EDTA and put in 7.5 ml chamber. 5 minutes after cells were all loaded, adjust speed to collect different fractions.

We use Backman elutriator, JE-5 rotor.

2.18 Expression and purification of tagged Survivin

Tagged Survivin expression construct:

Human Survivin cDNA, S-tag, SBP tag and 6XHis tag were amplified by PCR then cloned into pcDNA3.1 vector. Construct structure is shown in Figure 46.

2.19 Preparation of cell lysates

Before harvesting, cells were pre-treated with colcemid for 16 hours for HeLa or 12 hours for DT40. To prepare the lysates, HeLa cells were trypsinized, washed twice with PBS and resuspended in lysis buffer (50 mM Tris-HCl, 0.2 M NaCl, 1% NP-40, 0.5% deoxycholate, 1 mM PMSF, 20 mM β-ME, pH 8.0) supplemented with protease inhibitors, 1mM PMSF (Sigma), 1 μg/ml CLAP (chymostatin, leupeptin, antipain, pepstatin A, Sigma). After sonication, cellular debris was removed by centrifugation at 20,000 x g for 20 min at 4 °C.

2.20 Protein affinity pull-down

Cell lysates were fractionated by affinity chromatography. Firstly the lysates were incubated overnight on ImmunoPure™ Immobilized streptavidin beads (Pierce, Cat. 20227), washed twice with lysis buffer and once with washing
buffer (50 mM Tris-HCl, 0.1% NP-40, 0.4 M NaCl, 0.1 mM PMSF). The proteins were eluted from the streptavidin beads in the elution buffer (2 mM D-biotin in lysis buffer). This elution was then added onto S-protein agarose beads (Novagen, Cat. 68704-4). After washing, the beads were boiled in sample buffer. All samples were subjected to SDS-PAGE gel and then checked by immunoblotting, silver staining or Coomassie blue staining.
III. Cellular and molecular analysis of Survivin function
III. Cellular and molecular analysis of Survivin functions

3.1 Purpose of this study

Numerous studies including Survivin knockdown by RNAi have been reported (Beltrami et al., 2004; Carvalho et al., 2003; Jiang et al., 2001; Li et al., 1998; Mahotka et al., 1999; Mirza et al., 2002; Song et al., 2004; Temme et al., 2003). The varying efficiency of knockdown and the varying half-life of oligos end up producing a heterogeneous population that can be difficult to analyze. Thus, it is not easy to draw a confident conclusion in cell population studies using RNAi. Instead, studies must be done on a single cell basis, which is difficult and puts the results in doubt. In addition, RNAi studies of Survivin did not allow the study of Survivin structural and dominant-negative mutants. Survivin null mice have been made (Uren et al., 2000) (Zwerts et al., 2007), but these survivin knockout mice die in early embryonic development, which prevents their further analysis.

Conditional gene targeting in vertebrates has been successfully achieved in murine embryonic stem cells in culture, several human tissue culture cells and chicken DT40 cells. Here we tried to make a Survivin conditional knockout cell line in DT40 in order to analyze Survivin function in a null background. The big advantage of this system is that it supplies enough material for biochemistry and large cell population studies. A conditional knockout also makes it possible to analyze Survivin mutants confidently in a clear background lacking the wild-type protein.
3.2 Background of Survivin conditional knockout

Chicken DT40 B cells undergo homologous recombination at very high frequencies by far exceeding those of any described mammalian cell lines (Buerstedde and Takeda, 1991; Hudson et al., 2003; Ruchaud et al., 2002). Targeting vectors for deleting the Survivin gene in DT40 cells were constructed by two former lab members, Dr. Ciaran Morrison and Dr. Ana Carvalho. Their targeting constructs and strategy are depicted in Figure 13. This strategy allowed the deletion of the entire *survivin* coding region (725 bp) and its replacement by a selectable marker giving resistance to neomycin, histidinol or puromycin. The neomycin vector was used to target successfully the first *survivin* allele. The heterozygous cells were then transfected in order to express a conditional rescue construct under the control of a Tet off system. The second allele was targeted using the histidinol vector. A conditional knockout clone was obtained and analysed (this clone will be later referred in the manuscript as KO-1). During the analysis it appeared that despite a strong phenotype, the expression of the rescue in that clone, couldn't be totally repressed. This problem was thought to be due to the relatively high expression of the rescue compared to the endogenous levels (use of a strong CMV promoter).

This is when I joined the project with a new strategy.
Figure 13. **Targeting constructs and strategy.** Schematic representation of the targeting vectors containing a resistance cassette between two genomic regions, 5'- and 3'-arms flanking the chicken *survivin* gene locus. Red bar represents the 5' external probe used for southern blotting. Red arrows show the EcoR I restriction sites. Purple arrows show the Afl II restriction sites. The green arrows show the PflM I restriction sites. Double head arrows show the size of fragments digested by EcoR I, Afl II and PflM I respectively.
3.3.1 Targeting of the first Survivin allele

In order to target the first Survivin allele, wild type DT40 cells were transfected with the histidinol targeting vector. After drug selection, genomic DNA from histidinol resistant clones was extracted and digested by EcoR I or Afl II, and tested by Southern blotting using a 5'-external probe. The results confirmed that one Survivin allele of a clone was disrupted (Figure 14, -/+ lanes).

![Southern blot analysis of genomic DNA from wild type and heterozygous cells.](image)

**Figure 14.** Southern blot analysis of genomic DNA from wild type and heterozygous cells. The upper band corresponds to the histidinol locus (4.9 kb for the EcoR I digestion; 7 kb for the Afl II digestion); the lower band represents the wild type locus (4.1 kb for the Eco RI digestion; 5 kb for the Afl II digestion). WT wild type DT40 cells; -/+ heterozygous cells.

3.3.2 Design and analysis of a new Survivin rescue system

For the new Survivin conditional knockout, I redesigned the repression system (Tet-off) in order to express the Survivin rescue at levels close to the endogenous. To do so, the CMV promoter, driving the tTA transactivator, was replaced by the chicken survivin or kif4 promoter (Figure 15A). Three different tTAs (tTA2, tTA3 and tTA4) showing various strength (98%, 39% and 14% of the original tTA respectively) were modified in the same way and tested. The tTA2 driven by the kif4 promoter was made by Dr. Kumiko Samejima. She tested the strength of these different tTAs by transient co-expression with tet-O-Lamin A:GFP. Both tTA2 driven by kif4 and survivin...
promoter show levels of Lamin A:GFP expression lower than when using a CMV promoter.

3.3.3 Generating a stable conditional Survivin heterozygote

Heterozygous cells were co-transfected with tet-O :Survivin vector and tTA2 driven by either the *kif4* or *survivin* promoter. The *survivin* rescue cDNA has a 36 bp deletion in the 3'UTR, providing a way of distinguishing the exogenous from the endogenous transcripts by RT-PCR (Figure 15B). The tTA plasmids contain a neomycin resistance marker allowing selection of stable transfectants (2 mM geneticin). Resistant clones were tested for the expression of the rescue in the presence or absence of doxycycline by RT-PCR analysis (Figure 15 C). The results show two DNA fragments amplified from clones transfected by both tTA2 driven by *kif4* or *survivin* promoter and tet-O:Survivin, and only one fragment amplified from the wild type cell line or cell line transfected by only tTA2 driven by *kif4* or *survivin* promoter (Figure 15 C, upper panel). The upper (315 bp) band corresponds to the wild type *survivin* allele. The lower band (279 bp) represents the *survivin* rescue in which the 3' UTR bears a 36 bp deletion accounting for the size shift in the gel. The same clones were tested for their ability to repress the rescue in the presence of doxycycline (figure 15 C, lower panel). The results show that the transcription of the rescue Survivin was totally repressed in most of the clones. In parallel, I tested the system by transiently transfected tet-O:Lamin A-GFP into a heterozygote clone expressing the Survivin rescue in the presence or absence of doxycycline. FACS analysis showed that Lamin A:GFP was expressed and was totally shut off after adding doxycycline even though the transfection efficiency was low (Figure 15D). These results show that I generated heterozygous cell lines expressing a Survivin rescue of which expression can be regulated by doxycycline.
Figure 15. Expressing the rescue Survivin using the Tet-Off system before targeting of the second allele. A. The rescue constructs. The upper panel represents the tTA driven by CMV or KIF4 promoter. Lower panel
shows the *survivin* cDNA in PUHD plasmid. B. RT-PCR strategy to
distinguish the wild type and rescue Survivin. C. RT-PCR result to confirm
the expression (upper panel without doxycycline) and repression (lower
panel with doxycycline) of rescue Survivin. Lanes 1-8 are conditional
heterozygotes. Lane 9 is 100 bp DNA marker. Lane 10 is positive control
using chicken Survivin 3'UTR delete. Lane 11 is negative control. D. FACS
analysis of the expression of tTA and repression by transiently transfected
TetO:LaminA-GFP into heterozygotes expressing the rescue Survivin.

### 3.3.4 Targeting of the second Survivin allele

The second targeting vector (puromycin) was transfected into the
heterozygous cells expressing the rescue Survivin. *Survivin* being an
essential gene, resistant clones were tested for their ability to die after 3 days
in doxycycline. The clones that were dying in the presence of doxycycline
were selected as candidates for Southern blot analysis in order to
differentiate proper targeting events from random integration. After EcoR I
digestion of genomic DNA, specific targeting events were identified by the
absence of a wild type band (4.1 kb) and by the presence of a doublet (4.8
and 4.9 kb) corresponding to the histidinol and puromycin targeted alleles
(Figure 16A lane 2). This result was further confirmed by another Southern
blot using samples digested by PfilM I where the two targeted alleles are
further apart (7 kb for histidinol and 9.5 kb for puromycin targeted alleles)
(Figure 16B lane 2). I obtained two independent knockout clones, both
confirmed by Southern blotting (they are referred later in the manuscript as
KO2-1 and KO2-2).
Figure 16. Survivin conditional knockout cell line. A. The Survivin knockout cell line was confirmed by Southern blotting using EcoR I digestion. B. The knockout cell line further confirmed using PflMI I digestion. The probe used in these experiments is the 5'-terminal external probe shown in the figure 13 (red box).

3.3.5 Survivin rescue construct could be fully repressed, yielding complete knockout cells

To check the proper expression and repression of the Survivin rescue at the transcriptional and protein levels, the different knockout clones obtained were tested by real time PCR, Western blotting and immunofluorescence in the presence of doxycycline for increasing amounts of time. The levels of survivin transcripts were analysed by RT-PCR every 4 hours after the addition of doxycycline. The results were normalized against an actin control. Four hours after adding doxycycline, the transcription levels were already dramatically decreased in both KO1 and the two knockout clones I obtained KO2-1 and KO2-2 (Figure 17A). The mRNA level fell to less than 25% of time zero after 4 hours in doxycycline. After 24 hours, only 1% of transcripts remained. Compared with the wild type DT40 cells, Survivin protein in KO2 subclone 1 without doxycycline was at a level similar to wild type Survivin (Figure 17B). The protein levels were analysed after addition of doxycycline by Western blotting for both KO-1 and KO-2.1 using an affinity purified rabbit polyclonal antibody raised against the full-length
chicken Survivin protein (WCE43D)(Figure 17C). Both clones show Survivin being repressed but not fully in KO-1 compared to KO-2 in which the protein is nearly undetectable after 36 hours (Figure 17C, right panel lane 5). Note that the protein is over-expressed in KO-1 compared to the wild type (compare lane 1 and 2 in the left panel). In contrast, the Survivin levels in KO-2 are very similar to the wild type (compare lane 1 and 2 in the right panel). The repression of Survivin protein was confirmed by immunofluorescence staining on KO-2 cells using the same antibody. Survivin was no longer detectable in mitotic cells after 60 h in doxycycline (Figure 17D) compared with the clear centromeric signal in untreated cells.

IMPORTANT NOTE

The following results were obtained using either the KO-1 and/or KO-2. During the course of my PhD, I started analysing Survivin functions on KO-1 cells while I was concentrating my efforts in making the second knockout KO-2. In order to simplify the reading I will call the knockout cells:

- Survivin\textsuperscript{on} cells when cells are grown in absence of doxycycline
- Survivin\textsuperscript{off} cells when cells are treated with doxycycline

The length of treatment and the specific knockout clone (KO-1 or KO-2) will be specified in each figure.
Figure 17. Repression of the Survivin rescue. A & B. Real time PCR shows that Survivin was repressed after addition of doxycycline. This experiment used actin as control for normalisation. Error bars mean the ±S.D. from 4 different experiments. C. Immunoblotting analysis of Survivin repression after addition of doxycycline. Whole cell lysate (20 μg) from DT40
(WT) and Survivin\textsuperscript{OFF} cells (doxycycline 0 - 48 hours for KO-1; 0 - 60 hours for KO-2) was subjected to 12.5% SDS-PAGE and probed with affinity-purified polyclonal anti-Survivin antibody (WE43D). \(\alpha\)-tubulin was used as a loading control. D. Immunofluorescence staining with WE43D (red), \(\alpha\)-tubulin B512 (Green) and DAPI (blue) of Survivin knockout Survivin\textsuperscript{ON} and Survivin\textsuperscript{OFF} cells. Scale bar is 5 \(\mu\)m.

### 3.3.6 Survivin is essential for cell survival

Embryos from Survivin knockout mice die at 4.5 days post coitum (Uren et al., 2000). Interference of Survivin expression by antisense oligonucleotides caused cell death in the G2/M phase of the cell cycle (Li et al., 1999). In KO1 and KO2 cells we found that Survivin is essential for cell viability. Sixty hours after adding doxycycline, Survivin\textsuperscript{OFF} cells underwent apoptosis and the remaining cells became larger. On the contrary, Survivin\textsuperscript{ON} cells were still healthy and the population appeared homogenous (Figure 18A). Proliferation of both KO1 and KO2 cells stopped at 36 hours after addition of doxycycline. In contrast, the doubling time of the untreated cell lines was not changed (Figure 18B). Sixty hours after the addition of doxycycline, most cells had died. However, wild type, heterozygote and untreated KO cells grew exponentially. Looking at apoptotic cells by Annexin-V staining, we could detect apoptosis from 36 h reaching a plateau of 80% at 60 h (Figure 18C analyzed by Dr Ana Carvalho). Taken together these results show that Survivin is an essential protein, though its function remains unclear.
Figure 18. Survivin is an essential protein. A. Morphology of Survivin knockout cells with and without doxycycline after 60 hours under phase contrast microscopy. B. Growth curves of Survivin knockout KO1, KO2 and DT40 clones with or without doxycycline. C. Index of Annexin V positive cells after adding doxycycline to DT40, heterozygote and KO1 cells (data of Dr Ana Carvalho)
3.3.7 INCENP destabilization and mislocalization after Survivin repression

Survivin, as an important member of the chromosomal passenger complex, orchestrates mitosis together with INCENP, Aurora B and Borealin (Vagnarelli and Earnshaw, 2004). Members of the chromosomal passenger complex are dependant on each other for protein stability and localisation (Gassmann et al., 2004; Honda et al., 2003). Therefore I tested the stability and localisation of INCENP in Survivin\textsuperscript{OFF} cells. After 60 h in the presence of doxycycline, INCENP protein was nearly undetectable in Survivin\textsuperscript{OFF} cells by Western blotting (Figure 19A). This result suggests that Survivin may affect the stability of the chromosomal passenger complex. This result was confirmed by the immunofluorescence staining of INCENP. INCENP localization was impaired in Survivin\textsuperscript{OFF} cells (Figure 19B). No INCENP was detected on centromeres in prometaphase of Survivin\textsuperscript{OFF} cells, whereas in Survivin\textsuperscript{ON} cells INCENP was normally localized between the two CENP-H: GFP spots labelling the kinetochores (Figure 19B).

Aurora B kinase is the enzymatic core of the chromosomal passenger complex. One important function of the complex is the modification of Histone H3 tails. The phosphorylation on Ser10 of Histone H3 is considered a hallmark of Aurora B activity (Fischle et al., 2005; Hirota et al., 2005). Here we asked whether Survivin was required for Aurora B activity by looking at Histone H3 phosphorylation levels. The Ser10- phosphorylated histone H3 levels decreased when Survivin was shut off (Figure 19A). This change was not caused by a decrease in the fraction of mitotic cells. The mitotic index of Survivin\textsuperscript{OFF} cells showed no significant difference compared to wild type or Survivin\textsuperscript{ON} cells (Figure 21B). We could still detect residual levels of this H3 modification even at late time points when most cells were dead. By immunofluorescence, the difference in phospho H3 staining was visible tough
Figure 19. INCENP expression and localization are impaired in Survivin knockout cells. A. Western blotting of Survivin, INCENP and Ser10 phospho-H3 in the knockout cell line. Survivin, INCENP and Ser10 phospho-H3 were probed with WE43D, 1186 antibodies. α-tubulin was used as loading control.
B. Immunofluorescence staining of CENP-H-GFP (green), INCENP (red) in Survivin\textsuperscript{ON} (upper panel) and Survivin\textsuperscript{OFF} (lower panel) cells (data of Dr Ana Carvalho).

Figure 20. Levels of Ser10 phosphorylation on histone H3 are reduced in Survivin knockout cells. A. Ser10–phosphorylated histone H3 (green) was stained in Survivin\textsuperscript{ON} (upper panel) and Survivin\textsuperscript{OFF} (lower panel) cells (KO2). B. INCENP and Ser10–phosphorylated histone H3 in KO1. Upper
panel Survivin\textsuperscript{ON}; lower panel Survivin\textsuperscript{OFF} (data of Dr Ana Carvalho). Scale bar is 5 μm.

3.3.8 Cells could initiate and traverse mitosis without Survivin.

Survivin expression is cell-cycle related, showing peak levels at G2/M phase (Fukuda and Pelus, 2001; Li et al., 1998). A study of the role of Survivin in CD34\textsuperscript{+} cells indicated that it is involved in promoting CD34\textsuperscript{+} cell cycle entry (Fukuda et al., 2002). In our system I showed that Survivin\textsuperscript{OFF} cells could initiate mitosis (Figure 21A and Figure 22). The figure shows a slight increase in mitotic index in both knockouts after adding doxycycline for 48 or 60 hours when compared to the wild type cells (Figure 21B). The difference between KO cells treated with doxycycline 60 hours and KO cells untreated is significant (0.01<\textit{P}\textsuperscript{KO1}<0.02, 0.02<\textit{P}\textsuperscript{KO2}<0.05). Time lapse imaging of knockout cells 36 hours after adding doxycycline demonstrated that cells without Survivin could enter and complete mitosis (Figure 22 and movie 1 and 2). Cells with tetraploid or octaploid nuclei were able to enter mitosis again. Cells could achieve metaphase, anaphase and telophase without Survivin (movie 4).

Mouse Survivin null embryos showed disrupted microtubule formation, and failed to survive beyond 4.5 days post-coitum (Uren et al., 2000). In \textit{S. pombe}, Bir1 deletion mutants were unable to achieve a metaphase-to-anaphase transition because of a failure of spindle microtubule elongation (Uren et al., 1999). In budding yeast, a complex of the chromosomal passenger proteins Bir1 and Sli15 (Survivin and INCENP) was identified that links centromeres to microtubules. However, we carefully analysed the mitotic spindles in Survivin\textsuperscript{OFF} cells, which all looked normal (Figure 21A microtubule channels).
Figure 21. Mitosis without Survivin. A. Cells could initiate and complete mitosis without Survivin. Survivin\textsuperscript{ON} (upper two panels) and Survivin\textsuperscript{OFF} (lower two panels) cells were stained with WE43D (red), B512 (green) and DAPI (blue). Metaphase and anaphase are shown. B. Index of mitotic cells for...
DT40, KO1 and KO2 in the presence and absence of doxycycline. Different cell lines were labelled with different colour and patterns. Error bars ±S.D from 4 independent experiments. Scale bar is 5 µm.

Figure 22. Selected frames of representative live cell imaging performed on Survivin\textsuperscript{ON} and Survivin\textsuperscript{OFF} cells. White-black panel shows the H2B-RFP channel. The merged images of DIC (in red) and histone H2B-RFP (in green) are shown in the top rows. Numbers indicate the time of...
3.3.9 Survivin is required for mitotic spindle checkpoint after loss of tension.

Lens et al. reported that Survivin is required for a sustained spindle checkpoint in response of lack tension (Lens et al., 2003). U2OS cells escaped a taxol-induced arrest when Survivin was knocked down by RNAi. In order to analyse the spindle checkpoint behaviour in our system I used the microtubule-stabilizing agent taxol and microtubule-depolymerizing agent nocodazole on survivin-depleted cells. It has been reported that in the presence of taxol, microtubules can attach to kinetochores, but spindle tension is abolished (McEwen et al., 1997; Waters et al., 1998). Surprisingly, SurvivinOFF cells showed very similar mitotic index to SurvivinON cells and wild type cells when they were blocked for 12 hours in taxol or nocodazole (Figure 23A and Figure 24A, B). Apoptosis levels were analysed on the same samples by TUNEL staining. FACS analysis showed that these mitotic cells blocked by taxol or nocodazole were still healthy (Figure 24C and D). I did see a decrease of mitotic index after adding Taxol or nocodazole for 24 hours (Figure 23C and D). This result must have been caused by the death of arrested cells. Because the total viable cells were less than 10% of the viable populations 12 hours after drug addition. This implies that the spindle checkpoint may still function in the absence of Survivin using a high dose taxol (>400 nM). However, SurvivinOFF cells were arrested by 10 nM taxol (Figure 24F). In our lab, similar results were obtained after INCENP depletion in DT40 cells. These results show that Survivin is required for spindle checkpoint arrest in response to lack of tension in DT40 cells.
Figure 23. Survivin is not required for spindle checkpoint after loss of tension. A and B. Cells were blocked in mitosis by Taxol or nocodazole. KO cells were grown with 0.5 μg/ml doxycycline for 36 hours, then drugs were added to final concentration 10 μM Taxol or 0.5 μg/ml nocodazole for another 12 hours. Mitotic indexes were scored after hypotonic swelling of the cells using 75 mM KCl buffer. C and D. The cells of A and B were grown for 24 hours after adding Taxol and nocodazole.
Figure 24. Cells arrested by Taxol and nocodazole in the absence of...
Survivin. A and B. Mitosis blocked by Taxol or nocodazole. KO cells grown with 0.5 μg/ml doxycycline for 36 hours, then taxol or nocodazole were added to final concentration 0.5 μM or 0.5 μg/ml respectively. Cells were harvested after another 11 hours. The mitotic index was scored after hypotonic swelling of the cells using 75 mM KCl buffer. Only living cells were counted. C and D. Samples from the same experiment as in A and B were analyzed by TUNEL staining. F. SurvivinOFF cells are arrested by taxol at 10 nM.

3.3.10 Cells fail Cytokinesis without Survivin

We found that a high percentage of cells became multinucleated after Survivin was switched off by adding doxycycline for 48 hours. This was consistent with previous reports (Carvalho et al., 2003; Li et al., 1999). Some cells had even more than four nuclei or eight spindle poles (Figure 25A). After adding doxycycline for 60 hours, multinucleated cells accumulated to more than 50%, compared to about 5% of the SurvivinON cells and wild type DT40 cells (Figure 25B; P<0.001). Live cell imaging of elutriated SurvivinOFF cells showed chromosome segregation or cytokinesis failure (Figure 25C). Separation of the sister chromatids failed in some SurvivinOFF cells. These cells entered anaphase but exited before telophase, though cleavage furrows were formed. Sometimes the two sister chromosomes were drawn back and formed larger single nuclei (Figure 25C and movies 3-1, 3-2, 4). These cells were scored as cells with single nuclei for the mitotic index measurement in Figure 25B. This explained why many cells did not become multinucleated after Survivin was shut off, as instead many may have formed large single nuclei. In some cells the two daughter nuclei could not manage to separate from each other (Figure 22 and movie 2). The ingression of the cleavage furrow was normal in these cells. Our movie clearly showed two linked daughter cells at late telophase in which abscission failed. The two daughter cells fused to form a binucleated cell.
Figure 25. Cells depleted of Survivin failed Cytokinesis. A. Cells with multiple nuclei and multiple spindles. Survivin\textsuperscript{OFF} cells were stained with WE43D (red), $\alpha$-tubulin B512 (Green) and DAPI (blue). Upper panel shows an octoploid interphase cell and lower panel shows a mitotic cell with at least 6 spindle poles (microtubule channel). B. Multinucleation index of DT40, KO1
and KO2 in the presence or absence of doxycycline. Error bars mean the ±S.D. from 3 independent experiments. C. Selected frames of representative live cell imaging performed on Survivin\textsuperscript{OFF} cells. DIC is shown in the top rows and H2B-RFP is shown in the lower row. Numbers indicate the time of image collection in hours: minutes. Survivin\textsuperscript{OFF} cells frequently show chromosome segregation defects giving rise to cells with larger nuclei. Scale bar is 5 nm.

Our movies showed 35 cells failed in cytokinesis in 41 mitotic Survivin\textsuperscript{OFF} cells (KO1; 32 in 40 of KO2). No SurvivinON cells were observed to fail in cytokinesis (10 mitotic cells). More data is needed to elucidate the mechanism of why cells fail cytokinesis without Survivin.

### 3.3.11 Cell death in the absence of Survivin.

As one of the IAP family members, Survivin attracted the attention of scientists interested in apoptosis and cancer research. It was reported that down regulation of Survivin by RNAi caused spontaneous apoptosis (Ling et al., 2004; Pennati et al., 2004). Knockdown of Survivin using shRNA reduced the clonogenic survival of human sarcoma cell lines. Here we tried to answer whether removing Survivin from cells affects their response to apoptotic stimuli. Survivin conditional knockout cells were treated with doxycycline for 30 or 36 hours in order to repress the expression of the rescue cDNA. These cells were then incubated with etoposide for another 3 hours. Cell were collected and analysed by TUNEL staining. Results showed no difference between Survivin\textsuperscript{ON} or Survivin\textsuperscript{OFF} cells (Figure 26A). Other studies have shown that exposure to staurosporine increased the ratio of caspase-3-positive embryos when they were treated with antisense Survivin oligonucleotides (Kawamura et al., 2003). However, Survivin knockout cells pretreated with doxycycline for 30 hours are not more sensitive to staurosporine than Survivin\textsuperscript{ON} and wild type DT40 cells (Figure 26B). We also tried incubating Survivin\textsuperscript{OFF} (KO1 adding doxycycline for 36 hours) cells with
0.5 μM Taxol or nocodazole for 12 hours. FACS analysis showed that slightly fewer Survivin<sup>OFF</sup> cells were TUNEL positive when treated with taxol (9%) or nocodazole (8%) than Survivin<sup>OFF</sup> cells that were not treated (18%) (Figure 26C).

Figure 26. Cells depleted in Survivin are not more sensitive to...
apoptosis. A. TUNEL staining shows no difference between cells pre-treated with or without doxycycline incubated with etopside for 3 hours. Index of TUNEL positive cells was shown for KO1. Columns are labelled in different colour for different treatments. Etoposide was shown as e. B. Index of TUNEL positive cells treated with staurosporine. KO1 cells were pre-treated with doxycycline for 30 hours before adding staurosporine. Time that cells were incubated with staurosporine is shown at the bottom of panel B. Str represents staurosporine. C. Taxol and nocodazole have no effect on Survivin\textsuperscript{OFF} cells apoptosis. KO cells were grown with 0.5 μg/ml doxycycline for 36 hours, then were added to final concentration 0.5 μM Taxol or 0.5 μg/ml nocodazole for another 12 hours. Controls include wild type DT40 and KO1 with and without doxycycline. T represents taxol and N represents nocodazole.

There is no significant difference in the percentage of TUNEL positive between Survivin\textsuperscript{ON} and Survivin\textsuperscript{OFF} cells that were both treated with Taxol or nocodazole. The same experiment was also done using KO2 cells. Though a large percentage of cells treated with Taxol or nocodazole became apoptotic, the percentage of TUNEL positive cells is similar to KO2 cells treated only by doxycycline (Figure 26C and Figure 24C, D). This is because KO2 cells become unhealthy after adding doxycycline for 36 hours. Our results demonstrate that removing Survivin does not change the sensitivity of cells to the chemotherapeutic drugs tested.

However, I found that G\textsubscript{2}/M Survivin\textsuperscript{OFF} cells die much quicker than G\textsubscript{1} cells. Cells that were grown with doxycycline for 36 hours were synchronized by elutriation. Every 2 hours apoptotic cells were scored. Meanwhile samples were taken for apoptosis analysis by Annexin V and PI staining (Figure 27A and B). G\textsubscript{1} (Fraction 2) and S (Fraction 3) populations of cells grew happily for another 12 hours after synchronization, though at this time point quite a lot of cells had passed the G\textsubscript{2}/M peak. G\textsubscript{2}/M (Fraction 4 and Fraction 5) cells...
Figure 27. Survivin$^{\text{OFF}}$ cells die in interphase after becoming tetraploid. A, B, C and D. G$_2$/M cells died much sooner than G$_1$ cells. A. FACS profiles of the cell populations before and after synchronisation by elutriation are shown. Cells growing with doxycycline for 36 hours were synchronized, stained by PI and analyzed by FACS. B. FACS profiles of the cell populations 14 hours after being synchronized by elutriation (50 hours after adding doxycycline). C and D. Cell death after synchronisation scored by counting...
percentage of healthy cells (C) or the percentage of annexin positive cells (D). Levels of Survivin in the different fractions. The same number of cells were loaded for each lane. Film exposure time was overnight. “t” represents KO2 total cell population without doxycycline. f2, f3 f4 and f5 are the elutriation fractions. F. Multinucleation index of different fractions (Figure F is data of Dr Ana Carvalho).

died much quicker (Figure 27C and D). Western blotting analysis showed that the level of Survivin in the different fractions was the same (Figure 27E). Furthermore, we found that most cells had become multinucleated before they died (Figure 27F). Interestingly, Survivin<sub>OFF</sub> cells did not die in G2/M phase. Our movies (Figure 25 C; Movie 4 & 5) showed that cells died in interphase after failing cytokinesis. This cell death could not be delayed by the caspase inhibitor z-VAD-fmk.

### 3.3.12 GFP-tagged chicken and human Survivin can rescue the knockout

Interestingly, the knockout cell line could be totally rescued by expression of wild type GFP-tagged chicken Survivin or its human homolog. Chicken Survivin cDNA fused at its C-terminus to GFP and under control of the CMV promoter was transfected and stably expressed in our knockout cell lines. Expression of the GFP-tagged Survivin was verified by immunofluorescence staining and Western blotting using the WE43D affinity purified antibody. These cells grew normally in the presence or absence of doxycycline (Figure 28 A). The growth curves show that these cell lines proliferate similarly to wild type DT40 cells. GFP-tagged Survivin localized properly in prometaphase, metaphase, anaphase and telophase, colocalizing with endogenous INCENP (Figure 28 A’). Similarly, GFP-tagged human Survivin can localise properly in Survivin<sub>OFF</sub> cells and rescue life (Figure 28 B and B’). Our results show that GFP-tagged chicken and human Survivin could complement the Survivin knockout phenotype.
Figure 28. GFP-tagged Survivin can complement the loss of Survivin. A & A' Chicken Survivin-GFP can rescue the knockout phenotype. A. Growth curves of DT40, KO1 and KO1:ggSurvivin-GFP cells. A’. GgSurvivin-GFP...
colocalizes with INCENP (red) as detected by WE1186 antibody. B & B’ Knockout cells could also be rescued by human Survivin. Scale bar is 5 µm.

3.3.13 Survivin DD72, 73AA mutant still functions like wild type Survivin
The Survivin mutant (h DD70, 71AA) disrupts its interaction with Aurora B and causes multinucleation in HeLa cells (Cao et al., 2006). The equivalent mutation in chicken Survivin (DD72, 73AA) was introduced into our chicken Survivin knockout cells. Western blotting using WE43D antibody corroborated the expression of the Survivin mutant fused to GFP at its C-terminus (Figure 29 B). 36 hours after the wild type Survivin cDNA was switched off, the Survivin ^OFF^ cells begun undergoing apoptosis. However, Survivin ^OFF^ cells expressing the DD72, 73AA mutant Survivin grew normally in the presence and absence of doxycycline (Figure 29 C). The mutant cell lines had a very similar doubling time to wild type DT40. Therefore, in chicken the Survivin DD72, 73 AA mutant could completely rescue the knockout cells. Intriguingly, Survivin DD72 & 73AA localizes diffusely during prophase and metaphase (Figure 30 b and d), although this mutant moves to the midzone and concentrates at the midbody in telophase. Wild type Survivin fused to GFP localized perfectly in prophase and metaphase (Figure 30 a). Sometimes over-expression of a protein could impair its correct localization. To verify that the mislocalization was not due to overexpression, I checked the Survivin level by immunoblotting. The results showed that there was no Survivin DD72, 73AA overexpression compared to the wild type Survivin in the stable cell lines (around 3 times less)(Figure 31A). We then checked for an effect of the DD72, 73AA mutant on the spindle checkpoint. Survivin DD72, 73AA mutant cells were incubated with 10 µm/ml taxol for 12 hours after adding doxycycline 60 hours. Mitotic cells accumulated to 95% for the mutant cell lines and the wild type DT40, whether the rescue Survivin was switched off or on (Figure 31B and C). This results show that the mitotic spindle checkpoint still functioned when only the Survivin DD72, 73AA
mutant was expressed.

Figure 29. DD72.73AA mutant. A. Diagram showing Survivin DD72, 73AA mutant. B. Immunoblot showing the mutant expression and repression of the
rescue Survivin together with INCENP levels. Membrane was incubated with anti-Survivin WE43D, anti-INCENP 3D3 and anti α-tubulin B512 (Same amount as sample was loaded. Endogenous Survivin and GFP tagged Survivin mutant were exposed in one film). C. Growth curve confirming that the Survivin DD72&73AA mutant could rescue a Survivin knockout.

Figure 30. Localization of GFP-tagged DD72, 73AA mutant Survivin and INCENP. a. GFP-tagged wild type chicken Survivin and INCENP (probed by WE1186) localizes properly in Survivin<sup>OFF</sup> cells. b and d. Both DD72, 73AA
mutant Survivin (GFP channel) and INCENP (left channel) show a diffuse localization in metaphase in SurvivinON and SurvivinOFF cells. Scale bar is 5 μm.

Figure 31. The Survivin DD72, 73 AA mutation does not impair the spindle checkpoint. A. Western blot showing the levels of Survivin. B and C. The DD72, 73AA mutant has no affect on the spindle checkpoint. Error bars represent ±S.D. Wild type DT40 and cells expressing Survivin mutants
were pretreated with doxycycline for 60 hour then treated with 10 μM Taxol (B) or 0.5 μg/ml nocodazole for another 12 hours (C). Mitotic index were performed after hypotonic treatment using 75 mM KCl buffer.

3.3.14 Survivin mutants reported to be pre-apoptotic

More data on the potential anti-apoptotic role of Survivin has come from many structural, biochemical and mutant studies. These studies revealed that Survivin and Smac/DIABLO bind to each other (Muchmore et al., 2000; Sun et al., 2005). Survivin's interaction with Smac/DIABLO also had been demonstrated by co-immunoprecipitation (Kim et al., 2006; McNeish et al., 2005). Analysis of the D71A and D53A mutants of hSurvivin suggested that Survivin functions as an anti-apoptotic protein (Li et al., 1999; Muchmore et al., 2000; Song et al., 2004). Transfection of HeLa cells with Survivin mutants (D71A and D53A) caused spontaneous apoptosis, However, when our cell lines stably expressing chicken Survivin D55A (equivalent to hD53A) or DD72, 73AA (corresponding to hDD70, 71AA) mutants were grown in the presence of doxycycline, we saw no effect on their growth (Figure 29C and Figure 32C). These cells proliferated as well as wild type DT40 cells and the GFP-tagged ggSurvivin D55A mutant localized properly (Figure 32D).
Figure 32. Survivin D55A mutant. A. Diagram showing D55A mutant location. Mutant site is labeled in pink. B. Immunoblot analysis of SurvivinON: Survivin-GFP and SurvivinOFF: Survivin-GFP lysates. Expression of D55A mutant fused with GFP and repression of rescue Survivin were confirmed by
Immunoblotting using anti Survivin antibody WE43D. The presence of equal amount of tubulin in various lanes serves as a loading control. C. Growth curve of D55A mutant. D55A-GFP complements the knockout phenotype. Survivin<sup>OFF</sup>: Survivin-GFP and Survivin: D55A-GFP cells proliferate normally. D. Immunofluorescence staining of cells expressing the D55A mutant. INCENP was stained with WE1186 (red). Survivin D55A and INCENP localized normally in mitosis. Scale bar is 5 μm.

3.3.15 T36 mutants have no effect on cell proliferation

It was reported that Survivin was phosphorylated on Thr34 by the mitotic kinase p34cdc2-cyclin B1 (O'Connor et al., 2000). Inducible expression or adenoviral delivery of non-phosphorylatable Survivin Thr34-Ala, described as a dominant-negative, resulted in caspase-9-dependent apoptosis and anticancer activity in vivo (Grossman et al., 2001). In other studies, inhibition of Survivin phosphorylation on Thr34 by the cyclin-dependent kinase inhibitor flavopiridol resulted in loss of Survivin expression, and non-phosphorylated Survivin T34A exhibited accelerated clearance as compared with wild-type Survivin (Altieri, 2003). We constructed the chicken Survivin mutants T36A and T36E corresponding to human T34. Survivin T36A and T36E fused to GFP at the 3’ terminus were stably expressed in knockout cells (Figure 33B). These cell lines grew normally either with or without doxycycline. T36A/E mutants could completely rescue the viability of cells when the endogenous wild type Survivin was removed (Figure 33C). GFP-tagged Survivin mutants T36A and T36E could localize correctly on centromeres in metaphase and at the midzone in anaphase (Figures 34 and 35).
Figure 33. T36 mutants have no effect on cell proliferation. A. Diagram showing the Thr36 mutant location. B. Expression of mutants fused with GFP. Lysates from Survivin^ON^ and Survivin^OFF^ cells were probed with WE43D. C. Growth curves of DT40, T34A and T34E. These two mutants could rescue Survivin knockout.
Figure 34. Survivin mutant T36A localizes properly. Survivin KO1 cells that express the GFP-tagged Survivin T36A mutant were stained for INCENP WE1186 (red, left lane 1) and DAPI (blue, lane 3). Scale bar is 5 μm.
Figure 35. **Survivin mutant T36E localizes properly.** Survivin KO1 cells that express the GFP-tagged Survivin T36E mutant were stained with WE1186 (red) and DAPI (blue). Scale bar is 5 µm.

3.3.16 **Zinc binding residues are crucial for Survivin function**

Crystal structure studies of Survivin demonstrated that four strictly conserved Zn$^{2+}$-binding residues, Cys 57, Cys 60, His 77, and Cys 84, form a zinc finger, that is important to stabilize the structure of the BIR domain (Chantlat 2000; Verdecia 2000). Muchmore’s study showed that two other residues, Glu76 and His77, interact with Zn$^{2+}$ to mediate dimerization of Survivin (Muchmore 2000). In our lab, Survivin C86A (corresponding to
human C84A) and C59A (corresponding to human C57A) mutants were made by site-directed mutagenesis (Figure 36A). These mutants were stably transfected into KO-1. Immunoblots substantiated their expression (Figure 36 and 38). WE43D Survivin antibody recognized both the mutated Survivin fused to GFP (50 kDa) and the rescue Survivin (16 kDa) (Figure 36B). Experiments demonstrated that these mutants could not rescue the Survivin knockout cells. Cell lines stably expressing these Survivin mutants had similar growth curves compared to the knockout in the presence of the expressed wild type cDNA. After 46 hours in doxycycline, cells carrying the mutant Survivin started to die and very few viable cells were found after 60 hours (Figure 36C). Immunofluorescence studies of these Survivin mutant cell lines showed that mutated Survivin does not localize properly in mitosis. As opposed to wild type Survivin, neither mutant protein localized to the centromeres (Figure 37d, Figure 38D-a) or spindle midzone (Figure 37c) when the rescue Survivin was shut off. Both mutant proteins were also mislocalized in metaphase and telophase (Figure 37 b, c and Figure 38D-a, b) in SurvivinON cells. These results suggest these two mutant proteins are not able to interact with wild type Survivin and could not to be targeted to the proper location. Furthermore, we found that Survivin C86A and C59A could not target INCENP to centromeres or the midzone (Figure 37d, e and Figure 38 D-c, d), though INCENP localized properly when the rescue Survivin was still on (Figure 37b, c and Figure 38D-a, b). Previous studies showed that the human C84A mutant was dominant-negative (Li et al., 1998), but our results did not confirm this. When the rescue Survivin was still on, the cell lines stably expressing C86A or C59A grew normally. In SurvivinON cells, these Survivin mutants did not affect other chromosomal passengers, such as INCENP localization (Figure 37 and Figure 38D). Our experiments demonstrated that the BIR domain is crucial for Survivin function, especially the Zinc binding residues, which may play an important role for Survivin structure and interaction with its partners.
Figure 36. Zinc binding residue C59A mutant. A. Diagram showing the location of mutant amino acid C59. B. Immunoblot showed the repression of rescue Survivin and expression mutant Survivin C59A fused with GFP (in one film). C. C59A mutant could not rescue the Survivin knockout.
Figure 37. Localization of Survivin mutant C59A-GFP and INCENP. C59A-GFP cannot localize properly in the presence or absence of doxycycline. INCENP staining is diffuse in mitotic SurvivinOFF cells. KO1 expressing wild type chicken Survivin as control (a). INCENP was stained with WE1186. Note that INCENP could not localize properly when C59A/GFP was the only Survivin Spicer in the cells (left panel of d and e). Scale bar is 5 μm.
Figure 38. Zinc binding residue C86A mutant. A. Diagram showing the amino acid C86. B. Immunoblot showing the repression of rescue Survivin and expression mutant Survivin C86A fused with GFP. C. The C86A mutant could not rescue the Survivin knockout. D. C86A-GFP cannot localize properly in the presence or absence of doxycycline. INCENP is mislocalized.
in mitotic Survivin\textsuperscript{OFF} cells. INCENP was stained with WCE1186.

2.4 17. The linker region between the BIR and C-terminal $\alpha$-helix of Survivin is essential for its function

Structural studies revealed hydrophobic contacts and hydrogen bonds in the linker region of Survivin (residues 82-102) (Chantalat et al., 2000). Structural studies on human Survivin by nuclear magnetic resonance also revealed that it is a bow tie-shaped homodimer in solution (Sun et al., 2005). Therefore, I tested whether the linker region was important for Survivin function. Two mutants were made in the linker domain between the BIR domain and the C-terminal $\alpha$-helix of chicken Survivin (Figure 39A). One mutant, L98AV100A, corresponds to human Survivin L96AL98A. In the other, L104 and L106 were both mutated to Alanines (corresponding to human Survivin L102AL104A). The residue L98, V100, L104 and L106 are conserved amongst \textit{Danio rerio}, \textit{Gallus gallus} and \textit{Homo sapiens} (Figure 12). L98AV100A could totally rescue the Survivin knockout. In the absence or presence of doxycycline L98AV100A showed growth rates similar to the Survivin\textsuperscript{ON} knockout cell line at the standard DT40 growth temperature of 39 °C (Figure 40A). The doubling time of L98AV100 in Survivin\textsuperscript{OFF} cells was about 1.5 hours longer than Survivin\textsuperscript{ON} cells (calculated from growth curves to be about 10.5 hours). The doubling time of L104AL106A in Survivin\textsuperscript{OFF} cells (about 17.5 hours) is longer than Survivin\textsuperscript{ON} cells (12 hours)(Figure 40B), although L104AL106A could also rescue growth of the knockout cells. The expression of mutated Survivin and the repression of rescue Survivin were confirmed by Western blotting (Figure 39B 39°C lanes 4, 5, 8 and 9).
Figure 39. Expression of Survivin linker region mutants in KO. A. Schematic representation of the L98A V100A mutation. Mutated amino acids are shown in colour. B. Western blot using WCE43D confirmed the expression and repression of Survivin. Wild type DT40 as control lane 1; knockout as control lane 2 and 3; L98AV100A lane 4 and 5; KO1: WT
Survivin-GFF lane 6 and 7; and L104AL106A-GFP lane 8 and 9. Endogenous Survivin and GFP-tagged Survivin were shown in different panels. 39°C blot was probed in one experiment. In order to use the same loading sequence as 41°C panel, the photograph 39°C blot was cut and arranged as 41°C panel. C. IP from KO and L98AV100A using antibody against GFP. Samples were probed by WCE43D. KO as control.

Next I checked the localization of these two mutants fused to GFP at their C-termin. In SurvivinON cells, Survivin L98AV100A-GFP appeared diffuse in metaphase, anaphase and telophase cells at 39°C (Figure 41a, c and e), but this did not affect the localization of INCENP. This was consistent with the report that human Survivin L96AL98A could not localize in mitosis (Knauer et al., 2006a). However I found that L98AV100A-GFP could slightly concentrate on midbodies in some KO-2 SurvivinON cells, in which the levels of rescue Survivin were 10% lower than wild type DT40 cells. More interestingly, L98AV100A could target properly in mitosis when the rescue Survivin was switched off at 39°C (Figure 41b, d and f). The GFP signal was very clear at centromeres, the spindle midzone and midbody. Similar phenotypes were observed for L104AL106A mutant at 37 °C (Figure 42).

Strikingly, neither mutant could rescue the Survivin knockout at 41 °C. Cells expressing both mutants started dying 48 hours after doxycycline addition, that is 24 hours later than the knockout (Figure 40C). In contrast, wild type DT40 and knockout cells stably expressing wild type Survivin fused to GFP grew normally. Also the Survivin mutant cell lines could grow as well as the wild type DT40 cell line at 41 °C in absence of doxycycline. Immunofluorescence staining revealed that L98AV100A and L104AL106A could not localize properly at 41 °C in either the absence or presence of doxycycline (Figure 43 and 44). Both Survivin mutants and INCENP could not target to the centromeres, the midzone and the midbodies in SurvivinOFF cells (Figure 43c, e and Figure 44c & e) at 41 °C, whereas the GFP-tagged
wild type localized perfectly at 41°C in the presence of doxycycline (Figure 43a and Figure 44a). When the Survivin containing the linker mutations did not target properly at 41 °C, cells became multinucleated (Figure 44e) or exhibited multiple spindles (Figure 43c and Figure 44c). INCENP still localized properly in SurvivinON cells (Figure 43 b, d, and f; Figure 44 b, d, and f) at 41 °C. To confirm whether these mutants are temperature sensitive, we firstly grew L98AV100A at 37°C for several days with doxycycline then transferred cells to 43°C. Under these conditions, KO cell expressing the L98AV100A mutant stopped proliferation and died (Figure 45). So I tried to find out whether these mutants abolish dimerization. Co-IP using anti-GFP antibody still could pull down endogenous Survivin from cell lines stably expressing L98AV100A grown at 39°C and 41°C (Figure 39C). Because the IP operation was done on ice, whether the monomers reform dimers after being cooled down is not known. Collectively these studies showed that the linker region is important for Survivin function. When the L98, V100, L104 or L106 were mutated to A, Survivin becomes temperature sensitive and loses its function.
Figure 40. Linker regions are essential for Survivin function. A. L98AV100A growth curve at 39 °C. Survivin\textsuperscript{OFF}: L98AV100A-GFP cells proliferate normally. Their doubling time is 12 hours compared with the Survivin\textsuperscript{ON} 10.5 hours. B. L104AL106A growth curve at 37 °C. Survivin\textsuperscript{OFF}: L104AL106A grew slower than wild type cells. The doubling time was 18 hours compared to 12 hours for Survivin\textsuperscript{ON} (calculated from growth curve). Nonetheless, this mutant could rescue the knockout. C. L98AV100A growth curve at 41 °C. Survivin\textsuperscript{OFF} cell number increased until 36 hours after addition doxycycline but cells then started to die after 48 hours. However, wild type DT40 and Survivin\textsuperscript{OFF}: GgSurvivin-GFP cells grew normally. D. L104AL106A growth curve at 41 °C. These cells had similar phenotypes.
Survivin L98AV 100A-GFP in KO2 grown at 39°C
Figure 41. Localization of Survivin mutant L98AV100A and INCENP at 39 °C. Cells were fixed with 4% PFA after adding doxycycline 48 hours then were stained for INCENP (WE1186) (lane 1), and DAPI (lane 3). Scale bar is 5 μm.
Survivin L104AL106A-GFP grown at 37 °C

Figure 42. Localization of Survivin mutant L104AL106A and INCENP at 37 °C. INCENP was stained with WE 1186 (left panel). Scale bar is 5 μm.
Figure 43. Localization of Survivin mutant L98AV100A and INCENP at 41 °C. KO cells expressing tagged wild type Survivin are shown as control. INCENP was stained with WE1186 (red). Line is 1. Scale bar is 5 μm.
Survivin L104AL106A-GFP grown at 41°C. KO cells expressing tagged wild type Survivin are shown as control. INCENP was stained with WE1186 (red). Lane is 1. Scale bar is 5 µm.
3.3.15 Predicted phosphorylation sites in Survivin are dispensable

Residues T34 and T117 were reported to be phosphorylated. In addition, T21, T48, S81 and T97 are predicted to be phosphorylated by various kinases. I therefore made chicken Survivin mutants T23A, S50A, S83A and T99A corresponding to the putative human phosphorylation sites. These mutants fused to GFP at their C-terminus were stably transfected into Survivin knockout cells. However, after visual examination, all cell lines stably expressing these mutants proliferated normally in presence or absence of doxycycline. So all these mutants could completely rescue Survivin^{OFF} cells, and none of these putative phosphorylation events is essential for Survivin function.
Attempts to identify interactors of Survivin
IV Attempts to identify interactors of Survivin

4.1 Background

Survivin, which plays a critical role to target the chromosomal passenger complex to centromeres, midzone and midbodies in mitosis, is very important for mitosis. Loss of function of Survivin results in degradation and mislocalization of other chromosomal passengers, failure of cytokinesis and death (Carvalho et al., 2003; Klein et al., 2006; Vader et al., 2006a). However, the mechanism underlining these phenotypes is not well understood. The identification of new or known proteins interacting directly or indirectly with Survivin would help to elucidate the different regulation pathways and functions the protein may be involved in.

Several methods are commonly used to screen for novel protein-protein interactions. These include the yeast two-hybrid assay and affinity purification. The yeast two-hybrid system was first described by Fields and Song (Fields and Song, 1989) and has been used extensively since then to identify novel protein-protein interactions (Hannon et al., 1993; Li and Fields, 1993). The main benefit of the yeast two-hybrid system is that it is a eukaryotic in vivo assay, therefore proteins are more likely to be properly folded and post-translationally modified. It is a very sensitive technique, and can therefore detect weak or transient protein interactions (as well as many false positives). Furthermore, this method can detect proteins of relatively low abundance (Yang et al., 1995).

Another commonly used method to screen for novel protein interactions is affinity purification followed by mass spectrometry. Co-immunoprecipitation is one type of affinity purification, but it is limited by specificity and the expensive price of antibodies. Therefore epitope tags have become general tools for specific and efficient native protein purification (for a review of affinity tags, see Terpe, 2003). The general approach consists of expressing the tagged protein in cells in order to pull it down along with any proteins that
bind to it. To decrease the binding of unspecific proteins, tandem affinity purification (TAP) tags have been developed (Rigaut, Shevchenko et al. 1999; Cheeseman and Desai 2005; Gavin, Aloy et al. 2006; (Puig et al., 2001). The principle behind the TAP tag approach is to express the target protein fused to two distinct purification tags in the host cell. The tagged protein plus any protein bound to it is isolated by the binding of the first tag to an appropriate affinity matrix, and unbound proteins are washed away. Then the tagged protein is released from the first matrix by cleavage or competitive elution before going through a second round of purification using the second tag ending up in a further enrichment of the protein. The original TAP tag utilized protein A (its Z domain binds to IgG) and calmodulin-binding peptide (CBP binds calmodulin) (Cheeseman and Desai, 2005). To date, many other tags have been conjugated into TAP systems, including 6X"His" (binds Ni), HA (hemagglutinin binds anti-HA antibody), S tag (binds s-protein), Biotin-tag (binds avidin or steptavidin), myc peptide (binds anti-myc antibody) and streptavidin binding peptide (SBP binds streptavidin). One of the benefits of this method is that the purification is carried out from cell extracts. The proteins are, therefore, more likely to be correctly post-translationally modified. This technique identifies proteins interacting with their partners in their normal cellular compartment unlike the GAL4 based two-hybrid system in which the proteins are targeted to the nucleus. For this reason, the TAP tag method has a low false positive rate.

In our lab, we developed an S-SBP tandem affinity purification tag and an S-SBP-6His triple affinity purification (TrAP) tag, based on the facts that the S-tag, 6Xhis and SBP tags are small and easy to handle, and that good monoclonal antibodies against S-tag and SBP tag are available. Therefore, these tags appeared to be very useful tools for both looking at the localization of the tagged protein in vivo and purifying it in vitro.

I therefore chose to use a TAP system to try isolating Survivin interactors.
4.2 Purpose of this study and experimental strategy

Through the identification of Survivin interactors, I expect to get a clearer idea on how the protein is regulated and how it functions during mitosis and apoptosis.

For that purpose, I expressed tagged human Survivin in a HeLa cell as well as in the SurvivinOFF DT40 cells. The two approaches have different advantages. The data analysis from a human cell over-expression and purification will be facilitated by the availability of an extended human proteomics database (less developed for chicken). On the other hand expressing the tagged protein at endogenous levels in a null background would ensure that most of the protein is incorporated at the right place in the cell and interact only with its normal designated partners (fewer false positives).

4.3 Tagging and purifying strategies

The "S-tag" is a short fragment of bovine pancreatic ribonuclease A that binds to its ligand, the S-protein, with a $K_d$ of $1.1 \times 10^{-7}$ M (at 25 $^\circ$C, 50 mM NaAc, pH 6.0, 100 mM NaCl). S-tag fusion proteins can be purified rapidly by affinity chromatography using immobilized S-protein (Raines, 1998). The SBP tag, a synthetic sequence isolated from a random peptide library, has a high affinity for streptavidin ($K_d = 2 \times 10^{-9}$ M), and can be effectively eluted with biotin (Figure 46). The "His" tag is the most commonly used purification tool because of its strong affinity for Nickel. Therefore we developed a tandem tag combining the "S-tag" and SBP tag, or combining the "S", SBP and "His" tags. Two or three rounds of purification can be done employing
this tandem affinity tag, in order to remove as much as possible of unspecific binding proteins.

A

6Xhis: HHHHHH

S-tag and TEV: KETAAAKFERQHMDSENLYFQG

SBP tag: MDEKTTGWRGHHVVEGLAGELEQLRARLEHHPQGQREPSGCGKLG

B

Figure 46. Affinity purification. A, Sequence of S-tag, streptavidin binding peptide tag (SBP tag) and His tag. B, Diagram describing the purification strategies both in DT40 Survivin^{OFF} cells and HeLa cells.
Purified samples from cells that stably express recombinant tagged Survivin are analysed by SDS-PAGE gel electrophoresis with subsequent mass spectrometry identification of protein complex components. Ultimately the complex components will be characterized and their function analysed.
4.4 Results and Discussion

4.4.1 Stable cell lines expressing recombinant tandem-tagged Survivin in Survivin knockout cells and HeLa cells.

Human Survivin fused to the S-SBP tandem tag at C-terminus was stably expressed in KO1 cells (Figure 46A), and the expression in these cell lines was confirmed by Western blot using WE43D anti-ggSurvivin antibody. Tagged human Survivin can fully rescue Survivin^{OFF} cells (Figure 28B an B'). Meanwhile, HeLa cells were stably transfected with human Survivin fused to S, SBP and 6Xhis tag (Figure 46A). Immunofluorescence staining on HeLa cells expressing tagged Survivin showed that it could colocalize properly with INCENP and Aurora B (Figure 48).

![Diagram showing expression constructs of tagged human Survivin](image)

**Stably expressed in KO1**

**Stably expressed in HeLa**

Figure 47. Expression constructs of tagged human Survivin. Upper diagram shows the construct expressed in Survivin KO1 cells. The lower diagram represents the construct transfected into HeLa cells: purple Survivin; cyan diamond S-tag; blue SBP tag; Grey 6XHis tag.
Figure 48. Localization of tagged human Survivin in HeLa cells. Mitotic HeLa cells expressing tagged Survivin were fixed and stained with anti S tag antibody (red) and passenger antibodies (green) shown. DNA was stained with DAPI in blue.

4.4.2 Affinity purification and mass spectrometry of tagged Survivin.

The procedure for affinity purification of tagged Survivin is described in the Materials and Methods section. The purified samples from HeLa cells were checked by Western blots incubated with anti-Survivin, Aurora B and Borealin antibodies. Aurora B and Borealin were pulled down together with the tagged Survivin from HeLa cells (Figure 49A). This result showed that the chromosomal passenger complex was still in the sample after purification. To test the quality of my purifications, sample SDS-PAGE gel was tested using
Figure 49. **Purification from HeLa expressing TrAP tagged human Survivin.** A. Samples were tested by Western blot using Aurora B, Survivin and Borealin antibodies. B, The same samples as panel A were checked by silver staining. At the top of each panel show the concentration of NaCl in buffer. P pellet; Su supernatant; US flow through streptavidin beads; ES elution from streptavidin beads; RS material remaining on streptavidin beads after elution; M protein marker; UN flow through from Ni beads; EN elution
from Ni beads; RN material remaining on Ni beads after elution. Red squares show the tagged Survivin.

silver staining. An increased purification quality was obtained using 400 mM NaCl in the lysis buffer compared to 200 mM NaCl, however I was concerned that high salt concentration may break the complex. Therefore only the purification prepared using 200 mM salt was sent for mass spectrometry for both HeLa and DT40 cells. After two rounds of purification samples from HeLa cells and from DT40 cells became cleaner (Figure 49 and Figure 50 B). Because we did not have antibodies against chicken Aurora B and Borealin, the samples from DT40 were probed with S antibody.

Figure 50. Tagged human Survivin expression and purification from KO1. A. S-SBP tagged human Survivin can rescue the chicken Survivin\textsuperscript{OFF} phenotype. KO1 is the control. B. Samples purified from KO1 (control) and KO1 expressing tagged human Survivin were detected by silver staining. Red box shows the tagged Survivin (32 kDa).
4.4.3 Result of mass spectrometry

Proteins identified by mass spectrometry are displayed in Tables 4 and 5; respectively, for the DT40 cell pull-down and HeLa cell pull-down. From the DT40 pull-down, Survivin and INCENP were identified. Mass spectrometry for another purification from DT40 cells using LAP tag (Localization and affinity purification, S-tag plus IP against GFP) (Cheeseman and Desai, 2005) recognized Borealin (Done by Dr Gassmann). However, except for Survivin, other passengers were not identified in the HeLa cell pull-down, though they were recognized by Western blotting. The explanation for this result is not clear. It may be caused by a sensitivity problem at the mass spectrometry level.

Tubulin subunits were also identified from the DT40 pull-down. Microtubule associated proteins myosin regulatory light chain, vimentin, and coiled-coil domain containing 87 were recognized from the HeLa cell pull-down. Actin and actin-dependent regulator SWI were identified from both DT40 and HeLa cell purifications. Actin is a contractile ring component, but till now, no data have reported whether it interacts with Survivin. This may suggest that Survivin regulates cytokinesis by interacting with these MAPs or contractile ring proteins, or these may be non-specific contaminants.

In the DT40 cell pull-down and HeLa cell pull-down, the chaperone Heat shock protein 70 (HSP70), was identified. It has been claimed that Survivin protein levels are regulated by heat shock proteins (Raslova, Kauffmann et al. 2007; (Diakos et al., 2007; Fortugno et al., 2003). This result would need further experimentation to confirm the interaction with Survivin.

Another interesting protein identified from DT40 cell pull-down using tagged human Survivin is P32 of splicing factor 2 (HABP1/p32/gC1qR/SF2), which also plays an important role in genome stability (Li and Manley, 2005).
Loss of splicing factor ASF/SF2 induces G2 cell cycle arrest and apoptosis (Li et al., 2005b). In preliminary experiments, tagged chicken and human p32 can pull-down Survivin from HeLa cells (Figure 51). Further experiments are still needed to confirm their interaction.

Table 9. Mass spectrometry result of pull-down from KO1 expressing tagged human Survivin

<table>
<thead>
<tr>
<th>Acc Num</th>
<th>Mass</th>
<th>Peptide/Protein Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>gi</td>
<td>502145</td>
<td>16389</td>
</tr>
<tr>
<td>gi50759965</td>
<td>70871</td>
<td>17 heat shock protein</td>
</tr>
<tr>
<td>gi45384506</td>
<td>96397</td>
<td>17 INCENP class II</td>
</tr>
<tr>
<td>gi50759965</td>
<td>70870</td>
<td>11 hypothetical protein, similar to MreN/PtsA</td>
</tr>
<tr>
<td>gi50758074</td>
<td>20367</td>
<td>9 similar to p32 subunit of splicing factor SF2</td>
</tr>
<tr>
<td>gi45383806</td>
<td>50157</td>
<td>4 translation elongation factor 1alpha1</td>
</tr>
<tr>
<td>gi51471192</td>
<td>32782</td>
<td>2 hypothetical protein XP_498536 [Homo sapiens] salivary proline-rich</td>
</tr>
<tr>
<td>gi45383776</td>
<td>28888</td>
<td>3 proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>gi50802640</td>
<td>19904</td>
<td>3 similar to beta actin</td>
</tr>
<tr>
<td>gi50748105</td>
<td>42992</td>
<td>3 similar to 26S protease regulatory subunit 6A</td>
</tr>
<tr>
<td>gi29788785</td>
<td>49671</td>
<td>3 beta 5-tubulin; beta lb tubulin [Homo sapiens]</td>
</tr>
<tr>
<td>gi45384338</td>
<td>49671</td>
<td>3 beta 5-tubulin [Gallus gallus]</td>
</tr>
<tr>
<td>gi50732950</td>
<td>44736</td>
<td>2 Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like do</td>
</tr>
<tr>
<td>gi50730111</td>
<td>59251</td>
<td>2 similar to Probable ubiquitin carboxyl-terminal hydrolase FAF-X (Ubiquitin 1)</td>
</tr>
<tr>
<td>gi50750628</td>
<td>120001</td>
<td>2 similar to SWI/SNF-related matrix-associated actin decpenedet regulator of</td>
</tr>
<tr>
<td>gi45383966</td>
<td>24427</td>
<td>2 ras-like protein</td>
</tr>
<tr>
<td>gi50734929</td>
<td>59725</td>
<td>3 similar to hypothetical MGC76252/TCP1</td>
</tr>
<tr>
<td>gi50803534</td>
<td>42014</td>
<td>2 similar to actin</td>
</tr>
<tr>
<td>gi45383077</td>
<td>46371</td>
<td>2 translation initiation factor 4A</td>
</tr>
<tr>
<td>gi4502491</td>
<td>31361</td>
<td>2 complement component 1, qsubcomponent binding protein prexursor</td>
</tr>
<tr>
<td>gi45382259</td>
<td>66737</td>
<td>2 dead-box RNA helicase(interact with INCENP)</td>
</tr>
<tr>
<td>gi50804057</td>
<td>50609</td>
<td>3 similar to ATP synthase</td>
</tr>
<tr>
<td>gi45384238</td>
<td>83187</td>
<td>2 CFR-associated protein p70</td>
</tr>
<tr>
<td>gi11415030</td>
<td>11367</td>
<td>2 histone H4</td>
</tr>
</tbody>
</table>

Table 9. Red accession numbers show the proteins identified both in DT40 TAP and LAP purification. Proteins description in blue represents protein recognized in both DT40 and HeLa TAP pull-down.
Table 10. Mass spectrometry result of pull-down from HeLa expressing tagged human Survivin

<table>
<thead>
<tr>
<th>Accession</th>
<th>Mass (Da)</th>
<th>Peptides</th>
<th>Score</th>
<th>Protein Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IP0055471</td>
<td>82245</td>
<td>12</td>
<td>647</td>
<td>Junction plakoglobin</td>
</tr>
<tr>
<td>IP002890078</td>
<td>64442</td>
<td>10</td>
<td>637</td>
<td>keratin 4 (desmosome)</td>
</tr>
<tr>
<td>IP00025753</td>
<td>114670</td>
<td>10</td>
<td>563</td>
<td>Desmoglein-1 precursor (desmosome)</td>
</tr>
<tr>
<td>IP00021439</td>
<td>42092</td>
<td>6</td>
<td>341</td>
<td>Actin, cytoplasmic 1</td>
</tr>
<tr>
<td>IP00007547</td>
<td>10885</td>
<td>4</td>
<td>224</td>
<td>Protein S100-A8</td>
</tr>
<tr>
<td>IP00022434</td>
<td>73881</td>
<td>4</td>
<td>220</td>
<td>ALB protein</td>
</tr>
<tr>
<td>IP00027462</td>
<td>13291</td>
<td>4</td>
<td>180</td>
<td>Protein S100-A9</td>
</tr>
<tr>
<td>IP000219018</td>
<td>36070</td>
<td>3</td>
<td>251</td>
<td>Glyceroldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>IP00006874</td>
<td>22324</td>
<td>3</td>
<td>199</td>
<td>Peroxiredoxin-1</td>
</tr>
<tr>
<td>IP00006210</td>
<td>16720</td>
<td>3</td>
<td>164</td>
<td>Isoform 1 of Baculoviral IAP repeat-containing protein 5</td>
</tr>
<tr>
<td>IP00454373</td>
<td>11229</td>
<td>3</td>
<td>139</td>
<td>Histone H4</td>
</tr>
<tr>
<td>IP00350376</td>
<td>76926</td>
<td>3</td>
<td>96</td>
<td>Protein-glutamine gamma-glutamyltransferase E precursor</td>
</tr>
<tr>
<td>IP00068000</td>
<td>22725</td>
<td>2</td>
<td>112</td>
<td>hypothetical protein LOC124229 (HRP773 salivary</td>
</tr>
<tr>
<td>IP00033494</td>
<td>19624</td>
<td>2</td>
<td>111</td>
<td>Myosin regulatory light chain</td>
</tr>
<tr>
<td>IP00036485</td>
<td>50451</td>
<td>2</td>
<td>95</td>
<td>Elongation factor 1-alpha 1</td>
</tr>
<tr>
<td>IP00023078</td>
<td>28557</td>
<td>2</td>
<td>81</td>
<td>Late envelope protein 7</td>
</tr>
<tr>
<td>IP00007425</td>
<td>94916</td>
<td>2</td>
<td>65</td>
<td>desmocollin 1 isoform Dsc1b preproprotein (desmosome)</td>
</tr>
<tr>
<td>IP000247167</td>
<td>30842</td>
<td>2</td>
<td>73</td>
<td>CDNA FLJ41947, clone PLACE6019932, moderately similar</td>
</tr>
<tr>
<td>IP00021428</td>
<td>42366</td>
<td>1</td>
<td>27</td>
<td>Actin, alpha skeletal muscle</td>
</tr>
<tr>
<td>IP00018278</td>
<td>13369</td>
<td>1</td>
<td>75</td>
<td>Histone H2A</td>
</tr>
<tr>
<td>IP00007797</td>
<td>15366</td>
<td>1</td>
<td>70</td>
<td>Fatty acid-binding protein, epidermal</td>
</tr>
<tr>
<td>IP00007547</td>
<td>23864</td>
<td>1</td>
<td>68</td>
<td>Myosin light polypeptide 6A</td>
</tr>
<tr>
<td>IP000221088</td>
<td>22504</td>
<td>1</td>
<td>65</td>
<td>40S ribosomal protein S9</td>
</tr>
<tr>
<td>IP00021828</td>
<td>11190</td>
<td>1</td>
<td>58</td>
<td>Cystatin B (cysteine protease inhibitor)</td>
</tr>
<tr>
<td>IP000216975</td>
<td>35874</td>
<td>1</td>
<td>53</td>
<td>Isoform 2 of Tropomyosin alpha-4 chain (actin binding)</td>
</tr>
<tr>
<td>IP000418471</td>
<td>53545</td>
<td>1</td>
<td>51</td>
<td>Venustin (HAP)</td>
</tr>
<tr>
<td>IP000376798</td>
<td>20468</td>
<td>1</td>
<td>51</td>
<td>ribosomal protein L11</td>
</tr>
<tr>
<td>IP000027547</td>
<td>11391</td>
<td>1</td>
<td>50</td>
<td>Dermcidin precursor</td>
</tr>
<tr>
<td>IP000221050</td>
<td>43166</td>
<td>1</td>
<td>39</td>
<td>Isocitrate dehydrogenase (NAD) subunit gamma, mitochondrial p</td>
</tr>
<tr>
<td>IP00019120</td>
<td>15302</td>
<td>1</td>
<td>33</td>
<td>CDNA FLJ41893, clone BRAH3005923</td>
</tr>
<tr>
<td>IP00000230</td>
<td>32715</td>
<td>1</td>
<td>47</td>
<td>Tropomyosin 1 alpha chain isoform 2</td>
</tr>
<tr>
<td>IP000761105</td>
<td>49010</td>
<td>1</td>
<td>45</td>
<td>similar to Adz2b CG6983-PA, isoform A isoform 3 (transcripts)</td>
</tr>
<tr>
<td>IP000366808</td>
<td>52261</td>
<td>1</td>
<td>45</td>
<td>Conoecosomin precursor</td>
</tr>
<tr>
<td>IP000038356</td>
<td>25640</td>
<td>1</td>
<td>43</td>
<td>25 kDa protein (Arginase)</td>
</tr>
<tr>
<td>IP000222204</td>
<td>44594</td>
<td>1</td>
<td>42</td>
<td>Serpin B3</td>
</tr>
<tr>
<td>IP000419424</td>
<td>26503</td>
<td>1</td>
<td>42</td>
<td>IGKVI-5 protein (antigen binding)</td>
</tr>
<tr>
<td>IP000031549</td>
<td>101218</td>
<td>1</td>
<td>41</td>
<td>Isoform 3A of Desmoscin-3 precursor</td>
</tr>
<tr>
<td>IP000152881</td>
<td>218321</td>
<td>1</td>
<td>40</td>
<td>Shroom-related protein (F-acin-binding protein)</td>
</tr>
<tr>
<td>IP000219575</td>
<td>53155</td>
<td>1</td>
<td>35</td>
<td>Bioenzymic hydrolase</td>
</tr>
<tr>
<td>IP000767531</td>
<td>148778</td>
<td>1</td>
<td>34</td>
<td>similar to Cyclin G-associated kinase (interact with cyclin G and )</td>
</tr>
<tr>
<td>IP000008849</td>
<td>8779</td>
<td>1</td>
<td>34</td>
<td>Small proline-rich protein 2G (structural molecule activity)</td>
</tr>
<tr>
<td>IP000033583</td>
<td>46646</td>
<td>1</td>
<td>32</td>
<td>Serpin B12 (serine (or cysteine) proteinase inhibitor)</td>
</tr>
<tr>
<td>IP000166200</td>
<td>40619</td>
<td>1</td>
<td>32</td>
<td>Gasdermin (unknown)</td>
</tr>
<tr>
<td>IP000137669</td>
<td>49845</td>
<td>1</td>
<td>31</td>
<td>Alpha-ename, lung specific</td>
</tr>
<tr>
<td>IP000168899</td>
<td>68568</td>
<td>1</td>
<td>29</td>
<td>DBF4 homolog B isoform 1 (regulator of the CDC7-like 1 protein)</td>
</tr>
<tr>
<td>IP000293188</td>
<td>21999</td>
<td>1</td>
<td>28</td>
<td>Isoform 1 of p63 apoptosis effector related to PMP-22</td>
</tr>
<tr>
<td>IP000345999</td>
<td>106343</td>
<td>1</td>
<td>27</td>
<td>Tyrosine-protein kinase transmembrane receptor ROR2 precursor</td>
</tr>
<tr>
<td>IP000387020</td>
<td>26943</td>
<td>1</td>
<td>26</td>
<td>Myocardin-2</td>
</tr>
<tr>
<td>IP000294405</td>
<td>25388</td>
<td>1</td>
<td>26</td>
<td>Cardiotrophin-like cytokine factor 1 precursor</td>
</tr>
<tr>
<td>IP000446354</td>
<td>13792</td>
<td>1</td>
<td>25</td>
<td>CDNA FLJ18058, clone NOVAR2000952 (unknown)</td>
</tr>
<tr>
<td>IP000302151</td>
<td>96707</td>
<td>1</td>
<td>24</td>
<td>Coiled-coil domain containing 47 (MAP)</td>
</tr>
</tbody>
</table>

Table 10. Proteins identified from HeLa TAP pull-down. Interesting proteins are labelled in colour.
Figure 51. Primary result of pull-down using TrAP tagged human and chicken P32 transiently expressing in HeLa cells. Whole cell lysates were incubated with streptavidin beads for 30 minutes. After two wash, streptavidin beads were boiled for gel samples. WCL whole cell lysate; US flow through streptavidin beads (loaded 25% amount of cells as other sample); X empty lane; SA material binding to streptavidin beads; SA material binding to S-protein beads from US.
Discussion
V Discussion

Survivin in mitosis

Survivin functions together with INCENP, Aurora B and Borealin (Carmena and Earnshaw, 2003; Ruchaud et al., 2007; Vagnarelli and Earnshaw, 2004), and studies have confirmed their physical interaction (Gassmann et al., 2004; Klein et al., 2006; Sampath et al., 2004). Tagged Survivin can pull down INCENP, Aurora B, Borealin and endogenous Survivin (unpublished data). Borealin can also co-IP INCENP, Survivin and Aurora B (Gassmann et al., 2004). In our DT40 knockout I found that when Survivin was repressed, INCENP could not localize properly as previously described (Carvalho et al., 2003) (Figure 20 an 21). In addition to the influence on INCENP, after Survivin was removed, Aurora B kinase function was impaired, as the level of Ser10—phosphorylated Histone 3 clearly decreased (Figure 19). These results are consistent with previous reports showing that INCENP and Aurora B function and mitotic localization require Survivin (Carvalho et al., 2003; Gassmann et al., 2004; Honda et al., 2003; Vader et al., 2006a). The binding sites in INCENP for Aurora B, Survivin and Borealin, respectively, have been mapped; but the interactions between Aurora B, Survivin and Borealin are still being dissected. In vitro pull-down and co-IP previously indicated that human Survivin residues D70 and D71 (corresponding to chicken D72 and D73) are involved in the binding of Survivin to Aurora B (Cao et al., 2006). In the Survivin\textsuperscript{ON} or Survivin\textsuperscript{OFF} cells, I also found GFP-tagged Survivin-DD72, 73AA and INCENP to be diffusely localized in prometaphase and metaphase. However, this did not affect Survivin\textsuperscript{OFF} proliferation. Furthermore, in those cells the levels of Histone 3 phosphorylated at Ser10 looked similar to those of Survivin\textsuperscript{ON} cells showing that Aurora B activity was not affected by those mutations (data not shown).

It remains to be determined whether this double mutation abolishes the interaction between Survivin and Aurora B in our system. Interestingly, with
Zinc finger amino acid mutations, Survivin cannot locate either INCENP or itself properly in mitosis, and is not able to rescue the Survivin knockout (Figure 36 and 37). One possible reason is that Zinc finger mutants cannot form dimers with wild type Survivin, so they cannot be targeted. Another possibility is that the mutated Survivin abolishes interaction with other proteins and that it is not a dimer. In summary, Survivin is essential for the chromosomal passenger complex localization and function (both spatially and temporally).

Another important question is whether Survivin is necessary for cells to enter mitosis. Our movies using tagged H2B-RFP and micrographs of cells stained with DAPI showed that Survivin\textsuperscript{OFF} cells accomplished the G2-M transition and achieved anaphase. Some tetraploid Survivin\textsuperscript{OFF} cells could still enter mitosis again (Figure 39A). Of course, this result does not mean that the centromere and midzone targeting of the chromosomal passenger complex is not important for its function later in mitosis. Chromosomal passengers targeting to the centromeres may be involved in the cytokinesis signal transmission (Buvelot et al., 2003; Earnshaw and Bernat, 1991; Wheatley et al., 2001a). However the mechanisms of chromosomal passenger action in mitosis are still not completely clear.

**Survivin and the spindle checkpoint**

A number of studies have shown that chromosomal passenger proteins Aurora B and Survivin affect the spindle checkpoint (Carvalho et al., 2003; Lens et al., 2003). When microtubule tension is lost due to paclitaxel treatment, cells in which Survivin was knocked down by RNAi exited mitosis prematurely (Carvalho et al., 2003; Lens et al., 2003). Similarly, in yeast, Aurora B/Ipl1 is required in the spindle checkpoint in response to lack of tension (Biggins and Murray, 2001). When Aurora B/Ipl1 was inhibited by antibodies, ZM447439 or hesparadin, mammalian cells could not activate the
spindle checkpoint in the absence of tension (Ditchfield et al., 2003; Hauf et al., 2003; Kallio et al., 2002). In our system (Figure 23) a large percentage of cells did not arrest in mitosis after incubation with 400 nM Taxol or 0.5 μg/ml nocodazole for 24 hours. This is true for wild type DT40 as well as Survivin$^{\text{ON}}$ and Survivin$^{\text{OFF}}$ cells. However, the index of mitotic cells after adding 400 nM Taxol or nocodazole for 12 hours was totally different. I found that more than 90% of the cells were arrested in mitosis, although 60% of Survivin$^{\text{OFF}}$ cells were dying at this time point. Interestingly, I found that fewer cells remained alive after adding the drugs for 24 hours than after adding the same drugs for 12 hours. So it is likely that the increase in the percentage of interphase cells is caused by the death of many arrested mitotic cells. Strikingly, only 50% Survivin$^{\text{OFF}}$ cells are arrested in mitosis when cells treated with 10 nM taxol, but 90% SurvivinON cells were arrested in mitosis. Taken together our results suggest that Survivin is apparently required for activating the spindle assembly checkpoint in lower dose taxol (5 nM-50 nM). High concentration taxol might activate another shckpoint pathway independent Survivin.

**Survivin is required for cytokinesis**

Our experiments and other reports (Carvalho et al., 2003; Lens et al., 2003; Li et al., 1999) have demonstrated that knockout or knockdown of Survivin leads to the accumulation of multinucleated cells (Figure 39). This result is similar to the phenotype in C. elegans embryos in which Survivin was knocked down by RNAi (Fraser et al., 1999; Speliotes et al., 2000). Some reports have argued that multinucleated cells were not observed, but these results may stem from poor RNAi efficiency. Incomplete cytokinesis has been observed in some naturally occurring developmental processes. Bone marrow megakaryocytes are terminally differentiated cells, that skip late anaphase and cytokinesis during endomitosis. Studies have revealed that megakaryocytes apparently lack Survivin at all stages of the endomitotic cell cycle (Zhang et al., 2004), though Aurora B was expressed and localized...
normally at prophase in polyploidizing mouse bone marrow megakaryocytes (Zhang et al., 2004). Vascular smooth muscle cells, another polyploidizing cell line, exhibit a similar phenotype (Nagata et al., 2005). It appears that development and differentiation can be regulated by adjusting the level of Survivin. However, the mechanism by which Survivin regulates cytokinesis is elusive.

Many studies have tried to solve the mechanism of cytokinesis. Rappaport et al. had noticed that microtubules and the midzone play an important role in the delivery of the cytokinesis signals (Cao and Wang, 1996; Murata-Hori et al., 2002; Murata-Hori and Wang, 2002a; Rappaport, 1961; Rappaport, 1996). However, in our experiments, cleavage furrow formation and ingression in anaphase appeared normal in Survivin:\textsuperscript{OFF} cells (Figure 22 and 25 and movie 4). The failure of cytokinesis caused by lack of Survivin happened later in the process. Why knocking out Survivin caused failure in cytokinesis is complex and hard to answer. There are more than 20 known core proteins involved in cytokinesis, including the chromosomal passenger complex (Glotzer, 2005); and there are more than 160 polypeptides that participate in building midbodies (Skop et al., 2004). During the transition from metaphase to anaphase and then telophase, certain proteins are recruited and transferred to the centromere, midzone and midbody. In addition to chromosomal passenger proteins themselves (Adams et al., 2001b; Cooke et al., 1987; Gassmann et al., 2004; Skoufias et al., 2000), other proteins interacting with them also need to be recruited. MKLP-1, the regulator of mitotic spindle and cytokinesis, failed to localize to the spindle midzone and midbody in cells depleted of Survivin by RNAi (Yang et al., 2004). INCENP is also required for recruiting MKLP-1 to the spindle midzone/midbody (Zhu et al., 2005), and phosphorylation of MKLP1 by Aurora B is necessary for cytokinesis (Gruneberg et al., 2006; Guse et al., 2005). Aurora B knockdown leads to Taxins being lost from the midbody (Delaval et al., 2004). Ect2 localization requires both Aurora B and MKLP1
Survivin is essential for cell life
Survivin null mouse embryos become polyploid and fail to survive beyond 4.5 days post coitum (Uren et al., 2000). In tissue culture, interference with Survivin expression by antisense oligonucleotides caused cell death in the G2/M phase of the cell cycle (Li et al., 1999). On the other hand, another report argued that euploid human cells could survive in the absence of Survivin. Apoptosis was not detected using terminal deoxynucleotidyltranferase-mediated dUTP nick end labeling (TUNEL) assays (Yang et al., 2004).

From our experiments, cells did not die before they became tetraploid. Some tetraploid cells were able to survive another cell cycle (Figure 25A). Propidium iodide (PI) staining showed that a large population of cells passed G2/M (Figure 27B), but these multinucleated cells died by apoptosis later in interphase. Survivin was undetectable in terminally differentiated and quiescent cells (Ambrosini et al., 1997), so it appeared not to be required for survival of these cells. Our experiment showed that Survivin is essential for dividing cells, though I do not have data that can explain exactly why the tetraploid cells die.

Survivin and cell death
Survivin has been implicated in protecting cells from apoptosis (Altieri, 2003; Wheatley and McNeish, 2005). Studies have reported that overexpression of Survivin increased resistance of cancer cells to radiation...
(Chakravarti et al., 2004; Lu et al., 2004) and cancer therapy agents like etoposide, staurosporine or paclitaxel (Dohi and Altieri, 2005; Temme et al., 2003). However, our results did not show any increased sensitivity of Survivin\textsuperscript{OFF} cells to etoposide, staurosporine or taxol relative to wild type or Survivin\textsuperscript{ON} cells (Figure 26). I also found that Survivin\textsuperscript{OFF} cells synchronized by elutriation died after becoming multinucleated and that this death could not be blocked by z-VAD-fmk or the JNK kinase inhibitor SP600125. Cell death occurring here appeared to be caspase-independent, and if it is occurring specifically because Survivin is absent, then Survivin's role cannot be functioning primarily as a caspase inhibitor.

Studies on Survivin structure (Muchmore et al., 2000; Sun et al., 2005), biochemistry (Kim et al., 2006; McNeish et al., 2005) and mutants (Li et al., 1999; Liu et al., 2004a; Mesri et al., 2001; Muchmore et al., 2000; O'Connor et al., 2000; Song et al., 2004; Wall et al., 2003) have argued for a role of Survivin in apoptosis. Several Survivin mutants have been reported to contribute to apoptosis. However, our experiments showed those mutants, including T36A/E, D55A and DD7273AA could all rescue the Survivin knockout and that cell proliferation was the same as in wild type DT40 cells (Figure 29, 32 & 33). These results were clear, considering the null background, and consistent with an earlier report (Wheatley et al., 2007). The difference from published studies might be because different cells have different regulation pathways or because these mutants have no effect on Survivin function.

Our results indicating the lack of role for Survivin in cell death regulation are very similar to those reported in \textit{S. pombe} (Rajagopalan and Balasubramanian, 1999), \textit{C. elegans} (Fraser et al., 1999), \textit{Drosophila} (Jones et al., 2000) and \textit{Xenopus} (Bolton et al., 2002). Hence, the death of Survivin knockout cells is more likely the consequence of abnormal nuclear processes rather than loss of inhibition of caspases or Smac. This suggests that
therapies utilizing antisence Survivin oligos or Survivin mutants need to be thought through more carefully.

Several Survivin structural elements are important.

The BIR domain of Survivin is conserved among organisms and also among BIR domain containing proteins. The Zinc finger amino acids are also completely identical from yeast to human (Ambrosini et al., 1997; Verdecia et al., 2000). The expression of human Survivin C84A in HeLa cells resulted in abnormal mitosis and apoptosis (Li et al., 1999). These results are similar to those obtained with our SurvivinOFF cells to some extent. When C59 or C86 (corresponding to human C57 and C84) were mutated to A, Survivin was totally mislocalized and could not target INCENP in mitosis (Figure 37 and 38). When endogenous Survivin was switched off, C59A and C86A led to the same phenotypes as seen with the knockout SurvivinOFF cells. However, C59A and C86A mutants did not appear to be dominant negative. These two mutants did not affect the growth of SurvivinON cells. This might be because the mutations not only caused the morphology collapse of the Survivin Zinc finger and loss of function, but also directly abolished its interaction with other chromosomal passengers.

Another important motif in Survivin is the linker region. The Survivin linker region between the BIR domain and the C-terminal α-helix (residues 89-102) is highly conserved among vertebrates (Figure 11). This region, together with the N-terminal region and the α4 helix, are implicated in Survivin dimerization. Residues F93, L96, L98 and L102 (human Survivin) are especially important for dimer interaction (Chantalat et al., 2000; Verdecia et al., 2000). Crystal structure and nuclear magnetic resonance studies previously revealed that purified Survivin can form a dimer (Chantalat et al., 2000; Sun et al., 2005; Verdecia et al., 2000). Song et al have reported that Survivin forms a dimer in vivo (Song et al., 2004). Here I found that the linker
region is essential for Survivin function. Mutations of L98 and V100, or L104 and L106 (Corresponding to human L94, L96, L102 and L104 respectively) in chicken Survivin led to cessation of cell proliferation and cell death at 41°C (Figure 40). The Survivin mutant L98AV100A could not form a stable 3-D structure in NMR analysis (Xuemei Yuan, Personal communication). These results agree with the predicted structure based on crystallographic and NMR studies. Others have studied mutations at some of the same sites. The single amino acid mutant Survivin L98A could localize in HeLa cells (Colnaghi et al., 2006), but the double site mutant L96AL98A could not target to the centromere, midzone or midbody and caused mislocalization of other chromosomal passenger complex components (Knauer et al., 2006a). Our results show that Survivin L98AV100A and L104AL106A were all diffusely localized and could not concentrate at the centromeres, midzone and midbody when endogenous Survivin was still ON at 39°C. However, INCENP could localize perfectly in mitosis in the same cells (Figure 41 and 43). This result revealed that these two mutants are not dominant-negative. Surprisingly, when endogenous Survivin was switched off, these Survivin mutants could target well and function at 39°C. These data demonstrated that these mutants are able to function but are not able to compete with wild type Survivin. The conflict between this result and that of Knauer and Colnaghi might be attributed to different levels of expression of endogenous and exogenous Survivin. Interestingly, these mutants lose their function when the temperature increases to 41°C (Figure 40, 43 and 44). Phenotypes similar to the Survivin\textsuperscript{OFF} knockout cells were observed: mislocalization of Survivin and INCENP, multinucleated cells, multiple spindle poles. The double site human Survivin mutant in the same region, F101AL102A, was not able to dimerize (Engelsma et al., 2007). This demonstrated that these amino acids might take part in the dimerization and that the dimerization is crucial for Survivin function. There is also a possibility that these mutations cause Survivin to lose its interactions with other proteins, but more detailed biochemical data will be needed to confirm this hypothesis.
The Survivin linker region was also reported to be involved in the CRM1 exporting pathway that actively traffics proteins out of the nucleus (Colnaghi et al., 2006; Knauer et al., 2006a; Rodriguez et al., 2006; Rodriguez et al., 2002). There is no active nuclear export signal found in chicken Survivin, though chicken Survivin has a sequence resembling the conserved consensus motif (\textsuperscript{98}LTVQEFLKL\textsuperscript{106}). The chicken Survivin mutant P95A does accumulate inside the nucleus in interphase. Furthermore, it does not affect Survivin localization or function in mitosis. More data is needed to determine why mutations in the linker region cause Survivin to lose function.

Survivin and microtubule organization

Chromosomal passengers have been verified as essential for organization of the mitotic spindle. In \textit{S. pombe}, Bir1 deletion mutants were unable to finish the metaphase-to-anaphase transition because of a failure of spindle microtubule elongation (Uren et al., 1999). When Survivin was knocked out in mice or knocked down by RNAi in RPE cells, the organization of microtubules was disrupted (Uren et al., 2000; Yang et al., 2004). Similarly to Survivin, two other chromosomal passengers, Borealin and INCENP, are also required for chromatin-Induced microtubule stabilization and spindle assembly (Gassmann et al., 2004; Sampath et al., 2004). For these reports, no rescue experiment for the spindle formation was done, or else rescue was unsuccessful (Canovas and Guadagno, 2007). However, in my experiment I did not find microtubules to be abnormal in metaphase and anaphase, either in diploid or tetraploid Survivin\textsuperscript{OFF} cells. Because there is no midbody found in Survivin\textsuperscript{OFF} cells, microtubule structure is untraceable in later mitosis. In summary our experiments did not show any obvious changes in microtubule organization prior to telophase.

Is Survivin phosphorylated?
Post-translation modification of proteins plays an important role in regulation of their functions; hence many people have focused on Survivin modifications. Studies have identified that Survivin is phosphorylated by Aurora B on Thr117 and on Thr34 by CDK1 in human (O'Connor et al., 2000; Wheatley et al., 2004). However our knockout DT40 cells stably expressing T23A, T36A/E, S50A, S83A or T99A (corresponding to human T21, T34, T48, S81 and T97) grew normally and fully rescued the knockout phenotype. Our experiments suggest that T23, T36, S50, S83 and T99 might not be phosphorylated, or else phosphorylation on these sites is not necessary for cell proliferation. The roles of the Survivin phosphorylation remain to be identified.

**Do other proteins interact with Survivin besides chromosomal passengers?**

The interaction between Survivin and other chromosomal passengers has been confirmed (Gassmann et al., 2004; Klein et al., 2006; Sampath et al., 2004). Co-IP also verified that Survivin binds Ufd1 and hFAM (Vong et al., 2005). Survivin was reported to make a 250 kDa complex with other proteins including chromosomal passengers in mitosis. However, in interphase it is found in a 490 kDa complex as measured by sucrose gradient sedimentation, and superose 6 gel filtration isolated a 900 kDa complex (Bolton MA 2002). To sum up these data, I would ask whether there are other proteins interacting with Survivin, and whether different proteins interact with Survivin in different cell cycle phases? I think that proteomics studies on Survivin will help answer these questions.
General Conclusions and Perspectives

Survivin is a key regulator of mitosis and its over-expression in a wide variety of tumours makes it an important target in the understanding of cancer genesis. The Survivin knockout cells provided an excellent tool to study the protein function in vivo. Taken together our results showed that Survivin is essential for the completion of mitosis. However our experiments failed to provide evidence for a role of Survivin as an anti-apoptotic factor. The expression of Survivin mutated at specific residues in a Survivin wild type null background helped us dissecting the role of its different domains. Using this approach we showed that the Zinc finger and linker region are required for Survivin function during mitosis. Knockout cells stably expressing tagged Survivin were used for initial preliminary efforts to identify its interacting proteins. The Mass spectrometry analysis of tandem affinity purified tagged Survivin in the knockout cells identified several potential interactors including microtubule associated proteins and others of possible interest. Further analysis of these results may provide us with a better idea on how for example Survivin targets the chromosomal passenger complex to centromeres or how it is involved in completion of cytokinesis. There are still many questions to answer and getting our hands on any of these answers would help the field of cancer therapy development.

Taken all together, these results indicate that the Survivin knockout cell line has important advantages over the Survivin RNAi strategy, and it will constitute an important tool for the further study of Survivin function. Not only does it provide the possibility to explore the roles of Survivin mutants and different isoforms, but it could also be used for comparative biochemical analysis of uniform cell populations expressing or totally lacking Survivin. Although the knockout approach is more time-consuming than RNAi, it
provides a stable and more reliable option to achieve a completely null background.
References


Mollinari, C., Reynaud, C., Martineau-Thuillier, S., Monier, S., Kieffer, S., Garin, J., Andreassen, P. R., Boulet, A., Goud, B., Kleman, J. P., and...


186
methylation behaviours of sixteen genes that may contribute to
carcinogenesis of astrocytoma. BMC Cancer 4, 65.
C. elegans cell death gene ced-3 encodes a protein similar to mammalian
Differential regulation of CENP-A and histone H3 phosphorylation in G2/M. J
Cell Sci 114, 653-661.
quantity and localization of Aurora-B/AIM-1 and survivin during
megakaryocyte polyploidization and the consequences of Aurora-B/AIM-1-
Zhao, J., Tenev, T., Martins, L. M., Downward, J., and Lemoine, N. R.
(2000). The ubiquitin-proteasome pathway regulates survivin degradation in
Zhou, J., Yao, J., and Joshi, H. C. (2002). Attachment and tension in the
the spindle midzone/midbody by INCENP is essential for midbody formation
and completion of cytokinesis in human cells. Biochem J 389, 373-381.
Zwerts, F., Lupu, F., De Vriese, A., Pollefyty, S., Moons, L., Altura, R. A.,
cell survivin causes embryonic defects in angiogenesis, cardiogenesis, and