Characterisation of Caspase-2 Function in the DNA Damage Response and Tumour Suppression

A thesis submitted in total fulfilment of the requirements of the degree of Doctor of Philosophy

by

Joseph Puccini
Bachelor of Health Sciences (Honours)

School of Medicine, Discipline of Medicine
Faculty of Health Sciences, The University of Adelaide
Adelaide, South Australia

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Abstract

Caspases are a family of cysteine proteases that have essential functions in the regulation of apoptosis and inflammation. Despite being the most evolutionarily conserved caspase, the physiological functions of caspase-2 remain poorly defined. This is partly because caspase-2 knockout (Casp2\(^{-/-}\)) mice show no overt phenotype and only limited, tissue-specific apoptotic defects. Previous work from our laboratory has provided the first direct evidence demonstrating a role for caspase-2 in tumour suppression and protection against cellular transformation. However, the molecular mechanisms by which caspase-2 exerts these functions were not clearly defined.

In order to characterise the tumour suppressor function of caspase-2, the processes and pathways disrupted in caspase-2-deficient cells were investigated. Analysis of serially-passaged mouse embryonic fibroblasts (MEFs) demonstrated that caspase-2-deficiency promoted escape from replicative senescence which coincided with impaired induction of cyclin-dependent kinase inhibitors. Consistent with the increased proliferation rate of primary Casp2\(^{+/}\) MEFs, spontaneously-immortalized Casp2\(^{-/-}\) MEFs that had escaped replicative senescence also displayed an enhanced proliferative capacity compared to their wild type counterparts. These findings suggest that caspase-2 regulates cell proliferation which may contribute to its ability to protect against cellular transformation. Furthermore, serially-passaged Casp2\(^{+/}\) primary MEFs and Eµ-Myc/Casp2\(^{-/-}\) lymphomas showed enhanced aneuploidy, demonstrating that loss of caspase-2 promotes genomic instability (GIN). Treatment of Casp2\(^{+/}\) MEFs with ionizing radiation (IR) resulted in persistent DNA damage and defective cell cycle
checkpoint regulation, suggesting that \textit{caspase-2}-deficient cells have an impaired ability to efficiently respond to and repair DNA damage. Further analysis revealed that \textit{caspase-2}-deficient MEFs and E\textsubscript{\mu}-Myc lymphomas displayed defective activation of p53 and its downstream targets following IR treatment. Therefore, an attenuated p53 response may contribute to defective DNA damage response (DDR) signalling and GIN in \textit{caspase-2}-deficient cells.

In order to further investigate the extent and specificity of caspase-2 function in tumour suppression using an independent tumour model, \textit{Atm}\textsuperscript{+/−} and \textit{Casp2}\textsuperscript{+/−} mice were inter-crossed to generate \textit{Atm}\textsuperscript{−/−}\textit{Casp2}\textsuperscript{−/−} mice. Initial characterization revealed that \textit{caspase-2}-deficiency enhanced growth retardation and caused perinatal lethality in \textit{Atm}\textsuperscript{−/−} mice. A comparison of tumour susceptibility demonstrated that \textit{Atm}\textsuperscript{−/−}\textit{Casp2}\textsuperscript{−/−} mice developed lymphomas with a dramatically increased onset and penetrance compared to \textit{Atm}\textsuperscript{−/−} mice, providing additional evidence supporting a tumour suppressor function for caspase-2. Furthermore, \textit{Atm}\textsuperscript{−/−}\textit{Casp2}\textsuperscript{−/−} lymphomas showed an increased proliferation rate and enhanced oxidative damage compared to \textit{Atm}\textsuperscript{−/−} lymphomas. Moreover, lymphomas and pre-malignant lymphocytes derived from \textit{Atm}\textsuperscript{−/−}\textit{Casp2}\textsuperscript{−/−} mice displayed enhanced aneuploidy, linking the function of caspase-2 in the maintenance of genomic stability to its tumour suppressive activity.

Overall, this thesis provides novel insights into the physiological functions of caspase-2, highlighting its roles in the regulation of cell proliferation, the DDR, maintenance of genomic stability and tumour suppression.
Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Joseph Puccini

May 2014
The following publications have resulted from work performed during the period of this candidature.

**Publications included in thesis:**


**Publications non included in thesis:**


Awards

**Best Poster Prize** at ANZSCDB Meeting  
(Adelaide, 2011)

**National Travel Award**  
(South Australian Pathology Medical Staff Specialist Fund, 2011)

**Conference Travel Scholarship** to attend the 20th Euroconference on Apoptosis  
(European Cell Death Organization, 2012)

**Faculty of Health Sciences Postgraduate Travelling Fellowship**  
(University of Adelaide, 2012)

**Best Student Oral Presentation** at ANZSCDB Meeting  
(Adelaide, 2012)

**International Travel Award**  
(South Australian Pathology Medical Staff Specialist Fund, 2012)

**Beat Cancer Project Travel Grant**  
(South Australian Health and Medical Research Institute, 2013)

**Discipline of Medicine Travel Grant**  
(University of Adelaide, 2013)

**Best Student Oral Presentation** at ANZSCDB Meeting  
(Adelaide, 2013)
Conference Attendance

The 1st Australian Workshop on Cell Death (Oral Presentation)
Lindeman Island, Queensland (2011)

ANZSCDB Meeting (Poster)
Adelaide, South Australia (2011)

Faculty of Health Sciences Postgraduate Research Conference (Poster)
Adelaide, South Australia (2012)

20th Euroconference on Apoptosis - European Cell Death Organization (Poster)
Rome, Italy (2012)

ANZSCDB Meeting (Oral Presentation)
Adelaide, South Australia (2012)

Cell Growth and Proliferation Gordon Research Conference (Poster)
West Dover, Vermont, USA (2013)

Cell Growth and Proliferation Gordon Research Seminar (Oral Presentation)
West Dover, Vermont, USA (2013)

ANZSCDB Meeting (Oral Presentation)
Adelaide, South Australia (2013)
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# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>S3BP1</td>
<td>p53 binding protein 1</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>8-hydroxy-2'-deoxyguanosine</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>AML</td>
<td>acute myelogenous leukaemia</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APAF-1</td>
<td>apoptotic protease activating factor 1</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>AT</td>
<td>ataxia telangiectasia</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>ataxia telangiectasia and Rad3-related</td>
</tr>
<tr>
<td>BAK</td>
<td>Bcl-2 homologous antagonist/killer</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl-2-associated protein X</td>
</tr>
<tr>
<td>BCL</td>
<td>B cell lymphoma</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 homology 3</td>
</tr>
<tr>
<td>BID</td>
<td>BH3-interactingdomain death agonist</td>
</tr>
<tr>
<td>BN</td>
<td>binucleated</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BRCA1</td>
<td>breast cancer 1</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BUB1</td>
<td>budding uninhibited by benzimidazoles 1</td>
</tr>
<tr>
<td>C</td>
<td>Celsius</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase activation and recruitment domain</td>
</tr>
<tr>
<td>Cat</td>
<td>catalase</td>
</tr>
<tr>
<td>CBMN</td>
<td>cytokinesis-block micronucleus assay</td>
</tr>
<tr>
<td>CDC25</td>
<td>cell division cycle 25</td>
</tr>
<tr>
<td>Cdk</td>
<td>cyclin-dependent kinase</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CHK</td>
<td>checkpoint kinase</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre(s)</td>
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<tr>
<td>Cy</td>
<td>cyanine</td>
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<tr>
<td>Cys</td>
<td>cysteine</td>
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<td>Cyt c</td>
<td>cytochrome c</td>
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DAB diaminobenzidine
DAPI 4′,6′-diamidino-2-phenylindole
DD death domain
DDR DNA damage response
DED death effector domain
DEPC diethylpyrocarbonate
DGR dorsal root ganglion
DIABLO direct IAP-binding protein with low PI
DMEM Dulbecco’s Modified Eagles Medium
DNA deoxyribonucleic acid
dSB double-strand break
dsDNA double-stranded DNA
DTT dithiothreitol
dUDP deoxyuridine diphosphate

e embryonic day
ECF enhanced chemifluorescence
ECL enhanced chemiluminescence
ECM epithelial-mesenchymal transition
EDTA ethylenediaminetetraacetic acid
EGFR epidermal growth factor receptor
ELISA enzyme-linked immunosorbent assay
EMT extracellular matrix
FADD FAS-associated protein with death domain
FASL FAS-ligand
FBS foetal bovine serum
FC flow cytometry
FISH fluorescence in situ hybridisation

grams(s)
G6PD glucose-6-phosphate-dehydrogenase
GADD45 growth arrest and DNA damage 45
gDNA genomic DNA
GIN genomic instability
Gy gray

hour(s)
HDM2 human double minute 2
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
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<tr>
<td>IB</td>
<td>immunoblot</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
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<td>IgG</td>
<td>immunoglobulin G</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<td>iMEF</td>
<td>immortalised mouse embryonic fibroblast</td>
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<td>IR</td>
<td>ionizing radiation</td>
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<tr>
<td>kDa</td>
<td>kilodalton(s)</td>
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<td>litre(s)</td>
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<td>M</td>
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<td>myeloid cell leukaemia 1</td>
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<td>MDC1</td>
<td>mediator of DNA damage checkpoint 1</td>
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<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<td>MutL homolog 1</td>
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<td>mmol</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<td>MMTV</td>
<td>mouse mammary tumour virus</td>
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<td>MN</td>
<td>micronucleus</td>
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<td>MOMP</td>
<td>mitochondrial outer membrane permeabilisation</td>
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<td>MRN</td>
<td>MRE11-RAD50-NBS1</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<td>NGF</td>
<td>nerve growth factor</td>
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<td>NLS</td>
<td>nuclear localisation sequence</td>
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<td>nM</td>
<td>nanomolar</td>
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<tr>
<td>nm</td>
<td>nanometer(s)</td>
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<tr>
<td>OD</td>
<td>optical density</td>
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<td>P</td>
<td>passage</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>phosphate buffered saline-Tween-20</td>
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<td>PCD</td>
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<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>paraformaldehyde</td>
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<td>propidium iodide</td>
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<td>phosphoinositide 3-kinase</td>
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<td>pentose phosphate pathway</td>
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<td>peroxiredoxin 3</td>
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<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
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<td>PUMA</td>
<td>p53-upregulated modulator of apoptosis</td>
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<td>PVDF</td>
<td>polyvinylidene fluoride</td>
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<td>qPCR</td>
<td>quantitative PCR</td>
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<td>retinoblastoma</td>
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<td>ribonuclease</td>
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<td>reverse osmosis</td>
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<td>reactive oxygen species</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
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<td>room temperature</td>
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<td>SA-β-gal</td>
<td>senescence-associated β-galactosidase</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>serine</td>
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<td>SESN</td>
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</table>
SFE  signal-free end
siRNA  small interfering RNA
SMAC  second mitochondria-derived activator of caspases
SOD2  superoxide dismutase 2
SSB  single-strand break
SV40  simian virus 40

TAE  Tris-acetic acid-EDTA
TBS  Tris-buffered saline
TBS-T  Tris-buffered saline/Tween-20
TdT  terminal deoxynucleotidyl transferase
TEMED  tetramethylethylenediamine
TNFSF  tumour necrosis factor superfamily
TP53  tumour protein 53
TRAIL  tumour necrosis factor-related apoptosis-inducing ligand
TRP53  transformation-related factor protein 53
TUNEL  terminal deoxynucleotidyl transferase dUTP nick end labeling

UV  ultraviolet

v/v  volume per volume
VEGFA  vascular endothelial growth factor A

wk  week(s)
w/v  weight per volume
WT  wild type

xg  times the force of gravity
X-gal  5-bromo-4-chloro-3-indolyl-β-D-galactosidase

yr  year(s)
°  degrees
µg  micrograms
µL  microlitre
µM  micromolar
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**Chapter 1**
Sections 1.1 – 1.4 are unpublished and provide a review of the literature relevant to the themes of this thesis. Section 1.5 provides a general introduction to caspase-2 which is comprised of part of a published review article:


**Chapter 2**
This chapter details the experimental procedures conducted throughout this thesis and expands on the materials and methods from the published articles presented in chapters 3 and 4.

**Chapter 3**
This chapter includes unpublished data as well as work published in:


**Chapter 4**
This chapter is comprised of work published in:


**Chapter 5**
This chapter is comprised of unpublished work that provides an overall discussion linking the findings from chapters 3 and 4 and also incorporates parts of a published review article:

Chapter 1

General Introduction
## Statement of Authorship

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<tr>
<th>Name of Co-Author</th>
<th>Dr. Loretta Dorstyn</th>
</tr>
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<th>Name of Co-Author</th>
<th>Prof. Sharad Kumar</th>
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1.1. Apoptosis

Apoptosis is a highly conserved mode of programmed cell death (PCD) that plays an essential role in metazoan development by sculpting structures and deleting obsolete cells (Fuchs and Steller, 2011; Kerr et al., 1972). Apoptosis is also important for normal cell turnover as well as the removal of damaged or infected cells, thereby contributing to the maintenance of tissue homeostasis and immunity (Fuchs and Steller, 2011). Autophagy and necroptosis are alternative modes of PCD that also contribute to development and tissue homeostasis (Fuchs and Steller, 2011). However, only apoptosis will be further discussed in this thesis.

Genetic and epigenetic aberrations that cause deregulation of the apoptotic machinery can perturb the balance between life and death at the cellular level and contribute to various pathological states (Fuchs and Steller, 2011; Thompson, 1995). For example, increased apoptotic activity in neurons is associated with the pathophysiology of Alzheimer’s disease (Hyman and Yuan, 2012). Conversely, insufficient apoptosis can promote inappropriate survival and expansion of potentially malignant cells harbouring oncogenic mutations, which can lead to cancer (Cotter, 2009; Ouyang et al., 2012).

During apoptosis, cells undergo distinct biochemical and morphological changes that ultimately lead to the ordered dismantling of cells. One of the earliest morphological changes that occurs is cytoplasmic shrinkage caused by the degradation of cytoskeletal...
components (Hacker, 2000). This breakdown in cellular structure is accompanied by chromatin condensation and nuclear fragmentation which is executed by specific DNA endonucleases that are activated during apoptosis (Hacker, 2000). Once internal cellular structures have been condensed and degraded, they are encapsulated into membrane-bound vesicles (called apoptotic bodies) through a process known as membrane blebbing (Hacker, 2000). Apoptotic bodies are then engulfed and digested by phagocytic cells such as macrophages (Hacker, 2000). Through this process, cells can be destroyed and removed without causing leakage of their intracellular contents. This is distinct to necrosis which leads to the release of pro-inflammatory molecules that can damage neighbouring cells (Fink and Cookson, 2005). Therefore, unlike necrotic cell death, apoptosis is an ‘immunologically silent’ mechanism of cell death (Fink and Cookson, 2005).

The biochemical and morphological hallmarks of apoptosis are choreographed by a network of pro-apoptotic and anti-apoptotic proteins that oppose each other’s function (Luthi and Martin, 2007; Taylor et al., 2008). These regulatory proteins coordinate the activation of a family of proteases called caspases whose function will be discussed further in the following section.
1.2. Caspases

Caspases are a family of aspartate-specific cysteine proteases (Kumar, 2007). To date, 14 mammalian caspases have been identified (11 in the human genome) (Kumar, 2007). The members of the caspase family are evolutionarily conserved (from worms to humans) reflecting their essential role in metazoan development (Kumar, 2007).

Caspases can be grouped according to their structure and biological functions. Caspase-1, -4, -5 and -11 are known as the inflammatory caspases (Martinon and Tschopp, 2007; Riedl and Shi, 2004). They are involved in the processing and activation of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and IL-18 and are important for coordinating the inflammatory response (Martinon and Tschopp, 2007).

Murine caspase-12 is also a member of the inflammatory caspase family (Martinon and Tschopp, 2007), however its evolutionary function is not conserved since its human orthologue is a pseudo-caspase (Lamkanfi et al., 2004). The second group of caspases are known as the apoptotic caspases and function as the initiators (caspase-2, -8, -9, -10) or effectors (caspase-3, -6, -7) of apoptosis (Kumar, 2007; Riedl and Shi, 2004). The biological function of caspase-14 has not been elucidated; however it has been implicated in epithelial cell differentiation (Eckhart et al., 2000). For the purpose of this thesis, only the apoptotic caspases will be discussed.

Like many proteases, caspases are synthesised as inactive precursors called zymogens which facilitates strict regulation of their activity (Kumar, 2007; Riedl and Shi, 2004). The structure of caspase zymogens consists of a C-terminal catalytic domain and an N-
terminal prodomain (Kumar, 2007). The catalytic domains of caspases are comprised of a small and large subunit containing the catalytic cysteine residue within the active site (Kumar, 2007; Riedl and Shi, 2004). The prodomains of initiator and effector caspases differ with respect to their structure and function (Kumar, 2007; Riedl and Shi, 2004). Initiator caspases have large prodomains that contain protein-protein interaction motifs such as the caspase activation and recruitment domain (CARD) of caspase-2 and caspase-9 and the death effector domains (DEDs) of caspase-8 and caspase-10 (Kumar, 2007). These protein-protein interaction domains of initiator caspases permit their recruitment and dimerisation into complexes that stimulates their catalytic activity (Kumar, 2007; Shi, 2002). Following dimerisation, the prodomains of initiator caspases are removed by autoproteolytic cleavage to promote an active enzyme conformation (Kumar, 2007; Shi, 2002). In contrast, effector caspases have short prodomains that can be cleaved by upstream initiator caspases (Kumar, 2007; Shi, 2002). Proteomic studies have revealed that once activated, effector caspases cleave hundreds of essential proteins that mediate the degradation of cellular components, leading to the cell shrinkage and destruction associated with the effector phase of apoptosis (Luthi and Martin, 2007; Taylor et al., 2008).

1.2.1. Caspase activation pathways: intrinsic and extrinsic apoptosis

There are two distinct pathways by which apoptosis can proceed, namely the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway (Fig. 1.1) (Riedl and Shi, 2004). These two pathways are defined by the nature and source of the apoptotic stimuli a cell receives.
The intrinsic pathway is activated by cellular stresses such as DNA damage or reactive oxygen species (ROS) (Riedl and Shi, 2004). These intracellular stimuli lead to the activation of the pro-apoptotic B cell lymphoma 2 (Bcl-2) family proteins, Bcl-2 homologous antagonist/killer (BAK) and Bcl-2-associated protein X (BAX) which oligomerise and form pores in the outer mitochondrial membrane (Wei et al., 2001). This induces mitochondrial outer membrane permeabilisation (MOMP), which stimulates the release of mitochondrial pro-apoptotic molecules such as cytochrome c (cyt c) and second mitochondria-derived activator of caspases/direct IAP-binding protein with low PI (SMAC/DIABLO) (Kluck et al., 1997; Kroemer et al., 2007). Under basal conditions, BAK/BAX function is antagonised by anti-apoptotic members of the Bcl-2 family such as BCL-2 and BCL-XL, thereby inhibiting MOMP and blocking apoptosis (Fletcher et al., 2008). Inhibition of apoptosis is also achieved by BCL-2-mediated neutralisation of pro-apoptotic Bcl-2 homology 3 (BH3)-only proteins that can directly activate BAK/BAX (Fig. 1.1) (Kuwana et al., 2005; Letai et al., 2002).

In response to apoptotic stimuli, pro-apoptotic proteins promote caspase activation by inhibiting anti-apoptotic regulators and activating other pro-apoptotic effectors. SMAC/DIABLO sequesters anti-apoptotic proteins of the inhibitor of apoptosis (IAP) family which, under basal conditions, block apoptosis by directly inhibiting caspase activation (Deveraux et al., 1999; Du et al., 2000; Srinivasula et al., 2001; Verhagen et al., 2000). BH3-only proteins such as p53-upregulated modulator of apoptosis (PUMA) stimulate MOMP by sequestering anti-apoptotic Bcl-2 family members, thereby relieving inhibition of BAK/BAX (Fig. 1.1) (Chen et al., 2005; Willis et al., 2007).
Figure 1.1. Extrinsic and intrinsic apoptosis pathways. The intrinsic (mitochondrial) pathway is activated by intracellular stimuli such as DNA damage which leads to PUMA induction. PUMA sequesters anti-apoptotic BCL-2 and BCL-X\(_L\), permitting BAX/BAK oligomerisation and MOMP causing the release of Cyt c which binds APAF-1 and promotes the formation of the procaspase-9 activation complex called the apoptosome. Activated caspase-9 then processes and activates downstream effector caspses which carry out the execution phase of apoptosis. SMAC/DIABLO released from the mitochondria sequesters IAP proteins thereby relieving inhibition of caspase activation. The extrinsic (death receptor) pathway is engaged upon death receptor-death ligand interactions that trigger the oligomerisation of death-receptor complexes at the plasma membrane. This stimulates the recruitment and activation of procaspase-8 monomers via adaptor proteins such as FADD. Activated caspase-8 then processes the downstream effector caspase, caspase-3. The extrinsic pathway can connect with the intrinsic pathway through caspase-8-mediated cleavage of BID which leads to BAK/BAK activation and MOMP. Adapted from Ashkenazi, 2002.
When released from the mitochondria, cyt c associates with apoptotic protease activating factor 1 (APAF-1) (Jiang and Wang, 2000). This binding event causes a conformational change that facilitates APAF-1 oligomerisation and formation of a heptameric complex (known as the apoptosome) which functions as an activation platform for procaspase-9 (Jiang and Wang, 2000; Li et al., 1997; Zou et al., 1999). Binding of procaspase-9 monomers to the apoptosome stimulates their proteolytic activity and subsequent activation by autoprocessing (Li et al., 1997; Zou et al., 1999). Activated caspase-9 then cleaves and activates the downstream effector caspases (caspase-3, -6, and -7) which in turn process downstream proteins that execute apoptosis (Fig. 1.1) (Cain et al., 1999; Zou et al., 1999).

The extrinsic pathway is engaged upon binding of extracellular ligands to transmembrane death receptors (Riedl and Shi, 2004). Several types of death receptors (e.g. Fas) and ligands (e.g. FasL) have been identified which belong to the tumour necrosis factor super family (TNFSF) (Aggarwal, 2003). Each death receptor has cognate death ligands and specific adaptor molecules which facilitate the assembly of specific intracellular signalling complexes (Aggarwal, 2003). Therefore, different combinations of death ligands and adaptors give rise to specificity in death receptor signalling. In addition to the ligand binding domain in their extracellular region, death receptors contain death domains (DDs) in their intracellular tails that allow interactions with DD-containing adaptor molecules. Ligand binding stimulates the oligomerisation of death receptors at the plasma membrane (e.g. Fas-FasL interaction) leading to the formation of homotrimeric death receptor complexes (Aggarwal, 2003). Adaptor
molecules such as Fas-associated protein with death domain (FADD) are then recruited to the intracellular DDs of specific death receptor complexes (Fig. 1.1) (Chinnaiyan et al., 1995; Lavrik et al., 2008). These adaptor molecules also contain DEDs which recruit DED-containing initiator caspases (procaspase-8 or procaspase-10) and triggering their activation by dimerisation-dependent autoprocessing (Fig. 1.1) (Medema et al., 1997).

In addition to downstream effector caspases, activated caspase-8 and caspase-10 can also cleave BH3 interacting-domain death agonist (BID) (Li et al., 1998; Luo et al., 1998; Milhas et al., 2005). This results in a truncated form of BID (tBID) which interacts with BAK/BAX, thereby stimulating MOMP and triggering procaspase-9 activation (Fig. 1.1) (Korsmeyer et al., 2000). Through this mechanism, death receptor-mediated activation of BID provides a connection between the intrinsic and extrinsic pathways.

As discussed previously, apoptosis represents an important defence strategy that protects organisms against tumourigenesis (Cotter, 2009; Ouyang et al., 2012). Using knockout mouse models, specific caspases such as caspase-2 (see 1.5) and caspase-8 have been shown to function as tumour suppressors (Ho et al., 2009; Krelin et al., 2008; Teitz et al., 2000). The role of apoptosis in tumour suppression is exemplified by cancer cells which frequently acquire mutations that cause inactivation and over expression of positive and negative regulators of apoptosis, respectively (Delbridge et al., 2012). In the following section, the properties of cancer cells and the molecular mechanisms of tumourigenesis will be described.
1.3. Cancer

Cancer is a complex group of diseases characterised by excessive and uncontrollable cell proliferation (Hanahan and Weinberg, 2011). The most recent statistics show that cancer accounted for approximately 29.8% of all deaths in Australia, making it a leading cause of mortality (Australian Bureau of Statistics, 2011). Several environmental factors including diet, infection and exposure to carcinogens are associated with an increased risk of developing cancer (Anand et al., 2008). In addition, genetic determinants such as the inheritance of cancer-promoting alleles are also known to contribute to cancer predisposition (Anand et al., 2008). Through decades of research we have gained an enormous amount of knowledge and insight into the aetiology of cancer at the cellular and molecular levels. This has led to development of advanced diagnostics and therapeutics that have dramatically improved the prognosis and quality of life of cancer patients over the last 2 decades. However, understanding the complex mechanisms of drug resistance, tumour cell heterogeneity and the identification of prognostic biomarkers remain major challenges in the field.

When cells acquire mutations in genes that regulate the spatial and temporal dynamics of cell survival, growth and division, they can lose their ability to accurately control these fundamental processes and become cancerous (Hanahan and Weinberg, 2011). This multistep process by which cells become cancerous is termed oncogenesis and involves a multitude of genetic and epigenetic changes. The most frequent changes observed in tumour cells are those affecting the regulation, expression and function of
two classes of genes known as tumour suppressor genes and oncogenes (Hanahan and Weinberg, 2011; Lee and Muller, 2010), whose function will be described in the following section.

1.3.1. Tumour suppressor genes and oncogenes

Given their fundamental role in regulating the molecular events of oncogenesis, understanding the function of tumour suppressor genes and oncogenes is at the crux of cancer research. In general, tumour suppressor genes provide a barrier that protects against cellular transformation by antagonizing pathways that positively regulate cell growth, proliferation and survival (Lee and Muller, 2010; Sherr, 2004). Tumour cells frequently display deletions, epigenetic silencing and loss-of-function mutations in these genes (Hanahan and Weinberg, 2011; Lee and Muller, 2010). Examples of tumour suppressor genes that are commonly inactivated in human cancers include those that function in apoptosis such as tumour protein 53 (TP53) and its transcriptional targets BAX and PUMA (Delbridge et al., 2012; Olivier et al., 2010).

In general, the biological functions of both alleles of a tumour suppressor gene must be lost in order for the protein it encodes to lose its tumour suppressor function (Knudson, 2001; Lee and Muller, 2010). Therefore, mutations in tumour suppressor genes tend to act recessively (Lee and Muller, 2010). However, when cells lose the function of a single allele of a tumour suppressor gene, inactivation of the remaining functional allele is often selected for (Knudson, 2001; Sherr, 2004). In contrast, losing the function of a single allele of a haploinsufficient tumour suppressor gene can
abolish its tumour suppressor activity (Payne and Kemp, 2005; Sherr, 2004). Monoallelic mutations in such genes act dominantly, because the reduced level of protein or biological activity produced from a single functional allele is insufficient to function as a tumour suppressor (Payne and Kemp, 2005).

Proto-oncogenes are the counterparts of tumour suppressor genes and generally oppose their functions (Croce, 2008). Therefore, as with loss of tumour suppressor genes, cancer cells have a strong selective pressure for genetic and epigenetic changes that lead to overexpression and gain-of-function of proto-oncogenes (Croce, 2008; Hanahan and Weinberg, 2011). The resultant deregulated, cancer-promoting gene is termed an oncogene. In contrast to tumour suppressor genes, proto-oncogenes commonly encode proteins that positively regulate cell growth, division and survival pathways (Croce, 2008; Hanahan and Weinberg, 2011). Examples of such oncogenes that are frequently over-expressed in human tumours include growth-promoting factors such as RAS and MYC and anti-apoptotic regulators such as BCL-2 (Croce, 2008; Hanahan and Weinberg, 2011). Oncogenes can also promote tumourigenesis by enabling other malignant properties such as invasion and angiogenesis (Hanahan and Weinberg, 2011) (see 1.3.2).

There are various mechanisms by which proto-oncogenes can be converted into cancer-promoting oncogenes. Amplification of chromosomal regions containing proto-oncogenes and epigenetic activation are both common mechanisms that lead to the overexpression of proto-oncogenes (Croce, 2008). Specific chromosome translocation
events that place proto-oncogenes under the transcriptional control of constitutively active enhancer elements are also a frequent occurrence in many cancers (e.g. MYC translocations in Burkitt’s lymphoma) (Croce, 2008; Taub et al., 1982).

As a consequence of alterations to the expression and function of tumour suppressor genes and proto-oncogenes, cells acquire malignant properties that promote tumourigenesis. These capabilities that tumour cells acquire have been termed the ‘hallmarks of cancer’. Examples of how deregulation of tumour suppressor genes and proto-oncogenes give rise to the hallmarks of cancer will be discussed in the following section.

1.3.2. Hallmarks of cancer

In 2000, Hanahan and Weinberg proposed the six hallmarks of cancer: replicative immortality; sustaining proliferative signals; evasion of growth suppressors; resisting cell death; inducing angiogenesis and invasion and metastasis (Hanahan and Weinberg, 2000). More recently, 4 additional hallmarks of cancer have been identified (see 1.3.2.7), giving rise to the 10 unifying hallmarks of the cancer phenotype (Fig. 1.2) (Hanahan and Weinberg, 2011). Understanding each of these hallmarks has expanded our understanding of the mechanisms responsible for initiating and driving tumourigenesis. Furthermore, targeting these cancer-promoting capabilities has led to the development of various classes of anti-cancer agents (Luo et al., 2009).
Figure 1.2. The hallmarks of cancer. The 10 unifying features of cancer cells. These interdependent capabilities enable the malignant properties that drive tumourigenesis. From Hanahan and Weinberg, 2011.
1.3.2.1. Replicative immortality

The ability to divide indefinitely is a well-documented property of cancer cells (Hanahan and Weinberg, 2011). Normally, specialised structures at the ends of chromosomes, called telomeres, restrict cells from limitless replication (Deng et al., 2008). With each division cycle telomeres become progressively shorter until they eventually reach a critical length (Harley et al., 1990). When this occurs, cells are either eliminated by apoptosis or enter a state of irreversible growth arrest known as senescence (Deng et al., 2008). These processes are tumour suppressive as they prevent telomere dysfunction which can lead to DNA damage and oncogenic chromosomal aberrations (Blasco et al., 1997; Feldser and Greider, 2007). One way in which cancer cells circumvent this restriction is by reactivating telomerase, the enzyme that catalyses telomere elongation. Telomerase expression, which is virtually undetectable in most cell types, has been shown to be significantly increased in the majority of human tumours (Kim et al., 1994; Shay and Wright, 1996). By maintaining telomere length cancer cells are able to prevent telomere shortening, thereby enabling escape from replicative senescence or apoptosis. Cells that overcome these barriers and acquire the ability to divide indefinitely are said to be immortalised.

1.3.2.2. Sustaining proliferative signalling

Excessive, uncontrolled proliferation is an obligate feature of cancer cells. Normally, cells are instructed to divide only when stimulated with specific growth-promoting signals. Cancer cells bypass this requirement by acquiring mutations in proto-oncogenes that encode proteins that positively regulate cell cycle transitions and
growth (Hanahan and Weinberg, 2011). Hence, cancer cells frequently display mutations in growth factor receptors and mitogen-activated kinases that lead to their overexpression or render them constitutively active (Croce, 2008). Examples of such proto-oncogenes that are commonly mutated in human tumours include epidermal growth factor receptor (EGFR) and its downstream signalling kinase RAF (Croce, 2008). These mutant receptors and signalling kinases are strongly selected for by cancer cells because of their ability to amplify growth signalling, thereby providing a potent proliferative advantage. Specifically targeting the active forms of these proteins with small molecule inhibitors has proved very effective for the development of anti-proliferative chemotherapeutic agents (Luo et al., 2009).

### 1.3.2.3. Evasion of growth suppressors

Cells are also restricted from unscheduled proliferation by a network of regulatory proteins that suppress cell growth and division (Sherr, 2004). Many of these proteins function by antagonizing mitogen-activated protein kinases and proteins that drive cell cycle progression (Sherr, 2004). These negative regulators of cell growth and proliferation are commonly encoded by tumour suppressor genes. Examples of such tumour suppressor genes frequently inactivated in human tumours include, phosphatase and tensin homolog (PTEN) and retinoblastoma (RB), which are well-known negative regulators of the phosphoinositide 3-kinase (PI3K) pathway and G1/S phase transition of the cell cycle, respectively (Hanahan and Weinberg, 2011; Sherr, 2004). Through loss-of-function of these tumour suppressors, cancer cells become insensitive to the antagonistic signals that normally restrict growth and proliferation.
Cells that display self-sustained proliferation and insensitivity to anti-growth signals subsequently acquire a hyperproliferative phenotype.

### 1.3.2.4. Resisting cell death

Cells displaying hyperproliferation experience replicative stress and associated DNA damage due to rapid, unscheduled cell division that becomes uncoupled from checkpoint surveillance mechanisms which normally protect against genotoxic stress (Kastan and Bartek, 2004; Zeman and Cimprich, 2014). Like other forms of cellular stress, DNA damage can induce cell death by apoptosis. Cancer cells often overcome this tumour suppressor barrier by establishing mechanisms to evade apoptosis (Delbridge et al., 2012). Cancer cells can become insensitive to cell death through loss-of-function mutations and epigenetic silencing of pro-apoptotic tumour suppressor genes (e.g. Bcl-2 interacting mediator of cell death [BIM] and PUMA) and overexpression of anti-apoptotic proto-oncogenes (e.g. myeloid cell leukaemia 1 [MCL1] and BCL-2) (Cotter, 2009; Delbridge et al., 2012). By virtue of their ability to circumvent apoptosis, cells harbouring DNA damage and mutations are permitted to survive and proliferate, allowing the transmission of mutations to daughter cells. This promotes the accumulation of additional mutations that drive oncogenesis (Cotter, 2009; Hanahan and Weinberg, 2011).

### 1.3.2.5. Inducing angiogenesis

Like normal cells, cancer cells require nutrients and oxygen as well as means to excrete metabolic waste products. Solid tumours are often comprised of unordered aggregates
of aberrant cells that lack the normal architecture and vasculature of healthy tissues. This can result in tumour cells having impaired access to nutrients and oxygen. In order to overcome this, cancer cells switch on genes that reactivate angiogenesis (the formation of new blood vessels) such as vascular endothelial growth factor A (VEGF-A) (Ferrara et al., 2003; Weis and Cheresh, 2011). VEGF-A is frequently overexpressed or ectopically activated in tumour cells leading to induction of pro-angiogenic signalling which promotes tumour vascularisation (Hanahan and Folkman, 1996; Weis and Cheresh, 2011). Therefore, is it not surprising that tumour vascularisation is a characteristic associated with advanced-stage, aggressive tumours and poor clinical outcome (Fontanini et al., 1997; Frank et al., 1995; Hollingsworth et al., 1995). This has prompted the development of therapies that target pro-angiogenic signalling (such as anti-VEGF-A neutralising antibodies) which have been successful in impairing vascularisation and growth of solid tumours (Ferrara et al., 2007; Kim et al., 1993).

1.3.2.6. Invasion and metastasis

Another distinct hallmark of cancer cells is their ability to invade neighbouring tissues (Hanahan and Weinberg, 2011). This capability is facilitated by induction of an epithelial-mesenchymal transition (EMT), a process required for programming developmental events and wound-healing (Nieto, 2009). This multi-step process promotes increased cell motility by downregulating the expression of cell-to-cell adhesion molecules such as E-Cadherin and secretion of extracellular matrix- (ECM) degrading enzymes such as matrix metalloproteinases (MMP) (Dumont et al., 2008; Kessenbrock et al., 2010; Onder et al., 2008). Hence, these factors that promote
tumour cell EMTs and motility are associated with an invasive phenotype. Cancer cells that detach from a primary tumour can enter the circulation (via blood vessels and lymphatic vessels) and metastasise to secondary organs. Cells that become uncoupled from their tissue of origin lose survival signals from ECM proteins (such as integrins) and die through a process known as anoikis (Desgrosellier and Cheresh, 2010). Therefore, metastatic tumours that colonise secondary organs arise from the ability of cancer cells to escape their tissue of origin and survive and proliferate in foreign tissues (Kim et al., 2012; Simpson et al., 2008).

**1.3.2.7. Hallmarks of cancer: the next generation**

By virtue of increasingly sophisticated high-throughput technologies, the last decade of cancer research has seen major advancements in our understanding of the molecular events that coordinate oncogenesis. This has led to the identification of 4 additional hallmarks of cancer: avoiding immune destruction; tumour-promoting inflammation; deregulating cellular energetics and genomic instability (GIN) (Hanahan and Weinberg, 2011). Despite only a relatively superficial understanding of the mechanisms responsible for these hallmarks, their contribution to tumourigenesis is now becoming increasingly appreciated.

It has been known for many years that the immune system contributes to tumour suppression through the surveillance and clearance of cancer cells. There is a growing body of evidence which suggests that cancer cells undergo various adaptations that enable evasion of immune detection and destruction (Grivennikov et al., 2010).
However, the precise cellular and molecular events operating between tumour cells and the immune system remain poorly defined. The immune system can also contribute to tumour progression by promoting inflammation. Within tumours, necrotic cells release pro-inflammatory molecules that recruit immune cells such as macrophages and neutrophils (DeNardo et al., 2010; Grivennikov et al., 2010). These inflammatory cells have been shown to interact with cancer cells and secrete factors that enable angiogenesis, survival, proliferation and invasion (DeNardo et al., 2010; Grivennikov et al., 2010). Therefore, the immune system is a double-edged sword having both tumour-suppressing and tumour-promoting capabilities.

The idea that cancer cells undergo metabolic reprogramming was proposed more than 50 years ago by Otto Heinrich Warburg (Warburg, 1956). He observed that cancer cells have a tendency to preferentially switch from aerobic to anaerobic glucose metabolism, even in the presence of oxygen; a phenomenon now referred to as the Warburg Effect (Warburg, 1956). These metabolic changes are associated with the increased glucose uptake and utilisation that is characteristic of cancer cells (Ward and Thompson, 2012). There is now strong evidence demonstrating that cancer cells undergo metabolic reprogramming which leads to deregulated cellular energetics (Ward and Thompson, 2012).

The deregulated expression and activity of many metabolic enzymes (such as isocitrate dehydrogenase 1, pyruvate kinase M2 and phosphoglycerate dehydrogenase) has recently been observed in human cancers, supporting the link between deregulation of
cellular energetics and tumourigenesis (Cohen et al., 2013; Israelsen et al., 2013; Locasale et al., 2011). However, we are only now starting to understand the molecular intricacies and implications of these alterations. It is thought that these metabolic changes are necessary for cancer cells to meet the large bioenergetic demands required to support rapid growth and proliferation (Ward and Thompson, 2012). This is exemplified by the selective sensitivity of cancer cells to agents that inhibit catabolic, energy-producing pathways (Dang, 2012; Zhao et al., 2013). As such, attention is now being focused on developing inhibitors that target these vulnerabilities in cancer cells.

GIN is another well-established property of cancer cells that was observed over a century ago (Holland and Cleveland, 2009). As with metabolic alterations observed in cancer cells, the molecular mechanisms underlying GIN are only now beginning to emerge. GIN can refer to various structural lesions such as DNA breaks, deletions and translocations or numerical alterations where cells lose or gain copies of whole chromosomes (aneuploidy) (Abbas et al., 2013; Pfau and Amon, 2012). Cancer cells frequently display these abnormalities which have been proposed to contribute to tumour cell heterogeneity and the adaptive potential that fuels tumour cell evolution (Abbas et al., 2013; Gordon et al., 2012; Pfau and Amon, 2012). Furthermore, GIN has been associated with poor prognosis and survival in multiple tumour types (Bakhoum et al., 2011; Carter et al., 2006; Walther et al., 2008). High-throughput analysis of tumours has revealed that alterations to the expression and function of components that regulate DNA repair (e.g. breast cancer 1 [BRCA1]) and chromosome segregation (e.g. mitotic arrest deficient 2 [MAD2] and budding uninhibited by benzimidazoles 1
[\text{BUB1}] \text{ give rise to GIN (Weaver and Cleveland, 2006). Research in this area is now being directed towards determining whether GIN is a driving force or a consequence of tumourigenesis and how this cancer-specific property might be exploited therapeutically.}
1.4. The DNA Damage Response

Cells are constantly exposed to agents that cause DNA damage. In addition to exogenous sources of genotoxic stress such as solar UV radiation, by-products of normal cell metabolism can also cause DNA damage (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). There are various forms of DNA damage a cell can sustain including single-stranded breaks (SSBs), double-stranded breaks (DSBs), inter- and intra-strand DNA crosslinks and DNA adducts (Jackson and Bartek, 2009). Accumulation of DNA damage is thought to contribute to the ageing process and is the molecular basis of oncogenesis (Jackson and Bartek, 2009). Given that cellular homeostasis depends on error-free transmission of genetic information, cells have evolved elaborate molecular mechanisms capable of repairing damaged DNA, collectively referred to as the DNA damage response (DDR) (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). The DDR is executed by an intricate network of proteins, many of which possess tumour suppressor functions (Liang et al., 2009). In response to DNA damage, these networks are engaged and cooperate to sense, transduce and repair damaged DNA.

During a DDR, DNA damage is sensed by proteins that bind to DNA lesions and assemble into multi-protein complexes. These complexes contain proteins that transduce signals to downstream effectors which initiate DNA repair and cell cycle arrest pathways (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). Crosstalk between the cell cycle and DNA repair machinery is critical for orchestrating an efficient response to DNA damage. Cell cycle arrest is an important aspect of the DDR.
as it gives adequate time for damaged DNA to be repaired (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). If the damage is extensive or irreparable, cells are eliminated by apoptosis to prevent the transmission of potentially deleterious mutations to daughter cells (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). As discussed previously, such cells carrying DNA damage that escape apoptosis are the precursors of cancerous cells.

The link between the DDR and tumourigenesis is exemplified by germline mutations in human DNA repair genes such as *BRCA1* and MutL homolog 1 (*MLH1*) which are known to cause an increased predisposition to certain types of cancers (Ciccia and Elledge, 2010). Due to their increased proliferation rates and compromised genome surveillance, cancer cells are often hypersensitive to agents that induce DNA damage (Helleday *et al.*, 2008; Lord and Ashworth, 2012). This weakness provides a therapeutic window which has been exploited for the development of chemotherapeutic agents that activate DNA damage-induced cell death in tumours (Helleday *et al.*, 2008; Lord and Ashworth, 2012). Various classes of drugs that induce DNA damage are currently used for the treatment of different types of cancers including DSB-inducers (e.g. etoposide), DNA intercalators (e.g. doxorubicin), DNA cross-linkers (e.g. cisplatin) and alkylating agents (e.g. cyclophosphamide) (Helleday *et al.*, 2008; Lord and Ashworth, 2012).

Mutations that cause defects in the DDR not only increase the risk of developing cancer but are also associated with drug resistance and poor prognosis (Bouwman and
Jonkers, 2012; Lord and Ashworth, 2012). Therefore, understanding how cells respond to DNA damage is not only important to enhance our knowledge of the mechanisms operating during tumourigenesis, but also for improving therapeutic strategies. The function of two important components of the DDR, ATM and p53, are discussed in more detail in the following sections.

1.4.1. ATM

Ataxia-telangiectasia (AT) is a rare, autosomal recessive human disease characterised by growth retardation, infertility, neurological defects, immune deficiency and an increased predisposition to cancer (Lavin, 2008). AT is caused by loss-of-function, germline mutations in the ATM (ataxia-telangiectasia mutated) gene which encodes a large, multi-domain serine/threonine protein kinase, essential for cell cycle checkpoint regulation and DNA repair (Savitsky et al., 1995; Stracker et al., 2013). Atm knockout (Atm−/−) mice recapitulate many clinical features of AT such as growth retardation, infertility and also develop spontaneous thymic lymphomas (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996). Therefore, they are a widely-used model of both AT and T cell lymphomagenesis. Although Atm−/− mice are viable, two-independent studies have demonstrated that knockin mice expressing catalytically inactive forms of ATM display embryonic lethality (Daniel et al., 2012; Yamamoto et al., 2012). Interestingly, these knockin mice emulate the defective DNA repair and GIN phenotypes characteristic of Atm−/− mice. These findings suggest that ATM has important functions in development and maintenance of genomic stability that are independent of its catalytic activity.
ATM has a well-established role in DSB signalling and is an important mediator of DNA damage-induced cell cycle arrest and DNA repair (Stracker et al., 2013). Under basal conditions, ATM exists as inactive homodimers and in response to DSBs, a DNA-binding complex called the MRN complex (comprised MRE11, RAD50 and NBS1) tethers ATM to DNA break sites (Fig. 1.3) (Bakkenist and Kastan, 2003; Lee and Paull, 2004, 2005). ATM is recruited to the MRN complex via NBS1 and this interaction promotes ATM autophosphorylation on Ser1981 and its dissociation into active monomers (Bakkenist and Kastan, 2003; You et al., 2005). Upon activation, ATM phosphorylates protein substrates that inhibit cyclin-dependent kinase (Cdk) complexes (which drive cell cycle progression), thereby causing cell cycle arrest (Malumbres and Barbacid, 2009).

Checkpoint kinase 1 (CHK1), CHK2 and the tumour suppressor p53 (see 1.4.2.) are three important mediators of ATM-dependent cell cycle checkpoint activation (Fig. 1.3) (Banin et al., 1998; Canman et al., 1998; Chaturvedi et al., 1999; Gatei et al., 2003; Matsuoka et al., 2000). CHK1 and CHK2 initiate cell cycle arrest by phosphorylating and inhibiting cell division cycle 25 (CDC25) family members which drive cell cycle progression via their ability to activate Cdk complexes (Falck et al., 2001; Malumbres and Barbacid, 2009). CHK1 and CHK2 can also promote cell cycle arrest by directly phosphorylation and activating p53, which in turn upregulates the Cdk inhibitor p21^{CIP1/WAF1} (Fig. 1.3) (Hirao et al., 2000; Shieh et al., 2000; Xiong et al., 1993).

In addition to cell cycle arrest, ATM is also important for activating the DNA repair machinery (Cremona and Behrens, 2013). ATM-dependent phosphorylation of histone H2AX on Ser139 (known as γH2AX) is a critical upstream event required for the
Figure 1.3. The ATM DNA damage pathway. The MRN complex is recruited to DNA breaks and forms a bridge between DNA ends. ATM is recruited to the MRN complex which facilitates its autophosphorylation and dissociation into active monomers. Activated ATM then phosphorylates histone H2AX which serves as a docking site for the scaffold protein MDC1 which in turn recruits more MRN complex (leading to amplification of ATM signalling) and DNA repair factors such 53BP1 and BRCA1. ATM-mediated activation of CHK1 and CHK2 promotes cell cycle checkpoint activation and p53-dependent apoptosis.
recruitment and retention of DNA repair complexes to DSBs (Burma et al., 2001; Paull et al., 2000). Following ATM activation, \( \gamma \)H2AX accumulates at sites flanking DSBs and recruits the scaffold protein mediator of DNA damage checkpoint 1 (MDC1) which recruits more MRN complex and other ATM substrates that function in DNA repair, such as p53 binding protein 1 (53BP1) and BRCA1 (Fig. 1.3) (Eliezer et al., 2009; Lou et al., 2003; Lou et al., 2006; Spycher et al., 2008; Stucki et al., 2005; Wu et al., 2008). Therefore, in addition to recruitment of DNA repair complexes to DSB sites, ATM-mediated phosphorylation of histone H2AX is critical for the amplification of DNA damage signalling (Bonner et al., 2008; Lou et al., 2006).

ATM can also be activated by oxidative stress independently of the MRN complex and DSBs (Guo et al., 2010). Despite the overlapping functional outcomes of ATM activation by these different stimuli, the mechanisms of ATM activation in each case are molecularly distinct. Under conditions of oxidative stress, ATM monomers become covalently linked by an intermolecular disulphide bond which forms following oxidation of a conserved cysteine residue (Cys2291) (Guo et al., 2010). ATM also contributes to antioxidant defence by activating glucose-6-phosphate dehydrogenase (G6PD), the rate-limiting enzyme in the pentose phosphate pathway (PPP) (Cosentino et al., 2011). Upregulation of the PPP stimulates the production of nicotinamide adenine dinucleotide phosphate (NADPH) which has a critical role in the regeneration of antioxidant molecules such as glutathione (Cosentino et al., 2011). Moreover, ATM can indirectly engage the antioxidant defence system by activating p53, which in turn upregulates the expression of various antioxidant enzymes (see 1.4.2 and Fig. 1.4). A

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role for ATM in the regulation of redox homeostasis is further supported by the increased oxidative damage observed in tissues from AT patients and Atm-deficient mice, which is thought to contribute to the pathologies associated with AT (Barlow et al., 1999; Kamsler et al., 2001; Watters, 2003).

1.4.2. The p53 pathway

The tumour suppressor protein p53 (encoded by the transformation-related protein 53 [Trp53] and TP53 genes in mice and humans, respectively) is a transcription factor that plays a central role in regulation of the DDR (Junntila and Evan, 2009; Meek, 2009). Consistent with an essential role in tumour suppression, TP53 is one of the most frequently mutated genes in human tumours (Meek, 2009; Olivier et al., 2010). In addition to direct inactivation of p53, deregulation of upstream modulators of p53 function is also frequently observed in human tumours (Junntila and Evan, 2009). Furthermore, extensive GIN in tumours is strongly correlated with loss of p53 function (Fujiwara et al., 2005; Hanel and Moll, 2012). Therefore, p53 has been labelled as the ‘guardian of the genome’.

In addition to DNA damage, p53 is activated in response to multiple stimuli including ROS, metabolic stress, oncogene activation and hypoxia (Meek, 2009). Therefore, p53 forms a central axis of the cell’s stress response system. In unstressed cells, p53 is ubiquitinated by the E3 ubiquitin ligase, MDM2 (mouse double minute 2 [HDM2 in humans]), targeting it for degradation by the 26S proteasome (Fig. 1.4) (Haupt et al., 1997; Kubbutat et al., 1997). In addition to degradation of p53, MDM2 also suppresses
Figure 1.4. The p53 pathway. In unstressed cells, p53 activity is inhibited by MDM2-mediated ubiquitination (and subsequent degradation by the 26S proteasome) and nuclear export. MDM2 also inhibits the transcriptional activity of p53. In response to oncogenic stress, p19^ARF^ stabilises p53 by neutralizing MDM2. Oxidative stress and DNA damage activates ATM which directly phosphorylates p53, disrupting its interaction with MDM2. ATM also promotes p53 stabilisation by phosphorylating MDM2 and inhibiting its interaction with p53. Phosphorylated p53 transactivate multiple target genes that mediate cycle arrest, DNA repair, apoptosis or antioxidant defence.
p53 function by steric inhibition of its transcriptional activity and by promoting its nuclear export (Momand et al., 1992; Oliner et al., 1993; Tao and Levine, 1999a). Given its critical function as a negative regulator of p53 stability and activity, overexpression of HDM2 by gene duplication events is a frequent occurrence in many types of human tumours (Wade et al., 2013). As discussed in 1.4.1, ATM-mediated phosphorylation of p53 on Ser18 (Ser15 in humans) is critical for p53 stabilisation and accumulation in response to DNA damage (Canman et al., 1998). ATM also indirectly promotes p53 stabilisation by phosphorylating MDM2 on Ser394 (Ser395 in humans) which disrupts its interaction with p53, thereby relieving MDM2-mediated inhibition (Fig. 1.4) (Maya et al., 2001). Therefore, ATM is a key upstream activator of the p53-dependent DDR.

Another critical modulator of p53 stability and activity is the tumour suppressor, p19ARF (p14ARF in humans) (Sherr, 2006). p19ARF indirectly stabilises p53 by sequestering MDM2 and promoting its nuclear export and degradation (Fig. 1.4) (Pomerantz et al., 1998; Tao and Levine, 1999b; Zhang et al., 1998). Importantly, p19ARF is specifically engaged upon oncogenic stress signals (such as activation of oncogenic RAS and MYC) independently of DNA damage (Sherr, 2006). Given that persistent oncogenic signalling is an obligate feature of cancer cells (Luo et al., 2009), p19ARF-dependent p53 stabilisation has been suggested to be a key mechanism of p53 activation in tumour cells (Sherr, 2006). As with Mdm2 overexpression, tumour cells commonly circumvent p53-mediated cell cycle arrest and apoptosis through inactivation of p19ARF (Kim and Sharpless, 2006; Sherr, 2006).
Following stabilisation and accumulation in response to stress signals, activated p53 transactivates multiple target genes encoding effectors of the p53 response (Meek, 2009; Menendez et al., 2009). In response to specific cellular stress conditions, cells activate distinct p53-inducible transcriptional programmes that are determined by the nature and extent of the insult (Menendez et al., 2009). The target gene repertoire of p53 includes genes involved in various biological functions that protect against genotoxic and oxidative stress including cycle arrest (14-3-3σ and p21<sup>CIP1/WAF1</sup>), DNA repair (growth arrest and DNA damage 45 [GADD45] and proliferating cell nuclear antigen [PCNA]), antioxidant defence (sestrin 1 [SESN1], SESN2 and superoxide dismutase 2 [SOD2]) and apoptosis (BAX, PUMA and NOXA) (Budanov et al., 2002; el-Deiry et al., 1993; Hermeking et al., 1997; Hussain et al., 2004; Kastan et al., 1992; Miyashita and Reed, 1995; Nakano and Vousden, 2001; Oda et al., 2000; Velasco-Miguel et al., 1999; Xu and Morris, 1999). Another canonical p53 target gene is its negative regulator, Mdm2 (Barak et al., 1993). p53-mediated transactivation of Mdm2 is a critical node of the p53 regulatory circuit that is important for establishing a negative feedback loop that terminates the p53 response (Wu et al., 1993).
1.5. Caspase-2

Along with caspase-1, caspase-2 was one of the first discovered mammalian homologues of Caenorhabditis elegans CED-3 (Kumar et al., 1994; Kumar et al., 1992). Given the function of caspase-1 (IL-1β-converting enzyme) in IL-1β maturation, caspase-2 was hailed as the caspase that was more likely to functionally emulate CED-3 in mammalian cell death (Kumar, 1995; Miura et al., 1993). However, as we now know, there are multiple caspases in mammals, some with distinct functions in cell death and inflammation (Kumar, 2007). Surprisingly, caspase-2 does not sit in either camp, having no general role in apoptosis or inflammation (Kumar, 2007). The fact that caspase-2 is the most evolutionarily conserved of mammalian caspases but yet is dispensable for most cases of cell death (apoptotic and non-apoptotic) remains puzzling.

Caspase-2 is synthesised as a zymogen and is activated via a dimerisation-dependent, autocatalytic cleavage mechanism (Baliga et al., 2004). Procaspase-2 is made up of a C-terminal catalytic domain containing the active site (Cys320) and an N-terminal CARD, which mediates protein–protein interactions and facilitates recruitment into activation platforms (Fig. 1.5) (Baliga et al., 2004). Upon proximity-induced oligomerisation via its CARD, caspase-2 becomes partially active (Butt et al., 1998). Autoprocessing then occurs between the small and large subunits of the catalytic domain yielding a fully active enzyme (Baliga et al., 2004). Further processing results in removal of the N-terminal CARD, generating a fully mature tetramer (Baliga et al., 2004). Caspase-2 activation has also been shown to be regulated by phosphorylation mediated by
Figure 1.5. Structure of procaspase-2. Schematic of the domain structure of the caspase-2 zymogen showing the N-terminal CARD domain and the small (p12) and large (p19) subunits of the C-terminal catalytic domain. The nuclear localisation sequence (NLS), phosphorylation sites, catalytic cysteine (Cys30) and processing sites are also indicated (see main text).
various kinases, including calcium/calmodulin-dependent protein kinase II (Ser164), protein kinase CK2 (Ser157) and CDK1 (Ser340) (Fig. 1.5) (Andersen et al., 2009; Nutt et al., 2005; Shin et al., 2005).

Caspase-2 is unique among the caspase family in that it possess characteristics of both initiator and effector caspases. For example, the long CARD-containing prodomain of caspase-2 is structurally reminiscent of other initiator caspases (Kumar, 2007). Consistent with a role in apoptosis initiation, biochemical studies have shown that caspase-2 can act upstream of MOMP and caspase-9 activation via BID cleavage (Bonzon et al., 2006; Robertson et al., 2002; Wagner et al., 2004). Also, like other initiator caspases, caspase-2 activation initially occurs by dimerisation (Baliga et al., 2004). However, based on its cleavage specificity and its ability to be processed downstream of initiator caspases, caspase-2 functionally emulates other effector caspases (Paroni et al., 2001; Samraj et al., 2007; Thornberry et al., 1997). The conflicting structural and functional properties of caspase-2 have led to it being classified as both an initiator and effector caspase. The differential functions of caspase-2 in apoptosis initiation and execution are most likely stimulus and context-specific. Another unique feature of caspase-2 is its constitutive nuclear localisation, a property that has been shown to be dependent on its nuclear localisation sequence (NLS) located in the prodomain (Fig. 1.5) (Baliga et al., 2003; Colussi et al., 1998).

Caspase-2 has been implicated in apoptosis induced by multiple intrinsic and extrinsic stimuli including DNA damage, TNF-related apoptosis-inducing ligand (TRAIL), ROS and
cytoskeletal disruption (Braga et al., 2008; Ho et al., 2008; Madesh et al., 2009; Olsson et al., 2009; Shin et al., 2005; Sidi et al., 2008; Vakifahmetoglu et al., 2006; Wagner et al., 2004) (Fig. 1.6). However, as discussed in 1.5.1, deletion of caspase-2 in mice does not lead to a phenotype that would support a broad function for this caspase in apoptosis (Bergeron et al., 1998).

### 1.5.1. Caspase-2 knockout mice

Studies in caspase-2 knockout (Casp2^−/−) have mice failed to provide any conclusive evidence that caspase-2 is essential for apoptosis (Bergeron et al., 1998; Marsden et al., 2004; O’Reilly et al., 2002). Casp2^−/− mice have excess numbers of germ cells in their ovaries, and caspase-2-deficient oocytes have been reported to be somewhat resistant to apoptosis induced by chemotherapeutic drugs (Bergeron et al., 1998). However, most cell types in wild type and Casp2^−/− mice, including thymocytes and dorsal root ganglion (DRG) neurons, show comparable levels of cell death in response to various cytotoxic stimuli (O’Reilly et al., 2002).

In addition, previous studies from our laboratory and other groups have demonstrated that Casp2^−/− mice show signs of premature ageing such as reduced bone volume, alopecia and shortened maximal lifespan (Shalini et al., 2012; Zhang et al., 2007). Furthermore, Casp2^−/− mice are noticeably thinner as they age and display increased oxidative damage in tissues and impaired antioxidant defence (Shalini et al., 2012; Zhang et al., 2007).
Figure 1.6. The function of caspase-2 in cell death. Cellular stress induced by DNA damage, cytoskeletal disruption or ROS can lead to alternative pathways of caspase-2 activation. In response to cytoskeletal disruption or ROS, the activation of caspase-2 occurs upstream of MOMP. Following DNA damage, caspase-2 is activated downstream of ATM to induce apoptosis. ATM also activates p53 and MOMP via PUMA and BAX/BAK activation, leading to Cyt c release and formation of the APAF-1 apoptosome, which recruits and activates caspase-9, leading to caspase-3 activation. SMAC/DIABLO release from mitochondria during MOMP blocks IAP function to facilitate the caspase activation cascade. Once activated, the key effector caspase, caspase-3, also cleaves caspase-2, which potentially provides an amplification loop for the caspase activation cascade.
Overall, Casp2\(^{-/-}\) mice have provided only limited insight into the physiological functions of this enigmatic caspase. Compensatory mechanisms have been proposed to account for the lack of an overt phenotype (Kumar, 2009). However, as discussed throughout this thesis, context-dependent, fine-tuning functions of caspase-2 may explain these observations in Casp2\(^{-/-}\) mice.

1.5.2. Caspase-2 – the anti-cancer connection

As discussed previously, apoptosis is a critical tumour-suppressive process that prevents survival and clonogenic expansion of potentially malignant cells carrying deleterious mutations (Cotter, 2009; Ouyang et al., 2012). Given their essential role as regulators of apoptosis initiation and execution, caspases have been proposed to possess tumour suppressor functions (Olsson and Zhivotovsky, 2011). However, only caspase-2 and caspase-8 have been experimentally demonstrated to have such a role (Ho et al., 2009; Krelin et al., 2008; Teitz et al., 2000). As alluded to previously and discussed in a number of recent reviews (Fava et al., 2012; Kumar, 2009), previous studies focusing on the role of caspase-2 in apoptosis have yielded somewhat contradictory findings, questioning the significance of caspase-2 in PCD. More recently, attention has been focused on its potential non-apoptotic functions. There are several lines of evidence that implicate caspase-2 in human cancers. Much of the earlier work comes from studies investigating haematological malignancies. For example, the human gene encoding caspase-2 is located in a region of chromosome 7 (7q34-35) that is frequently deleted in leukaemias (Kumar et al., 1995). In addition to the identification of caspase-2 somatic mutations in gastric and colorectal cancers (Kim et
al., 2011b), reduced expression of caspase-2 has been correlated with chemotherapeutic drug resistance in childhood acute lymphoblastic leukaemia (ALL) (Holleman et al., 2005). In line with these findings, reduced caspase-2 protein levels have been associated with poor prognosis and outcome in patients with acute myelogenous leukaemia (AML) and ALL (Estrov et al., 1998; Faderl et al., 1999). Furthermore, analysis of caspase-2 expression from publicly available microarray data has demonstrated that, in addition to various haematological malignancies, caspase-2 is downregulated in multiple solid tumours such as hepatocellular and invasive breast carcinomas, ovarian adenocarcinomas and glioblastomas (Ren et al., 2012). The loss of caspase-2 locus and expression in tumours is consistent with a putative tumour suppressor function. Paradoxically, somatic mutations of caspase-2 are rare in various human cancers (Kim et al., 2011a). Therefore, direct mutational inactivation of caspase-2 (unless deleted, as in haematological malignancies carrying chromosome 7q deletions/aberrations) is unlikely to explain its reduced expression or loss of function in human tumours. However, other mechanisms such as miRNA-mediated or epigenetic silencing of caspase-2 cannot be ruled out.

1.5.3. Caspase-2 function in protecting against cellular transformation

Clinical studies implicating caspase-2 in human cancers have prompted further investigation into its potential function in tumour suppression. There is growing evidence to suggest that caspase-2 is an important barrier that protects against cellular transformation. Studies from our laboratory have shown that loss of caspase-2 slightly increases the rate of proliferation in primary and E1A/Ras-transformed MEFs,
suggesting defective cell cycle regulation in caspase-2-deficient cells (Ho et al., 2009). Consistent with their increased proliferation rate, E1A/Ras-transformed Casp2\textsuperscript{−/−} MEFs displayed an increased ability to form colonies in soft agar (Ho et al., 2009). Interestingly, this increased proliferative capacity and enhanced ability of anchorage-independent growth in E1A/Ras-transformed Casp2\textsuperscript{−/−} MEFs coincided with an increased tumourigenic potential in nude mice (Ho et al., 2009). These observations link the functions of caspase-2 in the regulation of growth and proliferation with its ability to protect against transformation. Importantly, the catalytic activity of caspase-2 has been shown to be required for its ability to regulate proliferation and suppress transformation (Ren et al., 2012).

### 1.5.4 Caspase-2 as a tumour suppressor – evidence from mouse models

On the basis of in vitro studies describing a role for caspase-2 in protecting against cellular transformation, loss of caspase-2 function would be predicted to enhance tumour susceptibility in vivo. Contrary to this prediction, caspase-2 deletion is not sufficient to induce spontaneous tumourigenesis in mice (Shalini et al., 2012). However, when crossed into an oncogenic background, the function of caspase-2 in tumourigenesis becomes apparent. We have previously shown that deletion of caspase-2 in E\textsubscript{µ}-Myc transgenic mice accelerates lymphomagenesis, providing the first direct line of evidence for a tumour suppressor function for caspase-2 (Ho et al., 2009). These results with E\textsubscript{µ}-Myc/Casp2\textsuperscript{−/−} mice have also been confirmed by an independent study from another laboratory (Manzl et al., 2012). Interestingly, loss of even a single allele of caspase-2 is sufficient to augment lymphomagenesis in this model, suggesting
that caspase-2 may be a haploinsufficient tumour suppressor (Ho et al., 2009). These observations are consistent with the reduced expression of caspase-2 in human cancer, which may also be associated with loss of heterozygosity.

The extent of caspase-2 in tumour suppression has been further explored using various models of carcinogen-induced tumourigenesis. However, caspase-2 failed to suppress tumourigenesis induced by ionizing radiation (IR) or 3-methylcholanthrene (Manzl et al., 2012). These findings suggest that caspase-2 is not a general tumour suppressor and may indicate a context-specific function.

Recently it was shown that deletion of caspase-2 caused a modest increase in tumour onset in the mouse mammary tumour virus (MMTV)-Neu transgenic model of breast carcinoma (Parsons et al., 2013). Interestingly, this was only observed in multiparous mice with no significant differences in tumour onset observed between MMTV-Neu/Casp2+/+ and MMTV-Neu/Casp2−/− nulliparous mice. Unlike female nulliparous mice, mammary glands from female multiparous mice undergo extensive proliferation and differentiation during pregnancy and lactation (Strange et al., 1992). Given that loss of caspase-2 augments tumourigenesis only in multiparous mammary glands, caspase-2 function in protecting against cellular transformation appears to become more important in highly proliferative tissues that experience increased replicative and oncogenic stress, compared with tissues with a lower proliferative activity. These studies show that not only does the tumour suppressor function of caspase-2 extend beyond Myc-driven malignancies, but that it is also a suppressor of epithelial tumours.
Taken together, studies from mouse models have demonstrated that the function of caspase-2 in tumour suppression becomes important under conditions of oncogenic stress rather than suppressing tumourigenesis at the level of initiation. This is supported by the fact that Casp2⁻/⁻ mice do not develop spontaneous tumours, but caspase-2 deficiency in tumour-prone mice can potentiate tumourigenesis.

1.5.5. **Possible mechanism(s) of tumour suppression by caspase-2**

As discussed above, *in vivo* mouse models have been instrumental in establishing caspase-2 as a tumour suppressor. Given its functions in protecting against cellular transformation and suppressing tumour development, it is reasonable to speculate that these two functions are interdependent. Attention has now been focused on identifying and characterizing the mechanisms by which caspase-2 exerts these functions. Given that cancer cells frequently display aberrant proliferation, GIN and an impaired response to DNA damage (Abbas *et al.*, 2013; Hanahan and Weinberg, 2011), involvement of caspase-2 in the regulation of these processes likely contributes to its function in suppressing transformation and tumourigenesis.
1.6. Project Aims

The physiological functions of caspase-2 have remained enigmatic since its discovery 2 decades ago (Kumar et al., 1994). However, as discussed, recent work from our laboratory has demonstrated that caspase-2 is a tumour suppressor, providing new insights into the biological function of caspase-2 (Ho et al., 2009). At the beginning of this study, the mechanisms by which caspase-2 exerts its tumour suppressor function were undefined. Therefore, the aim of this study was to characterise the role of caspase-2 in tumour suppression by defining the processes and pathways disrupted by loss of caspase-2.

**The primary aims of this thesis were to:**

1) Characterise the function of caspase-2 in the DDR and protection against cellular transformation.

2) Use a non-Myc driven mouse model of lymphomagensis to investigate the extent and specificity of caspase-2 in tumour suppression.
Chapter 2

Materials and Methods
2.1. Chemical Reagents

All chemical reagents used were of analytical grade (or the highest grade obtainable).

Table 2.1. Chemical reagents used in this study

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>3, 3'-diaminobenzidine (DAB)</td>
<td>VectaStain</td>
</tr>
<tr>
<td>4',6'-diamidino-2-phenylindole (DAPI)</td>
<td>Roche</td>
</tr>
<tr>
<td>Acetic acid (glacial)</td>
<td>Chem-Supply</td>
</tr>
<tr>
<td>Acrylamide/Bis solution</td>
<td>Bio-Rad</td>
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<tr>
<td>Agarose</td>
<td>AppliChem</td>
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<tr>
<td>Ammonium chloride (NH₄Cl)</td>
<td>Sigma-Aldrich</td>
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<tr>
<td>Ammonium persulphate (APS)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Biotinylated 16-dUTP</td>
<td>Roche</td>
</tr>
<tr>
<td>Bouin’s solution</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Bradford dye reagent</td>
<td>Bio-Rad</td>
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<td>MPU (SA Pathology)</td>
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<td>Sodium dodecyl sulphate (SDS)</td>
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<td>GIBCO</td>
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<tr>
<td>β-mercaptoethanol</td>
<td>GE Healthcare</td>
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</table>

*Chapter 2: Materials and Methods*
2.2. Mice

2.2.1. Mouse husbandry

All animals were maintained in specific pathogen-free conditions in a 12:12 light/dark cycle and treated in accordance with protocols approved by the SA Pathology/Central Northern Adelaide Health Services Animal Ethics Committee. After weaning at postnatal day 18-21, mice were separated according to gender with food and water provided ad libitum. Casp2\(^{-/-}\) (O'Reilly et al., 2002), E\(\mu\)-Myc (Adams et al., 1985) and Atm\(^{+/}\) (Elson et al., 1996) mice have been previously described and backcrossed to a C57BL/6 background for 10-20 generations. Mice displaying signs of tumour burden such as hunched posture, weight loss, ruffled coats and respiratory distress were humanely sacrificed by carbon dioxide asphyxiation or cervical dislocation and tumour presence was confirmed by autopsy.

2.2.2. Breeding schemes

**Caspase-2:** Casp2\(^{+/}\) and Casp2\(^{-/-}\) littermates were generated from Casp2\(^{+/}\) x Casp2\(^{+/}\) intercrosses.

**E\(\mu\)-Myc:** Male E\(\mu\)-Myc mice were crossed with Casp2\(^{-/-}\) females to generate E\(\mu\)-Myc/Casp2\(^{+/}\) mice. E\(\mu\)-Myc/Casp2\(^{+/}\) males were then crossed again with Casp2\(^{-/-}\) females to generate E\(\mu\)-Myc/Casp2\(^{-/-}\) mice. Because differences in lymphoma onset
have been observed between male and female Eµ-Myc mice, the Eµ-Myc transgene was only propagated through males.

**Atm**: Because Atm\(^{-/-}\) mice are infertile, Atm\(^{+/+}\) mice were inter-crossed to generate Atm\(^{-/-}\) offspring. Atm\(^{+/+}\) mice were then crossed with Casp2\(^{-/-}\) mice to generate compound heterozygotes (Atm\(^{+/+}\)Casp2\(^{+/+}\)), which were subsequently crossed with Casp2\(^{-/-}\) mice to obtain Atm\(^{+/+}\)Casp2\(^{-/-}\) offspring. Atm\(^{+/+}\)Casp2\(^{-/-}\) mice were then inter-crossed to generate the following genotypes: Atm\(^{+/+}\)Casp2\(^{-/-}\); Atm\(^{+/+}\)Casp2\(^{-/-}\); Atm\(^{-/-}\)Casp2\(^{-/-}\).

PCR genotyping was performed to detect the Eµ-Myc transgene and Casp2 and Atm knockout alleles (see 2.3.1).

### 2.2.3. Tissue extraction and histology

Mouse tissues were harvested and snap frozen in liquid nitrogen or fixed in 10% v/v neutral buffered formalin for 24-48 h. Snap frozen tissues were stored at -70°C. Fixed tissues were processed and embedded in paraffin, sectioned and stained with haematoxylin and eosin by the Department of Surgical Pathology, SA Pathology (Adelaide, SA) by standard procedures. Digital images were acquired using a NanoZoomer (Hamamatsu).

### 2.2.4. Whole-mount embryos

Females were set up in timed pregnancies (see 2.2.2) and monitored twice daily for the
presence of a copulation plug at which time they were designated as embryonic day 0.5 (E0.5). Pregnant females were humanely sacrificed at E18.5 by cervical dislocation. Embryos were extracted, decapitated and heads and bodies fixed in Bouin’s solution for 2 days, followed by daily washes in 70% v/v ethanol for 7 days. Fixed embryos were processed and embedded in paraffin, sectioned and stained with haematoxylin/eosin by the Department of Surgical Pathology, SA Pathology (Adelaide, SA). Digital images were acquired using a NanoZoomer (Hamamatsu).

2.2.5. Blood analysis

Total blood lymphocyte counts were measured in peripheral blood extracted from tumour-laden mice via cardiac puncture immediately following carbon dioxide asphyxiation. Automated analysis and blood smears were performed by the Department of Clinical Pathology, SA Pathology (Adelaide, SA).
2.3. Nucleic Acid Analysis

2.3.1. Genomic DNA extraction for genotyping

Genomic DNA (gDNA) was extracted from mouse tail tips using the RED Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich) according to the manufacturer’s instructions. PCR was performed using 2 µL of gDNA, 100 ng of each primer (see table 2.2) and 10 µL of RED Extract-N-Amp PCR Mix in a final volume of 20 µL. Reactions without gDNA were used as a negative control for each PCR run.

2.3.2. Polymerase chain reaction (PCR)

The following thermocycler conditions were used for the indicated genotyping protocols:

**Caspase-2** - 94°C for 3 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min and a single elongation step of 72°C for 5 min;

**Eµ-Myc** – 94°C for 40 sec followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 1 min and a single elongation step of 72°C for 5 min;

**Atm** - 94°C for 3 min followed by 35 cycles of 94°C for 45 sec, 64°C for 45 sec, 72°C for 1 min followed by a single elongation step of 72°C for 5 min.
2.3.3. **DNA gel electrophoresis**

PCR-amplified DNA fragments were analysed by electrophoresis in 2% w/v agarose gels made in TAE buffer (40 mM Tris, 20mM glacial acetic acid, 1 mM EDTA, pH 8.0). 100 base pair ladder (New England BioLabs) was used as molecular weight standards. Samples were electrophoresed at 100 volts (V) in TAE buffer for 30-45 min. DNA was stained with ethidium bromide solution (2 μg/ml) for 10-15 min and visualised under UV light (254nm) using a transilluminator (UVItec).

Table 2.2. Sequences of forward (F) and reverse (R) primers used for genotyping.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences (5’-3’)</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase-2</td>
<td>GCAGTGAACAGAAGGAGGTGCC</td>
<td>Wild type and targeted alleles (R)</td>
</tr>
<tr>
<td></td>
<td>TTAAGAAGGGTGAGAACAGAG</td>
<td>Targeted allele (F)</td>
</tr>
<tr>
<td></td>
<td>TCATCCAAGCATGTCGTGGAGG</td>
<td>Wild type allele (F)</td>
</tr>
<tr>
<td>Eµ-Myc</td>
<td>CAGCTGGCGTAATAGCGAAGAG</td>
<td>Transgene (F)</td>
</tr>
<tr>
<td></td>
<td>CTGTGACTGTTGAGTACTCAACC</td>
<td>Transgene (R)</td>
</tr>
<tr>
<td></td>
<td>CTAGGCCACAGAATTGAAAGATCT</td>
<td>Untargeted allele (F)</td>
</tr>
<tr>
<td></td>
<td>GTAGGTGGAAATTCTAGCATCATCC</td>
<td>Untargeted allele (R)</td>
</tr>
<tr>
<td>Atm</td>
<td>TGTCAGTGTAAAGCTATTGTGC</td>
<td>Wild type and targeted alleles (F)</td>
</tr>
<tr>
<td></td>
<td>AAGGTGTAGATAGGTAGCGCATTT</td>
<td>Wild type allele (R)</td>
</tr>
<tr>
<td></td>
<td>AACGAGATCACGAGCTCTGTTCC</td>
<td>Targeted allele (R)</td>
</tr>
</tbody>
</table>

All genotyping primers were purchased from GeneWorks.
2.3.4. RNA extraction and quantification

Total RNA was extracted from cell pellets or frozen thymic lymphoma tissue using TRIZOL® reagent. Cells and tissues were lysed in 1 mL of TRIZOL® reagent and incubated for 5 minutes at room temperature (RT, 20-23°C). TRIZOL® lysates were then stored at -70°C overnight and RNA preparation conducted according to the manufacturer’s instructions. RNA was resuspended in diethylpyrocarbonate- (DEPC) treated water and digested with RNase-free DNase I (Sigma-Aldrich) according to the manufacturer’s instructions. RNA was then quantified using a NanoDrop1000™ spectrophotometer (Thermo Scientific).

2.3.5. First strand cDNA synthesis

First strand cDNA synthesis was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosciences) with 1 µg of oligo-dT primers (GeneWorks) and 1 µg of total RNA in the presence of recombinant RNase inhibitor (Life Technologies) according to the manufacturer’s instructions. Reactions (final volume 20 µL) were cycled using the following thermocycler conditions: 25°C for 10 min, 37°C for 2 h and 85°C for 5 min. Negative control reactions (without reverse transcriptase) were included for each RNA sample.

2.3.6. Quantitative PCR (qPCR)

Quantitative PCR (qPCR) was performed on a Rotor-Gene™ 3000 (Corbett Life Science/Qiagen) using RT² Real-Time SYBR® Green/ROX PCR Master Mix (Qiagen) as per the manufacturer’s instructions. cDNA was diluted 1:10 with sterile DEPC-treated
water. Reactions were cycled using the following thermocycler conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Each gene was analysed in 2-3 independent PCR runs. Expression was normalised to β-actin by the 2^−ΔΔCt method using Rotor-Gene™ 6000 Software (v1.7, Corbett Life Science/Qiagen). Control reactions (without reverse transcriptase or template) were included in each PCR run. Amplification specificity was assessed by melt curves analysis and electrophoresis of PCR products (see 2.3.3). The amplification efficiency of each primer pair used for qPCR analysis (see Table 2.3) was between 95-110%.

Table 2.3. Sequences of primers used for qPCR analysis of mouse genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences (5’-3’)</th>
<th>Source</th>
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<tr>
<td>β-actin</td>
<td>F: GATCATTGCTCCTCCTGAGC</td>
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<tr>
<td></td>
<td>R: AGTCCGCCTAGAAGCATTG</td>
<td></td>
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<tr>
<td>Cat</td>
<td>F: TCTTCGTCGCCAGTCTTCC</td>
<td>Designed using Primer-BLAST</td>
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<tr>
<td></td>
<td>R: CCTGGTCGCTTTGTAATGG</td>
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</tr>
<tr>
<td>p16INK4a</td>
<td>F: CGGTCGTACCCCCAGTTCAG</td>
<td>(Krishnamurthy et al., 2006)</td>
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<tr>
<td></td>
<td>R: GCACCGTAGTGGAGCAGGAG</td>
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<tr>
<td>p19ARF</td>
<td>F: TGAGGCTAGAGGATCTTGAAG</td>
<td>(Krishnamurthy et al., 2006)</td>
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<tr>
<td></td>
<td>R: GTGAACGTTGCCATCATCATC</td>
<td></td>
</tr>
<tr>
<td>p21CIP1/WAF1</td>
<td>F: AGTGTGCGTGGTGTCCCTTCGC</td>
<td>(Kwon et al., 2003)</td>
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<tr>
<td></td>
<td>R: ACACCAGTGGCAGACAGC</td>
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<tr>
<td>Prdx3</td>
<td>F: GCTCTGTCCCTGCTGTGTC</td>
<td>Designed using Primer-BLAST</td>
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<tr>
<td></td>
<td>R: AGGCTGACTTCTTTGGGCG</td>
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<tr>
<td>Puma</td>
<td>F: ATGCCCTCCTCACCTCTATCT</td>
<td>(McKenzie et al., 2010)</td>
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<tr>
<td></td>
<td>R: AGCACAGGATTGCTGGAGCCG</td>
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<td>Sens1</td>
<td>F: CCGTGTTCTGCTCACACAC</td>
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<td>R: CATTTACGGAAGCATTTCCGTC</td>
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<td>Sens2</td>
<td>F: TAGCCTGCAGCGCTCACCTAT</td>
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<td>R: TATCTGATGCAAAGACGCA</td>
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<td>Sod2</td>
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<td>(Shalini et al., 2012)</td>
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<tr>
<td></td>
<td>R: TGTCCTGGGCATTAGCATT</td>
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</table>

All qPCR primers were purchased from GeneWorks.

Chapter 2: Materials and Methods
2.3.7. 8-hydroxy-2'-deoxyguanosine (8-OHdG) ELISA

DNA oxidation was measured by quantifying 8-OHdG (8-hydroxy-2'-deoxyguanosine) levels using the DNA/RNA Oxidative Damage EIA Kit (Caymen Chemical, Cat# 589320) according to the manufacturer’s instructions. 8-OHdG levels (expressed as pg/µg of DNA) were measured in gDNA extracted from frozen primary lymphoma tissue. Tissues were digested in buffer containing 50 mM Tris-HCl, 50 mM EDTA pH 8.0, 0.125% w/v SDS and 800 µg/mL proteinase K (Millipore) for 3 h at 55°C. gDNA was extracted and purified as described in 2.3.8. Purified gDNA was digested with 1 unit of nuclease P1 (Sigma-Aldrich) for 1 h at 37°C, treated with 5 units of calf intestinal alkaline phosphatase (New England Biolabs) for 30 min at 37°C then incubated for 10 min at 100°C. Samples were quantified using a NanoDrop1000™ spectrophotometer (Thermo Scientific). A FLUOstar Optima microtitre plate reader (BMG Labtek) was used to measure optical density (OD) at 405 nm. Samples (diluted 1:50) and standards were assayed in duplicate.

2.3.8. Phenol/chloroform purification of DNA

To purify gDNA for 8-OHdG measurements (see 2.3.7), phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 mM Tris (pH 8.0) was added to digested tissues and mixed by inverting. Samples were centrifuged at 2000 x g for 10 min and upper aqueous layers transferred to new tubes. DNA was precipitated with 2 volumes of ethanol and 0.1 volumes of 3M (pH 4.6) sodium acetate for 5 minutes at RT. DNA pellets were washed twice with 70% v/v ethanol and centrifuged at 10,000 x g for 5 min before resuspending in DEPC-treated water and dissolving for 15 min at 55°C.
2.4. Protein Analysis

2.4.1. Protein extraction

Cell pellets or frozen primary lymphoma tissues were homogenised in lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% v/v nonyl phenoxypolyethoxylethanol, 0.5% w/v sodium deoxycholate and 0.1% w/v SDS) in the presence of Halt™ protease/phosphatase inhibitor cocktail (Thermo Scientific). Cell/tissue lysates were incubated for 30-60 minutes at 4°C then freeze-thawed 2-3 times (using liquid nitrogen and ice) before centrifugation at 13,000 x g for 10 minutes. Protein lysates were then transferred to new tubes and stored at -20°C or -70°C. Protein lysates were analysed by SDS polyacrylamide gel electrophoresis (PAGE) as described in 2.4.3 or used for protein carbonyl measurements (see 2.4.6).

2.4.2. Protein concentration quantification

Concentrations of protein lysates were determined used the Bradford Protein Assay (Bio-Rad) in a 96-well microtitre plate. Bovine serum albumin (BSA, Sigma-Aldrich) standards (0.1-0.5 mg/mL) were used to establish a standard curve. 200 µL of diluted (1:4) Bradford dye reagent (Bio-Rad) was added to 10 µL of BSA standards or 10 µL of diluted samples (1:10 for cell lysates, 1:50 for tissue lysates). A FLUOstar Optima microtitre plate reader (BMG Labtek) was used to measure OD at 595 nm. Samples and standards were assayed in triplicate and protein concentrations calculated from the standard curve.
2.4.3. **SDS-PAGE and electrotransfer**

Total protein lysates (30-50 µg) were denatured by boiling for 15 min in protein loading buffer (50 mM Tris pH 6.8, 100 mM DTT, 1% w/v SDS, 0.05% w/v bromophenol blue and 10% v/v glycerol. Resolving (10-15%) and stacking (5%) polyacrylamide gels were cast using the following reagents: resolving gel - 10-15% v/v acrylamide/Bis solution (37.5:1), 0.375 M Tris pH 8.8, 0.1% w/v SDS, 0.1% w/v APS and 0.05% v/v TEMED; stacking gel - 5% v/v acrylamide/Bis solution (37.5:1), 0.125 M Tris pH 6.8, 0.1% w/v SDS, 0.1% w/v APS and 0.1% v/v TEMED. Polyacrylamide gels were placed in Hoefer minigel tanks (Amersham Biosciences) with protein electrophoresis buffer (25 mM Tris, 250 mM glycine and 0.1% w/v SDS). 4–15% Mini-PROTEAN® TGX™ precast gradient polyacrylamide gels (Bio-Rad) were also used for SDS-PAGE analysis. Protein lysates were electrophoresed at 20 milliamperes (mA) per gel and 200 V. Precision Plus Protein™ Kaleidoscope™ Standards (Bio-Rad) were used as molecular weight markers.

Electrophoresed proteins were transferred to methanol-soaked polyvinylidene fluoride (PVDF) membranes (GE Healthcare) using a Hoefer semi-dry transfer apparatus (Amersham Biosciences) and whatman filter paper pre-soaked in protein transfer buffer (49 mM Tris, 39 mM glycine, 0.0375% w/v SDS and 20% v/v methanol). Proteins were electrotransferred onto PVDF membrane for 90 min at 130 mA and 50 V.

2.4.4. **Immunoblotting**

Following electrotransfer, PVDF membranes were incubated for 1-2 h on a rocker at RT in blocking buffer containing 5% w/v skim milk in TBS-T (25 mM Tris pH 7.5, 140 mM
NaCl, 2.7 mM KCl and 0.05% v/v Tween®20). Blocked membranes were then incubated with primary antibodies (see Table 2.4) diluted in blocking buffer overnight on a rocker at 4°C. After several washes in TBS-T, membranes were incubated with the following conjugated, species-specific secondary antibodies diluted 1:2000 in blocking buffer: IgG horseradish peroxidase (HRP)-conjugated whole antibodies (GE Healthcare), IgG alkaline phosphatase (AP)-conjugated whole antibodies (Millipore) or IgG Cy5-conjugated whole antibodies (GE Healthcare). HRP-conjugated secondary antibodies were detected using ECL™ substrate (enhanced chemiluminescence, GE Healthcare) and developed on X-ray film (Fujix) using a photo processor. AP-conjugated secondaries were detected using ECF™ substrate (enhanced chemifluorescence, GE Healthcare). Proteins detected using AP and Cy5-conjugated antibodies were visualised on a Typhoon 9410 (Molecular Dynamics) and analysed with ImageQuant software (GE Healthcare).

2.4.5. **Coomassie staining**

Following electrotransfer, polyacrylamide gels were stained with 0.25% w/v Coomassie Brilliant Blue solution for 30-60 min. Gels were then destained in 40% v/v methanol and 10% v/v glacial acetic acid for 1-2 h.

2.4.6. **Protein carbonyl measurements**

Protein oxidation was quantified using the Protein Carbonyl Colorimetric Assay Kit (Caymen Chemical, Cat# 10005020) according to the manufacturer's instructions. Protein carbonyl levels (expressed as nmoles/mg of protein) were measured in total
protein lysates prepared from frozen thymic lymphoma tissue or freshly isolated splenocyte and thymocyte cell pellets as described in 2.4.1. Protein concentration was determined using the Bradford Protein Assay (Bio-Rad) as described in 2.4.2. A FLUOstar Optima microtitre plate reader (BMG Labtek) was used to measure OD at 355 nm. Samples and controls were measured in triplicate.
Table 2.4. Primary antibodies used for immunoblotting (IB), immunocytochemistry (ICC), immunohistochemistry (IHC) and flow cytometry (FC).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host Species and Clonality</th>
<th>Clone and Supplier</th>
<th>Application and Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>mouse monoclonal</td>
<td>AC-15, Sigma-Aldrich</td>
<td>IB (1:2000)</td>
</tr>
<tr>
<td>Caspase-2</td>
<td>rat monoclonal</td>
<td>11B4, Walter and Eliza Hall Institute (Melbourne, VIC)</td>
<td>IB (1:500)</td>
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<td>CHK1</td>
<td>mouse monoclonal</td>
<td>DCS-310, Thermo-Scientific</td>
<td>IB (1:500)</td>
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<td>CD4-Alexa Fluor® 488</td>
<td>rat monoclonal</td>
<td>RM4-5, BD Biosciences</td>
<td>FC (1:100)</td>
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<td>CD8a-PE</td>
<td>rat monoclonal</td>
<td>53-6.7, BD Biosciences</td>
<td>FC (1:100)</td>
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<td>CD90.2-PE</td>
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<td>FC (1:100)</td>
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<td>KAP-1</td>
<td>rabbit polyclonal</td>
<td>Novus Biologicals</td>
<td>IB (1:1000)</td>
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2.5. **Tissue Culture and Cellular Analysis**

2.5.1. *Cell culture conditions*

Primary mouse embryonic fibroblasts (MEFs), spontaneously-immortalised MEFs (iMEFs), SV40-immortalised MEFs, lymphoma cell lines and primary splenocyte/thymocyte suspensions were cultured at 37°C in a humidified incubator at atmospheric oxygen with 10% carbon dioxide in complete medium: high glucose Dulbecco’s Modified Eagles Medium (DMEM, Sigma-Aldrich) supplemented with 10% v/v foetal bovine serum (FBS, JHR Biosciences), 50 µM β-mercaptoethanol, non-essential amino acid mix, 2 mM L-glutamine, 15 mM HEPES and 100 µM penicillin/streptomycin. SV40-immortalised MEFs were obtained from Prof. Andreas Villunger (Innsbruck Medical University, Austria). U2OS (human osteosarcoma cell line) cells were obtained from Sonia Dayan (The University of Adelaide, SA) and cultured at 37°C in a humidified incubator at atmospheric oxygen with 5% carbon dioxide in complete medium: high glucose DMEM supplemented with 10% v/v FBS, 2 mM L-glutamine, 15mM HEPES and 100 mM penicillin/streptomycin. EL4 (mouse thymic lymphoma cell line) cells were obtained from Dr. Simon Apte, (Queensland Institute of Medical Research, QLD) and cultured at 37°C in a humidified incubator at atmospheric oxygen with 5% carbon dioxide in complete medium: Roswell Park Memorial Institute medium (RPMI, Sigma-Aldrich) supplemented with 6% v/v FBS and 100 µM penicillin/streptomycin. To passage adherent cells, medium was removed and cells washed once in PBS. Cells were detached by incubation in 0.054% w/v trypsin (Difco) in Hank’s balanced salt solution (GIBCO) for 5-10 min at 37°C. Cells were then transferred
to complete medium and centrifuged for 5 min at 500 x g. Cell pellets were resuspended in complete medium and 4-6x10^5 cells (in 10-15 mL) reseeded into 75cm² tissue culture flasks (Greiner Bio-One). Non-adherent cells were passaged by collecting in a tube and centrifuging for 5 minutes at 500 x g. Cell pellets were then resuspended in complete medium and reseeded at 5x10^5 – 1x10^6 cells (in 5-6 mL) into 6-well plates (BD Falcon). Cell density and viability was calculated with a haemocytometer and trypan blue (0.8% w/v in PBS) dye exclusion.

2.5.2. Isolation of mouse embryonic fibroblasts (MEFs)

Primary MEFs were extracted from E13.5 embryos derived from pregnant mothers (from Casp2+/+ x Casp2+/+ and Casp2+/− x Casp2−/− crosses). Embryonic sacs were removed and transferred to a dish with warm, sterile PBS. Individual embryos were separated and detached from the placenta. Heads and livers were removed and embryo bodies transferred to 1.5 mL tubes with pre-warmed media. Embryos were homogenised with sterile, plastic pestles and dispersed by pipetting. Homogenates were added to 10 cm tissue culture plates (BD Falcon) containing 10ml of pre-warmed complete medium (see 2.5.1). MEFs were incubated at 37°C and allowed to adhere for 16-24 h. MEFs were washed once in PBS and overlayed with fresh, pre-warmed complete medium and cultured for a further 24 h. At the time of seeding, MEFs were labelled as passage 0 (P0). gDNA extracted from tail tips of embryos was used for genotyping (see 2.3.1). Primary MEFs at an early passage (P0–P4) for all experiments unless otherwise indicated. iMEFs were generated by serial passaging of primary MEFs in culture, using the 3T3 subculture method (Todaro and Green, 1963). Late passage
MEFs that were able to overcome replicative senescence and form colonies were expanded and termed immortalised.

2.5.3. **Isolation of lymphoma cell lines**

Freshly isolated lymphomas from Eµ-Myc and Atm⁻/⁻ mice were homogenised with a sterile, glass homogeniser in pre-warmed complete medium, filtering through a 40 µm nylon mesh and incubated with red blood cell lysis buffer (155 mM NH₄Cl, 10 mM NaHCO₃) for 5 min at RT. Primary lymphoma cells were routinely passaged for 3-4 wks until spontaneously-immortalised cell lines were established.

2.5.4. **Isolation of primary splenocytes and thymocytes**

Splenocyte and thymocyte suspensions were prepared from 13-14 wk old premalignant mice. Freshly isolated spleens and thymuses were homogenised with sterile, plastic pestles in media, filtered through a 40 µm nylon mesh and incubated with red blood cell lysis buffer (155 mM NH₄Cl, 10 mM NaHCO₃) for 5 min at RT. Freshly isolated splenocyte and thymocyte cell suspensions were stimulated with 5 µg/mL concanavalin A (Sigma-Aldrich) and 100 units/mL recombinant mouse interleukin-2 (Cell Signaling) for 48 and 24 h, respectively.

2.5.5. **Proliferation assays**

*Casp2⁺/⁺* and *Casp2⁻/⁻* iMEFs (1x10⁴) were seeded into 60mm dishes (BD Falcon) at day 0. Cells were harvested by trypsinisation every 24 h for 3 days and the number of viable cells counted by trypan blue exclusion with a haemocytometer.
2.5.6. Cryopreservation and thawing of cells

Log phase cell cultures were harvested, pelleted by centrifugation at 500 x g for 5 min and resuspended at a concentration of 2-3x10^6 cells/mL (adherent cells) or 8-10x10^6 cells/mL (non-adherent cells) in 1 mL of medium containing 20% v/v FBS and 10% v/v dimethyl sulphoxide (DMSO) into cryopreservation vials (Greiner Bio-One). Cells were frozen at -70°C then transferred to liquid nitrogen for long-term storage. To thaw cryopreserved cells, vials were incubated in a water bath (37°C), washed by centrifugation in pre-warmed media and seeded into tissue culture flasks.

2.5.7. Small interfering RNA (siRNA) transfections

U2OS cells (3x10^5) were seeded into 60 mm dishes (BD Falcon) in 2 mL of complete medium and allowed to adhere for 16-24 h. siRNA (final concentration of 50 nM) and 10 µL of TransIT-TKO® siRNA Transfection Reagent (Mirus) were diluted in 50 µL and 200 µL of Opti-MEM® (Sigma-Aldrich), respectively (per transfection) and incubated for 5 min at RT. The diluted siRNA and transfection reagent were then combined and incubated for a further 30 min at RT before being added dropwise to cells. After 30 h incubation at 37°C in the transfection complexes, the media was replaced and cells were allowed to recover for 16 h before treatment with 10 Gy γ-radiation (see 2.5.18) and harvesting for protein extraction (see 2.4.1). siRNAs were purchased from GenePharma: control siRNA (5’-UAAGGCUAUGAAGAGAUACTT-3’); human caspase-2 siRNA (5’-GUUGUUGAGCGAAUUGUUATT-3’).
2.5.8. **Immunophenotyping**

Freshly isolated thymic lymphoma cells (isolated as described in 2.5.3) were blocked in 10% v/v normal mouse serum/PBS (Sigma-Aldrich) for 30 min at RT. Cells were then incubated with conjugated primary antibodies (see Table 2.4) diluted 1:100 in 1% w/v BSA/PBS for 1 h on ice in the dark. After washing twice in 1% w/v BSA/PBS, cells were analysed live on an FC500 flow cytometer (Beckman Coulter). At least 20,000 events were recorded per sample. Data analysis was performed using FCS Express Flow Cytometry Research Edition Version 4 (DeNovo Software).

2.5.9. **Phospho-histone H3 (Ser10) staining and DNA content analysis**

Lymphoma cells (1x10⁶) were seeded into 6-well plates and cultured for 24 h before being treated with 10 µM menadione or exposed to 5 Gy γ-radiation (see 2.5.18). Primary MEFs (4x10⁵) and iMEFs/SV40-immortalised MEFs (2x10⁵) were seeded into 10 cm and 6 cm dishes (BD Falcon), respectively and cultured for 48 h before being exposed to 5 Gy γ-radiation. At the indicated times, cells were fixed in 70% v/v ethanol and stored at -20°C. For phospho-histone H3 (pH3) staining in MEFs, fixed cells were rehydrated by washing 2-3 times in PBS with centrifugation at 500 x g for 5 min. Cells were then permeabilised with 0.25% v/v Triton X-100/PBS for 10 min at RT then blocked in 1% w/v BSA/PBS for 1 h at RT. Cells were incubated with anti-pH3 (Ser10) primary antibody (see Table 2.4) diluted in blocking buffer, for 3 h at RT. Following washes in blocking buffer, cells were incubated with donkey anti-mouse Alexa Fluor® 488 secondary antibody (Life Technologies) diluted 1:1000 in blocking buffer, for 2 h at RT. Cells were again washed in blocking buffer before being incubated in staining
solution containing 40 µg/mL RNase A (Roche) and 25 µg/mL propidium iodide, for at least 3 h. Cells were stored at 4°C then analysed on an FC500 flow cytometer (Beckman Coulter). Cells with a sub-G1 DNA content were gated as apoptotic. pH3 positive cells with a 4N DNA content were labelled as mitotic. At least 50,000 events were recorded per sample. Cellular debris and doublets were excluded from the analysis based on non-linearity on a doublet discrimination plot. Data analysis was performed using Multicycle AV™ on FCS Express Flow Cytometry Research Edition Version 4 (DeNovo Software).

2.5.10. Preparation of chromosome spreads

Primary MEFs (see 2.5.2) and freshly-isolated primary lymphomas (see 2.5.3) or splenocyte/thymocyte cell suspensions (2.5.4) were incubated with 20 ng/mL Colcemid™ (Life Technologies) for 3-4 h. Cells were then swollen in hypotonic solution (0.075M KCl) for 15 min at 37°C then fixed immediately in freshly-prepared, ice cold methanol:glacial acetic acid solution (3:1) for 15 min at 37°C. Fixed cells were centrifuged at 500 x g for 10 min, washed 3-4 times in Carnoy's fixative then dropped onto glass slides and air dried overnight. MEF and lymphoma/splenocyte/thymocyte metaphase spreads were stained with 4% v/v Giemsa and 2 µg/mL 4’,6’-diamidino-2-phenylindole (DAPI), respectively. Coverslips were mounted with ProLong® Gold Antifade Reagent. Images were captured using an epifluorescence microscope as described in 2.5.17.
2.5.11. **Telomere fluorescent in situ hybridisation (FISH)**

FISH analysis was performed using the Telomere PNA FISH kit/Cy3 (DAKO) according to the manufacturers' instructions. Chromosome spreads were counterstained with 2 µg/mL DAPI. Coverslips were mounted with ProLong® Gold Antifade Reagent. Fluorescent images were captured using an epifluorescence microscope as described in 2.5.16 and manually merged using Photoshop 6.0 software (Adobe).

2.5.12. **Senescence associated β-galactosidase staining**

Senescence-associated β-galactosidase staining kit (Cell Signaling) was used according to the manufacturer’s instructions to identify senescent cells. Briefly, primary MEFs (1x10^5) were seeded onto glass coverslips in 35 mm dishes (BD Falcon) and allowed to adhere overnight. MEFs were then fixed for 10 min and rinsed thoroughly in PBS and incubated with the X-gal staining solution for 30 min at 37°C. The stained cells were washed with PBS and mounted in glycerol. Images were captured as described in 2.5.17.

2.5.13. **Gamma H2AX immunofluorescence**

Primary MEFs (1x10^5) were seeded onto glass cover slips in 35 mm dishes (BD Falcon) and allowed to adhere overnight before exposure to 10 Gy γ-radiation (see 2.5.18). Cells were fixed in 4% w/v paraformaldehyde for 15 min at 4°C, permeabilised with 0.25% v/v Triton X-100/PBS for 10 min at RT then blocked in 1% w/v BSA/PBS for 1 h at RT. Cells were incubated with anti-phospho-histone H2AX (Ser139) primary antibody (see Table 2.4) diluted in blocking buffer, overnight at 4°C. Following 3 washes in
blocking buffer, cells were incubated with donkey anti-rabbit Alexa Fluor® 488 secondary antibody (Life Technologies) diluted 1:1000 in blocking buffer, for 1 h at RT. Following antibody incubations and washes in PBS, cells were counterstained with 2 µg/ml DAPI for 5 min. Cover slips were mounted onto glass slides with ProLong® Gold Antifade Reagent. At least 300 cells were scored for each sample. Fluorescent images were captured using an epifluorescence microscope as described in 2.5.17.

2.5.14. Proliferating cell nuclear antigen (PCNA) IHC

Formalin-fixed, paraffin-embedded thymic lymphoma sections (5 µm) were deparaffinised in xylene (25 min at RT) and rehydrated in graded ethanol series (100, 90, 70, 50, 30% v/v for 2 min each at RT). Endogenous peroxidase activity was quenched with 3% v/v H₂O₂/PBS for 10 min at RT. A wax pen was used to draw a hydrophobic barrier around each tissue section. Tissue sections were then incubated for 1 h at RT with blocking buffer containing 5% v/v FBS/PBS-T before incubation with anti-PCNA primary antibody (see Table 2.4) diluted 1:250 in blocking buffer overnight at 4°C in a humidified chamber. For each tissue section, a negative control (without primary antibody) was included. After several washes in PBS-T, tissue sections were sequentially incubated with anti-mouse biotinylated secondary antibody diluted 1:250 in blocking buffer for 1-2 h (GE Healthcare) and Avidin/Biotin Complex reagent ( VectaStain ) for 30 min at RT in a humidified chamber. DAB (3, 3′-diaminobenzidine) peroxidase substrate was added (5 min at RT) for colour development followed by counterstaining with Mayer’s haematoxylin solution for 2-5 min at RT. Tissue sections were then rinsed in reverse osmosis (RO) water and dehydrated in graded ethanol
series (90, 100% v/v for 2 min each at RT). Slides were then cleared by immersing in xylene for 25 min at RT before mounting coverslips with DePex mounting media. Digital images were acquired using a NanoZoomer (Hamamatsu).

2.5.15. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) IHC

Formalin-fixed, paraffin-embedded thymic lymphoma sections (5 µm) were deparaffinised in xylene (25 min at RT) and rehydrated in graded ethanol series (100, 90, 70, 50, 30% v/v for 2 min each at RT). Tissue sections were incubated with 20 µg/mL proteinase K (Millipore) for 15 min at RT and endogenous peroxidase activity quenched with 1% v/v H₂O₂/PBS for 5 min at RT followed by washing in TBS (10 mM Tris, pH 8.0). A wax pen was used to draw a hydrophobic barrier around each tissue section. Following a 10 min pre-incubation with terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Tris pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride), tissue sections were incubated with reaction buffer containing 135 units/mL TdT enzyme (Promega) and 9 µM biotinylated 16-dUTP in TdT buffer for 1 h at 37°C. Negative control reactions (with TdT enzyme excluded from the reaction mixture) were included for each tissue section. Reactions were terminated by immersing sections twice in saline sodium citrate buffer (300 mM NaCl, 30 mM sodium citrate) for 15 min. After blocking with 2% w/v BSA/PBS for 10 min at RT, tissue sections were washed in PBS for 5 min at RT and incubated with HRP-conjugated streptavidin (VectaStain) for 30 min at RT. Colour development was achieved by incubating sections in DAB peroxidase substrate for 5 min at RT. Following washes in PBS, tissue sections
were counterstained with Mayer’s haematoxylin solution for 2-5 min at RT, rinsed in RO water and dehydrated in graded ethanol series (90, 100% v/v for 2 min each at RT). Slides were then cleared by immersing in xylene for 25 min at RT before mounting coverslips with DePex mounting media. Digital images were acquired using a NanoZoomer (Hamamatsu).

2.5.16. **Cytokinesis block micronucleus (CBMN) assay**

MEFs (1-2x10⁵) were seeded onto glass coverslips in 35 mm dishes (BD Falcon) and allowed to adhere overnight. Cells were left untreated or exposed to 5 Gy γ-radiation (see 2.5.18) and incubated for 24, 48 or 72 h. Cells were then treated with 4 µg/ml cytochalasin B and incubated a further 24 h before fixation in methanol:glacial acetic acid solution (3:1). Cells were stained with 2 µg/ml DAPI for 5 min and fluorescent images captured as described in 2.5.17. At least 500–1000 cells were counted per slide.

2.5.17. **Microscopy**

Bright-field and fluorescent images were captured using an epifluorescence microscope (model BX51; Olympus,) and camera (UCMAD3/ CVM300, Olympus). 100X images were visualised using an oil immersion lens with microscope oil (Olympus). Cells were visualised under 40X or 100X ULAPO objective lens with NA=1.5.

2.5.18. **Gamma irradiation**

Cells were irradiated using a blood product gamma irradiator (model IBL 437C) with a caesium-137 source at approximately 5 Gy/min (Bio International, France).
2.6. Statistical Analysis of Data

Non-linear regression curve fitting was used for statistical analysis of mouse growth curves. Analysis of expected and observed frequencies of mouse genotypes and karyotype distributions was performed by a Chi-squared test. A log-rank test and Kaplan-Meier analysis was used for comparison of mouse survival curves. Two-tailed, unpaired Student’s $t$-tests were used for all other analyses. Data are represented as mean ± SEM unless otherwise stated. $P<0.05$ was considered statistically significant. Throughout this thesis * denotes $P < 0.05$, ** denotes $P < 0.01$ and *** denotes $P < 0.001$. All statistical analyses were performed using Microsoft® Excel 2010 or GraphPad Prism (v5).
Chapter 3

Caspase-2 deficiency promotes aberrant DNA damage response and genomic instability
Statement of Authorship

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Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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<th>Dr. Loretta Dorstyn</th>
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<th>Joseph Puccini</th>
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<th>Dr. Claire H Wilson</th>
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<th>Dr. Sonia Shalini</th>
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<th>Sarah Moore</th>
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Chapter 3: Caspase-2 deficiency promotes aberrant DNA damage response and genomic instability
Chapter 3: Caspase-2 deficiency promotes aberrant DNA damage response and genomic instability
3.1. Abstract

Caspase-2 is an initiator caspase which has been implicated in apoptotic and non-apoptotic signalling pathways, including cell cycle regulation, DNA damage signalling and tumour suppression. We previously demonstrated that caspase-2 deficiency enhanced oncogene-induced cellular transformation and augments lymphomagenesis in the Eµ-Myc mouse model. Furthermore, Caspase-2 knockout (Casp2⁻/⁻) mouse embryonic fibroblasts (MEFs) show aberrant cell cycle checkpoint regulation and a defective apoptotic response following DNA damage. Disruption of cell cycle checkpoints often leads to genomic instability (GIN), which is a common phenotype of cancer cells and can contribute to cellular transformation. Here we show that caspase-2 deficiency resulted in increased DNA damage and GIN in primary MEFs. Casp2⁻/⁻ MEFs readily escaped replicative senescence in culture and exhibit increased micronuclei formation and sustained DNA damage following γ-irradiation. Cytogenetic analysis demonstrated that a loss of caspase-2 was associated with increased aneuploidy in both primary MEFs and Eµ-Myc lymphoma cells. We also show that loss of caspase-2 leads to defective transactivation of p53 target genes following DNA damage. These findings suggest that caspase-2 serves an important role in DNA damage signalling and maintaining genomic integrity which may contribute to its functions in protecting against cellular transformation and tumourigenesis.
3.2. Introduction

Caspase-2 is the most evolutionarily conserved caspase with highest sequence similarity to Caenorhabditis elegans caspase, CED-3 (Kumar et al., 1994). Caspase-2 has been shown to have a role in apoptosis induced by various stimuli, including heat shock, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), cytoskeletal-disrupting drugs and DNA damage (Bonzon et al., 2006; Ho et al., 2008; Ho et al., 2009; Shin et al., 2005; Sidi et al., 2008). However, the exact function of caspase-2 in apoptosis has remained unclear as caspase-2 knockout (Casp2\(^{-/-}\)) mice have no overt phenotype and only minor apoptotic defects in some cell types (Bergeron et al., 1998; Marsden et al., 2004; O’Reilly et al., 2002). Interestingly, Casp2\(^{-/-}\) mice have an abnormal abundance of oocytes and display premature ageing-related traits indicating that caspase-2 may have context-dependent functions (Braga et al., 2008; Shalini et al., 2012; Zhang et al., 2007). Recent studies have demonstrated possible non-apoptotic functions of caspase-2 in cell cycle checkpoint regulation and the oxidative stress response (Ren et al., 2012; Shalini et al., 2012; Sohn et al., 2011). The loss of caspase-2 has been associated with increased cell proliferation and defective cell cycle arrest in response to ionizing radiation (IR) (Ho et al., 2009; Ren et al., 2012; Sohn et al., 2011). In addition, a role for caspase-2 in a checkpoint kinase 1- (CHK1) inhibited, ataxiatelangiectasia mutated (ATM) DNA damage response (DDR) pathway has been suggested in p53-deficient cells (Ho et al., 2009; Sidi et al., 2008).
Cell cycle progression is tightly regulated by a series of checkpoints to safeguard against DNA damage induced by stresses such as replication and free radicals or by IR and cytotoxic drugs (Kastan and Bartek, 2004; Malumbres and Barbacid, 2009). Checkpoint activation is regulated through the activity of cyclin-dependent kinases (CDKs) and by CDK inhibitors, including $p19^{ARF}$, $p16^{INK4a}$ and $p21^{CIP1/WAF1}$ which induce senescence and have been implicated in cell immortalisation (Carnero et al., 2000). Improper segregation of chromosomes during mitosis or excessive and irreparable damage to DNA activates cell cycle checkpoints that lead to cell cycle arrest, thereby facilitating DNA repair or apoptosis to remove damaged cells (Ciccia and Elledge, 2010; Vitale et al., 2011).

The activation of the DDR pathway components ATM and ATR (ataxia telangiectasia and Rad3-related) and its substrates checkpoint kinases 1 and 2 (CHK1, CHK2) are critical for cell cycle arrest and repair of DNA breaks (Ciccia and Elledge, 2010). The activation of ATM leads to the phosphorylation and activation of histone H2AX, which is recruited to sites of DNA damage (Burma et al., 2001; Cremona and Behrens, 2013). This is followed by activation of multiple downstream targets, including CHK1, CHK2 and p53, leading to cell cycle checkpoint activation and DNA repair (Cremona and Behrens, 2013). Activation of p53 is tightly regulated by ATM and CHK2-mediated phosphorylation and stabilisation (Canman et al., 1998; Hirao et al., 2000) and by mouse double minute 2 (MDM2) mediated degradation (Haupt et al., 1997; Kubbutat et al., 1997). In addition, the tumour suppressor $p19^{ARF}$ sequesters and inhibits MDM2 and allows transactivation of a number of p53 target genes that control cell growth or
apoptosis (Menendez et al., 2009; Riley et al., 2008; Tao and Levine, 1999b).

Inaccurate DNA repair or aberrant cell cycle checkpoint surveillance results in accumulation of DNA damage and GIN (Ciccia and Elledge, 2010; Kastan and Bartek, 2004). Many human diseases are caused by mutations in cell cycle checkpoint genes such *ATM* (ataxia-telangiectasia), *ATR* (Seckel syndrome) and *CHK2* (Li–Fraumeni syndrome) and can manifest as GIN and an increased susceptibility to cancer (Caldecott, 2008; Cimprich and Cortez, 2008; Lavin, 2008; Srivastava et al., 1990). The activation of the DDR is therefore critical to maintain genome stability, but the mechanisms that determine the choice between activation of DNA repair, cell cycle arrest and/or apoptosis are not fully understood.

Given that previous studies suggested a function for caspase-2 in cell cycle regulation, proliferation and tumour suppression (Ho et al., 2009; Ren et al., 2012; Sohn et al., 2011), we tested whether loss of caspase-2 leads to GIN. In this study, using primary and immortalised MEFs from wild type (*Casp2*+/+) and *Casp2*−/− mice, we demonstrate that cells lacking caspase-2 display enhanced DNA damage and aneuploidy in culture. We also show that caspase-2 deficiency results in reduced p53 activation and consequently reduced p53-upregulated modulator of apoptosis (*Puma*) and *p21*<sub>^CIP1/WAF1</sub> expression in response to DNA damage. Thus, our data show that caspase-2 is important in maintaining genome stability and provides a possible mechanistic basis for its function as a tumour suppressor.
3.3. Results

3.3.1. Casp2−/− MEFs readily escape replicative senescence and immortalise in culture

Through the accumulation of oxidative and genotoxic stress, primary MEFs undergo replicative senescence in culture (Parrinello et al., 2003). From our previous studies, we have shown that Casp2−/− primary MEFs exhibit an increased proliferation rate in culture compared to Casp2+/+ primary MEFs (Ho et al., 2009). Therefore, we assessed whether caspase-2 deficiency affects the proliferative capacity and senescence onset of primary MEFs over serial passages in culture. Under our experimental conditions, Casp2+/+ primary MEFs entered replicative senescence by passage 6-8 (P6-8) as detected by senescence-associated-β-galactosidase (SA-β-gal) activity (Fig. 3.1A). Interestingly, Casp2−/− primary MEFs showed no significant increase in the number of senescent cells at this time. This coincided with a lack of induction of key CDK inhibitors p19ARF, p16INK4a and p21CIP1/WAF1 that are associated with the onset of replicative senescence (Fig. 3.1B) (Bringold and Serrano, 2000; Carnero et al., 2000).

Through continuous serial passaging, we were able to readily isolate several Casp2−/− MEF clones that had escaped replicative senescence as a result of spontaneous immortalisation. Casp2+/+ MEFs also became immortalised but at a later passage (>P10). The spontaneously-immortalised MEFs (iMEFs) were characterised by their ability to be continually passaged and their faster growth rate compared with primary MEFs. Consistent with observations in primary MEFs, all independently-generated
Chapter 3: Caspase-2 deficiency promotes aberrant DNA damage response and genomic instability

Fig. 3.1. Casp2⁻/⁻ MEFs readily escape replicative senescence. (A) Senescence was assayed by SA-β-galactosidase staining of Casp2⁺/⁺ (n=3-5) and Casp2⁻/⁻ (n=3-5) primary MEFs at the indicated passages. (B) Markers of cell cycle arrest and senescence, p19ARF, p16INK4a and p21CIP1/WAF1 were assessed by quantitative PCR. Expression is relative to β-actin. Data represented as mean ± SEM from at least three different batches of MEFs for each passage (*P<0.05 and **P<0.01, Student’s t-test). This experiment was performed by Dr. Loretta Dorstyn.
Casp2−/− iMEF clones showed significantly increased proliferation rates compared with Casp2+/+ iMEF clones (Fig. 3.2A). In contrast, the proliferation rate of Casp2+/+ and Casp2−/− simian virus 40 (SV40) T antigen-immortalised MEFs were not significantly different (data not shown). Given its potent effects on multiple pathways regulating cell growth and division, overexpression of the SV40 T antigen may masks the subtle effects of caspase-2 deficiency on cell proliferation.

In addition to their increased proliferation rate, Casp2−/− iMEFs appeared morphologically different, being less fibroblastic and more rounded compared with Casp2+/+ iMEFs (Fig. 3.2B). These morphological changes suggest that caspase-2 may regulate cytoskeletal architecture during the immortalisation process. Both Casp2+/+ and Casp2−/− iMEFs exhibited contact inhibition and were unable to grow in soft agar (data not shown), indicating that they were not transformed. Together, these findings indicate that loss of caspase-2 enhances cell proliferation and allows cells to readily overcome replicative senescence in culture.

3.3.2. DNA damage-induced cell cycle checkpoint analysis in Casp2−/− MEFs

Several studies have implicated caspase-2 in the regulation of DNA damage-induced cell cycle checkpoint responses (Ho et al., 2009; Sohn et al., 2011; Taghiyev et al., 2011). Therefore, we performed cell cycle analysis in primary, spontaneously-immortalised and SV40-immortalised MEFs. Cell cycle profiles were analysed in MEFs by DNA content analysis with propidium iodide staining and flow cytometry.
Fig. 3.2. Casp2\(^{-/-}\) MEFs readily immortalise in culture. (A) Proliferation rates of spontaneously-immortalised MEF (iMEF) clonal populations derived from Casp2\(^{+/+}\) and Casp2\(^{-/-}\) primary MEFs. Numbers indicate different clones. (B) Representative images (20X) of Casp2\(^{+/+}\) and Casp2\(^{-/-}\) iMEF clonal populations showing smaller and more rounded morphology of Casp2\(^{-/-}\) iMEFs.
In response to IR treatment, all Casp2<sup>+/+</sup> and Casp2<sup>-/-</sup> MEF derivatives accumulated in G2/M, indicating G2/M arrest (Fig. 3.3A-C). This coincided with a reduction in the G0/G1 populations and concomitant increases in the S phase populations at 4 h after IR treatment (Fig. 3.3A-C). However, the cell cycle distributions of Casp2<sup>-/-</sup> MEFs following IR treatment were indistinguishable from that of their Casp2<sup>+/+</sup> counterparts. Therefore, from this analysis, loss of caspase-2 does not appear to affect cell cycle checkpoint regulation in primary and immortalised MEFs under these conditions.

We next focused our attention specifically on the G2/M checkpoint. In order to further assess IR-induced G2/M checkpoint activation, mitotic indices were quantified in primary MEFs, iMEFs and SV40-immortalised MEFs following IR treatment. Phospho-histone H3 (Ser10) (pH3), a well-established mitotic marker (Wei et al., 1999) was used with flow cytometry to label mitotic cells. pH3 positive cells with a 4N DNA content were gated as mitotic. Primary MEFs and iMEFs displayed efficient G2 arrest 4 h after IR treatment, as indicated by the reduction in the percentage of mitotic cells (Fig. 3.4A-B). By 8 h after IR treatment, both primary MEFs and iMEFs recovered and re-entered mitosis as shown by the increase in mitotic index (Fig. 3.4A-B). However, no significant differences were observed between the ability of Casp2<sup>+/+</sup> and Casp2<sup>-/-</sup> primary MEFs and iMEFs to overcome IR-induced arrest and re-enter mitosis. As with primary MEFs and iMEFs, SV40-immortalised MEFs efficiently arrested in G2 following IR treatment (Fig. 3.4C). Interestingly, Casp2<sup>-/-</sup> SV40-immortalised MEFs showed a significantly higher percentage of mitotic cells for all time points following IR treatment (Fig. 3.4C). These data demonstrate that Casp2<sup>-/-</sup> SV40-immortalised MEFs were able to
Fig. 3.3. Cell cycle profiling in $\text{Casp2}^{+/+}$ and $\text{Casp2}^{-/-}$ MEFs. DNA content analysis in $\text{Casp2}^{+/+}$ and $\text{Casp2}^{-/-}$ MEF derivatives. Following treatment with ionizing radiation (IR), (A) early passage primary MEFs (n=4, 5 Gy), (B) spontaneously-immortalised MEFs (iMEFs) (n=2, 5 Gy) and (C) SV40-immortalised MEFs (n=3, 10 Gy) were stained with propidium iodide and the proportion of cells in G0/G1, S and G2/M quantified by flow cytometry. Values represent means from 2-3 independent experiments.
Fig. 3.4. Mitotic checkpoint analysis in Casp2+/+ and Casp2-/- MEFs. DNA damage-induced G2/M checkpoint analysis in Casp2+/+ and Casp2-/- MEF derivatives. Following treatment with ionizing radiation (IR), (A) early passage primary MEFs (n=4, 5 Gy), (B) spontaneously-immortalised MEFs (iMEFs) (n=2, 5 Gy) and (C) SV40-immortalised MEFs (n=3, 10 Gy) were labelled with anti-phospho-histone H3 (pH3) Ser10 and propidium iodide. The proportion of mitotic cells (pH3 positive cells with a 4N DNA content) was then quantified by flow cytometry. Data represented as mean ± SEM (*P<0.05 and **P<0.001, Student’s t-test). (C) taken from Puccini (2009), Honours Thesis, The University of Adelaide.
overcome IR-induced G2 arrest more readily compared with Casp2\textsuperscript{+/+} controls. Consistent with another study using oncogene-transformed MEFs (Ren et al., 2012), these findings indicate that loss of caspase-2 disrupts the integrity of the IR-induced G2/M checkpoint in SV40-immortalised MEFs.

3.3.3. Loss of caspase-2 results in increased DNA damage

The increased proliferative capacity and enhanced ability to overcome replicative senescence in Casp2\textsuperscript{-/-} primary MEFs led us to assess whether loss of caspase-2 results in increased accumulation of DNA damage. Therefore, we assessed the extent of DNA damage following IR treatment over an 8 h time course in primary MEFs using γH2AX immunofluorescence, a well-established marker of double-strand breaks (DSBs) (Burma et al., 2001). Under these conditions, we did not observe significant levels of cell death (data not shown) which can lead to false positives because of apoptosis-associated DNA fragmentation. One hour after IR treatment, γH2AX levels increased significantly with 80–85% of cells showing a strong γH2AX signal (Fig. 3.5A-B). While, the number of γH2AX positive cells decreased over time following IR treatment, a significantly increased proportion of Casp2\textsuperscript{-/-} primary MEFs were γH2AX positive (42% and 39% at 6 and 8 h, respectively) compared with Casp2\textsuperscript{+/+} primary MEFs (31% and 19% at 6 and 8 h, respectively) (Fig. 3.5A-B). These observations indicate that Casp2\textsuperscript{-/-} primary MEFs exhibit persistent DNA damage following IR treatment, possibly due to premature cell cycle re-entry and/or inefficient DNA repair.
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Fig. 3.5. Loss of caspase-2 leads to sustained DNA damage following IR treatment. Early passage Casp2+/+ (n=4) and Casp2−/− (n=4) primary MEFs were treated with ionizing radiation (IR, 10 Gy) and analysed at the indicated times. (A) Representative fluorescent images (20X) of cells stained with anti-phospho-histone H2AX Ser139 (γH2AX) antibody and DAPI. (B) Quantitation of γH2AX positive cells. For each experiment, at least 300 cells were scored per sample. Data represented as mean ± SEM (*P<0.05, Student’s t-test).
Next we assessed this persistent DNA damage over a longer time period using the cytokinesis-block micronucleus (CBMN) assay to quantifying the frequency of micronuclei (MN). MN can originate from DNA breaks, chromosome fragments or lagging chromosomes during aberrant cell division and are therefore a robust marker for assessing DNA damage (Fenech, 2002, 2007). Following IR treatment, primary MEFs and iMEFs were treated with cytochalasin B for 24 h to inhibit cytokinesis and allow MN quantification following a single nuclear division. As expected, Casp2\(^{+/−}\) primary MEFs displayed a significantly higher percentage of binucleated (BN) cells with MN compared with Casp2\(^{+/+}\) MEFs 24 h after IR treatment (Fig. 3.6A-B). Furthermore, this increase was sustained up to 72 h after IR treatment (Fig. 3.6A-B). A similar result was observed in Casp2\(^{−/−}\) iMEFs which displayed a more pronounced increase in IR-induced MN formation (Fig. 3.6B). Importantly, both Casp2\(^{−/−}\) primary MEFs and iMEFs exhibited a significant increase in the frequency of BN cells at all time points following IR treatment, compared with Casp2\(^{+/+}\) MEFs (Fig. 3.6C). These data further demonstrate the ability of Casp2\(^{−/−}\) MEFs to proliferate faster and overcome IR-induced cell cycle arrest. Additionally, the presence of MN suggests that Casp2\(^{−/−}\) MEFs more frequently enter mitosis with damaged DNA, leading to GIN.

### 3.3.4. Caspase-2 deficiency enhances aneuploidy

Aberrant proliferation and mitotic checkpoint control, as well as abnormalities in DSB repair lead to accumulative DNA damage and are common causes of GIN (Gordon et al., 2012). Given the enhanced proliferation rate and increased DNA damage observed in Casp2\(^{−/−}\) MEFs, we further investigated GIN caused by loss of caspase-2. Primary
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Fig. 3.6. Increased micronuclei in Casp2⁻/⁻ MEFs following IR treatment. Cells were treated with ionizing radiation (IR, 5 Gy) and at the indicated time points following IR (24, 48 and 72 h) cells were then incubated with cytochalasin B for 24 h. (A) Representative images (40X) of DAPI-stained primary MEFs with arrows showing binucleated cells containing MN (micronuclei). Scale bars = 10 µm. Percentage of (B) binucleated cells with MN and (C) binucleated cells as determined by microscopy of early passage primary MEFs (n=3) and spontaneously-immortalised MEFs (iMEFs, n=3) following IR. At least 500 cells were counted per time point for each genotype. Data represented as mean ± SEM (*P<0.05, **P<0.01 and ***P<0.001, Student’s t-test). This experiment was performed by Dr. Loretta Dorstyn.
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MEFs naturally become aneuploid overtime owing to the accumulative effects of oxidative and replicative stress experienced in culture (Wang et al., 2013). Therefore, Casp2\(^{+/+}\) and Casp2\(^{-/-}\) primary MEFs were used to determine how loss of caspase-2 affects genome stability in vitro. Analysis of metaphase chromosome spreads from serially-passaged primary MEFs revealed an increased frequency of aneuploidy in Casp2\(^{-/-}\) MEFs (Fig. 3.7A-B). Cells with a diploid (2C) chromosome number (40 in mice) were observed in 68.7% of Casp2\(^{+/+}\) P1 MEFs compared with only 50.3% in Casp2\(^{-/-}\) P1 MEFs (Fig. 3.7A-B). A significant reduction in the number of diploid cells was also observed in Casp2\(^{-/-}\) MEFs at P4 (28.9%) with a corresponding increase in the number of tetraploid (4C) cells (56.0%) compared with Casp2\(^{+/+}\) P4 MEFs (64.2% diploid and 30.6% tetraploid) (Fig. 3.7A-B). In addition, Casp2\(^{-/-}\) MEFs displayed a higher frequency of polyploidy (>4C) compared with Casp2\(^{+/+}\) MEFs at each passage (Fig. 3.7B). Importantly, the increased aneuploidy observed in Casp2\(^{-/-}\) MEFs became more pronounced at later passages (P6 and P8) demonstrating that loss of caspase-2 promotes aneuploidy overtime in culture.

Given that loss of caspase-2 enhances Eµ-Myc-induced lymphomagenesis (Ho et al., 2009), we also assessed ploidy in caspase-2-deficient Eµ-Myc tumours. Metaphases prepared from freshly isolated primary lymphomas were classified as either diploid, low-grade aneuploid (loss or gain of up to 9 chromosomes) or high-grade aneuploid (loss of gain of 10 or more chromosomes). Analysis of metaphases from Eµ-Myc lymphoma cells demonstrated that 46.0% and 54.0% of metaphases displayed diploid and low-grade aneuploid karyotypes, respectively (Fig. 3.8A-C). In contrast, only 13.7%
Fig. 3.7. *Casp2*−/− Primary MEFs exhibit increased aneuploidy. (A) Images of metaphase spreads (*Casp2*+/+ at 40X and *Casp2*−/− at 20X) from early (P1) and late (P4) passage *Casp2*+/+ and *Casp2*−/− primary MEFs with chromosome numbers indicated. (B) Quantitation of diploid (2C), triploid (3C), tetraploid (4C) and polyploid (>4C) metaphases from *Casp2*+/+ (n=3) and *Casp2*−/− (n=3) primary MEFs at the indicated passage numbers. Data represented as mean percentage of metaphases showing ploidy from three independent batches of MEFs of each genotype (*P<0.05 and ***P<0.001; Chi-squared test comparing *Casp2*−/− MEFs with passaged-matched *Casp2*+/+ MEFs). This experiment was performed with assistance of the Department of Molecular Pathology (SA Pathology, Adelaide, South Australia).
**Fig. 3.8. Caspase-2-deficient tumours exhibit increased aneuploidy.** (A) Images of metaphase spreads (100X) derived from Eµ-Myc (n=3) and Eµ-Myc/Casp2⁻/⁻ (n=3) primary lymphomas with chromosome number indicated. (B) Quantitation of diploid, low-grade aneuploid and high-grade aneuploid metaphases. Data represented as mean percentage of metaphases (**P<0.01, Chi-squared test). (C) Table showing chromosome numbers. This experiment was performed by Dr. Loretta Dorstyn.
of Εµ- Myc/Casp2−/− lymphoma cells displayed diploid karyotypes. This reduction in the frequency of diploid karyotypes in Εµ-Myc/Casp2−/− lymphoma cells coincided with a dramatic increase in the frequency of low-grade (82.4%) and high-grade (3.9%) aneuploid karyotypes (Fig. 3.8A-C). These data indicate that loss of caspase-2 leads to increased aneuploidy in tumour cells in vivo.

Given that Casp2−/− mice show signs of premature ageing (Shalini et al., 2012; Zhang et al., 2007), we tested whether the increased aneuploidy caused by caspase-2 deficiency was associated with this phenotype. Splenocytes are a convenient and widely-used system for cytogenetic analysis, therefore ploidy analysis was performed on concanavalin A-stimulated splenocytes isolated from aged (16-18 months) Casp2+/+ and Casp2−/− mice (an age at which Casp2−/− tissues show increased oxidative stress – Shalini et al., 2012). From this analysis, no statistically significant differences in karyotype distributions were observed between Casp2+/+ and Casp2−/− splenocytes (Fig. 3.9A-C). Therefore, loss of caspase-2 is not necessarily associated with increased aneuploidy in aged tissue.

### 3.3.5. Loss of caspase-2 attenuates p53 signalling

Increased proliferative capacity, decreased senescence and GIN have all been associated with loss of p53 function (Gordon et al., 2012; Tomasini et al., 2008). Given that these phenotypes are also associated with caspase-2 deficiency, the transactivation of canonical p53 target genes involved in cell cycle arrest (p21^{CIP1/WAF1}) and apoptosis (Puma) were assessed in Casp2+/+ and Casp2−/− primary MEFs following
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Fig. 3.9. Ploidy analysis in primary Casp2<sup>+/+</sup> and Casp2<sup>−/−</sup> splenocytes. (A) Fluorescent images of metaphase spreads (100X) derived from splenocytes isolated from 16-18 month-old Casp2<sup>+/+</sup> (n=3) and Casp2<sup>−/−</sup> (n=3) mice with chromosome numbers indicated. (B) Quantitation of diploid, low-grade aneuploid, high-grade aneuploid metaphases. Data represented as mean percentage of metaphases. (C) Table showing chromosome numbers.

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Fig. 3.10. Defective transactivation of p53 target genes in Casp2°/° MEFs. Quantitative PCR analysis of p21CIP1/WAF1 and Puma transcript levels from early passage Casp2°/+ (n=3-4) and Casp2°/° (n=3-4) primary MEFs treated with ionizing radiation (IR, 10 Gy) and analysed over a time course. Expression is relative to β-actin levels. Data represented as mean ± SEM (*P<0.05 and **P<0.01, Student’s t-test). This experiment was performed by Dr. Loretta Dorstyn.
IR-induced DNA damage. From this analysis, we observed a significantly reduced transcript levels of $p21^{\text{CIP1/WAF1}}$ and $\text{Puma}$ in $\text{Casp2}^{-/-}$ MEFs compared to $\text{Casp2}^{+/+}$ MEFs following IR treatment (Fig. 3.10). These findings demonstrate that loss of caspase-2 attenuates p53-mediated signalling following DNA damage. To directly assess the activation of p53 protein, total and phosphorylated p53 (Ser18) and p21 protein levels were analysed by immunoblotting in primary MEFs following IR treatment. Although total p53 protein levels increased by 4 h in both $\text{Casp2}^{+/+}$ and $\text{Casp2}^{-/-}$ MEFs as expected, there was a marked reduction in p53 protein levels following IR treatment in $\text{Casp2}^{-/-}$ MEFs (Fig. 3.11). Similarly, there was a significant reduction in the levels of phosphorylated p53 (Ser18) and p21 levels in $\text{Casp2}^{-/-}$ MEFs compared with $\text{Casp2}^{+/+}$ MEFs (Fig. 3.11).

Next, we investigated p53-mediated DDR signalling in lymphoma cell lines isolated from Eµ-MyC and Eµ-MyC/$\text{Casp2}^{-/-}$ mice. Interestingly, Eµ-MyC/$\text{Casp2}^{-/-}$ lymphoma cells also showed significantly reduced induction of p53, phospho-p53 (Ser 18) and p21 protein compared with Eµ-MyC lymphoma cells (Fig. 3.12). This further supports a function for caspase-2 in regulating p53 activation following DNA damage and indicates that reduced p53 activity may contribute to the enhanced GIN and tumourigenesis in caspase-2-deficient Eµ-MyC mice.

To confirm if a defective p53 response was specifically due to loss of caspase-2, we assessed p53 activation following siRNA-mediated knockdown of caspase-2 in a human cell line (U2OS) which carries wild type p53. Consistent with our observations in
Fig. 3.11. Defective p53 activation in Casp2⁻/⁻ MEFs. Immunoblot analysis of phospho-p53 (Ser18), total p53 and p21 protein levels from early passage primary Casp2⁺/⁺ and Casp2⁻/⁻ MEFs following treatment with ionizing radiation (10 Gy). β-actin is included as a loading control. This experiment was performed by Dr. Loretta Dorstyn.
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**Fig. 3.12.** Defective p53 activation in caspase-2-deficient E\(\mu\)-Myc tumours. Immunoblot analysis of phospho-p53 (Ser18), total p53 and p21 protein levels in E\(\mu\)-Myc (n=2) and E\(\mu\)-Myc/Casp2\(^{-/-}\) (n=3) lymphoma cell lines following treatment with ionizing radiation (10 Gy). β-actin is included as a loading control.
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Fig. 3.13. Defective p53 activation in caspase-2-depleted U2OS cells. U2OS cells were transfected with scrambled control siRNA (siCNTRL) or caspase-2 siRNA (siCASP2) then treated with ionizing radiation (10 Gy). Protein levels of phospho-p53 (Ser15), p53 and p21 were assessed by immunoblotting. Efficiency of caspase-2 knockdown was >90%. β-actin is included as a loading control.
Casp2\(^{-/-}\) MEFs, caspase-2-depleted U2OS cells also showed significantly reduced levels of total p53 protein, phospho-p53 (Ser15) and p21 following IR treatment (Fig. 3.13). In further experiments we used Nutlin-3a, a specific MDM2 inhibitor that disrupts the p53-MDM2 interaction, thus enhancing p53 stability (Vassilev et al., 2004). Interestingly, we found that Nutlin-3a treatment (10 \( \mu \)M) did not restore the defective induction of p53 and p21 protein in Casp2\(^{-/-}\) primary MEFs, indicating that the effect of caspase-2 on p53 regulation may be independent of MDM2 (Fig. 3.14). Together these findings indicate that caspase-2 is required for efficient p53 activation and signalling following DNA damage and the impairment of p53 activity in caspase-2-deficient cells may, at least in part, be responsible for the observed increase in DNA damage and aneuploidy.
Fig. 3.14. Pharmacological inhibition of MDM2 does not restore defective p53 induction in Casp2+/− MEFs. Early passage Casp2+/+ and Casp2−/− primary MEFs were treated with the MDM2 antagonist, Nutlin-3a (10 µM) for the indicated times. p53 and p21 protein levels were then analysed by immunoblotting. β-actin is included as a loading control.
3.4. Discussion

In this study we demonstrated that loss of caspase-2 leads to increased GIN in primary MEFs and Eµ-Myc tumour cells. In addition, caspase-2 deficiency leads to defective p53 signalling following IR-induced DNA damage. These findings may explain how loss of caspase-2 leads to an increased propensity for oncogene-mediated transformation and tumourigenesis (Ho et al., 2009). Furthermore, we showed that Casp2−/− MEFs readily undergo spontaneous immortalisation in culture and that Casp2−/− SV40-immortalised MEFs show a DNA damage-induced G2/M checkpoint defect. Previous reports have also implicated a role for caspase-2 in DNA damage-induced cell cycle checkpoint regulation (Ho et al., 2009; Parsons et al., 2013; Ren et al., 2012; Sohn et al., 2011). As a consequence of defective checkpoint regulation and a weakened DDR, Casp2−/− MEFs may accumulate more DNA damage following IR treatment and over serial passaging in culture, which can contribute to increased aneuploidy.

Casp2−/− primary MEFs displayed a significantly higher level of DNA damage as indicated by the increased presence of MN and γH2AX foci compared with Casp2+/+ primary MEFs, following IR treatment. γH2AX recruitment to DSBs is important for initiating DNA repair and clearance of γH2AX is necessary for cell cycle progression (Keogh et al., 2006). Our data suggest that loss of caspase-2 may lead to defective cell cycle checkpoint maintenance which can promote escape from IR-induced arrest and subsequent premature re-entry into the cell cycle. This would lead to inefficient DNA repair and the accumulation of IR-induced DNA damage. Mitotic entry in the presence
of unrepaired DNA damage can cause errors in chromosome segregation that leads to GIN and aneuploidy (Gordon et al., 2012; Lobrich and Jeggo, 2007). These findings indicate that caspase-2 function may be required to initiate an efficient response to DNA damage to prevent GIN.

Although Casp2−/− primary MEFs displayed an increase proliferation rate and enhanced DNA damage and aneuploidy, based on cell cycle profiling experiments, loss of caspase-2 did not appear to disrupt cell cycle checkpoint regulation following IR in primary MEFs. Our previous findings have, however, demonstrated defective IR-induced arrest in Casp2−/− primary MEFs at 24 h, following a higher dose (10 Gy) of IR (Ho et al., 2009). These different experimental conditions may have been sufficient to induce a cell death response, and may explain why Casp2−/− MEFs (which were shown to be more resistant to IR-induced apoptosis), were able to readily evade IR-induced arrest (and subsequent apoptosis), permitting cell cycle re-entry in the presence of unrepaired DNA damage. Based on these observations, we cannot rule out the possibility that the apoptotic activity of caspase-2 may contribute to its function in maintaining genomic stability. Furthermore, p53 function may be an important determinant of caspase-2-mediated apoptosis. Therefore, further investigation is required to determine the relative contributions of apoptotic and non-apoptotic pathways to the tumour suppressor function of caspase-2.

Interestingly, when combined with SV40 T antigen overexpression in MEFs, loss of caspase-2 resulted in a pronounced G2/M checkpoint defect. The SV40 T antigen is
known to abrogate the function of various tumour suppressors (such as p53 and Rb) that control cell cycle checkpoint integrity, leading to GIN (Ahuja et al., 2005). Therefore, in such sensitised backgrounds, cells may become more dependent on caspase-2 function for orchestrating an efficient DDR and activating cell cycle checkpoints. This is consistent with the notion that the effects of caspase-2 deficiency become apparent under conditions of oncogenic and replicative stress (see 5.3).

We have previously shown that an absence of caspase-2 enhances the development of Eμ-Myc-induced B-cell lymphomas (Ho et al., 2009). Our studies here extend on these findings and show that Eμ-Myc/Casp2−/− lymphoma cells exhibited enhanced aneuploidy. The expression of activated oncogenes such as Ras and Myc has been shown to induce GIN (Woo and Poon, 2004). For example, E1A/Ras-transformed Trp53−/− MEFs display increased GIN caused by elevated reactive oxygen species (ROS) production (Woo and Poon, 2004). We have previously reported that caspase-2 deficiency leads to increased ROS and oxidative stress in tissues from aged mice (Shalini et al., 2012) but whether this is the reason for the increased GIN in Casp2−/− primary cells and tumours is unclear. Based on cytogenetic analysis of splenocytes derived from aged Casp2−/− mice (which do not display increased aneuploidy), the premature ageing phenotype caused by caspase-2 deficiency may not be linked to caspase-2 function in maintaining genomic stability. This suggests that the increased aneuploidy caused by caspase-2 deficiency is specifically associated with cells that typically display enhanced levels of background GIN, such as serially-passaged MEFs and tumour cells. This is consistent with caspase-2 exerting its tumour suppressor
activity through its function in maintaining genomic stability.

We also show that Casp2\(^{-/-}\) primary MEFs readily escaped replicative senescence in culture and this was associated with a lack of induction of the CDK-inhibitors \(p16^{INK4a}\), \(p19^{ARF}\) and \(p21^{CIP1/WAF1}\). This, together with the increased GIN associated with loss of caspase-2, may provide a significant driving force that renders Casp2\(^{-/-}\) cells more susceptible to immortalisation and oncogene-induced transformation. Consistent with these findings, Casp2\(^{-/-}\) primary MEFs become aneuploid more readily than Casp2\(^{+/+}\) MEFs upon co-expression of E1A and oncogenic Ras (Ho et al., 2009), indicating that oncogene-induced GIN is exacerbated in the absence of caspase-2.

Our studies, using Casp2\(^{-/-}\) primary MEFs also revealed that loss of caspase-2 leads to defective activation of p53 following IR treatment. Consistent with this, caspase-2-depleted U2OS cells and caspase-2-deficient Eµ-Myc lymphoma cells also displayed an attenuated p53 response following IR. These findings demonstrate that caspase-2 contributes to the regulation of the p53-mediated DDR. A recent study demonstrated that caspase-2 cleaves MDM2 \textit{in vitro} (Oliver et al., 2011). This cleavage resulted in removal of the C-terminal RING domain (required for p53 ubiquitination), thereby enhancing p53 stability. Thus, according to this model, following DNA damage, caspase-2 has a role in a positive feedback loop that inhibits MDM2, enhancing p53 stability and activity. While consistent with our observations of reduced p53 function in caspase-2-deficient cells, our experiments with the MDM2 antagonist, Nutlin-3a, indicate that caspase-2 may also regulate p53 in an MDM2-independent manner.
Further work is required to delineate the mechanisms by which caspase-2 regulates p53. This is likely to be highly context-dependent with the nature and extent of DNA damage being important determinants of caspase-2-mediated regulation of p53.

Inconsistent with a role in tumour suppression, Casp2−/− mice do not display an increased incidence of spontaneous tumours with age (Shalini et al., 2012). Also, we and others do not observe an increased susceptibility to lymphoma development following repeated low-dose IR treatment of Casp2−/− mice (our unpublished data, Manzl et al., 2012). These observations suggest that there are other limiting factors in aneuploidy-induced tumourigenesis (Li et al., 2010) and the function of caspase-2 as a tumour suppressor may become important specifically under conditions of oncogenic stress. Although Casp2−/− cells display reduced p53 activation following DNA damage, we do not detect a complete loss of p53 function. Following oncogenic and replicative stress, decreased p53 function in Casp2−/− cells would enhance survival of genomically unstable cells and augment stress-induced chromosomal damage by allowing DNA damage to persist unchecked. Together, this would facilitate increased cellular transformation by oncogenes (such as Ras and Myc) and enhance the ability of transformed cells to become tumourigenic (Ho et al., 2009).

Our findings thus provide evidence that loss of caspase-2 results in a defective DDR and as a consequence, cells lacking caspase-2 accumulate DNA damage and become aneuploid more readily. These findings provide direct evidence that caspase-2 has an important role in the maintenance of genome stability, potentially via regulating p53.
function. Furthermore, our data suggest that caspase-2 may function by fine-tuning key tumour suppressor networks that are important for eliminating cells with genetic aberrations, thereby preventing cellular transformation and oncogenesis (Fig. 3.15). A fundamental question that remains is whether caspase-2 regulates p53 by directly targeting MDM2 in vivo or whether caspase-2 indirectly regulates p53 through other tumour suppressor genes (e.g. p19^ARF and p16^INK4a) or oncogenes (e.g. Ras and Myc). In addition, determining how broadly the tumour suppressor function of caspase-2 extends is another important aspect which warrants further investigation.
**Fig. 3.15.** Caspase-2 in growth signalling and DNA damage response pathways. Inappropriate activation of oncogenic signalling pathways and DNA damage lead to cellular stress that promotes cellular transformation. In response to oncogenic stress and DNA damage, tumour suppressor pathways are activated, which mediate anti-proliferative responses such as apoptosis, senescence and cell cycle arrest. These cellular responses form critical barriers that protect against cellular transformation and tumourigenesis. Caspase-2 has been shown to regulate multiple components of these pathways controlling proliferation and the response to DNA damage.
Chapter 4

Loss of caspase-2 augments lymphomagenesis and enhances genomic instability in Atm-deficient mice
## Statement of Authorship

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### Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

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<th>Name of Principal Author (Candidate)</th>
<th>Joseph Puccini</th>
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<td>Contribution to the Paper</td>
<td>Experimental design, performed experiments, data analysis, wrote manuscript and edited manuscript.</td>
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<th>Name of Co-Author</th>
<th>Dr. Sonia Shalini</th>
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<th>Dr. Anne K Voss</th>
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<td>Name of Co-Author</td>
<td>Dr. Magtouf Gatei</td>
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<th>Dr. Claire H Wilson</th>
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<th>Dr. Devendra K Hiwase</th>
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<th>Prof. Martin F Lavin</th>
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<td>Name of Co-Author</td>
<td>Dr. Loretta Dorstyn</td>
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<td>Experimental design, overall supervision of project, co-wrote manuscript and acted as corresponding author.</td>
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<th>Name of Co-Author</th>
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4.1. Abstract

Caspase-2, the most evolutionarily conserved member of the caspase family, has been shown to be involved in apoptosis induced by various stimuli. Our recent work indicates that caspase-2 has putative functions in tumour suppression and protection against cellular stress. As such, the loss of caspase-2 enhances lymphomagenesis in Eμ-Myc transgenic mice, and caspase-2 knockout (Casp2\(^{-/-}\)) mice show characteristics of premature aging. However, the extent and specificity of caspase-2 function in tumour suppression is currently unclear. To further investigate this, ataxia telangiectasia mutated knockout (Atm\(^{-/-}\)) mice, which develop spontaneous thymic lymphomas, were used to generate Atm\(^{-/-}\)Casp2\(^{-/-}\) mice. Initial characterisation revealed that caspase-2 deficiency enhanced growth retardation and caused synthetic perinatal lethality in Atm\(^{-/-}\) mice. A comparison of tumour susceptibility demonstrated that Atm\(^{-/-}\)Casp2\(^{-/-}\) mice developed tumours with a dramatically increased incidence compared with Atm\(^{-/-}\) mice. Atm\(^{-/-}\)Casp2\(^{-/-}\) lymphoma cells displayed an increased proliferative capacity and extensive aneuploidy that coincided with elevated oxidative damage. Furthermore, splenic and thymic T cells derived from premalignant Atm\(^{-/-}\)Casp2\(^{-/-}\) mice also showed increased aneuploidy. These observations suggest that the tumour suppressor activity of caspase-2 is linked to its function in the maintenance of genomic stability and suppression of oxidative damage. Given that ATM and caspase-2 are important components of the DNA damage response and antioxidant defence system, which are essential for the maintenance of genomic stability, these proteins may synergistically function in tumour suppression by regulating these processes.

Chapter 4: Loss of caspase-2 augments lymphomagenesis and enhances genomic instability in Atm-deficient mice
4.2. Introduction

The physiological functions of caspase-2 have remained elusive since its discovery two decades ago (Kumar et al., 1994; Kumar et al., 1992; Wang et al., 1994). This is partly due to the lack of a clear phenotype in caspase-2 knockout (Casp2\(^{-/-}\)) mice which display only minor, tissue-specific apoptotic defects (Bergeron et al., 1998). We have previously demonstrated that loss of caspase-2 accelerates lymphomagenesis in Eμ-Myc transgenic mice, providing the first experimental evidence for a tumour suppressor function (Ho et al., 2009). Consistent with a role in tumour suppression, loss of caspase-2 expression and function has been detected in various human cancers and has been associated with poor prognosis and drug resistance (Estrov et al., 1998; Faderl et al., 1999; Hofmann et al., 2001; Holleman et al., 2005; Kim et al., 2011b; Kumar et al., 1995). The findings presented in chapter 3 also demonstrated that Casp2\(^{-/-}\) mouse embryonic fibroblasts (MEFs) display aberrant proliferation, persistent DNA damage and cell cycle checkpoint defects. Furthermore, Casp2\(^{-/-}\) primary MEFs and lymphomas derived from Eμ-Myc/Casp2\(^{-/-}\) mice exhibited increased aneuploidy, suggesting that caspase-2 is important for the maintenance of genomic stability. Chapter 3 also presents data demonstrating that attenuated p53 induction and activity in Casp2\(^{-/-}\) MEFs following DNA damage may contribute to these defects.

Strict regulation of cell cycle progression and preservation of genomic integrity are essential for maintaining cell homeostasis and preventing cellular transformation (Abbas et al., 2013; Ciccia and Elledge, 2010). This is exemplified in cancer cells, which
frequently display elevated basal levels of DNA damage, genomic instability (GIN) and aberrant proliferation (Abbas et al., 2013; Hanahan and Weinberg, 2011). To protect against these detrimental effects, cells have evolved complex tumour suppressor networks to eliminate potentially malignant cells and prevent the transmission of mutations (Hanahan and Weinberg, 2011). Ataxia telangiectasia (AT) mutated (ATM) kinase is a core component of these pathways and has an essential role in sensing DNA double-strand breaks (DSBs) and transducing downstream signals that activate the DNA repair and cell cycle checkpoint machinery (Shiloh and Ziv, 2013). ATM has also been shown to be directly activated by oxidative stress through a mechanism distinct to that of activation by DSBs (Guo et al., 2010). Upon activation by reactive oxygen species (ROS), ATM can stimulate the production of the antioxidant molecule nicotinamide adenine dinucleotide phosphate (NADPH), thereby contributing to regulation cellular redox homeostasis during oxidative insult (Cosentino et al., 2011). Interestingly, Casp2−/− mice show premature aging-related traits, including oxidative tissue damage and a reduced ability to tolerate stress, implicating caspase-2 in the regulation of the antioxidant stress response (Shalini et al., 2012; Zhang et al., 2007).

Given the overlapping functions of ATM and caspase-2 in the DNA damage response (DDR) and oxidative defence system, which are important for maintaining genomic stability, we generated Atm/Caspase-2 double knockout (Atm−/−Casp2−/−) mice to investigate whether caspase-2 and Atm genetically and functionally interact in tumour suppression. Atm−/− mice recapitulate many clinical features of the rare human disease AT, such as growth retardation, infertility, and hypersensitivity to IR (Barlow et al.,
1996; Elson et al., 1996). As in AT patients, Atm\(^{-/-}\) mice are also prone to spontaneous lymphomagenesis (Barlow et al., 1996; Elson et al., 1996). Here we show that caspase-2 deficiency exacerbates somatic growth retardation and causes perinatal lethality in Atm\(^{-/-}\) mice. Importantly, we demonstrate that loss of caspase-2 dramatically increases the incidence of lymphomagenesis in Atm\(^{-/-}\) mice. Furthermore, thymic lymphomas derived from Atm\(^{-/-}\)Casp2\(^{-/-}\) mice displayed an increased proliferative capacity, extensive aneuploidy and elevated oxidative damage. These results demonstrate that caspase-2 is a suppressor of lymphomas in Atm\(^{-/-}\) mice and provide further direct evidence for a tumour suppressor function for caspase-2. Importantly, we also demonstrate that this function is linked to the maintenance of genomic stability, shedding new light on the physiological significance of caspase-2 in protecting against tumourigenesis.
4.3. Results

4.3.1. Caspase-2 deficiency enhances growth retardation and causes perinatal lethality in Atm−/− mice

To further investigate the role of caspase-2 in tumour suppression, we generated Atm−/−Casp2−/− mice. This was achieved by inter-crossing Atm+/−Casp2−/− mice (Fig. 4.1). Consistent with previous studies (Barlow et al., 1996; Elson et al., 1996), expected Mendelian ratios were observed from Atm+/− × Atm+/− intercrosses (Table 1). Interestingly, analysis of progeny from Atm+/−Casp2+/− intercrosses revealed that only 23 (10%) Atm−/−Casp2−/− mice were recovered at birth from a total of 230 offspring, a 60% reduction from the expected frequency (25%; Table 1). However, analysis of genotype frequencies from Atm+/−Casp2+/− intercrosses at embryonic day 18.5 (E18.5) demonstrated that Atm+/−Casp2+/− embryos were present at the expected Mendelian ratios (Table 1). Furthermore, Atm+/−Casp2+/− embryos were alive at E18.5 as determined by their response to toe pinch stimulus. These observations indicate that Atm+/−Casp2+/− offspring die during or soon after birth. To assess the reason for this lethality, we carefully examined serial sections of wild type, Atm+/−, Casp2+/− and Atm−/−Casp2+/− E18.5 embryos. However, we failed to observe any specific phenotype in Atm−/−Casp2+/− embryos (Figs. 4.2 and 4.3).

To assess somatic animal growth, wild type, Atm+/−, Casp2+/−, and Atm−/−Casp2+/− mice were weighed weekly from 2 to 18 wk of age. Consistent with previous studies (Barlow et al., 1996; Elson et al., 1996), we observed a 20% to 25% reduction in the body
Chapter 4: Loss of caspase-2 augments lymphomagenesis and enhances genomic instability in Atm-deficient mice

Fig. 4.1. Generation of Atm$^{+/}$Casp2$^{-/-}$ mice. (A) Schematic of breeding schemes used to generate Atm$^{+/}$Casp2$^{-/-}$ mice.
Table 4.1. Genotype frequencies from mouse intercrosses

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<th>Genotype</th>
<th>Observed</th>
<th>Expected</th>
<th>P-value</th>
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<tr>
<td>Cross: Atm(^{+/−}) x Atm(^{+/−}) (n=284) [Birth]</td>
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<tr>
<td>Atm(^{+/−})</td>
<td>74 (26.1%)</td>
<td>71 (25.0%)</td>
<td></td>
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<tr>
<td>Atm(^{+/−})</td>
<td>155 (54.6%)</td>
<td>142 (50.0%)</td>
<td>0.0853</td>
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<tr>
<td>Atm(^{−/−})</td>
<td>55 (19.3%)</td>
<td>71 (25.0%)</td>
<td></td>
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<tr>
<td>Cross: Atm(^{+/−}) Casp2(^{+/−}) x Atm(^{+/−}) Casp2(^{+/−}) (n=230) [Birth]</td>
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<td></td>
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<tr>
<td>Atm(^{+/−}) Casp2(^{+/−})</td>
<td>70 (30.4%)</td>
<td>57.5 (25.0%)</td>
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<td>Atm(^{+/−}) Casp2(^{+/−})</td>
<td>137 (59.6%)</td>
<td>115 (50.0%)</td>
<td>0.000001</td>
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<tr>
<td>Atm(^{−/−}) Casp2(^{+/−})</td>
<td>23 (10.0%)</td>
<td>57.5 (25.0%)</td>
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<tr>
<td>Cross: Atm(^{+/−}) Casp2(^{+/−}) x Atm(^{+/−}) Casp2(^{+/−}) (n=62) [E18.5]</td>
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<td></td>
</tr>
<tr>
<td>Atm(^{+/−}) Casp2(^{+/−})</td>
<td>16 (25.8%)</td>
<td>15.5 (25.0%)</td>
<td></td>
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<tr>
<td>Atm(^{+/−}) Casp2(^{+/−})</td>
<td>32 (51.6%)</td>
<td>31 (50.0%)</td>
<td>0.908</td>
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<tr>
<td>Atm(^{−/−}) Casp2(^{+/−})</td>
<td>14 (22.6%)</td>
<td>15.5 (25.0%)</td>
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Observed and expected numbers of offspring generated from Atm\(^{+/−}\) x Atm\(^{+/−}\) crosses at birth and from Atm\(^{+/−}\) Casp2\(^{+/−}\) x Atm\(^{+/−}\) Casp2\(^{+/−}\) crosses at birth and embryonic day 18.5 (E18.5). Expected and observed frequencies are indicated in parentheses. Statistical comparison was performed by Chi-squared test.
weights of male and female $Atm^{-/-}$ mice compared with sex-matched, wild type littermates (Fig. 4.4A-C). Male and female $Atm^{-/-}Casp2^{-/-}$ mice displayed a further 10% to 15% reduction in body weight compared with sex-matched, $Atm^{-/-}$ controls (Fig. 4.4A–C). This additive growth defect was observed before weaning and persisted throughout adulthood. Also, $Casp2^{-/-}$ mice displayed a minor growth defect compared to wild type mice, although this trend only reached statistical significance in females (Fig. 4.4B). Taken together, these data demonstrate that combined loss of caspase-2 and $Atm$ exacerbates growth retardation and causes perinatal lethality in mice. Although the growth retardation phenotype in $Atm^{-/-}$ mice has been known for many years (Barlow et al., 1996; Elson et al., 1996), the mechanism of this defect remains unknown. Similarly, examination of $Atm^{-/-}Casp2^{-/-}$ mice did not provide any phenotypic clues to the enhanced growth retardation. From our observations, the majority of $Atm^{-/-}Casp2^{-/-}$ mice were frail at birth, unable to compete for feeding and routinely culled by their mothers. Thus, although the mechanism of growth retardation remains to be established, the perinatal lethality may simply be a result of weak constitution of some of the $Atm^{-/-}Casp2^{-/-}$ pups.

### 4.3.2. Combined loss of caspase-2 and $Atm$ augments lymphomagenesis

We have previously shown that caspase-2 deficiency accelerates lymphomagenesis in Eμ-Myc transgenic mice (Ho et al., 2009). Other reports show that caspase-2 deficiency does not appear to affect tumour development induced by low dose $\gamma$-radiation or 3-methylcholanthrene (Manzl et al., 2012). More recently, it was shown that deletion of caspase-2 in murine mammary tumour virus ($MMTV$)-Neu transgenic mice
Fig. 4.2. Gross morphology of Atm\(^{-/-}\)Casp2\(^{-/-}\) embryos. Representative images of haematoxylin and eosin-stained, embryonic day 18.5 sagittal sections of the indicated genotypes. Representative images of corresponding live embryos are also shown. Scale bar = 5 mm.
Chapter 4: Loss of caspase-2 augments lymphomagenesis and enhances genomic instability in Atm-deficient mice

Fig. 4.3. Morphology of Atm<sup>-/-</sup> Casp2<sup>-/-</sup> embryonic tissues. Representative images (20X) of haematoxylin and eosin-stained, embryonic day 18.5 sagittal sections of brain, heart, liver, lung, thymus, intestine, kidney, and skin of the indicated genotypes. Scale bar = 100 μm.
accelerated tumourigenesis, showing that caspase-2 is also a suppressor of epithelial tumours (Parsons et al., 2013). To further investigate the tumour suppressor function of caspase-2, specifically in the context of the DDR and oxidative stress pathways, cohorts of \( \text{Atm}^{+/} \) and \( \text{Atm}^{+/} \text{Casp2}^{+/} \) mice were generated and monitored for tumour development over a 1 yr period. Under pathogen-free conditions, 12 of 38 \( \text{Atm}^{+/} \) mice (31.6%) developed spontaneous tumours with an average onset of 30.8 wk (Fig. 4.5A-B). Notably, loss of caspase-2 dramatically enhanced the incidence of tumourigenesis in \( \text{Atm}^{+/} \) mice. Within 1 yr, 14 of 22 \( \text{Atm}^{+/} \text{Casp2}^{+/} \) mice (63.6%) developed tumours, with an average onset of 25.9 wk (Fig. 4.5A-B). Both \( \text{Atm}^{+/} \) and \( \text{Atm}^{+/} \text{Casp2}^{+/} \) mice predominately developed lymphomas of the thymus, with no differences observed in the histopathology of these lymphomas between genotypes (Fig. 4.5C). We also observed a small proportion of non-thymic lymphomas in both genotypes, which presented as hepatosplenomegaly and tumours of the lymph nodes (Fig. 4.5B). Wild type and \( \text{Casp2}^{+/-} \) mice in a C57BL/6 background do not develop spontaneous tumours within 1 y under the same pathogen-free conditions (Shalini et al., 2012). Both \( \text{Atm}^{+/} \) and \( \text{Atm}^{+/} \text{Casp2}^{+/} \) lymphomas were highly aggressive and infiltrated various secondary sites including the lungs, kidney, liver and peripheral blood (Fig. 4.6A). Peripheral blood lymphocyte counts were not significantly different between tumour-laden \( \text{Atm}^{+/} \) and \( \text{Atm}^{+/} \text{Casp2}^{+/} \) mice indicating comparable tumour burdens (Fig. 4.6B).

Immunophenotyping and histological analysis revealed that thymic lymphomas derived from \( \text{Atm}^{+/} \) and \( \text{Atm}^{+/} \text{Casp2}^{+/} \) mice were predominantly composed of immature CD4⁺CD8⁺ lymphoblastic T cells (also expressing the pan T-cell marker
Fig. 4.4. Caspase-2 deficiency enhances growth retardation in Atm-deficient mice. Male and female mice were weighed weekly from 2 to 18 wk of age. Growth curves showing mean body weights for (A) females and (B) males of indicated genotypes. Data represented as mean ± SEM (**P<0.01 and ***P<0.001, non-linear regression analysis). (C) Representative images of 6-wk-old male mice showing size differences between genotypes.
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Fig. 4.5. Caspase-2 deficiency accelerates lymphomagenesis in Atm−/− mice. (A) Kaplan–Meier plot comparing percentage tumour-free survival in cohorts of Atm−/− (n=38) and Atm−/−Casp2−/− (n=22) mice (P=0.0077, log rank test). (B) Comparison of tumour types, incidence, and onset. (C) Representative images of Atm−/− and Atm−/−Casp2−/− thymic lymphomas and haematoxylin and eosin-stained stained thymic lymphoma sections (40X) showing tumour cell morphology. Scale bar = 50 μm. No spontaneous tumour development was observed in wild type or Casp2−/− mice over a 1 yr period in the same pathogen-free animal facility.
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Fig. 4.6. Metastasis of Atm<sup>−/−</sup> and Atm<sup>−/−</sup>Casp2<sup>−/−</sup> thymic lymphomas. (A) Representative images (10X) of haematoxylin and eosin-stained sections of lung, kidney, brain and liver from tumour-laden mice showing metastatic infiltration. Liver, lung, and kidney showed extensive metastatic infiltration, whereas no metastasis was observed in the brain. Scale bar = 200 μm. (B) Peripheral blood lymphocyte counts and representative images of blood films (40X) from tumour-laden Atm<sup>−/−</sup> (n=7) and Atm<sup>−/−</sup>Casp2<sup>−/−</sup> (n=7) mice. Each point represents an individual mouse. Data presented as mean ± SEM.
CD90.2; Fig. 4.7A-B). Therefore, although caspase-2 deficiency did not appear to affect the metastatic potential, immunophenotype or morphology of thymic lymphomas, loss of caspase-2 dramatically increased the incidence and onset of tumourigenesis in Atm\(^{-/-}\) mice.

### 4.3.3. Caspase-2 deficiency confers a proliferative advantage in Atm\(^{-/-}\) lymphomas

As caspase-2 has been suggested to contribute to cell cycle regulation (Manzl et al., 2012; Parsons et al., 2013; Sohn et al., 2011) and Casp2\(^{-/-}\) MEFs proliferate at a faster rate than their wild type counterparts (Ho et al., 2009), we assessed the proliferative capacity of Atm\(^{-/-}\)Casp2\(^{-/-}\) lymphomas. The proportion of proliferating cells, as determined by proliferating cell nuclear antigen (PCNA) expression, was significantly increased in Atm\(^{-/-}\)Casp2\(^{-/-}\) thymic lymphomas (81.5%) compared with Atm\(^{-/-}\) thymic lymphomas (69.3%; Fig. 4.8A-B). Interestingly, we observed comparable numbers of apoptotic cells in Atm\(^{-/-}\) and Atm\(^{-/-}\)Casp2\(^{-/-}\) thymic lymphomas as assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunohistochemistry (IHC) (Fig. 4.9A-B). These data demonstrate that the loss of caspase-2 does not affect apoptosis but enhances proliferation in Atm\(^{-/-}\) lymphomas, suggesting that proliferative defects may contribute to the accelerated tumourigenesis observed in Atm\(^{-/-}\)Casp2\(^{-/-}\) mice.

Given that ATM and caspase-2 are both involved in regulation of the DDR, we next assessed how Atm\(^{-/-}\) and Atm\(^{-/-}\)Casp2\(^{-/-}\) thymic lymphoma cells responded to genotoxic
Chapter 4: Loss of caspase-2 augments lymphomagenesis and enhances genomic instability in Atm-deficient mice

Fig. 4.7. Immunophenotyping of Atm$^{-/-}$ and Atm$^{-/-}$Casp2$^{-/-}$ thymic lymphomas. Flow cytometry analysis of CD4, CD8a, and CD90.2 (Thy1.2) expression in freshly isolated Atm$^{-/-}$ (n=3) and Atm$^{-/-}$Casp2$^{-/-}$ (n=6) thymic lymphomas. (A) Representative dot plots and histograms (unshaded histogram represents negative control) and (B) quantification of expression of indicated markers. Data presented as mean ± SEM.
Chapter 4: Loss of caspase-2 augments lymphomagenesis and enhances genomic instability in Atm-deficient mice

Fig. 4.8. Loss of caspase-2 enhances proliferation in Atm<sup>−/−</sup> lymphomas. (A) Representative images (40X) and (B) quantification of proliferating cell nuclear antigen (PCNA) immunohistochemistry performed on thymic lymphoma sections derived from Atm<sup>−/−</sup> (n=5) and Atm<sup>−/−</sup>Casp2<sup>−/−</sup> (n=5) mice. Scale bars = 50 μm. A total of 900 to 1,500 cells were blind-counted over four separate fields of view for each tumour. Data presented as mean ± SEM (**P<0.01, Student t-test).
Fig. 4.9. Loss of caspase-2 does not affect cell death in Atm⁻/⁻ lymphomas. (A) Representative images (40X) and (B) quantification of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) immunohistochemistry performed on thymic lymphoma sections derived from Atm⁻/⁻ (n=7) and Atm⁻/⁻Casp2⁻/⁻ (n=10) mice. Scale bars = 50 μm. A total of 900 to 1,500 cells were blind-counted over four separate fields of view for each tumour.
stress. Primary cell lines isolated from Atm−/− and Atm−/−Casp2−/− thymic lymphomas were cultured and treated with 5 Gy IR (to induce DSBs) and the phosphorylation of a number of ATM substrates then assessed by immunoblotting (Fig. 4.10). As expected, in response to DSBs, phosphorylation of downstream effectors involved in DNA repair and cell cycle arrest (CHK1, SMC1 and KAP-1) were significantly ablated in Atm−/− lymphoma cells (Fig. 4.10). However, we did not observe any further impairment in the activation of these proteins in Atm−/−Casp2−/− lymphoma cell lines. As the loss of caspase-2 is associated with impaired p53 signalling (chapter 3), we assessed p53 and p21 protein levels in Atm−/− and Atm−/−Casp2−/− primary thymic lymphomas. However, we did not observe any differences between genotypes, suggesting that loss of caspase-2 does not affect p53 activity in Atm-deficient lymphomas (Fig. 4.11). We also examined the sensitivity of Atm−/− and Atm−/−Casp2−/− thymic lymphoma cell lines to IR and the ROS inducer menadione and observed comparable levels of apoptosis in response to these stimuli (Fig. 4.12A-B). Taken together, these data demonstrate that loss of caspase-2 does not affect DNA damage or ROS-induced apoptosis in Atm−/− lymphomas in vitro.

4.3.4. Atm−/−Casp2−/− lymphomas and premalignant T lymphocytes display enhanced aneuploidy

To assess whether caspase-2 influences genomic stability in Atm−/− lymphomas, we performed cytogenetic analysis on freshly isolated thymic lymphomas. Metaphases from tumour cells were categorised as diploid (40 chromosomes), low-grade aneuploid (loss or gain of as many as 9 chromosomes), or high-grade aneuploid (loss or gain of
Figure 4.10. DNA damage response in $Atm^{-/-}$ and $Atm^{-/-}Cas2^{-/-}$ thymic lymphoma cell lines. Immunoblot analysis of SMC1, phospho-SMC1 (Ser957), KAP-1, phospho-KAP-1 (Ser824), CHK1 and phospho-CHK1 (Ser317) protein levels in $Atm^{-/-}$ (n=2) and $Atm^{-/-}Cas2^{-/-}$ (n=3) lymphoma cell lines following treatment with ionizing radiation (5 Gy) at the indicated time points. Untreated and irradiated (5 Gy) EL4 cells (mouse thymoma cell line) were used as a positive control. β-actin is shown as a loading control. Immunoblots were performed by Dr. Magtouf Gatei.
Chapter 4: Loss of caspase-2 augments lymphomagenesis and enhances genomic instability in Atm-deficient mice

Fig. 4.11. p53 activity in Atm⁻/⁻ and Atm⁻/⁻ Casp2⁻/⁻ thymic lymphomas. Immunoblot analysis of p53, p21 and caspase-2 protein levels in Atm⁻/⁻ (n=6) and Atm⁻/⁻ Casp2⁻/⁻ (n=8) thymic lymphoma extracts. β-actin is shown as a loading control.
Fig. 4.12. Caspase-2 deficiency does not affect DNA damage- or ROS-induced cell death in Atm\(^{-/-}\) thymic lymphoma cell lines. Flow cytometry analysis of apoptosis in Atm\(^{-/-}\) (n=2-3) and Atm\(^{-/-}\)Casp2\(^{-/-}\) (n=3) primary thymic lymphoma cell lines following treatment with (A) ionizing radiation (IR, 5 Gy) and (B) 8 h treatment with vehicle (DMSO) or menadione (Men, 10 μM). Cells with a sub-G1 DNA content were gated as apoptotic. Data presented as mean ± SEM.
10 or more chromosomes). It has previously been shown that thymic lymphomas derived from *Atrx* mice are mostly diploid with a mean chromosome number of 40 (Liyanage *et al.*, 2000). Consistent with this, the majority of metaphases (82.9%) from all *Atrx* thymic lymphomas analysed showed diploid karyotypes, with only 14.6% and 2.5% of metaphases displaying low- and high-grade aneuploidy, respectively (Fig. 4.13A-C). In contrast, *Atrx*/*Casp2* thymic lymphomas displayed a significant increase in the proportion of metaphases with low-grade (58.9%) and high-grade (13.9%) aneuploidy, which coincided with a marked reduction in the proportion of diploid metaphases (27.2%; Fig. 4.13A-C). These observations provide direct evidence that loss of caspase-2 promotes GIN in vivo.

To determine whether loss of genomic stability contributes to the enhanced lymphomagenesis in *Atrx*/*Casp2* mice, cytogenetic analysis was performed on concanavalin A-stimulated splenic and thymic T lymphocytes derived from 13 to 14 wk old premalignant mice. Karyotype distributions of *Atrx* splenocytes and thymocytes were indistinguishable from the wild type and *Casp2* controls (Fig. 4.14A-C). Consistent with primary lymphomas, we observed a reduced frequency of diploid metaphases and concomitant increase in the proportion of aneuploid metaphases in premalignant *Atrx*/*Casp2* splenocytes and thymocytes compared with *Atrx* controls (Fig. 4.14A-C). These observations demonstrate that loss of caspase-2 promotes GIN even before tumour. As *Casp2* MEFs and lymphomas derived from Eμ-Myc/*Casp2* mice have been shown to exhibit reduced telomere length (Dorstyn *et al.*, 2012), we performed fluorescence in situ hybridization (FISH) to assess telomeres...
Chapter 4: Loss of caspase-2 augments lymphomagenesis and enhances genomic instability in Atm-deficient mice

Fig. 4.13. Enhanced genomic instability in Atm<sup>-/-</sup>Casp2<sup>-/-</sup> thymic lymphomas. (A) Representative fluorescent images (100X) of DAPI-stained metaphase spreads (with chromosome counts indicated) from Atm<sup>-/-</sup> and Atm<sup>-/-</sup>Casp2<sup>-/-</sup> thymic lymphomas. (B) Quantification of metaphases showing frequencies of diploid, low-grade aneuploid and high-grade aneuploid karyotypes in thymic lymphomas derived from Atm<sup>-/-</sup> (n=3) and Atm<sup>-/-</sup>Casp2<sup>-/-</sup> (n=8) mice. A total of 80 metaphases were counted per mouse. Data represented as mean percentage of metaphases (***P<0.001, Chi-squared test). (C) Table showing chromosome counts.

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Fig. 4.14. Enhanced genomic instability in pre-malignant $Atm^{-/-}Casp2^{-/-}$ lymphocytes. Quantification of metaphases showing frequencies of diploid, low-grade aneuploid, and high-grade aneuploid karyotypes in concanavalin A-stimulated (A) splenocytes and (B) thymocytes derived from wild type (n=4), $Casp2^{-/-}$ (n=4), $Atm^{-/-}$ (n=3), and $Atm^{-/-}Casp2^{-/-}$ (n=5) premalignant mice (13-14 wk old). A total of 50 metaphases were counted for splenocytes and thymocytes per mouse. Data represented as mean percentage of metaphases (**P < 0.001, Chi-squared test). (C) Table showing chromosome counts in splenocytes and thymocytes.
in thymic lymphomas. However, we did not observe any significant differences in telomere loss between \( \text{Atm}^{+/} \) and \( \text{Atm}^{+/}\text{Casp2}^{+/} \) thymic lymphomas (Fig. 4.15A-B), suggesting that telomere dysfunction does not contribute to GIN in this model.

### 4.3.5. Increased oxidative damage in \( \text{Atm}^{+/}\text{Casp2}^{+/} \) lymphomas

Aneuploidy is known to alter cellular energy metabolism and cause increased ROS production (Gordon et al., 2012). Given that ATM and caspase-2 have both been implicated in the oxidative stress response and \( \text{Atm}^{+/}\text{Casp2}^{+/} \) lymphomas exhibit extensive aneuploidy, we assessed oxidative damage in thymic lymphomas derived from \( \text{Atm}^{-/} \) and \( \text{Atm}^{-/}\text{Casp2}^{-/} \) mice. Protein carbonyls and 8-hydroxy-2'-deoxy guanosine (8-OHdG) are the products of oxidatively modified protein and DNA, respectively, and are robust markers for oxidative damage (Kasai, 1997; Levine et al., 1994). We observed significantly increased levels of protein carbonyls and 8-OHdG in \( \text{Atm}^{-/}\text{Casp2}^{-/} \) thymic lymphomas, compared with \( \text{Atm}^{+/} \) thymic lymphomas (Fig. 4.16A-B). Premalignant \( \text{Atm}^{+/} \) splenocytes and thymocytes displayed increased protein carbonyl levels compared with wild type controls; however, we did not observe any differences in protein carbonyl levels between \( \text{Atm}^{+/} \) and \( \text{Atm}^{+/}\text{Casp2}^{+/} \) premalignant splenocytes and thymocytes (Fig. 4.17A-B). These observations imply that the increased oxidative damage observed in \( \text{Atm}^{+/}\text{Casp2}^{+/} \) thymic lymphomas is acquired at the malignant stage, rather than in premalignant cells.

In response to oxidative stress, cells up-regulate a highly conserved network of antioxidant genes to neutralise ROS and prevent oxidative damage.
Fig. 4.15. Caspase-2 deficiency does not affect telomere length in Atm⁻/⁻ thymic lymphomas. (A) Representative fluorescent images (100X) of metaphase spreads stained with a Cy3-conjugated PNA-telomere probe (red) and DAPI (blue). Yellow asterisks indicate chromosomes with signal-free ends (SFEs), with magnified images shown (inset). (B) Quantification of chromosomes with SFEs in Atm⁻/⁻ (n=3) and Atm⁻/⁻Casp2⁻/⁻ (n=4) thymic lymphomas. At least 50 metaphases were counted per tumour. Data presented as mean ± SEM.
Chapter 4: Loss of caspase-2 augments lymphomagenesis and enhances genomic instability in Atm-deficient mice

Fig. 4.16. Increased oxidative damage in $A tm^{+/−} C a s p 2^{+/−}$ thymic lymphomas. (A) 8-hydroxy-2′-deoxy guanosine (8-OHdG) and (B) protein carbonyl levels in $A tm^{−/−}$ (n=6–7) and $A tm^{+/−} C a s p 2^{+/−}$ (n=10) thymic lymphomas. Each point represents an individual tumour (***P < 0.001, Student t-test).
(Kohen and Nyska, 2002). The mitochondrial-localized antioxidant enzymes manganese superoxide dismutase (Sod2), peroxiredoxin III (Prdx3), and catalase (Cat) form an essential axis of these pathways (Kohen and Nyska, 2002). In addition, cells also up-regulate the stress-responsive genes of the sestrin family to mediate antioxidant defence (Budanov, 2011). Not surprisingly, the increased oxidative stress observed in Atm−/−Casp2−/− lymphomas coincided with a significant increase in the transcript levels of these antioxidant genes (Fig. 4.18.). Although a trend was observed, the difference in Cat levels did not reach statistical significance (Fig. 4.18). Moreover, we also observed increased transcript levels of Sesn1 and Sesn2, further indicating the presence of elevated oxidative stress in Atm−/−Casp2−/− lymphomas (Fig. 4.18). These results demonstrate that ATM and caspase-2 cooperate to suppress oxidative stress in tumour cells.
Fig. 4.17. Oxidative damage analysis in premalignant lymphocytes. Protein carbonyl measurements in (A) splenocytes and (B) thymocytes derived from premalignant wild type (n=4), Casp2⁻/⁻ (n=4), Atm⁻/⁻ (n=3) and Atm⁻/⁻Casp2⁻/⁻ (n=5) mice. Data presented as mean ± SEM (*P < 0.05 and **P < 0.01 – compared with wild type controls, Student t-test). This experiment was performed by Dr. Sonia Shalini.
Fig. 4.18. Upregulation of antioxidant genes in \( \text{Atm}^{-/-}\text{Casp2}^{-/-} \) thymic lymphomas. Quantitative PCR analysis of superoxide dismutase 2 (\( \text{Sod2} \)), peroxiredoxin 3 (\( \text{Prdx3} \)), Sestrin 1 (\( \text{Sesn1} \)), Sestrin 2 (\( \text{Sesn2} \)), and Catalase (\( \text{Cat} \)) expression in \( \text{Atm}^{-/-} \) (n=6) and \( \text{Atm}^{-/-}\text{Casp2}^{-/-} \) (n=10) thymic lymphomas. Expression is relative to \( \beta \)-actin levels. Data represented as mean ± SEM (*\( P < 0.05 \) and **\( P < 0.01 \), Student \( t \)-test).
4.4. Discussion

In this study, we used $\text{Atm}^{-/-}$ mice as a non–oncogene-driven model of spontaneous thymic lymphomagenesis and showed that deletion of $\text{caspase-2}$ enhanced the incidence of lymphomagenesis in $\text{Atm}^{-/-}$ mice. We also observed increased proliferation, aneuploidy and oxidative damage in $\text{Atm}^{-/-}\text{Casp2}^{-/-}$ lymphomas. Furthermore, our work uncovered that loss of $\text{caspase-2}$ enhances growth retardation and causes perinatal lethality in $\text{Atm}^{-/-}$ mice.

Several DNA repair deficient mouse strains have been created in an $\text{Atm}$-null background to study their function in vivo. Similar to $\text{Atm}^{-/-}\text{Casp2}^{-/-}$ mice, many of these double knockout strains are synthetic lethal, including $\text{Trp53}^{-/-}\text{Atm}^{-/-}$, $\text{Parp1}^{-/-}\text{Atm}^{-/-}$, $\text{Lig4}^{-/-}\text{Atm}^{-/-}$ and $\text{DNA-PKcs}^{-/-}\text{Atm}^{-/-}$ mice (Gurley and Kemp, 2001; Lee et al., 2000; Menisser-de Murcia et al., 2001; Xu et al., 1998). Although histological inspection of embryonic tissues did not reveal any obvious anomalies that could explain the death of $\text{Atm}^{-/-}\text{Casp2}^{-/-}$ mice, it is likely that enhanced growth retardation contributes to the lethality in newborn pups. Previous studies have reported increased levels of ROS and chronic oxidative stress in tissues from $\text{Atm}^{-/-}$ mice (Barlow et al., 1999; Kamsler et al., 2001). Aged $\text{Casp2}^{-/-}$ mice have also been shown to have an impaired antioxidant defence system and elevated levels of ROS which is believed to cause premature aging (Shalini et al., 2012; Zhang et al., 2007). Therefore, it is possible that combined loss of $\text{Atm}$ and $\text{caspase-2}$ exacerbates oxidative damage, leading to enhanced retardation of somatic growth and perinatal lethality in $\text{Atm}^{-/-}\text{Casp2}^{-/-}$ mice.
In support of this hypothesis that a poor response to oxidative stress might cause growth retardation, mutation of genes involved in the oxidative stress response have previously been shown to cause developmental retardation in humans (Basel-Vanagaite et al., 2012).

Given the overlapping functions of ATM and caspase-2 in the DDR, antioxidant defence and tumour suppression, we hypothesised that deletion of both these genes would accelerate tumourigenesis. Indeed, we show that deletion of caspase-2 dramatically enhanced the incidence of lymphomagenesis in Atm\(^{-/-}\) mice. These important findings demonstrate that caspase-2 is also a suppressor of non-Myc-driven lymphomagenesis.

Consistent with previous studies (and the results in chapter 3) showing that loss of caspase-2 is associated with enhanced cell proliferation (Ho et al., 2009; Parsons et al., 2013), we observed an increased proliferation index in Atm\(^{-/-}\)Casp2\(^{-/-}\) lymphomas. These observations suggest that caspase-2 deletion, when combined with other oncogenic lesions, confers a proliferative advantage. Taken together, these data indicate that caspase-2 may function in cell cycle regulation, which may partly contribute to its tumour suppressor function. Importantly, caspase-2 deficiency did not affect DSB signaling or apoptosis induced by genotoxic and oxidative stress in Atm\(^{-/-}\) lymphoma cell lines. Also, we provide evidence that loss of caspase-2 had no effect on p53 signaling in Atm\(^{-/-}\) lymphomas. Although contradictory to the findings in chapter 3, this lack of cooperation between caspase-2 and p53 in Atm\(^{-/-}\) tumours is not surprising considering the role of ATM as a central upstream regulator of p53 activity. Therefore,
it is likely that any potential defects in p53 signalling caused by loss of caspase-2 are masked by the effects of Atm-deficiency. In addition, defective cell death pathways are unlikely to contribute to the increased tumour incidence in $Atm^{+/}Casp2^{-/-}$ mice. However, we cannot rule out the possibility that caspase-2 deficiency renders premalignant $Atm^{-/-}Casp2^{-/-}$ cells harbouring aneuploid karyotypes resistant to cell death.

In this study, we also demonstrate that caspase-2 deficiency is associated with GIN in vivo. Importantly, we observed increased aneuploidy even in premalignant lymphocytes providing evidence that loss of genomic stability is not merely a consequence, but rather a cause of the enhanced tumourigenesis in $Atm^{-/-}Casp2^{-/-}$ mice. This finding further support the idea that caspase-2 may exert its tumour suppressive activity through its function in maintaining genomic stability.

There is strong evidence that aneuploid cells experience proteotoxic stress as a result of imbalances in gene dosage, which leads to the generation of ROS (Gordon et al., 2012). In line with this, we observed increased oxidative damage to DNA and proteins in $Atm^{-/-}Casp2^{-/-}$ lymphomas as assessed by 8-OHdG and protein carbonyl levels, respectively. Interestingly, we did not observe increased oxidative damage in lymphocytes derived from premalignant $Atm^{-/-}Casp2^{-/-}$ mice, compared with $Atm^{-/-}$ controls. This suggests that excessive oxidative damage appears to follow aneuploidisation and is a phenotype acquired in malignant cells.
Elevated levels of ROS is a feature associated with cancer cells, and there is mounting evidence to suggest that cancer cells experiencing oxidative stress undergo extensive reprogramming of cellular processes that regulate redox homeostasis (Gordon et al., 2012). It has been suggested that this reprogramming may represent an adaptive response that maintains ROS levels below a toxic threshold (while still inflicting oxidative damage), thereby permitting survival of tumour cells experiencing chronic oxidative stress (Gordon et al., 2012). In line with this, the observed increase in oxidative damage in \(\text{Atm}^{-/-}\text{Casp2}^{-/-}\) lymphomas coincided with up-regulation of various stress-responsive genes involved in ROS detoxification. Interestingly, a recent study showed that elevated levels of ROS in aneuploid cells triggers an ATM-dependent response that protects against aneuploidy-induced tumourigenesis (Li et al., 2010). In this study, the levels of ROS were also shown to correlate with the extent of aneuploidy (Li et al., 2010). Our data further predict that aneuploidy and oxidative damage induced by \textit{caspase}-2 deficiency in the absence of \textit{Atm} would allow these damaged cells to acquire additional oncogenic lesions more readily. These observations are consistent with a model in which ATM and caspase-2 operate in parallel tumour suppressor pathways that protect against oxidative stress and aneuploidy (Fig. 4.19). Therefore, disruption of these pathways may explain the enhanced incidence of lymphomagenesis in \(\text{Atm}^{-/-}\text{Casp2}^{-/-}\) mice.

One of the major open questions in the field is whether the functions of caspase-2 in the DDR, maintenance of genomic stability and tumour suppression are solely based on its role in apoptosis, or whether caspase-2 regulates these processes through non-
apoptotic mechanisms. Nonetheless, our work here shows that loss of caspase-2 in a sensitised background promotes GIN and oxidative damage that promotes tumourigenesis. Consistent with these observations, our previous studies demonstrate that, although caspase-2 deficiency renders primary MEFs more susceptible to oncogene-induced transformation and increases the tumourigenic potential of transformed cells (Ho et al., 2009), loss of caspase-2 is not sufficient to initiate spontaneous tumourigenesis in vivo (Shalini et al., 2012). Taken together, these findings provide further evidence that one of the primary functions of this enigmatic caspase is protection against cellular stress conditions that promote tumourigenesis (see 5.3).
Chapter 4: Loss of caspase-2 augments lymphomagenesis and enhances genomic instability in Atm-deficient mice

Fig. 4.19. A model for caspase-2 and ATM function in tumour suppression. ATM prevents DNA damage induced by oncogenic and replication stress through its well-established functions in DNA repair and cell cycle arrest. Therefore, in the absence of ATM, cells accumulate DNA damage, which promotes mutagenesis and tumourigenesis. Caspase-2 is important for suppressing aneuploidy and the associated oxidative stress, thereby providing a protective barrier against tumourigenesis in Atm-deficient cells. Therefore, combined deletion of Atm and caspase-2 promotes aneuploidy and exacerbates oxidative stress, thereby accelerating tumourigenesis.
Chapter 5

General Discussion
# Statement of Authorship

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## Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate’s thesis.

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5.1. Work Leading to this Study

Caspase-2 was discovered in our laboratory 2 decades ago (Kumar et al., 1994; Kumar et al., 1992). Since then, biochemical studies have successfully defined the enzymatic properties of caspase-2, including its substrate specificity and structure (Baliga et al., 2004; Schweizer et al., 2003; Tang et al., 2011). Elucidating the physiological significance of caspase-2 on the other hand has proven to be much more challenging. Caspase-2 knockout (Casp2−/−) mice were generated in order to facilitate this and have been a valuable tool for investigating the function of caspase-2 in vivo (Bergeron et al., 1998; O'Reilly et al., 2002). However, previous studies have failed to reveal an essential role for caspase-2 in apoptosis.

Prior to this thesis, a seminal publication from our laboratory provided the first direct experimental evidence supporting a tumour suppressor function for caspase-2. This study employed the Eµ-Myc transgenic mouse model and showed that caspase-2 deficiency accelerated Myc-induced lymphomagenesis (Ho et al., 2009). Importantly, loss of a single allele of caspase-2 was sufficient to augment lymphomagenesis in this model. Furthermore, loss of caspase-2 was also associated with an enhanced susceptibility to oncogene-induced transformation in mouse embryonic fibroblasts (MEFs) (Ho et al., 2009). Despite follow up studies that provided additional evidence supporting a role for caspase-2 in tumour suppression, the molecular mechanisms that explained its tumour suppressor activity were not clearly defined (Kim et al., 2011b; Manzl et al., 2012; Ren et al., 2012; Yoo et al., 2011). These previous studies, in
combination with earlier clinical studies implicating caspase-2 in human cancers (Estrov et al., 1998; Faderl et al., 1999; Hofmann et al., 2001; Holleman et al., 2005; Kumar et al., 1995) formed the framework for the current study in which the function of caspase-2 in tumour suppression was further investigated.
5.2. Major Findings of this Thesis

The data presented in chapter 3 extends upon our previous work and provides mechanistic insights into the function of caspase-2 in protecting against cellular transformation and tumourigenesis. Unlike immortalised or transformed cell lines which frequently display aberrant proliferation cell cycle checkpoint regulation, primary cells present a more robust model system to study the events that drive cellular transformation. Chapter 3 presents data using Casp2\(^{-/-}\) primary MEFs to demonstrate that caspase-2 regulates cell proliferation and the DNA damage response (DDR). Furthermore, caspase-2 was shown to have an important function in the maintenance of genomic stability both in primary MEFs and in E\(\mu\)-Myc lymphomas. Importantly, evidence is presented implicating caspase-2 in the modulation of p53 function.

These findings from chapter 3 were then extended in chapter 4 using an in vivo approach. To this end, Atm\(^{-/-}\) mice were employed as a non-Myc-driven lymphoma model to further investigate the tumour suppressor function of caspase-2. Surprisingly, combined loss of caspase-2 and Atm caused perinatal lethality and enhanced growth retardation in mice. While these phenotypes were investigated in this thesis, determining their exact mechanisms was beyond the scope of this study and will subsequently require further analysis. Given that ATM and caspase-2 have overlapping functions in antioxidant defence and the DDR, it is tempting to speculate that the observed lethality and growth retardation phenotypes are linked to increased DNA
damage and oxidative stress caused by combined deletion of these genes. Treating \( \textit{Atm}^{+/-}\textit{Casp2}^{+/-} \) mice (or pregnant mothers) with antioxidants such as N-acetylcysteine could be used to test the involvement of oxidative stress in these phenotypes. The results presented in chapter 4 also demonstrate that loss of \textit{caspase-2} dramatically enhances the incidence of lymphomagenesis in \( \textit{Atm}^{-/-} \) mice, providing additional evidence for a tumour suppressor function for caspase-2. Furthermore, \( \textit{Atm}^{-/-}\textit{Casp2}^{-/-} \) lymphomas displayed an increased proliferative capacity and enhanced oxidative damage. Consistent with the increased genomic instability (GIN) observed in Eµ-Myc/Casp2\(^{+/-}\) lymphomas (chapter 3), \( \textit{Atm}^{-/-}\textit{Casp2}^{-/-} \) lymphomas also displayed extensive aneuploidy, furthering demonstrating that caspase-2 is required to maintain genomic stability \textit{in vivo}.

In the following sections the potential mechanisms by which caspase-2 may exert its tumour suppressor function will be discussed in the context of the findings from this thesis and previously published studies.

### 5.2.1. Regulation of cell growth and proliferation

In line with aberrant growth signalling and increased susceptibility to cellular transformation in the absence of caspase-2 (Ho \textit{et al.}, 2009), \( \textit{Casp2}^{-/-} \) primary MEFs were found to rapidly undergo spontaneous immortalisation in culture. This suggests that loss of \textit{caspase-2} is associated with deregulation of cell proliferation. This tendency to rapidly immortalise in culture most likely stems from the ability of \( \textit{Casp2}^{-/-} \) MEFs to readily escape replicative senescence. Interestingly, escape from senescence
coincided with reduced expression of the anti-proliferative cyclin-dependent kinase (CDK) inhibitors \( p19^{\text{ARF}} \), \( p16^{\text{INK4b}} \) and \( p21^{\text{CIP1/WAF1}} \) in late passage (P6-P8) \( \text{Casp}2^{-/-} \) MEFs. In addition to their well-established function in regulation of senescence onset, these CDK inhibitors have been implicated in tumour suppression (Abbas and Dutta, 2009; Chin et al., 1998). Therefore, caspase-2 may protect against cellular transformation by regulating senescence, a known tumour suppressive mechanism that prevents the propagation of cells harbouring potentially harmful mutations or experiencing oncogenic stress (Campisi, 2001). In line with these findings, caspase-2-deficient tumours from \( \text{E}^{\mu}\text{-Myc} \), \( \text{Atm}^{-/-} \) and mouse mammary tumour virus (MMTV)-Neu transgenic mice display an increased proliferation rate (Manzl et al., 2012; Parsons et al., 2013). Therefore, disruptions in pathways controlling growth and proliferation may account, at least in part, for the increased susceptibility of caspase-2-deficient cells to spontaneous immortalisation and oncogene-induced transformation. Therefore, involvement of caspase-2 in the processes may also contribute to its function in tumour suppression.

### 5.2.2. The DNA damage response (DDR)

The data presented in this thesis provides strong evidence implicating caspase-2 in the regulation of the DDR. However, a lack of known substrates together with contradictory findings has made it difficult to elucidate the physiological relevance and mechanistic basis of caspase-2 function in the DDR. On the basis of data presented in this thesis and in other studies, it is clear that the specificity of caspase-2 function in the DDR is highly context-dependent. Cell type, in addition to the nature and severity
of damage sustained by a cell, seem to be important determinants of the extent of
caspase-2 involvement in the response to cellular stress. Nevertheless, there is clear
experimental evidence demonstrating that caspase-2 can regulate the p53-dependent
DDR (Baptiste-Okoh et al., 2008; Oliver et al., 2011; Parsons et al., 2013). Following
treatment with ionizing radiation (IR), Casp2\textsuperscript{-/-} MEFs and E\textsubscript{m}-Myc/Casp2\textsuperscript{-/-} lymphomas
show impaired induction of p53 target genes involved in cell cycle arrest (\textit{p21}\textsuperscript{CIP1/WAF1})
and apoptosis (\textit{Puma}). Impaired p53 activity in the absence of caspase-2 may partly
explain cell proliferation defects, enhanced GIN and attenuated DDR signalling in
caspase-2-deficient cells.

The ubiquitin ligase mouse double minute 2 (MDM2), a negative regulator of p53 (see
1.4.2), was recently identified as a caspase-2 substrate (Oliver et al., 2011). Caspase-2-
mediated cleavage of MDM2 in response to DNA damage generated an MDM2
fragment that stabilised p53 via a positive feedback loop (Oliver et al., 2011). This
mechanism has been proposed to explain the reduced p53 activity and aberrant DDR
observed in \textit{Casp2\textsuperscript{-/-}} MEFs. However, in chapter 3, evidence for MDM2-independent
modulation of p53 activity by caspase-2 is presented. Therefore, the way in which
caspase-2 controls p53 activity may be more complex than originally anticipated.

Paradoxically, combined deletion of \textit{Trp53} and \textit{caspase-2} does not affect tumour onset
in mice (Manzl et al., 2013), challenging the involvement of p53 signalling in caspase-2-
mediated tumour suppression. However, the lack of cooperation between caspase-2
and p53 in tumourigenesis is not surprising given the dominant effects of p53-
 deficiency which most likely mask any subtle defects caused by \textit{caspase-2}-deficiency.
In addition, caspase-2 has also been implicated in p53-independent pathways, highlighting the complexity of caspase-2 in the modulation of the DDR. Checkpoint kinase 1 (CHK1) was shown to suppress a caspase-2-dependent apoptosis pathway in p53-deficient cells in zebrafish (Sidi et al., 2008). Interestingly, this pathway was also shown to be conserved in human cancer cells lines. CHK1 inhibition restored sensitivity to IR-induced apoptosis independent of mitochondrial involvement and caspase-3 (Sidi et al., 2008). This pathway is dependent on ATM-mediated phosphorylation of p53-induced death domain protein (PIDD), triggering the RIP-associated protein with a death domain (RAIDD) recruitment and assembly of the PIDDosome complex, leading to caspase-2 activation (Ando et al., 2012). However, a recent study has challenged the requirement of caspase-2 in this pathway in mammalian cells (Manzl et al., 2013). Nonetheless, this CHK1-suppressed pathway has been proposed to be a mechanism of apoptosis that ensures deletion of cells that sustain DNA damage in the presence of compromised checkpoint surveillance (Ando et al., 2012; Sidi et al., 2008). Importantly, these findings highlight that some potentially unidentified functions of caspase-2 may become apparent only under very specific experimental or physiological conditions.

The data presented in chapter 3 demonstrates that caspase-2 affects the function of multiple proteins that are known to regulate cell proliferation and apoptosis (see Fig. 3.15). On the basis of available in vitro data, caspase-2 most likely fine-tunes multiple pathways that converge on the regulation of the DDR and cell proliferation. Therefore, one of the primary functions of caspase-2 may be to ensure that a robust response is triggered and maintained under specific stress conditions. Given the nuclear functions
of various cell cycle and DDR regulators, involvement of caspase-2 in these processes may explain its constitutive nuclear localisation. Therefore, changes in the sub-cellular localisation of caspase-2 in response to specific stimuli may permit differential access to the cytoplasmic apoptotic machinery and nuclear DDR components, reflecting its bimodal functions in apoptotic and non-apoptotic pathways. Hence, a major open question that arises from this is whether the tumour suppressor function of caspase-2 is dependent on its apoptotic activity and/or non-apoptic processes. However, because of the apparently redundant function of caspase-2 in apoptosis and the extensive promiscuity in caspase signalling, this hypothesis has been difficult to test experimentally.

5.2.3 Maintenance of genomic stability

Given the inextricable link between deregulation of proliferation, GIN and tumourigenesis (Abbas et al., 2013; Malumbres and Barbacid, 2009), a role for caspase-2 in maintaining genomic stability may be the main mechanisms by which caspase-2 exerts its tumour suppressor function. The results presented in this thesis demonstrate that in serially-passaged primary MEFs, loss of caspase-2 promotes aneuploidy. These in vitro observations were extended and validated in vivo with lymphomas derived from Eμ-Myc/Casp2Δ/Δ and AtmΔ/ΔCasp2Δ/Δ mice, which also displayed an increased frequency of aneuploidy. Consistent with these data, increased GIN and aberrant mitoses were recently reported in breast epithelial tumours derived from MMTV-Neu/Casp2Δ/Δ mice (Parsons et al., 2013). Taken together, these important findings provide direct evidence that caspase-2-deficient tumours frequently display
aneuploidy, implicating caspase-2 in the maintenance of genomic stability \textit{in vitro} and \textit{in vivo}. Given that aneuploidy is a well-established hallmark of cancer, the ability of caspase-2 to prevent GIN is an attractive candidate mechanism that could potentially explain its tumour suppressor function.

In addition to its role in DNA damage-induced apoptosis, caspase-2 is also known to be required for cell death induced by cytoskeletal disruption (Ho \textit{et al.}, 2009; Vakifahmetoglu-Norberg \textit{et al.}, 2013). Interestingly, defective regulation of cytoskeletal components (especially the spindle apparatus) has been linked to impaired chromosome segregation and GIN (Gordon \textit{et al.}, 2012; Pfau and Amon, 2012). In line with these findings, caspase-2 has been implicated in a mode of cell death that is induced following aberrant mitoses, called mitotic catastrophe (Castedo \textit{et al.}, 2004; Mansilla \textit{et al.}, 2006). Despite the lack of a clear molecular definition for mitotic catastrophe, it has been proposed to be required for the deletion of aneuploid cells arising from abnormal mitotic events (Vitale \textit{et al.}, 2011). Hence, mitotic catastrophe is viewed as a tumour-suppressive mechanism that is important for maintaining genomic stability (Vitale \textit{et al.}, 2011). Therefore, it is plausible that caspase-2 is specifically required to execute this specialised cell death pathway in cells that have become aneuploid through defective mitotic processes such as improper chromosome segregation. This hypothesis fits with the increased frequency of aneuploidy observed in caspase-2-deficient MEFs and tumours. Hence, in the absence of caspase-2, aneuploid cells may persist, thereby promoting the acquisition of additional oncogenic lesions. In support of a role for caspase-2 in cell death induced by
aberrant mitotic events, epithelial tumours derived from $MMTV$-$Neu/Casp2^{-/-}$ mice displayed a striking increase in the rate of karyomegaly and multinucleation (Parsons et al., 2013). Further, $MMTV$-$Neu/Casp2^{-/-}$ tumours showed a high frequency of cells with aberrant mitoses displaying abnormal spindle asters, a defect not observed in $MMTV$-$Neu/Casp2^{+/+}$ tumours (Parsons et al., 2013).

The findings from chapter 3 also implicate caspase-2 in regulation of the DNA damage-induced G2/M checkpoint, which promotes G2 arrest and subsequent inhibition of mitotic entry in the presence of unrepaired DNA damage (Kastan and Bartek, 2004; Lobrich and Jeggo, 2007). Inappropriate mitotic progression of cells harbouring DNA breaks has been shown to cause aberrant mitoses leading to improper chromosome segregation and aneuploidy (Gordon et al., 2012; Lobrich and Jeggo, 2007). Consistent with these findings, increased mitotic indices were observed in caspase-2-deficient tumours derived from $E\mu$-$Myc$ and $MMTV$-$Neu$ transgenic mice (Manzl et al., 2012; Parsons et al., 2013). Together with the increased proliferation observed in $Atm^{-/-}/Casp2^{-/-}$ lymphomas, these findings provide evidence for impaired cell cycle progression in caspase-2-deficient cells, suggesting that caspase-2 may regulate these processes in vivo.

One of the major open questions in the field is whether GIN is a consequence or a driver of tumourigenesis. Analysis of pre-malignant lymphocytes from $Atm^{-/-}/Casp2^{-/-}$ mice in chapter 4 demonstrated that loss of caspase-2 promoted aneuploidy even before tumour onset. Therefore, not only do these findings demonstrate that caspase-
2 is important for maintaining genomic stability but also provide strong evidence that this function is linked to its tumour suppressor activity. The results presented in chapter 4 also demonstrated that loss of caspase-2 enhances oxidative stress in Atm\(^{-}\) lymphomas. Several studies have linked aneuploidy to reactive oxygen species (ROS) generation, both of which are known to promote tumourigenesis (Abbas \textit{et al.}, 2013; Gordon \textit{et al.}, 2012; Sosa \textit{et al.}, 2013). Given that caspase-2 is known to function in antioxidant defence (Shalini \textit{et al.}, 2012), suppression of oxidative damage may be linked to the function of caspase-2 in maintaining genomic stability and therefore contribute to its tumour suppressive activity.

Taken together, the results presented in this thesis and by others indicate that the functions of caspase-2 in the maintenance of genomic stability and cell cycle checkpoint regulation are potentially linked and contribute to its role in tumour suppression.
5.3. Conclusions and Perspectives

This thesis has characterised many previously undefined functions of caspase-2. The work presented here demonstrates that loss of caspase-2 promotes aneuploidy both in vitro and in vivo and that caspase-2-deficient cells have an impaired ability to respond to DNA damage. Following from our earlier study showing that caspase-2 is a suppressor of Eµ-Myc-induced lymphomagenesis, this raised the question as to whether caspase-2 is specifically involved in Myc-driven malignancies or whether its tumour suppressor function is relevant to other types of cancers. This was addressed using Atm⁻/⁻ mice as non-Myc-driven model of lymphomagenesis, which along with another studying using the MMTV-Neu breast cancer model, demonstrate that the tumour suppressor function of caspase-2 extends beyond Myc-driven lymphomas.

This thesis has uncovered many novel findings regarding caspase-2 function in tumourigenesis. However, there are still large voids in our knowledge regarding the mechanisms of its tumour suppression function. From this, several key questions arise that form the basis for future investigations. One of the most pressing issues that warrants further attention is whether caspase-2 maintains genome stability by functioning at the level of cell cycle checkpoint regulation or via a cell death-dependent mechanism such as mitotic catastrophe. Cytoskeletal-disrupting chemotherapeutic agents (such as vincristine and paclitaxel) have been shown to induce chromosomal aberrations in mice (Galmarini et al., 2007; Jagetia and Shrinath Baliga, 2002). These agents are also carcinogenic in mutant mouse strains that have
impaired spindle assembly checkpoint (SAC) function (Iwanaga et al., 2007). Therefore, treating Casp2\(^{-/}\) mice with such agents may provide a sensitised experimental system to test whether the tumour suppressor function of caspase-2 is directly dependent on its ability to maintain genomic stability. If caspase-2 is required for deleting genomically unstable cells, Casp2\(^{-/}\) mice would be expected to show increased chromosome instability and/or enhanced susceptibility to tumourigenesis induced by cytoskeletal disruptors. Crossing Casp2\(^{-/}\) mice into SAC mutant backgrounds (such as Bub1 and Mad2 transgenic mice) that display GIN and are prone to spontaneous tumourigenesis could also be used to test this hypothesis in vivo (Ricke et al., 2011; Sotillo et al., 2007). Importantly, identification of substrates that link caspase-2 to checkpoint signalling and the DDR will be an essential prerequisite for gaining a mechanistic understanding of its tumour suppressor function.

Although the catalytic activity is required for the apoptotic function of caspase-2, whether or not it is required for its tumour suppressive activity remains to be determined. A recent study attempting to address this issue demonstrated that the catalytic activity of caspase-2 was necessary to regulate proliferation and suppress transformation and tumour formation in nude mice (Ren et al., 2012). However, a major caveat of this study was the use of oncogene-transformed MEFs. As discussed previously, because transformed cells often display disrupted DDR and cell cycle checkpoint pathways, they are not a reliable model system for studying the events that occur during transformation. A transgenic knockin mouse expressing a catalytic mutant form of caspase-2 would present a more robust model system to assess the...
requirement of the catalytic activity of caspase-2 in tumour suppression *in vivo*. Similarly, generation of a knockin mouse expressing a nuclear localisation-deficient mutant form of caspase-2 could be used to determine whether its tumour suppressive activity is dependent on nuclear localisation. With the emergence of increasingly efficient genome editing technologies such as the CRISPR/Cas system, such transgenic knockin strains could be generated in much shorter time frames and at lower costs compared with conventional techniques (Cong *et al.*, 2013; Yang *et al.*, 2013).

Despite earlier observations in knockout mice that ruled out an essential physiological function for caspase-2 in developmental PCD and inflammation (Kumar, 2009; O'Reilly *et al.*, 2002), as discussed in this thesis, experimental evidence has begun to emerge supporting important physiological functions for caspase-2. In particular, the focus of this thesis was directed towards exploring the role of caspase-2 in suppressing tumourigenesis. Clinical observations together with the extensive *in vitro and in vivo* experimental evidence presented in this thesis have strengthened our understanding of the physiological functions of caspase-2 in disease, with clear roles in maintenance of genomic stability and the DDR. However, as discussed throughout this thesis and listed in Table 5.1, there are a number of caveats that caution against a major, essential and widespread function of caspase-2 in tumour suppression. Clearly, more studies with different *in vivo* models and further mechanistic studies directly linking caspase-2 to its apparently diverse modes of functions are required. It will also be of great interest to further investigate the relevance of caspase-2 function in tumour suppression in human cancers.
On the basis of our current understanding of caspase-2, one of its predominant functions appears to be protection against cellular stress. Therefore, in the absence of caspase-2, cells are unable to efficiently counteract challenges such as oxidative and oncogenic stresses, making caspase-2-deficient mice more susceptible to these stress conditions. This proposed model (Fig. 5.1) may explain the physiological basis upon which caspase-2 mitigates stress conditions, such as in the context of ageing and tumourigenesis.

A lack of known caspase-2 substrates has been a major hindrance to dissecting out the mechanisms that underlie the physiological functions of caspase-2. Moreover, substrate identification has been hampered by the highly context-dependent functions of caspase-2 and the extensive promiscuity in substrate specificity among members of the caspase family. However, with the advent of increasingly sophisticated proteomics technologies, identification of specific substrates linking caspase-2 to its physiological functions is keenly awaited.

Given the essential role of PCD in development and tissue homeostasis, a detailed understanding of the molecular pathways that regulate cell death has wide-ranging implications for many human diseases ranging from cancer to Alzheimer’s. Importantly, understanding the processes by which healthy cells become cancerous is at the crux of current cancer research. Overall, the findings from this thesis provide novel insights into the physiological functions of caspase-2, highlighting its function in the maintenance of genomic stability, regulation of the DDR and tumour suppression.
Table 5.1. Evidence implicating caspase-2 in tumour suppression

<table>
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<tr>
<th>Evidence</th>
<th>For</th>
<th>Against</th>
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<tr>
<td></td>
<td>Reduced levels of caspase-2 associated with drug resistance in childhood ALL (<em>Holleman et al.</em>, 2005)</td>
<td>Not all tumour types show reduced expression of caspase-2</td>
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<tr>
<td></td>
<td>Chromosomal locus containing human caspase-2 gene frequently deleted in blood cancers (<em>Kumar et al.</em>, 1995)</td>
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<td></td>
<td>Somatic mutations in some gastric tumours (<em>Kim et al.</em>, 2011b)</td>
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<tr>
<td><strong>In vitro studies (cell biology)</strong></td>
<td>Caspase-2-deficient cells display: Increased proliferation rate (<em>Ho et al.</em>, 2009; <em>Parsons et al.</em>, 2013; Chapter 3)</td>
<td>Caspase-2 is not essential for apoptosis induced by many stimuli</td>
</tr>
<tr>
<td></td>
<td>Aberrant cell cycle checkpoint regulation (<em>Ho et al.</em>, 2009; Chapter 3)</td>
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<tr>
<td></td>
<td>Increased genomic instability (<em>Ho et al.</em>, 2009; <em>Parsons et al.</em>, 2013; Chapters 3-4)</td>
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<td></td>
<td>Impaired DNA damage response (<em>Ho et al.</em>, 2009; <em>Parsons et al.</em>, 2013; Chapter 3)</td>
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<td></td>
<td>Increased susceptibility to immortalise and transform (<em>Ho et al.</em>, 2009; Chapter 3)</td>
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<tr>
<td><strong>In vivo studies (mouse models)</strong></td>
<td>Caspase-2 is a tumour suppressor in: <em>Eµ-Myc</em> transgenic mice (<em>Ho et al.</em>, 2009)</td>
<td>Aged <em>Casp2&lt;sup&gt;−/−&lt;/sup&gt;</em> mice do not have an increased incidence of tumours (<em>Shalini et al.</em>, 2012)</td>
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<td></td>
<td><em>MMTV-Neu</em> transgenic mice (<em>Parsons et al.</em>, 2013)</td>
<td>Not all tumour models are affected by loss of caspase-2</td>
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<td></td>
<td><em>Atm&lt;sup&gt;−/−&lt;/sup&gt;</em> mice (Chapter 4)</td>
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Figure 5.1. Caspase-2 fine-tunes cellular responses to protect against stress conditions. In the absence of caspase-2, cells become more susceptible to stress conditions. Therefore, when challenged, caspase-2 knockout animals have a reduced ability to counteract these adverse conditions, leading to enhanced oncogenic, oxidative, replicative and metabolic stress. At the phenotypic level, this manifests as premature ageing and increased tumour susceptibility.


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