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Impacts of DNA damage on the pericentriolar material and centriole duplication

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A thesis submitted to the National University of Ireland Galway for the degree of Doctor of Philosophy

September 2015

Supervisor: Prof. Ciaran Morrison
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Abbreviations

°C  degree Celsius
A   ampere
aa  amino acid(s)
Aki1 Akt kinase-interacting protein 1
AID auxin-inducible degron
Ana2 anastral spindle 2
APC/C anaphase promoting complex/cyclosome
APS ammonium persulphate
ATM ataxia telangiectasia, mutated
ATP adenosine-5’-triphosphate
ATR ATM-Rad3 related
ATRIP ATR-interacting protein
BER base excision repair
BLAST basic local alignment search tool
BLM bloom syndrome protein
bp  base pair(s)
BRCA1 breast cancer associated gene1
BRCA2 breast cancer associated gene 2
BSA bovine serum albumin
Bub budding uninhibited by benzimidazole
CAK cyclin activating kinases
CDK cyclin-dependent kinase
CDK5RAP2 cyclin-dependent kinase 5 regulatory associated protein 2
CHD chromodomain-helicase-DNA binding protein
cDNA complementary DNA
Chk  checkpoint kinase
CKI cyclin-dependent kinase inhibitor
C-NAP1 centrosomal NEK2-associated protein 1
CSG mCherry-SCC1-eGFP
C-terminus carboxy terminus
DAPI 4’, 6-diamidino-2-phenylindole
DISC1 disrupted in schizophrenia 1
DDR DNA damage response
DMSO dimethylsulfoxide
DNA-PK DNA-dependent protein kinase
DNA-PKcs DNA-PK catalytic subunit
dNTP deoxyribonucleotide-5’-triphosphate
D-PLP Drosophila pericentrin like protein
DSB  double-strand break
dsDNA double-stranded DNA
E. coli Escherichia coli
E2F adenovirus E2 promoter binding factor
ECL enhanced chemiluminescence
EDTA ethylenediaminetetraacetic acid
EGTA ethylene glycol tetraacetic acid
FACS fluorescence-activated cell sorting
FANCD2 Fanconi anaemia, complementation group D2
FBS foetal bovine serum
Fig  figure
FITC fluorescein isothiocyanate
<table>
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<tr>
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<th>Full Form</th>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPA</td>
<td>replication protein A</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAC</td>
<td>spindle assembly checkpoint</td>
</tr>
<tr>
<td>SAP</td>
<td>shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>SCC</td>
<td>Sister Chromatid Cohesion protein</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Ser/Thr</td>
<td>serine/threonine</td>
</tr>
<tr>
<td>Sgo1</td>
<td>shugosin 1</td>
</tr>
<tr>
<td>sSgo1</td>
<td>short shugoshin 1</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SMC</td>
<td>Structural Maintenance of Chromosomes</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>TAE</td>
<td>tris acetate EDTA</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription activator-like effector nucleases</td>
</tr>
<tr>
<td>TEMED</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Tev</td>
<td>protease of tobacco etch virus</td>
</tr>
<tr>
<td>TEV</td>
<td>Tev protease recognition sequence (aa sequence: EXXYXQG/S)</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>Wapl</td>
<td>wings apart-like</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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Declaration

I, Lisa Mullee, certify that this thesis is all my own work and I have not obtained a degree in this university or elsewhere based on this work. Certain figures in Chapter 5, which are clearly indicated in the figure legends, were performed in collaboration with Dr. Alicja Antonczak and Dr. Yifan Wang and are also published in our co-authored paper, “Opposing effects of pericentrin and microcephalin on the pericentriolar material regulate CHK1 activation in the DNA damage response”.

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Abstract
The centrosome consists of two barrel-shaped centrioles embedded in a proteinaceous pericentriolar material (PCM), a highly organised scaffold that serves as a platform for proteins that regulate spindle assembly, protein degradation and organelle trafficking. The large coiled-coil protein, pericentrin, is a major structural component of the PCM. Centrosome duplication is tightly controlled so that it happens once and only once per cell cycle. The caspase-like protease, separase, promotes centrosome duplication via cleavage of centriolar cohesin and pericentrin. Genotoxic stresses lead to centrosome amplification, a frequently observed feature in cancer. Pericentrin has been implicated in the control of DNA damage responses (DDR) through its interactions with checkpoint kinase 1 (CHK1) and microcephalin (MCPH1).

In this thesis, we explored the impacts of DNA damage on centrosome duplication, and the regulation of the PCM and separase in the DNA damage response. We developed a centrosomal-localising molecular sensor that detected separase activity at both the chromosomes and the centrosomes in early mitosis. A gene targeting approach to tag endogenous human separase for investigation into the direct regulation of separase in response to damage generated heterozygotes, which did not express the tagged protease. Homozygotes were not obtained, possibly due to unexpected indels occurring in targeted alleles. Investigations into the impact of ionizing irradiation on the PCM components pericentrin and CDK5RAP2 revealed a marked expansion in volume and a noticeable change in higher order organisation. PCM expansion was dependent on CHK1 kinase activity and was potentiated by MCPH1 deficiency. Furthermore, pericentrin deficiency or mutation of a separase cleavage site blocked DNA damage-induced PCM expansion. The extent of nuclear CHK1 activation after DNA damage reflected the level of PCM expansion, with a reduction in pericentrin-deficient or separase cleavage site mutant-expressing cells, and an increase in MCPH1-deficient cells that was suppressed by the loss of pericentrin. Deletion of the nuclear export signal of CHK1 led to its hyper-phosphorylation after irradiation and reduced centrosome amplification. Deletion of the nuclear localisation signal led to low CHK1 activation and low centrosome amplification. From these data we propose a feedback loop between the PCM and the nuclear DNA damage response in which CHK1 regulates pericentrin-dependent PCM expansion to control its own activation.
1 Introduction

1.1 The cell cycle
The cell cycle is the series of molecular events that occur in the duplication and transmission of the complete set of genetic material from a single mother cell to two genetically identical daughter cells. Each daughter cell is approximately half the size of the mother cell prior to division. Therefore cells must increase in mass and necessary components before they reach an optimal size and state to begin DNA duplication. Until that optimal size and supportive environment is achieved, a repressive system termed the restriction point prevents the expression of many genes required for cell cycle progression. To complete a cell cycle, eukaryotic cells must progress through four consecutive phases whose transitions are tightly regulated and subject to surveillance mechanisms. During the G (gap) 1 phase, cellular components are synthesized to overcome the restriction point and permit progression to S (synthetic) phase, in which DNA synthesis leads to chromosome replication. G2 is a relatively brief phase in which key enzymatic activities trigger the entry into M (mitosis) phase. During M phase, the duplicated DNA is segregated for equal distribution to the poles of the cell. Each daughter cell inherits one copy of the partitioned parental genetic material. Cytokinesis describes the physical division of the mitotic cell to become two distinct and intact daughter cells. The non-mitotic phases are collectively referred to as interphase (Morgan, 2007) (Fig 1.1).

1.1.1 DNA replication
DNA replication in eukaryotic cells requires the rapid and accurate synthesis of large amounts of DNA. DNA replication requires the assembly of pre-replication complexes (pre-RCs) at thousands of DNA sites termed replication origins. This assembly begins during the G1 phase of the cell cycle, until at the G1–S-phase transition, pre-RCs are converted into pre-initiation complexes, in which the replicative helicase is activated, leading to DNA unwinding and initiation of DNA synthesis (Mechali et al., 2010).
In order to initiate DNA replication, each origin of replication must be bound by an origin recognition complex of proteins (ORC) that remain bound to the DNA throughout the replication process. Also required are accessory proteins termed, ‘licensing factors’. Licensing factors accumulate in the nucleus during G1 of the cell cycle and include CDC-6 and CDC10-dependent transcript 1 (CDT-1), which bind to the ORC and are essential for coating the DNA with minichromosome maintenance (MCM) proteins. Only DNA coated with MCM proteins can be replicated. The loading of the MCM helicase complex, which is a hexamer of the six subunits MCM2–7, is the final step of the licensing reaction and can happen at origins with ORC, CDC6 and CDT1 already bound. Activating replication at the origin involves the assembly of a pre-initiation complex (pre-IC) and activation of the MCM helicase complex. Pre-IC formation is triggered by the
phosphorylation of multiple replication factors by DBF4-dependent kinase (DDK) and CDKs at the G1–S phase transition. Activation of the origin into a functional replisome occurs in the S phase and is termed origin firing. This two-step activation of replication origins: licensing of origins with the assembly of the pre-RC in the G1 phase, followed by their timely, organized activation during the S phase, is essential to prevent re-duplication within the same cell cycle (Fragkos et al., 2015).

The DNA helicase activity of the MCM complex untwists the two DNA strands that make up the helix that is to be copied. Once unwound, the separated strands form a Y shape at either side of the replication ‘bubble’, which are termed replication forks. The unzipped DNA strands are referred to as 3’ and 5’ strands, distinguished by the direction in which their component nucleotides join up. The 3’ DNA strand, also known as the leading strand, is directed to a DNA polymerase for use as a continuous template in the synthesis of the first daughter DNA helix. The 5’ strand of the original DNA double helix, known as the lagging strand, has the opposite 3’ to 5’ orientation and thus requires a more convoluted replication mechanism. As it emerges unwound from the helicase, the lagging strand is looped back on itself for presentation to a second DNA polymerase in the preferred 5’ to 3’ orientation. These replicated sections, effectively synthesised in reverse, are called Okazaki fragments. When one Okazaki fragment is complete, the synthesised section is released and the next loop is drawn back for replication. As replication nears completion, ‘bubbles’ of newly replicated DNA meet and fuse, finally forming two new molecules. When converging forks meet, termination occurs, thus preventing over-replication and genetic instability (Fragkos et al., 2015; Dalgaard et al., 2009).

1.1.2 Mitosis
Mitosis refers to a complex sequence of events that can be subdivided based on discrete steps towards the segregation of the duplicated chromosomes into their own distinct nuclei in preparation for cytokinesis. These subdivisions- prophase, prometaphase, metaphase, anaphase and telophase- are tightly regulated to ensure the integrity of cell division. At mitotic entry cyclins, A and B activate CDK1 triggering prophase events- chromosome condensation and centrosome-mediated mitotic spindle assembly (Furuno et al., 1999; Clute et al., 1999; Gong et al., 2007). Prometaphase is governed by cyclin B-Cdk1 and involves nuclear envelope breakdown, completion of chromosome condensation, spindle assembly and
centromeric attachment to the chromosomes via the kinetochore (Foley and Kapoor, 2013). Cyclin-Cdns have completed their primary functions by metaphase, by which time all sister chromatid pairs are bi-orientated on the spindle.

The spindle assemble checkpoint (SAC) is an intra-mitotic surveillance checkpoint that only permits progression to anaphase when all kinetochores are attached ensuring appropriate chromosome segregation (Sancar et al., 2004). Satisfaction of the SAC triggers APC/C-Cdc20-dependent ubiquitination of metaphase targets such as cyclin B and securin, releasing the cysteine protease separase from inactivation (Chestukhin et al., 2003). Separase and Polo-like kinase 1 (Plk1) orchestrate the cleavage of SCC1, the kleisin subunit of the cohesin complex, and subsequent removal of the cohesin rings that bind the sister chromatids together. Shortening of the attached microtubules draws the split sister chromatids to the poles of the cell (Foley and Kapoor, 2013). Telophase encompasses the de-condensing of the chromosomes into daughter nuclei and the reformation of their respective nuclear membranes (Larijani and Poccia, 2009).

Cytokinesis processes involve significant reorganisation of the cell’s cytoskeleton to ensure that nuclear and cytoplasmic components are appropriately segregated between the two nascent daughter cells. An actomyosin contractile ring is formed that constricts the plasma membrane resulting in two daughter cells connected by a cytoplasmic bridge. Cytokinesis is complete upon severance of this bridge (Glotzer, 2005).

1.1.3 Regulation of the cell cycle

The order and integrity of the cell cycle processes depend on a central regulatory system that organizes and coordinates the events to allow the cell to optimally navigate duplication and division. The core component of this complex regulatory system is the cyclin-dependent kinase (Cdk) family of ser/thr kinases. Cdk activity oscillates throughout the cell cycle in response to alterations in their binding of regulatory cyclin subunits as well as Cdk-activating kinase activity. Each cell cycle phase is governed by specific Cdks: Cdk 4 and Cdk6 (G1), Cdk 2 (G1, G1/S), and Cdk1 (M). In human cells, Cdk1 is the only cyclin-dependent kinase required for viability, being required for successful completion of M-phase (Santamaria et al., 2007; Satyanarayana et al., 2008). Cyclins can be divided into three major classes;
G1/S (cyclin D1-3, cyclin E), S (Cyclin A1-2), and M (cyclin B1, B2). The expression levels of cyclins oscillate during the cell cycle as they bind and activate their respective Cdk partners to trigger precise and specific cell cycle transitions via phosphorylation of a range of substrates (Malumbres et al., 2009). Just as the ‘on’ switch for Cdns is robust and defined, the ‘off’ switch, flipped by Cdk-inhibitory kinases (CKIs) that phosphorylate the Cdk subunit at inhibitory sites, contributes to the precise control. In addition to cyclin-Cdns, eukaryotic cell proliferation requires the ubiquitin-ligase activity from the multi-subunit anaphase-promoting complex or cyclosome (APC/C). The APC/C is activated in early mitosis when it ubiquitinates key regulatory substrates, targeting them for destruction and allowing the progression of M-phase (Peters, 2006).

While the cyclin-Cdns and the APC/C are the key players in cell cycle control, there are a number of additional proteins that contribute significantly to the strict regulation of events and transitions. In a basic outline of how these regulatory proteins direct cell cycle progression, appropriate intra- and extracellular signals initiate activation of G1-Cdns leading to the expression of G1/S specific genes including G1/S and S cyclins. The binding of Cdk2 by G1/S and S phase cyclins promotes the inhibition of CKIs and thus the activity of the cyclin-Cdns in both chromosome and centrosome duplication. Although the M phase Cdk1 can bind to M phase cyclin B and cyclin A from S phase, it remains inhibited by Wee1-related inhibitory kinase until activated by Cdc25 phosphatases that remove the inhibitory phosphoryl group. Active cyclin B-Cdk1 complex triggers organization of the chromosomes (Furuno et al., 1999; Clute et al., 1999). Upon correct equatorial alignment of the chromosomes, APC/C in complex with Cdc20 inactivates Cdk1 by degrading cyclin B, preventing further phosphorylation of Cdk1 substrates and facilitating sister chromatid separation and progression through the remainder of mitosis and cytokinesis. The suppression of Cdk activity prompts the APC/C complex to exchange its subunit Cdc20 for Cdh1, thus keeping cyclin levels diminished until the subsequent cycle initiates Cdk activity that in turn inhibits the APC/C (Rape and Kirschner, 2004; Peters, 2006).
1.2 The centrosome
Temporally linked to, and regulated by, cell-cycle progression are centrosomes. Both the centrosome and DNA duplication processes are coordinated by the same fundamental cell cycle regulators.

1.2.1 Overview
In 1876 Belgium, Edouard van Beneden sketched his observations of mitosis in a metazoan parasite of cephalopods, including dark dots that he labelled, ‘polar corpuscles’, which were present at each spindle pole. He later published pioneering studies in nematode eggs outlining the duplication of these dots during mitosis and proposed them to be permanent self-replicating organelles of the cell (van Beneden, 1887; Sluder, 2014). In Germany, Theodor Boveri followed van Beneden’s observations with his own publications describing bodies at spindle poles. Boveri termed these structures, ‘centrosomes’, and deemed them responsible for initiating and organising the mitotic spindle as well as for determining the plane of cell division to the centrosome (Boveri, 1887). Notably, Boveri also recognised that supernumerary centrosomes resulted in multipolarity and alterations to the genomic content of dividing cells (Boveri, 1888). The term centriole was also designated by Boveri to describe the dense staining dots observed within the centrosome bodies (Boveri, 1900).

The importance of the centrosome proposed by van Beneden and Boveri proved insightful given that it is now established as the principal microtubule-organising centre (MTOC) in animal somatic cells. In cycling cells, the centrosome coordinates the nucleation of microtubules, as well as regulating cell adhesion, motility, polarity, intracellular trafficking and organising the spindle poles in mitosis. In recent years, the centrosome has been implicated as a signalling hub for the integration and coordination of cell cycle, developmental, and DNA damage signalling networks (Kramer et al., 2004; Sluder, 2005; Arquint et al., 2014; Conduit et al., 2015).

1.2.2 Centrosome organisation
Centrosomes are small, membrane-free organelles, with well-understood roles in the control of microtubule dynamics in the cell. A mitotic centrosome contains two centrioles, distinctive barrel-shaped structures made up of nine sets of triplet microtubules symmetrically arranged around a central axis (Fig 1.2). The paired
centrioles of a mitotic centrosome are arranged at right angles to one another, arising from the position at which the new, ‘daughter’ centriole forms at the proximal end of the pre-existing ‘mother’ during normal centriole duplication. In the normal cell cycle, this orthogonal arrangement is lost prior to centriole duplication, when centrioles disengage through a process that involves separase and Plk1 and thus become licensed for duplication in the subsequent S phase (Pagan et al, 2015; Schöckel et al, 2011; Tsou & Stearns, 2006; Tsou et al, 2009). Centrioles display an asymmetry along their length, with the proximal ends containing microtubule triplets that taper to doublets at the distal ends (Kenney et al, 1997; Paintrand et al, 1992; Vorobjev & Chentsov Yu, 1982). The two centrioles within a mitotic centrosome are distinct: the distal ends of the mature, mother centrioles carry two distinguishing sets of appendages, which are absent from the daughter centriole. These appendages anchor cytoplasmic microtubules and contribute to primary cilium formation, a cellular process that requires centrioles, acting as the basal body for ciliation (Bornens, 2002; Graser et al, 2007).

1.2.3 The pericentriolar material

The centrioles are contained within the pericentriolar material (PCM), a proteinaceous lattice (Bobinnec et al, 1998). The PCM contains the key microtubule nucleation sites that involve gamma-tubulin in a tetrameric complex, the gamma-tubulin small complex (γTuSC), which is then arranged in a ring structure known as the gamma-tubulin ring complex (γTuRC) (Moritz et al, 2000; Wiese & Zheng, 2000; Zheng et al, 1995). The PCM increases in size and microtubule nucleation capacity as cells progress toward mitosis, a process termed centrosome maturation (Palazzo et al, 2000). The mother centriole associates with a PCM assembly that differs from the daughter centriole-associated PCM in terms of microtubule association and motility. Hence, the daughter centriole is highly mobile in the cytoplasm until the next round of centriole duplication is well underway in late S phase (Piel et al, 2000).
Until recently, the PCM was viewed as a relatively amorphous structure due to its indistinct morphology in electron microscopy experiments. However, work by a number of groups using sub-diffraction imaging revealed an underlying organisation to the PCM (Fu & Glover, 2012; Lawo et al, 2012; Mennella et al, 2012; Sonnen et al, 2012), consistent with earlier work that indicated it to be an ordered structure (Dictenberg et al, 1998). Thus, the PCM is currently viewed as an ordered array of proteins dynamically scaffolded on coiled-coil proteins, notably pericentrin/kendrin, CDK5RAP2/Cep215 and AKAP450/CG-NAP (Woodruff et al, 2014; Woodruff et al, 2015).

1.2.4 Centriolar satellites

Centriolar satellites are dynamic microtubule-associated structures that cluster around interphase centrosomes and basal bodies (Tollenaere et al, 2015). Components of the satellites include proteins that contribute to the regulation of centrosome duplication, and the satellites disperse after mitotic entry (Dammermann & Merdes, 2002; Firat-Karalar et al, 2014; Kubo & Tsukita, 2003). Centriolar satellites have also been reported to be involved in ciliogenesis (Tollenaere et al, 2015). Certain stresses, including UV irradiation and heat shock, cause a p38-dependent displacement of some satellite components (Villumsen et al, 2013), with the depletion of satellite proteins by siRNA treatment causing a p38-directed G1

**Figure 1.2 Key elements of the centrosome**

Diagram shows a duplicated pair of centrosomes, consisting of orthogonally-arranged centrioles in PCM. The principal structures of the centrosome are shown, with the PCM protein, pericentrin, indicated in blue as a radial component of the PCM. Inset shows the engaged centriole pair without the PCM, the recently identified ‘stalk’ proposed to promote engagement by linking the cartwheel at the base of the daughter centriole to the proximal end of the mother is indicated in yellow/brown. The proteinaceous linker that tethers the centrosomes together until mitosis is indicated in purple.
phase cell cycle arrest (Mikule et al, 2007). Current models for satellite function suggest that they act as regulatory structures through the sequestration or transport of key centrosome components (Kodani et al, 2015; Tollenaere et al, 2015). Excessive assembly of centriolar satellites has been described after genotoxic stress (Loffler et al, 2013). This poorly-understood process also occurs during extended S-phase delays that permit centrosome overduplication (Prosser et al, 2009). While recent data have implicated centriolar satellite components in centrosome amplification (Staples et al, 2014; Staples et al, 2012), the mechanistic details of how centriolar satellites regulate centrosome amplification or contribute to the DNA damage response remain to be defined.

1.3 Centrosome duplication

1.3.1 Centrosome cohesion

Centrosome duplication is a closely controlled process (Fig 1.3). It is normally linked to chromosome replication, being regulated through CDK2 activation and the coordinated activities of separase and PLK1 (Figure 2). During centrosome duplication in S phase, a centrosomal linker is assembled to connect the proximal ends of the two centrioles that have become disengaged after anaphase. The linker is primarily formed by two pools of C-NAP1 that anchor fibrous connections of rootletin to the proximal base of the centrioles, which will now serve as mothers (Bahe et al, 2005; Yang et al, 2006). Additionally, the human microcephaly protein Cep135, that is constitutively expressed at the proximal end of centrioles interacts with C-NAP1 in a manner reported to promote the attachment of the linker to the centrioles (Kim et al., 2008). Other proteins implicated in contributing to the linker itself include Cep68, LRRC45, and LGALS3BP (He et al., 2013; Fogeron et al., 2013). This proteinaceous linker provides centrosome cohesion, tethering the two existing centrioles together as they each nucleate an orthogonally-orientated procentriole that elongates laterally from their proximal end through S and G2 phase.

The assembly of a daughter centriole at the base of the pre-existing mother centriole in mammalian cells is thought to begin with CEP152 and CEP 192 recruiting PLK4 (Dzhindzhiev et al., 2010; Cizmecioglu et al., 2010; Kim et al., 2013; Novak et al., 2014). PLK4 activity triggers SAS6 and STIL recruitment and formation of the
cartwheel (Arquint et al., 2012; Kratz et al., 2015; Cottee et al., 2015). The cartwheel is named after its morphology, revealed in electron tomography analyses, in which it appears as a central hub from which nine radially symmetric spokes emanate to the microtubule triplets. Each spoke terminates in a pinhead that connects to the first microtubule of the triplet. The central hub and the proximal spoke structures are composed of nine SAS6 dimers (Guichard et al., 2010; Hirono, 2014). SAS6 and STIL then recruit CPAP to the distal end of the spokes, where it promotes microtubule assembly (Schmidt et al., 2009). Several proteins important for centriole duplication- CEP135, γ-tubulin and CP110- are also recruited to the base of the procentriole. During S and G2 phases, centrioles start to elongate and increase their length in CPAP-dependent manner (Kleylein-Sohn et al., 2007; Schmidt et al., 2009; Tang et al., 2009; Roque et al., 2012).

Figure 1.3 The centrosome duplication cycle.
Diagram shows disengaged centrioles exiting mitosis. Both of these centrioles are competent to serve as a mother for pro-nucleation of a daughter cell through G1, and elongation through S, prior to separation upon transition from G2 to M phase. Separation depends on NEK2-mediated dissolution of the proteinaceous linker. PLK1 and separase then license centrosomes, consisting of a mother and daughter pair, for disengagement in preparation for the next duplication cycle.
As cells progress towards M phase, procentrioles are modified by PLK1 to enable the acquisition of PCM proteins required for centrosome duplication and mitotic spindle formation in the next cycle (Wang et al, 2011). The NIMA-related kinase, NEK2, phosphorylates both C-NAP1 and rootletin in late G2 to promote their removal from the centrosomes, thus dissolving the linker and allowing centrosomes to move poleward in preparation for mitotic spindle formation (Fry et al, 1998; Mayor et al, 2000). This process of centrosome separation is functionally and temporally distinct from centriole disengagement, and is regulated by different mechanisms (Agircan et al, 2014; Tanenbaum and Medema, 2010).

1.3.2 Centriole disengagement

Cells exiting mitosis possess one centrosome, containing two physically detached, disengaged centrioles that are both embedded in PCM and competent for duplication. Ensuring that only one duplication event happens per cell division cycle involves a combination of a centriole-intrinsic block, along with limiting concentrations and activities of centriole assembly factors to restrict centriole production to one daughter per mother (Wong and Stearns, 2003). Promoters of centriole duplication such as PLK4, STIL and SAS-6 are tightly regulated to limit their activity to an appropriate window for procentriole formation (Arquint et al, 2012; Cunha-Ferreira et al, 2009; Rogers et al, 2009; Tsou and Stearns, 2006). To relieve the centriole-intrinsic block to duplication, the mother-daughter centriole pair must dissociate, losing their tightly engaged arrangement. This uncoupling event is termed centriole disengagement and is required for both the mother and daughter to nucleate a new procentriole in G1 (Tsou and Stearns, 2006).

While the precise molecular composition of the engagement link that promotes the tight orthogonal arrangement is unclear, a study in purified human centrosomes using cryoelectron tomography identified a physical ‘stalk’ connecting the cartwheel at the base of the procentriole to the proximal end of the mother centriole (Guichard et al, 2010). It is possible that this stalk plays a role in centriole engagement. Work in *Drosophila melanogaster* has implicated SAS-5/Ana2 and SAS-6 as components in centriole cohesion, perhaps in the form of this stalk-like structure (Stevens et al, 2010).
1.3.3 Regulators of centriole disengagement

Two key regulatory proteins, PLK1, a mitotic kinase, and the separase protease promote centriole disengagement in vertebrates. The earliest clue implicating separase in this process was the observation that it localises to the centrosome during mitosis (Chestukhin et al, 2003). The first demonstration of its role in disengagement revealed that purified human centrosomes underwent anaphase-specific disengagement when added to *Xenopus* egg extracts, a process that could be prevented by non-degradable forms of the separase inhibitors, cyclin B and securin (Gorr et al, 2005; Tsou and Stearns, 2006). The confirmation that separase proteolytic activity and PLK1 kinase activity co-ordinately promote centriole disengagement coincided with multiple reports of cohesin at the centrosomes (Gimenez-Abian et al, 2010; Gregson et al, 2001; Guan et al, 2008; Kong et al, 2009; Nakamura et al, 2009; Tsou et al, 2009; Wong and Blobel, 2008). The cohesin component, Scc1, is an established chromosomal substrate of separase. Compelling evidence for centriolar cohesin as a disengagement-specific substrate includes the expression of a non-cleavable Scc1 inhibiting disengagement and the engineering of independent protease cleavage sites into the cohesin subunits that enabled the manipulation of centriole engagement both in vitro and in vivo (Schöckel et al, 2011).

This centriolar cohesin model draws another parallel between the centriole disengagement regulation system and that of sister chromatid segregation. In addition to cyclin B and securin, Aki1 and astrin have been reported as inhibitors of centrosomal separase (Nakamura et al, 2009; Thein et al, 2007). Furthermore, shugoshin (SGO1), the ‘guardian spirit’ protein that blocks chromosomal cohesin from premature cleavage prior to anaphase, has a splice variant that localises predominantly to the centrosome. Chromosomal SGO1 functions by recruiting PP2A to the centromere to oppose PLK1 kinase activity. The splice variant, whose mRNA is missing exon 6 but contains exon 9 is known as small shugoshin (sSGO1) (Wang et al., 2006). The protein produced by the small shugoshin (sSGO1) splice variant requires PLK1-dependent phosphorylation on Ser154 for centrosome localisation, while disruption of the sSGO1 protein triggers centriole disengagement (Riedel et al, 2006; Wang et al, 2008; Hamasaki et al., 2014). A recent study by the Stemmann group has identified the peptide encoded by exon 9 as the centrosomal targeting
signal of hSgo1 (CTS). The findings of the study show that the CTS is not only necessary and sufficient to direct Sgo1 to the centrosomes but also prevents centromeric targeting of Sgo1. Premature centriole disengagement in response to Sgo1 depletion was suppressed by blocking cleavage of an engineered cohesin, further demonstrating that sSGO1 protects centriole engagement by PP2A-dependent protection of centrosomal cohesin from prophase pathway signalling (Mohr et al., 2015).

Collectively, these layers of regulation and localisation of cohesin support a significant role for cohesin in centriole engagement. However, a study in Drosophila found cohesin cleavage to be insufficient for centriole disengagement (Oliveira and Nasmyth, 2013), while in Caenorhabditis elegans (C. elegans), the requirement for cohesin cleavage in disengagement is limited to meiosis II, beyond which microtubule-dependent forces drive the separation of the adjacent centrioles (Cabral et al, 2013).

While clarification of the structure, mechanism and role of centriolar cohesin is still required, centrosomal functions of separase have been further confirmed by the identification of the PCM component, pericentrin, as a contributor to centriole engagement and as a novel substrate of the protease (Lee & Rhee, 2012; Matsuo et al, 2012). Further studies have confirmed pericentrin cleavage as a promoter of centriole disengagement and licensing and have implicated pericentrin cleavage-mediated removal of CDK5RAP2 from the PCM as an important factor in these processes (Barrera et al., 2010; Pagan et al., 2015). SCC1- and pericentrin-based molecular sensors localised to the centrosome have revealed separase-dependent cleavage at mid metaphase, preceding separase activity detected at the chromosomes. PLK1 deficiency reduced the ability of separase to cleave its SCC1, but not its pericentrin, substrate. These findings indicate that PLK1 may regulate separase via the protease’s substrate accessibility (Agircan and Schiebel, 2014). Interestingly, a recent study in HeLa cells has suggested that PLK1-mediated maturation of the daughter centriole and the recruitment of PCM leads to a physical distancing of the daughter centriole from the mother (> 80 nm) which is sufficient to permit reduplication of the mother centriole (Shukla et al, 2015). This presents an additional mechanistic element that must be incorporated into models for how PLK1 contributes to centriole duplication.
1.4 Separase and its substrates: SCC1 and Pericentrin

1.4.1 Separase

The caspase-like protease separase belongs to the family of Cys-endopeptidases, and is considered the universal trigger of eukaryotic anaphase (Kumada et al., 2006; Wirth et al., 2006). Separase is essential for viability, as evidenced by embryonic lethality in separase null mice and pronounced polyploidy in mouse embryonic fibroblasts (MEFs) lacking separase (Wirth et al., 2006). Separase is a large protein (233 kDa in humans) with a conserved histidine and cysteine residue forming the catalytic dyad of its C-terminal active site (Uhlmann et al., 2000). Once active, separase cleaves its substrates specifically after the arginine of an ExxR consensus sequence (Stemmann et al., 2001; Sullivan et al., 2004; Uhlmann et al., 1999). Separase plays a major role in the appropriate segregation of DNA by cleaving SCC1 to release sister chromatids from the constraints of the cohesin ring (Uhlmann et al., 1999). More recently it was discovered that separase also cleaves SCC1 and pericentrin at the centrosome where both substrates promote centriole engagement, as outlined in section 1.3.3. Other separase substrates reported include the meiotic Scc1-homolog Rec8 (Petronczki et al., 2003; Kudo et al., 2009) and the kinetochore-associated protein Slk19 in budding yeast (Sullivan et al., 2001; Stegmeier et al., 2002). In higher eukaryotes, active separase also cleaves itself, upon which the resultant separase fragments remain associated and catalytically active. Thus, cleavage is not required for separase activation, at least in vitro (Waizenegger et al., 2002; Zou et al., 2002). Thus, the functional relevance of this remains unclear.

Prior to its active role in anaphase promotion, separase is inhibited by mutually exclusive association with either the Cdk1-cyclin B1 complex or its chaperone securin (Funabiki et al., 1996; Stratmann and Lehner, 1996; Yamamoto et al., 1996; Zou et al., 1999; Stemmann et al., 2001; Gorr et al., 2005). Protein phosphatase 2A (PP2A) is also interacts with human separase (Holland et al., 2007), and has recently been reported to stabilise separase-associated securin by dephosphorylation. This action assists in preventing precocious activation of separase and refining the sudden separation of sister chromatids in anaphase (Hellmuth et al., 2014). For most of the cell cycle, separase activity is restricted by securin binding. Securin degradation by the APC/C occurs only after all chromatid pairs have aligned appropriately along the equatorial mitotic spindle and the mitotic spindle checkpoint has been satisfied. At
the onset of anaphase, separase activity cleaves Scc1, triggering poleward movement of the segregated sister chromatids in preparation for cytokinesis and the completion of cell division (Uhlmann et al., 2000; Oliveira and Nasmyth, 2010). Separase activation is activated in a highly coordinated manner and the protein is rapidly degraded by the APC/C shortly after cleaving its substrates, now known to include centriolar SCC1 and pericentrin (Oliveira and Nasmyth, 2010; Schöckel et al., 2011; Lee & Rhee, 2012; Matsuo et al, 2012).

1.4.2 SCC1
A complex called cohesin is responsible for sister chromatid cohesion (Michaelis et al., 1997; Guachi et al., 1997). Cohesin is composed of two structural maintenance of chromosomes proteins (SMC1 and -3), joined together via their hinge domains, and a single α kleisin (SCC1/Rad21) subunit that create a tripartite ring which encircles sister chromatids (Gruber et al., 2003). The Scc1 N-terminus binds Smc3 while the C-terminus of Scc1 binds Smc1. Associating subunits SA1/2 and Pds5A/B bind the ring and provide a platform for either WapI or sororin in a mutually exclusive manner (Nishiyama et al., 2010). Ring opening occurs by prophase-pathway-dependent phosphorylation through the activity of PLK1 and Aurora B, followed by separase-mediated cleavage (Zou et al., 1999; Hauf et al., 2001), an action that is countered by shugoshin-dependent PP2A recruitment (Kitajima et al; Riedel et al., 2006). At the onset of anaphase, separase cleaves the SCC1 subunit (Uhlmann et al., 1999), which opens the cohesin ring and causes it to dissociate from chromosomes.

Centriole disengagement requires combined Plk1 and separase activities (Tsou and Stearns, 2006; Tsou et al., 2009; Schöckel et al., 2011). The Stemmann group provided strong evidence for a role of SCC1 at the centrosome, showing that the overexpression of a non-cleavable Scc1 cohesin subunit prevents centriole disengagement, while ectopic cleavage of an engineered variant promotes it (Schöckel et al., 2011). Other cohesin subunits, including SMC1 and SMC-3, were also reported to localise to the centrosome (Gregson et al., 2001; Guan et al., 2008; Wong and Blobel, 2008; Kong et al., 2009; Beauchene et al., 2010). These findings demonstrate that centriolar cohesin shares many of the components that comprise the cohesin ring binding sister chromatids.
However, no comprehensive study demonstrating co-localisation of cohesin subunits at the same centrosomal substructure through expression of functional cohesin-fluorescent protein fusions or through immunostaining of the endogenous proteins exists, so the assembled form that cohesin takes at the centrosome is unknown.

**1.4.3 Pericentrin**

Pericentrin is a large, elongated coiled-coil molecule, encoded by the *PCNT* gene, and is a conserved component of the PCM of animal centrosomes (Doxsey et al., 1994; Flory et al., 2000; Li et al., 2001; Martinez-Campos et al., 2004). Pericentrin is localised to the centrosome by its C-terminal PACT domain. This conserved domain is sufficient for centrosomal localisation throughout the mammalian cell cycle (Gillingham and Munro, 2000). The *PCNT* gene is expressed as two distinct isoforms: PCNTB, a longer isoform (also referred to as Kendrin or PCNT2) and the shorter PCNTA isoform, which lacks the second coiled-coiled region as well as the C-terminus and thus the calmodulin-binding and PACT domains (Doxsey et al., 1994; Flory et al., 2000; Flory and Davis, 2003; Lee and Rhee, 2011; Li et al., 2001; Miyoshi et al., 2006a). In addition, an isoform of PCNTB, lacking the N-terminus, was described in mice and flies (Martinez-Campos et al., 2004; Miyoshi et al., 2006b). The functional relevance of these isoforms was incompletely understood, with the possibility that they merely represent alternative splice forms still relatively unexplored. A study by the Rhee group demonstrated the ability of the full length PCNTB isoform to rescue mitotic defects caused by the loss of both PCNTA and PCNTB in human cells, indicating that PCNTB is the relevant PCNT protein for centrosome biology and mitosis (Lee and Rhee, 2011). For clarity, the terms pericentrin or PCNT will henceforth refer to the full length PCNTB protein.

Dictenberg and colleagues first proposed that pericentrin forms a lattice structure that exhibits dynamic alterations in size between interphase and mitosis. As discussed in section 1.2.3, advances in imaging techniques have since revealed the higher order spatial organisation of the PCM and highlighted the major structural role of pericentrin at the centrosome. Pericentrin serves as a multifunctional scaffold for facilitating interactions of a myriad of protein and protein complexes (Delaval and Doxsey, 2009), perhaps most importantly for cell division γTuRCs and CDK5RAP2, providing a structural framework in which the MTOC activities take place (Dictenberg et al., 1998; Takahashi et al., 2002; Zimmerman et al., 2004;
Buchman et al., 2010). Consequently, pericentrin is required for PLK1-dependent centrosome maturation at the onset of mitosis when centrosomal levels of multiple PCM components increase several-fold (Khodjakov and Rieder, 1999; Haren et al., 2006; Lüders et al, 2006; Gomez-Ferreria et al, 2007; Lee and Rhee, 2011).

In addition to MTOC activity and recruitment of PCM components, pericentrin promotes many diverse cellular processes including cilia formation (Martinez-Campos et al., 2004; Jurczyk et al., 2004; Miyoshi et al., 2006b), recruitment of signalling proteins such as CHK1, MCPH1, PKA, CDK5RAP2, DISC1 and PKCβII to the centrosome (Chen et al., 2004; Diviani et al., 2000; Miyoshi et al., 2004; Matsuzaki and Tohyama, 2007; Tibelius et al., 2009; Buchman et al., 2010), and centriole disengagement (as discussed in section 1.3.3). Pericentrin may also have a nuclear function, which could involve its interaction with chromodomain helicase DNA-binding protein 3/4 (CHD3/4), components of the nucleosome remodelling deacetylase (NuRD) complex (Sillibourne et al., 2007). Interestingly, inhibition of the nuclear export protein, chromosome region maintenance 1 (CRM1), results in the accumulation of pericentrin in the nucleus (Keryer et al., 2003; Liu et al., 2009). Pericentrin contains an unusual tripartite nuclear localisation signal (NLS), in addition to five classic nuclear export signals (NESs) that likely promote its cytoplasmic localisation and nucleocytoplasmic shuttling during the cell cycle (Liu et al., 2010).

Overexpression of pericentrin leads to enlarged, abnormal centrosomes and disrupts mitotic spindle integrity, possibly through its sequestration of dynein, the microtubule motor required for assembly of pericentrin at centrosomes (Purohit et al., 1999; Young et al., 2000). Strikingly, such overexpression also promotes centriole duplication via a de novo process, which exists in addition to the standard mechanism by which centrioles normally duplicate from a pre-existing template (Khodjakov et al., 2002; La Terra et al., 2005). Of relevance to this thesis, pericentrin overexpression causes expansion of the PCM, facilitating the nucleation of multiple daughter centrioles independently of any spatial or numerical control from the mother centrioles (Loncarek et al, 2008; Wang et al., 2013). This finding suggests that the mother centriole’s principal role in centriole assembly may be the regulation and specification of a PCM scaffold, rather than the provision of a template (Loncarek et al, 2008). The recent demonstration of mother centriole
reduplication in response to Plk1-induced maturation of the daughter centriole via recruitment of PCM supports this theory (Shukla et al., 2015).

Notably, in *Drosophila* the Pericentrin-like protein (PLP) is essential for ciliogenesis but appears to be dispensable for mitosis (Martinez-Campos et al, 2004). In contrast, loss-of-function analyses in frog embryos, mouse models, chicken B lymphocytes, and human cells have demonstrated roles for pericentrin in mitotic and interphase centrosomal activity (Doxsey et al., 1994; Mikule et al., 2007; Zimmerman et al, 2004; Wang et al., 2013; Chen et al., 2014; Ma and Viveiros, 2014). Depletion of pericentrin in human and murine cells or Pcnt<sup>−/−</sup> mice results in formation of smaller bipolar mitotic spindles or, in some cases, monopolar spindles as well as delayed mitotic progression and diminished cell proliferation (Zimmerman et al, 2004; Chen et al., 2014). The small mitotic spindles in PCNT-depleted cells display a reduced number of astral MTs (Zimmerman et al, 2004), which are important for mitotic spindle positioning (Buchman & Tsai, 2007). The study by Akbarian and colleagues attributes this phenotype to the drastic reduction of three proteins (CDK5RAP2, Ninein and Centriolin) in the absence of pericentrin, indicating that pericentrin is a crucial molecular scaffold for this functionally-connected set of spindle pole proteins (Chen et al., 2014).

A number of studies link pericentrin dysfunction to the rare human autosomal recessive genetic disorders, microcephalic osteodysplastic primordial dwarfism type II (MOPD II; MIM 210720), and Seckel syndrome (SCKL, MIM 210600) (Rauch et al., 2008; Griffith et al., 2008; Delaval and Doxsey, 2009). MOPD II and SCKL share clinical features that include intrauterine and postnatal growth retardation, extremely short stature and a pronounced reduction in brain size relative to body size (microcephaly) (Majewski and Goecke, 1982; Majewski et al., 1982; Willems et al., 2010). While MOPD II is distinguished from SCKL by skeletal dysplasia and mild/absent mental retardation, the *PCNT-SCKL* mutations are comparable to those seen in MOPD II patients and would therefore elicit the same clinical diagnosis (Hall et al., 2004; Willems et al., 2010). Mutations in the ATR protein that cause Seckel syndrome were identified as a single hypomorphic mutation in the *ATR* gene that alters *ATR* splicing (O’Driscoll et al., 2003). *PCNT-SCKL* mutations were later identified as homozygous truncating mutations in *PCNT* resulting in the loss of pericentrin from the centrosome (Griffith et al., 2008; Piane et al., 2009).
Interestingly, cells derived from PCNT-SCKL patients exhibit defective DNA damage responses. PCNT-SCKL cell lines show impaired phosphorylation of ATR-dependent substrates and impaired G2/M checkpoint arrest (Griffith et al., 2008; Alderton et al., 2004).

1.5 The DNA damage response

1.5.1 DNA damage response networks

When cells suffer DNA damage, two tasks are prioritised; repairing the lesions in the DNA and communicating the presence of damaged DNA. Thus the DNA damage response (DDR) can be considered as two major activities, one that orchestrates the repair of the damage and upregulates the expression of genes involved in the repair process and one that halts cell cycle progression. This cell cycle delay is maintained as a DNA damage checkpoint until the DNA damage is repaired, or the cell is driven out of the cell cycle into apoptosis or senescence (Ciccia & Elledge, 2010; Jackson & Bartek, 2009). These responses require the activation of a number of signalling pathways, most of which occur in the nucleus, although in the case of the cell cycle delay, the impact is throughout the cell (Fig 1.4).

The nuclear response initiates within seconds of the genotoxic insult, with sensor proteins recognising DNA lesions and recruiting ATM (Ataxia telangiectasia, mutated) and ATR (ATM and Rad3 related)- large, serine-threonine kinases of the phosphatidylinositol 3-kinase-like protein kinase (PIKK) family, as well as members of the poly (ADP-ribose) polymerase (PARP) family- PARP1, 2 and 3 (Beck et al, 2014; Schreiber et al, 2006). The PIKK kinases initiate the major DDR signalling cascades by phosphorylating their effector proteins, large networks which include the CHK1 and CHK2 kinases, to amplify the DNA damage signal, impose a checkpoint delay and enlist and activate an array of repair proteins at the DNA lesion (Lukas et al, 2011). ATM and ATR signals also maintain the cell cycle arrest through the transcription-regulatory activity of p53, which causes increased levels of the CDK inhibitor, p21, as well as its numerous other targets (Batchelor et al, 2009). The PARP polymerases assemble a recruitment platform via the rapid and transient synthesis of poly-(ADP-ribose) (PAR) chains on target proteins at the sites of DNA
damage. In summary, nuclear DNA damage is converted into a biochemical signal that affects the entire cell through a complex network of interdependent pathways.

1.5.2 DNA damage checkpoints

Maintaining genomic integrity requires constant cellular vigilance against frequent genotoxic insults. To achieve this, eukaryotes have developed complex surveillance mechanisms, called checkpoints. The DNA damage checkpoints ensure that cell
cycle progression only occurs if the cell is adequately equipped for the next phase in cell duplication. This precaution prevents improper chromosome segregation and the proliferation of potentially harmful DNA mutations. The intricate network that activates checkpoints is composed of DNA damage sensors, signal transducers and numerous effector pathways. The fundamental regulators are the PIKKs- ATM, ATR and DNA-PK- whose many substrates mediate cell cycle arrest in G₁, S or G₂ phases (Fig 1.4) (Bartek and Lukas, 2007).

1.5.3 The G1/S checkpoint

In a canonical cell cycle, the activation of CDK2, via its dephosphorylation by CDC25A and association with cyclin E, permits progression from G₁ into S-phase. DNA damage acquired in G₁ activates a checkpoint to block cells from initiating the replication of damaged templates. Upon DNA damage ATM/ATR phosphorylate CHK1/, CHK2 which in turn phosphorylate CDC25A, targeting it for degradation (Mailand et al., 2000). Depletion of CDC25A leads to the accumulation of phosphorylated CDK2 and inhibits its association with cyclin E. Without active CDK2, the CDC45-mediated recruitment of polymerases to ORCs required for new origin firing does not occur, meaning that DNA replication is blocked (Falck et al., 2001; Jin et al., 2003). Degradation of CDC25A is also linked to sustained inhibitory phosphorylation of CDK4 that maintains G₁ arrest (Terada et al., 1995). If this rapidly instigated delay is insufficient for complete repair, p53 transcriptional activity invokes an extended delay via activation of p21 and subsequent inhibition of CDK2. The resulting loss of Rb phosphorylation, prevents the activity of transcription factor E2F (required for the transcription of S phase genes), thus preventing S phase entry (Hatakeyama and Weinberg, 1995; Brehm et al., 1999; Bartek et al, 2001).

1.5.4 The intra S checkpoint

DNA damage occurring in S phase, or evading the G1-S phase arrest, activates the intra-S phase arrest. Upon detection of DNA damage by sensor proteins, DNA synthesis is slowed or halted via two distinct pathways (Falck et al., 2002; Yazdi et al., 2002). The first is the ATM-CHK2-CDC25A-CDK2 pathway, where phosphorylation of CDC25A at Ser123 targets it for degradation, preventing CDK2 activation and the subsequent recruitment of CDC45 and DNA polymerase α to ORCs (Falck et al., 2001). The second, less defined, pathway inhibits replication via
ATM-mediated phosphorylation of SMC1 and FANCD2 that requires NBS1 (Nakanishi et al., 2002; Kim et al., 2002; Taniguchi et al., 2002). FANCD2 stimulates BRCA1 activation of GADD45 alpha and beta transcripts involved in checkpoint activation (Smith et al., 1994; Yazdi et al., 2002).

1.5.5 The G2/M checkpoint
The G2/M checkpoint is the final opportunity to repair any DNA damage that had arisen prior to initiation of the cell division process. In response to DNA damage, ATM/ATR-dependent phosphorylation of CHK1/CHK2 leads to phosphorylation of CDC25C, which then binds the 14-3-3 proteins, prompting sequestration of CDC25C in the cytoplasm, away from its substrate CDK1 (Peng et al., 1997; Kramer et al., 2004). WEE1, the inhibitory kinase of CDK1 is also up regulated and this, along with Cdc25C downregulation maintains the CDK1/Cyclin B complex in an inactive state and prevents entry into mitosis (O'Connell et al., 1997; Raleigh and O'Connell, 2000). CHK1/CHK2 also phosphorylate and inhibit PLK1 from activating CDC25C, further contributing to suppression of CDC25C activity (van Vugt et al., 2010). As in the G1/S checkpoint, p53 upregulates transcription of the gene coding for p21 (CDKNIA). p21 can inhibit CDK2/Cyclin A and CDK4/6/Cyclin D and this results in an inhibition of the target genes of E2F (Agarwal et al., 1995; Stark and Taylor, 2004). p21 can also directly inhibit the CDK1/Cyclin B complex, ensuring it remains in an inactive state (Obaya and Sedivy, 2002; Sancar et al., 2004).

1.5.6 The spindle assembly checkpoint
The spindle assembly checkpoint (SAC) prevents mis-segregation and aneuploidy by ensuring that each daughter cell inherits one complete complement of genetic material. The SAC monitors the attachment of kinetochores to the plus-ends of microtubules, verifying that all chromosomes are bi-orientated between separated spindle poles on the metaphase plate before segregation can happen. Prior to accurate attachment of every chromosome to the mitotic spindle, the SAC blocks the APC/C from recognizing cyclin B and securin by promoting the incorporation of the APC/C co-activator, CDC20, into a complex called the mitotic checkpoint complex (MCC) (Hagting et al., 2002). The MCC is composed of MAD2, BUBR1 and BUB3 checkpoint proteins that then form a complex with CDC20 (Sudakin et al., 2001). This block effectively prevents the polyubiquitylation and subsequent degradation of
cyclin B and securin. The persistence of securin serves to maintain separase in its inactive form, inhibiting the cleavage of the cohesin required for the separation of sister chromatids, thereby preventing entry into anaphase (Oliveira and Nasmyth, 2010). Inhibition of cyclin B degradation prevents CDK1 inactivation and therefore mitotic exit, adding another layer of regulation to the SAC (Brito and Rieder, 2006). The appropriate alignment of the chromosomes, along the equator in combination with achieving bipolar attachment, relieves the SAC and permits progression to anaphase (Sacristan and Kops, 2015).

1.6 DNA repair
Improper repair of damaged DNA leads to genomic instability, senescence and apoptosis (Lukas and Bartek, 2009). Defects in an organism’s capacity to repair damage to its DNA are associated with a predisposition to immunodeficiency, neurological disorders and cancer (Subba Rao, 2007; Thoms et al., 2007), highlighting the importance of repair pathways for preserving genome integrity.

1.6.1 Repair mechanisms
Depending on the type of DNA damage inflicted, cells invoke specific DNA repair pathways in order to restore the genetic information as faithfully as possible. Briefly, these conserved repair pathways include the nucleotide excision repair (NER) pathway, the mismatch repair (MMR) pathway, the base excision repair (BER) pathway, the non-homologous end joining (NHEJ) repair pathway, and the homologous recombination (HR) pathway (Friedberg et al., 1995; Ciccia and Elledge, 2010).

The nature of the DNA damage sustained by the cell dictates the repair mechanism invoked to either reverse the damage or engage the apoptotic machinery in the case of irreparable damage. NER is the primary pathway used to remove bulky DNA adducts induced by UV light, environmental mutagens, and chemical agents. Defects in NER are responsible for the extremely skin cancer-prone inherited disorder xeroderma pigmentosum (XP). The heterotrimeric complex of RPA, XPA and XPC, in combination with the transcription factor TFIIH (containing XPB and XPD) recognises the bulky DNA lesions. ATP-dependent unwinding of the DNA allows the formation of a pre-incision complex, providing a platform for dedicated endonucleases to excise a short single stranded DNA fragment flanking the damage.
site. DNA polymerases δ or ε merge the gaps by copying the intact strand (Scharer, 2013; Wood et al., 2001).

DNA replication errors are rectified by the MMR pathway. When polymerase misincorporations are not detected by proofreading activity, the mismatch is detected by the MSH2/MSH6 dimer. This heterodimer transitions to a sliding clamp role in an ATP-dependent manner, prior to recruiting second heterodimer comprised of MLH1 and PMS2. The thus assembled sliding clamp surveys the DNA until a break, such as a gap between Okazaki fragments, is located and so identifies the newly synthesised strand. The exonuclease EXO1 is then recruited for degradation of the newly synthesised strand back as far as the misincorporated base. The lengthy single stranded region this isolates is stabilised by RPA binding before restoration by DNA polymerases δ and/or ε (Spies and Fischel, 2015).

BER is employed to repair damaged bases arising from endogenous activities or environmental stresses. Oxidisation, reduction, alkylation, or deamination can all result in damaged bases which are excised from the DNA sugar-phosphate backbone by a dedicated glycosylase. The resultant abasic site is further processed by short-patch repair or long-patch repair carried out by additional glycosylases and endonucleases. The complementary strand provides a template for re-synthesis of the missing bases by DNA polymerase β prior to ligation by DNA ligase III-Xrcc1 (Krokan et al., 2013).

1.6.2 Double-strand break repair
Double-strand breaks (DSBs) pose the most high risk form of damage, given the potential for loss of genetic material or chromosomal translocations. The cell has two main strategies for dealing with DSBs- NHEJ and HR. NHEJ is the predominant repair pathway for DSBs in the $G_1$ and early S phase, while the requirement of an intact template strand for HR restricts its activity to late S and $G_2$ phase. NHEJ (re)joins two DSB ends by apposition, processing, and ligation without the use of extended homology to guide repair, so is thus considered to have high potential for error. Detection of a DSB by the binding of Ku, a heterodimer of two related proteins, KU70 and KU80, forms a ring to which the DNA-PKcs binds (Chiruvella et al., 2013). The binding of the DNA-PKcs stimulates other repair factors and the subsequent alignment of the exposed DNA ends. DNA ligase IV, in complex with
XRCC4, seals the aligned ends. NHEJ is also essential for the development of the immune system, due to its role in V(D)J recombination (Lieber, 2010, Chiruvella et al, 2013).

1.6.2.1 Homologous recombination

Homologous recombination provides an essential mechanism to repair both accidental and programmed double strand breaks (DSBs) during meiosis and mitosis. The physiological importance of HR is evident in the observations of genomic instability in HR-deficient cells and in the cancer predisposition and developmental defects associated with mutations in HR genes (Moynahan and Jasin, 2010). DSBs are life-threatening DNA lesions and HR is the most error-free repair mechanism for repairing DSBs, since it uses homologous sequence from the undamaged sister chromatid as a template for faithful repair. A limitation on HR as a repair mechanism is its requirement for this template sequence restricting HR to S and G2 phases of the cell cycle (Takata et al., 1998; Ciccia and Elledge, 2010). Of relevance to this thesis, HR has also been harnessed as a research tool in promoting genetic recombinations in response to induced DSBs at sites of interest in the genome (Robbins, 1993; Jasin, 1996; Sun and Zhao, 2013; Byrne et al., 2015).

Instigation of HR requires DSB recognition by the MRE11-RAD50-NBS1 (MRN) complex. In combination with CtBP-interacting protein (CtIP) and BRCA1, MRN resects DSBs, in an ATM-dependent manner, to generate short 3’-ssDNA tails flanking the break (Symington, 2014). These ssDNA overhangs become immediately coated with replication protein A (RPA). RPA is then displaced by RAD51, aided by mediator proteins RAD52 or BRCA2 as well as RAD51 paralogues, forming a filament that catalyses pairing with a homologous template and strand invasion. This pairing and exchange of strands between two homologous lengths of DNA are the key reactions in HR (San Filippo et al., 2008). Once assembled, the RAD51-ssDNA complex searches for a homologous sequence in the sister chromatid DNA and then promotes invasion of the ssDNA to the template dsDNA to form a displacement loop (D-loop). This process is promoted by RAD54, considered to be an important factor in the subsequent dissociation of RAD51 and the uncovering of the 3’OH group required for initiation of DNA synthesis (San Filippo et al., 2008).
DNA polymerases then extend the 3’ end of the invading strand using the donor strand as a template to replace nucleotides removed in resection from the lesion site. The newly repaired DNA is ligated to the resected invading strands, forming an interlinked Holliday junction (HJ) complex that must be disentangled to separate the repaired DNA from the template strand (Liu et al., 2004). These double HJ intermediates are either dissolved or are resolved into separate duplex molecules by the catalytic action of resolvases in order to complete repair. The resultant duplex molecules can be cross-over recombinants, although cells seem biased towards non-crossover resolution products (Mankouri et al., 2004; Liberi et al., 2005). This process involves the combined activity of the SGS1/BLM complex which promotes migration of the HJs, and the Top3-Rmi1 complex complex, which decatenates the interlocked strands between two HJs. These activities result in the final non-crossover duplexes (Wu and Hickson, 2003).

1.7 DNA damage response components at centrosomes

1.7.1 Key DDR proteins at the centrosome

Several of these interdependent pathways intersect at centrosomes. A number of key activities involved in the DDR have been described at centrosomes. Antibody cross-reactivity has been a particular issue in assigning robust centrosomal localisations to specific proteins, with relatively weak concentrations of proteins of interest occasionally being used to assert a centrosomal location (Arquint et al, 2014). However, there exists good evidence for the following DDR proteins being found at centrosomes, at least during part of the cell cycle: ATM, ATR, CHK1, CHK2, BRCA1, BRCA2, PARPs. In considering the relevance of these localisations, it is significant that centrosomes are key to the spatiotemporal regulation of the cell cycle delay that permits cells sufficient time to repair their DNA before division. The prophase activation of CDK1-cyclin B occurs first at centrosomes, prior to the potentiation of a wave of nuclear CDK1-cyclin activity that drives mitosis (Jackman et al, 2003). As discussed in section 1.1.3, this initial activation is controlled by centrosomal CDC25 phosphatase (Cazales et al, 2005; De Souza et al, 2000; Dutertre et al, 2004; Lindqvist et al, 2005). The CDC25 phosphatases are inhibited through phosphorylation by the CHK1 and CHK2 effector kinases in the DNA damage
response (Furnari et al, 1997; Sanchez et al, 1997; Uto et al, 2004), although there are several other kinases that also regulate CDC25 activity, including the Aurora and Polo families (Karlsson-Rosenthal & Millar, 2006). Thus, checkpoint imposition after DNA damage involves the translation of a nuclear DNA damage signal to the centrosome.

1.7.2 ATM, ATR and CHK2 at the centrosome
The apical ATM kinase has been described at mitotic centrosomes in human lymphoblastoid cells (Oricchio et al, 2006) and in HeLa cervical carcinoma cells (Zhang et al, 2007). However, the extent to which centrosomal ATM is activated, as determined by phosphorylation at serine-1981, is not clear because of potential non-specificity of the phospho-antibody used in microscopy (Oricchio et al, 2006; Suzuki et al, 2006). Similarly, ATR at mitotic centrosomes has been described at centrosomes in HeLa cells (Zhang et al, 2007), but the functional significance of this localisation is unclear. CHK2 localises to centrosomes throughout the cell cycle, before and after DNA damage, although not all studies have described CHK2 there (Chouinard et al, 2013; Golan et al, 2010; Kramer et al, 2004; Tsvetkov et al, 2003). Its roles at the centrosome in mammalian cells are not fully understood, but a centrosomal function for CHK2 has been established in syncytial Drosophila embryos, where DNA damage caused CHK2-dependent centrosome inactivation and apoptotic elimination of cells after incomplete chromosome segregation in mitosis (Takada et al, 2003).

1.7.3 CHK1 at the centrosome
An influential paper described centrosomal CHK1 as a mechanism of controlling CDC25B’s activation of CDK1-cyclin B during mitotic entry (Kramer et al, 2004), with subsequent work from the same group implicating this regulatory pathway as a component of the DDR (Loffler et al, 2007; Tibelius et al, 2009). The precise localisation of CHK1 is an important element of this model, so a significant difficulty arose when another group saw no centrosomal CHK1 and identified potential antibody cross-reactivity in a key reagent (Matsuyama et al, 2011). The initial study that described CHK1 at the centrosome used the DCS-310 anti-CHK1 monoclonal antibody in microscopy experiments on U2OS osteosarcoma cells and fractionation analyses of KE37 T-cell leukaemia extracts, with siRNA to control for specificity (Kramer et al, 2004). These authors also localised GFP-CHK1 to
centrosomes in U2OS cells during the unperturbed cell cycle (Kramer et al, 2004), although other workers have reported limited localisation with a similar approach in U2OS cells (Chouinard et al, 2013). Centrosomal CHK1 has been described using the DCS-310 antibody in human fibroblasts (Loffler et al, 2007), HeLa (Boutros & Ducommun, 2008), HCT116 colon carcinoma cells (Katsura et al, 2009) and mouse embryonic stem (ES) cells (Koledova et al, 2010).

Against these observations must be set the persistence of centrosomal signal seen with DCS-310 after Chk1 knockout in conditionally-null murine fibroblasts, the absence of centrosomal signal with myc-CHK1 in human DLD1 colon carcinoma cells or FLAG-CHK1 in HeLa cells and the identification of a cross-reactivity of DCS-310 with the centrosomal protein Cdc151 in HeLa cells (Matsuyama et al, 2011). However, other groups, using different antibodies, have described CHK1 at centrosomes in mouse ES cells (Niida et al, 2007), HeLa (Jilani et al, 2015; Zhang et al, 2007), primary murine neuroprogenitor cells (Gruber et al, 2011) and U2OS (Jilani et al, 2015; Kim et al, 2014). Phospho-CHK1 antibodies have detected centrosome signals in H1299 lung carcinoma cells and DLD1 colon carcinoma cells (Wilsker et al, 2008), mouse ES cells (Niida et al, 2007) and HeLa cells (Jilani et al, 2015). On balance, despite the need for caution interpreting results using the DCS-310 antibody, there appears to be strong evidence for a centrosomal population of CHK1, although this is small in comparison to the nuclear fraction and may vary with cell type.

1.7.4 p53 and its role at the centrosome
Several of the DDR signals converge on the transcriptional regulator and tumour suppressor, p53, whose activation in response to cellular stresses directs the transcription of large network of target genes to control cell cycle progression, DNA repair and, after severe genotoxic stress, apoptosis (Batchelor et al, 2009; Riley et al, 2008). Interestingly, p53 deficiency leads to marked centrosome amplification (Fukasawa et al, 1996). This appears to be in large part through the aberrant transcriptional regulation of gene products involved in centrosome duplication, with CDK2-cyclin E being one potential target (Mussman et al, 2000; Tarapore et al, 2001). However, p53 has also been localised to centrosomes in several studies (Brown et al, 1994; Tarapore & Fukasawa, 2002), implying that its functions in centrosome regulation are not limited to the nucleus. The recent description of how
p53 regulates the appropriate timing of centrosome separation through restraining Aurora A-mediated PLK1 activity provides one mechanism by which non-transcriptional activities of p53 can impact the centrosome (Nam & van Deursen, 2014). DNA damage-responsive p53 centrosome localisation suggests that such activities may be a component of the DDR (Tritarelli et al, 2004).

1.7.5 BRCA1: roles in the DNA damage response and at the centrosome

The findings summarised above indicate that the kinase network of the DDR involves centrosomally localised signals. Additional key activities of the DDR also occur at centrosomes. The product of the breast and ovarian tumour susceptibility gene, BRCA1, is a large protein that contributes to genome stability through its involvement in several protein complexes that regulate DNA repair activities, particularly homologous recombinational repair (Savage & Harkin, 2015). BRCA1 binds DNA and contains two eponymous phosphoprotein-binding BRCA1 C-terminal (BRCT) domains that allow the dynamic regulation of these DNA repair complexes. BRCA1 forms a ubiquitin E3 ligase complex with its interactors BARD1 and the recently-described OLA1 (Matsuzawa et al, 2014). This ubiquitin ligase activity targets γ-tubulin and modulates both centrosome activity in microtubule nucleation and centrosome number (Hsu et al, 2001; Sankaran et al, 2005; Starita et al, 2004). BRCA1 also localises to centrosomes and so can contribute directly to their regulation (Hsu & White, 1998; Lotti et al, 2002; Okada & Ouchi, 2003; Tarapore et al, 2012). Defects in BRCA1 function lead to centrosome amplification (Xu et al, 1999), as does loss of OLA1 (Matsuzawa et al, 2014), indicating another mechanism by which BRCA1 mutations may contribute to cancer development. An important open question is whether the centrosome-regulatory ubiquitin ligase activity of BRCA1 responds to DNA damage signals, or whether the nuclear signals that arise from BRCA1 dysfunction lead to centrosome overduplication.

1.7.6 PolyADP-ribose polymerases

Several members of the polyADP-ribose polymerase (PARP) family have been described at centrosomes- PARP-1 (Kanai et al, 2000), PARP-3 (Augustin et al, 2003) and a telomeric PARP, tankyrase (Smith & de Lange, 1999). The PARPs are abundant nuclear enzymes that catalyse the formation of polyADP-ribose chains on target proteins and the local generation of a DNA damage signal. PARP1 and
PARP3 are associated with centrosomes throughout the cell cycle (Augustin et al., 2003; Kanai et al., 2003) and their loss leads to dysregulation of centrosome number, although relevant centrosomal polyADP-ribosylation targets are not yet established. Also remaining to be determined is whether centrosome amplification in PARP mutants arises from defects in DNA repair or in centrosome control.

Tankyrase-1 polyADP-ribosylates and stabilises CPAP, an important regulator of centriole duplication, so that tankyrase-1 depletion also leads to aberrant centriole numbers (Kim et al., 2012). Interestingly, tankyrase-1 polyADP-ribosylation of Miki, a regulator of mitotic kinetics, at prometaphase allows the appropriate assembly of key elements of the mitotic PCM (Ozaki et al., 2012). These roles for tankyrase-1 indicate how a centrosomal polyADP-ribose signal might be involved in DNA damage responses, although the telomere localisation of tankyrase may restrict such a response to specific instances of telomere damage.

1.8 Centrosomal proteins in the DDR

Some direct roles in DNA repair have been ascribed to centrosome proteins. Centrin2 is a component of the proximal centriolar lumen that plays a direct role in nucleotide excision repair of base lesions in DNA and is required for centriole stability in lower organisms and for ciliogenesis in mammals (Dantas et al., 2012; Prosser & Morrison, 2015). Centrin2 forms and helps to stabilise a complex with the xeroderma pigmentosum group C gene product (XPC), a component of a key DNA damage recognition complex (Araki et al., 2001; Sugasawa et al., 1998). Centrin2 stimulates in vitro DNA repair activities and has been shown to be required for efficient NER (Dantas et al., 2011; Nishi et al., 2005). Notably, the bulk of cellular centrin is not associated with centrioles (Paoletti et al., 1996), so that this example may reflect entirely separate roles for centrin2, rather than a centrosomal protein modulating a nuclear repair function. Separation-of-function analyses will be required to address this issue.

CEP164 is a centriole distal appendage protein that is necessary for primary cillum formation (Cajanek & Nigg, 2014; Graser et al., 2007; Schmidt et al., 2012). It has been described as an ATM/ ATR interactor whose deficiency impairs activation of DNA damage response proteins and the G2-to-M checkpoint (Sivasubramaniam et al., 2008), as well as an XPA interactor that localises to the sites of UV-induced DNA
damage (Pan & Lee, 2009). Recent data have indicated proliferative defects in CEP164-deficient cells (Slaats et al, 2014), but the mechanisms by which CEP164 directly affects DNA repair activities are unknown.

1.9 Effects of DNA damage on centrosomes

1.9.1 DNA damage-induced alterations to centrosomes
DNA damaging treatment causes marked alterations in centrosome structures and allows centrosome duplication to occur outside S phase, leading to abnormal numbers of centrosomes within the cell. Increases in centrosome number are frequently observed in cancer and can have significant effects on genome stability and cell behaviour, affecting mitosis, cell migration, and potentially contributing to tumourigenesis (Ganem et al, 2009; Godinho & Pellman, 2014). DNA damage-induced centrosome amplification can also allow the formation of aberrant numbers of primary cilia (Conroy et al, 2012). Such supernumerary cilia can affect the activity of cilium-localised cell signalling pathways, indicating an additional potential impact of centrosome abnormalities on cellular function (Mahjoub & Stearns, 2012).

Current models for how these abnormalities arise in cycling cells invoke the dysregulation of the normal centrosome duplication process or the overexpression of particular components of the centrosome duplication machinery (Godinho & Pellman, 2014). In addition, a variety of genotoxic stresses can lead to altered centriole numbers. Abnormal amplification of centrosomes has been observed following DNA damage induced by irradiation (Dodson et al, 2007; Sato et al, 2000) or DNA replication stress (Balczon et al, 1995; Meraldi et al, 2002). Centrosome amplification has been described under a wide range of stresses that affect genome stability, in a broad range of cell types and species, so it appears to be a general response to DNA damage and to involve the same steps as normal centrosome duplication, albeit at a different stage in the cell cycle with respect to chromosome replication (Saladino et al, 2012).
1.9.2 Centrosome amplification
DNA damage-induced centrosome amplification requires CHK1 and CDK activity, with p53-deficient cells showing CHK1-dependent upregulation of CDK2 activity (Bourke et al, 2010; Bourke et al, 2007). An extensive literature details the pathways by which CDK2-cyclin E drives centrosome duplication during the normal cell cycle and overduplication when aberrantly expressed or during an extended S-phase delay (Brownlee & Rogers, 2013; Godinho & Pellman, 2014). These pathways involve the Aurora A, PLK2, PLK4 and ROCK kinases that ultimately direct SAS-6 function in forming new procentrioles. The pathways that allow centrosome overduplication during extended S-phase arrest and those that direct amplification after S-phase may be regulated differently. For example, it has been reported that CHK2 is required for HU-induced centrosome overduplication (Wang et al, 2015), although CHK2 deficiency does not affect centrosome amplification in Rad51C-deficient model (Katsura et al, 2009). These differences may be related to the status of the centrosomes at the time the cell cycle delay is incurred, in that S-phase centrosomes are already licensed for duplication, while those in G2 must re-acquire such a license. Whether amplified centrosomes induced at different stages in the cell cycle have different effects on the cell remains to be explored in detail.

1.9.3 Centrosome splitting
A significant initial effect of DNA damage on centrosomes, seen in both transformed and non-transformed cells, is the splitting of centrosomes into individual centrioles (Inanc et al, 2010; Saladino et al, 2009). Consistent with DNA damage affecting centrosome structures, a report of centrosome splitting after replicative stress demonstrated a loss of PCM integrity, although the individual centrioles were not characterised in detail (Hut et al, 2003). This splitting appears to be equivalent to the centriole disengagement that licenses duplication. A detailed recent analysis of the impact of doxorubicin treatment on nontransformed hTERT-RPE1 cells showed that centriole splitting involves both PLK1 and an APC/C-regulated activity (Douthwright & Sluder, 2014). The requirement for separase activity in licensing during the normal cell cycle and the fact that DNA damage-induced centriole splitting precedes reduplication of the centrioles implicates the protease as the likely candidate for this regulated activity (Inanc et al, 2010; Saladino et al, 2009).
1.9.4 DNA damage and PLK1 activity

DNA damage-induced splitting is potentiated by the loss of C-NAP1 or rootletin (Conroy et al, 2012), which suggests that losing centrosome cohesion affects centriole disengagement, although how this happens remains to be described. One candidate for an activity that plays a role in controlling centrosome cohesion and centriole disengagement after DNA damage is PLK1. PLK1 is an ATM and ATR target (Smits et al, 2000; van Vugt et al, 2001), whose inhibition after DNA damage impedes centrosome separation by blocking the activation of NEK2 (Fletcher et al, 2004; Mardin et al, 2011; Zhang et al, 2005). The requirement for PLK1 in centriole disengagement or centrosome amplification after DNA damage (Douthwright & Sluder, 2014; Inanc et al, 2010) implies a difference between how PLK1 directs centriole disengagement and the resolution of centrosome cohesion. This may be related to the cell cycle timing of these activities, to varying activities of PLK1 on different substrates, or to the involvement of additional, overlapping pathways, such as separase, in allowing disengagement after DNA damage (Prosser et al, 2012). Interestingly, centrosome amplification in BRCA1- and OLA1- deficient cells involved increased numbers of split centrioles, although it is unclear whether the split centrioles arose from a DNA damage response or from dysregulation of centrosome integrity (Ko et al, 2006; Matsuzawa et al, 2014).

It is clear that several signals- ATM/ ATR/ CHK1/ CHK2 kinase activity, BRCA1 ubiquitin ligase functions and polyADP-ribosylation by members of the PARP family- are involved in the regulation of centrosome duplication and function after DNA damage (Fig 1.5). Further studies are required to fully understand the physiological relevance of their involvement in this regulation. Despite the numerous studies advancing our knowledge of centrosome duplication, the relative contributions of separase-dependent cleavage of cohesin and pericentrin to centriole disengagement remain enigmatic. In this study we aim to explore the contributions of separase and its substrates, in particular, pericentrin, to this regulation.
Figure 1.5 Signalling between the nucleus and the centrosome in response to DNA damage.
Schematic outlines the known signalling pathways from the nucleus that impact on the centrosome, as well as the potential centrosome-mediated signalling effects on nuclear responses. The mechanistic details of centrosomal effects on the nuclear DDR remain to be explored in detail.
1.10 Aims of this thesis

In this study we aimed to better understand the impacts of DNA damage on the PCM and centriole duplication, using three distinct, though related, approaches.

1. Early in the research project we sought to elucidate the temporal and spatial activity of separase at the centrosome. In order to visualise and quantitate separase activity in vivo, we generated a centrosomal-localising molecular sensor. Separase activity, as detected by the sensor, was analysed by live cell microscopy.

2. To investigate the direct regulation of separase in response to DNA damage, we devised a strategy to insert an affinity tag into the 5’ end of the hESPL1 locus. To incorporate the tag sequence, we used TALENs to promote HR-dependent genetic recombination in hTERT-RPE1 cells.

3. Next we investigated the impacts of DNA damage on the PCM. We used three-dimensional structured-illumination (3D-SIM) and light microscopy to analyse pericentrin structure and centrosome amplification in response to ionising irradiation (IR). To better understand the regulation/dysregulation involved in centrosome abnormalities after DNA damage, the analysis of these features was extended to a number of DDR mutant cell lines. Non-cleavable pericentrin was generated to investigate the requirement for separase-mediated cleavage in IR-induced effects on the PCM and centrosome duplication. Given the central role of CHK1 in the DDR, we followed its activation after IR by immunoblot and immunofluorescence microscopy. In addition, we investigated the activation responses of nuclear export signal (NES) and nuclear localising signal (NLS) CHK1 mutants to IR.

The research presented in this thesis contributes to our understanding of the effects of DNA damage on the PCM and centriole engagement as well as the mechanisms that regulate these effects.
2 Materials and methods

2.1 Materials

2.1.1 Chemicals and solutions

Chemicals used throughout this study were of analytical grade and were purchased from Sigma-Aldrich (Arklow, Ireland), VWR (Bridgeport, CT, USA), Fisher (Loughborough, UK) or GE Healthcare Life Sciences (Little Chalfont, UK). All solutions were prepared using ddH$_2$O or Milli-Q purified water, Millipore (Billerica, MA, USA) and where appropriate, autoclaved prior to use. Organic solvents, alcohols and acids were supplied by Sigma (Arklow, Ireland), VWR or Fisher. Oligodeoxynucleotide primers were purchased from Sigma-Aldrich. RNA was prepared using the TRIzol reagent (Total RNA Isolation) obtained from Invitrogen (Carlsbad, CA, USA) and all solutions used for RNA work were treated with 0.1% diethylpyrocarbonate (DEPC). All common reagents and buffers used throughout this study are presented in Table 2.1 (listed in alphabetical order).

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Composition</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking solution (immuno-fluorescence microscopy)</td>
<td>1% BSA in 1x PBS. Filter sterilised and stored with 0.1% Sodium azide</td>
<td>For blocking cells and diluting antibody</td>
</tr>
<tr>
<td>Blocking solution (western blot)</td>
<td>1x PBS, 0.1% Tween-20, 5% dried milk</td>
<td>To minimise the unspecific binding of antibodies</td>
</tr>
<tr>
<td>DABCO</td>
<td>2.5% DABCO, 50mM Tris base pH 8, 90% Glycerol</td>
<td>For mounting slides</td>
</tr>
<tr>
<td>DEPC water</td>
<td>0.1% DEPC in ddH$_2$O</td>
<td>For RNA work</td>
</tr>
<tr>
<td>6x DNA loading dye</td>
<td>20% Sucrose, 0.1M EDTA pH 8.0, 1% SDS, 0.25% Bromophenol blue, 0.25% Xylene cyanol.</td>
<td>For loading of DNA samples on agarose gels</td>
</tr>
<tr>
<td>Immuno-fluorescence fixation solution</td>
<td>4% Paraformaldehyde in 1x PBS Methanol supplemented with 5 mM EGTA</td>
<td>For fixation of cells for immunofluorescence microscopy</td>
</tr>
<tr>
<td>LB (Luria-Bertani) broth</td>
<td>1% tryptone, 0.5% yeast extract, 1% NaCl, pH adjusted to 7.0 with 4 M NaOH</td>
<td>Bacterial culture medium</td>
</tr>
<tr>
<td>Permeabilisation buffer</td>
<td>0.15% Triton-X-100 in 1x CB or PBS</td>
<td>For permeabilisation of cells for immunofluorescence microscopy after PFA fixation</td>
</tr>
<tr>
<td>1x Phosphate buffered saline (PBS)</td>
<td>137mM NaCl, 2.7mM KCl, 1.4mM NaH$_2$PO$_4$, 4.3mM Na$_2$HPO$_4$, pH 7.4</td>
<td>For washing cells</td>
</tr>
</tbody>
</table>
2.1.2 Molecular Biology Reagents

All biological reagents used in DNA digestion and cloning reactions, such as restriction enzymes, DNA polymerase (Klenow Fragment I) and DNA ligase were purchased from New England Biolabs (Hitchin, UK). The DNA polymerases TaKaRa LA Taq, KOD and SigmaTaq used in PCR were purchased from Takara Shizo Co. Ltd. (Osaka, Japan), Novagen (Darmstadt, Germany) and Sigma-Aldrich,
respectively. Deoxyribonucleoside tri-phosphates (dNTPs) used in cloning were purchased from Sigma-Aldrich. Shrimp Alkaline Phosphatase (SAP) was purchased from USB (Cleveland, OH, USA). DNA and protein size markers were supplied by New England Biolabs, Fermentas (Glen Burnie, MD, USA) or BioRad (Hercules, CA, USA).

DNA transformation was performed in competent Escherichia coli (E. coli) Top10 cultures. The strain used throughout this project was F- mcrAΔ(mrr-hsdRNS-mcrBC) φ80lacZAM15 ΔlacX74deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(StrR) endA1 nupG. Plasmid DNA preparations were carried out using GenEluteTM Plasmid Miniprep Kit and Midiprep Kit (Endotoxin-free), both from Qiagen (Crawley, UK). DNA gel extraction was performed using QIAquick Gel Extraction Kit from Qiagen.

Commercial molecular biology kits commonly used in this study are tabulated below in Table 2.2.

<table>
<thead>
<tr>
<th>Table 2.2 Molecular biology kits used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name</strong></td>
</tr>
<tr>
<td>GenElute Mammalian Genomic DNA Miniprep Kit</td>
</tr>
<tr>
<td>GenElute Plasmid Miniprep Kit</td>
</tr>
<tr>
<td>Nucleobond Xtra Midi (Endotoxin-free)</td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit</td>
</tr>
<tr>
<td>QIAquickPCR Purification Kit</td>
</tr>
<tr>
<td>SigmaSpin Sequencing Reaction Clean-Up</td>
</tr>
<tr>
<td>Superscript First-Strand Synthesis for RT-PCR kit</td>
</tr>
<tr>
<td>High Capacity RNA-to-cDNA kit</td>
</tr>
</tbody>
</table>
Chapter 2. Materials and methods

A number of commercially available cloning and expression plasmids were used during the course of this project, as shown in Table 2.3

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Use</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM T-Easy</td>
<td>General cloning</td>
<td>Promega (Southampton, UK)</td>
</tr>
<tr>
<td>pBlueScript (SK/KS)</td>
<td>General cloning</td>
<td>Stratagene (La Jolla, CA, USA)</td>
</tr>
<tr>
<td>pEGFP-C1/N1</td>
<td>Expression in mammalian cells</td>
<td>Clontech (Palo Alto, CA, USA)</td>
</tr>
<tr>
<td>pcDNA 3.1</td>
<td>Expression in mammalian cells</td>
<td>Invitrogen Life technologies</td>
</tr>
<tr>
<td>pMS2747-H2B</td>
<td>Expression in mammalian cells</td>
<td>Olaf Stemmann (unpublished)</td>
</tr>
<tr>
<td>sfGFP</td>
<td>Expression in mammalian cells</td>
<td>Pedelacq et al., 2006</td>
</tr>
<tr>
<td>pANMerCreMer</td>
<td>Recombine loxP sites to recycle resistance cassettes</td>
<td>Arakawa et al., 2001</td>
</tr>
<tr>
<td>pSwitch</td>
<td>Regulatory vector for expression of the GAL4-DBD/Hpr-lbd/p65-AD gene fusion</td>
<td>Invitrogen Life technologies</td>
</tr>
<tr>
<td>pGene/V5- His A</td>
<td>Inducible expression vector for expression in mammalian cells</td>
<td>Invitrogen Life technologies</td>
</tr>
<tr>
<td>pTal.CMV.T7.v2. 023034-07</td>
<td>Expression of TALENs in mammalian cell</td>
<td>Cellectis Bioresarch (Cambridge, MA, USA)</td>
</tr>
<tr>
<td>pEX-A Affinity Tag</td>
<td>Cloning of specific sequence for targeting vector</td>
<td>Eurofins MWG/Operon (Luxembourg)</td>
</tr>
</tbody>
</table>

A number of primers were used to clone cDNAs as well as in a PCR based screen designed to identify successfully targeted clones (Section 2.4.4, Fig 4.5).

<table>
<thead>
<tr>
<th>Primer use</th>
<th>Primer #</th>
<th>Primer sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSG-Chibby (pMS2747-Chibby)</td>
<td>LIM 1F</td>
<td>GGCAGCGCATGCGGCTGCTTCGGGAGCAC</td>
</tr>
<tr>
<td></td>
<td>LIM 1R</td>
<td>GGCGCGCCCTTCTCCATCGGTTGGA</td>
</tr>
<tr>
<td>CSG-Centrin4</td>
<td>LIM 2F</td>
<td>ATATGGTACCATGCCGCTTCTACAGGAA</td>
</tr>
<tr>
<td></td>
<td>LIM 2R</td>
<td>ATAGGCAGCGCATATAAGCTCGTTTCCTTC</td>
</tr>
<tr>
<td>Linker-</td>
<td>LIM 3F</td>
<td>ACTGGTCTGTTCTCTCTATGTAGCAGGGAAG</td>
</tr>
</tbody>
</table>
The antibodies (Abs) used during the course of this study for western blot immune-detection (IB) and immunofluorescence microscopy (IF) are listed in Table 2.5 (primary Abs) and Table 2.6 (secondary Abs).

**Table 2.5 Primary antibodies used in this study**

<table>
<thead>
<tr>
<th>Antigen/reactivity</th>
<th>Clone/reference number</th>
<th>Host Species</th>
<th>Working dilution for IB</th>
<th>Working dilution for IF</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-tubulin</td>
<td>B512</td>
<td>Mouse polyclonal</td>
<td>1:10000</td>
<td>1:2000</td>
<td>Sigma</td>
</tr>
<tr>
<td>CDK5Rap2</td>
<td></td>
<td>Rabbit</td>
<td>-</td>
<td>1:1000</td>
<td>AR. Barr <em>et al.</em></td>
</tr>
</tbody>
</table>
### Table 2.6 Secondary antibodies used for this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Conjugation</th>
<th>Host Species</th>
<th>Dilution for IB</th>
<th>Dilution for IF</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IgG (H &amp; L)</td>
<td>Texas red</td>
<td>Goat</td>
<td>-</td>
<td>1:200</td>
<td>Jackson ImmunoResearch (Suffolk, UK)</td>
</tr>
<tr>
<td>Mouse IgG (H &amp; L)</td>
<td>FITC (fluorescein isothiocyanate)</td>
<td>Goat</td>
<td>-</td>
<td>1:200</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Mouse IgG (H &amp; L)</td>
<td>FITC</td>
<td>Donkey</td>
<td>-</td>
<td>1:200</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Mouse IgG (H &amp; L)</td>
<td>Alexa 594 – 488</td>
<td>Goat</td>
<td>-</td>
<td>1:1000</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Mouse IgG (H &amp; L)</td>
<td>HRP (horseradish)</td>
<td>Goat</td>
<td>1:10000</td>
<td>-</td>
<td>Jackson ImmunoResearch</td>
</tr>
</tbody>
</table>
Chapter 2. Materials and methods

<table>
<thead>
<tr>
<th>Rabbit IgG (H &amp; L)</th>
<th>peroxidase)</th>
<th>Goat</th>
<th>-</th>
<th>1:1000</th>
<th>Jackson ImmunoResearch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit IgG (H &amp; L)</td>
<td>FITC</td>
<td>Goat</td>
<td>-</td>
<td>1:1000</td>
<td>Molecular Probes (Life Technologies)</td>
</tr>
<tr>
<td>Rabbit IgG (H &amp; L)</td>
<td>Alexa 594 – 488</td>
<td>Goat</td>
<td>-</td>
<td>1:1000</td>
<td>Jackson ImmunoResearch</td>
</tr>
<tr>
<td>Goat IgG (H &amp; L)</td>
<td>HRP</td>
<td>Donkey</td>
<td>1:10000</td>
<td>-</td>
<td>Abcam (ab7125)</td>
</tr>
</tbody>
</table>

2.1.3 Tissue culture reagents and cell lines

All sterile plasticware used for tissue culture was obtained from Sarstedt (Numbrecht, Germany), Corning (Corning, NY, USA), Fisher and Sigma-Aldrich. The following media used in cell culture were obtained from Lonza (Cambridge, UK): Dulbecco’s modified eagle medium with Ham’s F12 nutrient mix (DMEM-F12) and Roswell Park Memorial Institute media (RPMI) 1640. Tissue culture reagents such as trypsin, foetal bovine serum (FBS), chicken serum, newborn calf serum (NCS), penicillin and streptomycin (pen/strep), HEPES, sodium pyruvate, glutathione, serum-free OptiMEM, dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. For siRNA knockdown, Oligofectamine was purchased from Invitrogen.

Table 2.7 Cell lines and growth conditions used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Source</th>
<th>Culture medium</th>
<th>Growth condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DT40</td>
<td>Chicken B-cell lymphoma cell line</td>
<td>Prof. Ciaran Morrison (NUI Galway)</td>
<td>RPMI-1640, 10% FBS, 1% chicken serum</td>
<td>39.5°C, 5% CO₂</td>
</tr>
<tr>
<td>hTERT-RPE1</td>
<td>Human retina epithelial cell line, non-transformed, telomerase immortalised</td>
<td>ATCC (Middlesex, UK)</td>
<td>DMEM F12, 10% FBS, 200 mM L-glutamine, 15 mM HEPES</td>
<td>39.5°C, 5% CO₂</td>
</tr>
</tbody>
</table>

Table 2.8 Antibiotics used for selection of stable cell lines

<table>
<thead>
<tr>
<th>Name of the drug</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blasticidin</td>
<td>25μg/ml</td>
</tr>
<tr>
<td>Geneticin (Invitrogen)</td>
<td>1-2mg/ml</td>
</tr>
<tr>
<td>G418 (Invivogen)</td>
<td>1-2mg/ml</td>
</tr>
</tbody>
</table>
Chapter 2. Materials and methods

<table>
<thead>
<tr>
<th>Drug</th>
<th>Working concentration</th>
<th>Solvent</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nocodazole</td>
<td>100 ng/ml</td>
<td>DMSO</td>
<td>Reversible activation of spindle assembly checkpoint</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(pro-metaphase arrest)</td>
<td></td>
</tr>
<tr>
<td>MG132</td>
<td>0.5 – 2 μg/ml</td>
<td>DMSO</td>
<td>Proteasome Inhibitor</td>
<td>Sigma</td>
</tr>
<tr>
<td>Mifepristone</td>
<td>10-100 nM</td>
<td>DMSO</td>
<td>Inducing agent</td>
<td>Invitrogen Life technologies</td>
</tr>
</tbody>
</table>

The different drugs that were used in this project for pharmacological treatment of DT40 and hTERT-RPE1 cells in different assays are described in Table 2.9.

For the treatment of cells with ionising radiation (IR), a $^{137}$Cs source at 23.5 Gy/min was used (Mainance Engineering, Hampshire, UK).

2.1.4 Computer software

DNA plasmid maps were created using pDRAW32 software (Acaclone, www.acaclone.com). DNA sequences were viewed using Chromas software (version 2.31, Digital River, Shannon, Ireland). For bioinformatic analyses, BlastN or BlastP (http://www.ncbi.nlm.nih.gov/BLAST), ClustalW (www.ebi.ac.uk/clustalw) and dbEST (http://www.ncbi.nlm.nih.gov/dbEST/) were used. Microscopy imaging was performed using an Olympus BX-51 or BX-81 microscope, driven by OpenLab software (version 5, Improvision, Emeryville, CA, USA) or Volocity v6.21 (Improvision/Perkin Elmer). Deconvolved images were saved as Adobe Photoshop images (version 7, San Jose, USA). Analysis of flow cytometry samples was carried out on FlowJo software version 7.6 (FlowJo, Ashland, OR, USA). Live cell microscopy was carried out on either a Deltavision microscope, controlled by Softworx software (Applied Precision, Issaquah, WA, USA) or a Ultraview VoX spinning disc confocal microscope (Perkin Elmer), controlled by Volocity software. Quantitation analysis of live cell was carried out on Image-Pro Plus (Media Cybernetics, Rockville, MD, USA). Statistical analysis of microscopy data was carried out on Prism 5 (Graphpad, La Jolla, CA, USA).
2.2 Nucleic acid methods
The molecular cloning techniques used in this project were carried out according to published protocols (Sambrook et al., 2001).

2.2.1 Plasmid DNA preparation
Mini and midi plasmid DNA preparations were carried out using the GenElute™ Plasmid Miniprep and Midiprep kits according to the manufacturer’s instructions. For mini preps, DNA was isolated from 2 ml of bacterial culture and resuspended in 50-100 µl of deionised water. For midi preps, DNA from 100 ml of bacterial culture was resuspended in 100-200 µl of deionised water.

2.2.2 Restriction digest of DNA
Restriction endonucleases were used in the presence of the provided buffer (1X final concentration) and BSA (0.1 mg/ml). The digestion was carried out at the manufacturer’s indicated temperatures on a thermo-stable heat block for 1 to 16 hours, depending on the amount of DNA to be digested. Where appropriate, enzymes were heat inactivated by incubating at 65°C for 20 minutes.

2.2.3 Preparation of DNA for Cloning
Digested DNA used for cloning was purified using the SigmaSpin Sequencing Reaction Clean-up columns (Sigma) to remove restriction endonucleases and traces of buffer. Digested plasmid DNA was dephosphorylated on the 5’ ends using shrimp alkaline phosphatase ((SAP) 1U, pmol of DNA ends) in SAP buffer at 37°C for 1 hour. SAP was then heat inactivated by incubating the reaction at 65°C for 20 minutes. Where necessary, Klenow DNA polymerase or T4 DNA polymerase was used to blunt 5’ and 3’ overhangs, as per the manufacturer’s instructions. The prepared plasmid and insert DNA were then verified by agarose gel electrophoresis. Ligations were carried out using T4 DNA ligase in 1X T4 DNA ligase buffer at 4°C overnight or at room temperature for 3 hours prior to transformation of competent E. coli cells. An excess of insert DNA was generally used (1:3 to 1:10, depending on the concentration of DNA, as calculated from agarose gel electrophoresis and/or from NanoDrop 2000c spectrophotometer analysis). Linearized plasmid DNA was precipitated before transfections by adding one 10th of volume of 3M NaOAc pH 5.2 and 1 volume of isopropanol. The sample was then mixed and the DNA pelleted at top speed for 10 minutes. The pellet was washed with 70% ethanol and pelleted
again. After removing the ethanol and letting the pellet air dry, the DNA was resuspended 30µl of MilliQ H$_2$O.

### 2.2.4 Preparation of Genomic DNA from Tissue Culture Cells
Genomic DNA was prepared from suspension and adherent cells to screen clones for potential targeting events by PCR based screening (Section 2.4.4). The cells from a confluent 24-well dish were taken for DNA preparation. The cells from confluent 6-well dishes were frozen down. The cells were pelleted at 160 g for 5 minutes and resuspended in 500 µl of ‘Tail’ Buffer (Table 2.1) containing 0.5 mg/ml proteinase K and incubated overnight at 37°C or for 3 hours at 55°C. The following day, cell lysates were vigorously shaken for 5 minutes at 37°C in a thermomixer, before 250 µl of 6 M (saturated) NaCl was added and the shaking was carried out again for 5 minutes. Precipitated proteins were removed by centrifugation at 16,100 g for 10 minutes. DNA was precipitated in isopropanol before washing in 70% ethanol and air drying for 5-15 minutes. DNA was resuspended in ~50µl MilliQ H$_2$O at 37°C.

### 2.2.5 Polymerase Chain Reaction (PCR)
PCRs were performed on a TGradient block (Biometra, Göttingen, Germany) or an Eppendorf Mastercycler Nexus Gradient (Hamburg, Germany) using Sigma Taq or KOD Hot Start polymerases (if high proofreading was required). The sequences of all the primers used in this study are shown in Table 2.4. Table 2.10 has examples of the PCR programmes and conditions used.

| Table 2.10 PCR conditions for Sigma Taq and KOD Hot Start (different annealing temperatures were used if necessary) |
|---|---|---|---|
| Reagent | Final concentration | PCR step | Temperature and duration |
| | Sigma Taq | KOD Hot Start | Sigma Taq | KOD Hot Start |
| PCR buffer | 1x | 1x | Initial denaturation | 94°C - 2min | 95°C - 2min |
| Mg$^{2+}$ | 1x (2 mM) | 1.5 - 2.25 mM | Denaturation | 94°C - 1min | 95°C - 20s |
| dNTP’s | 200 µM | 200 µM | Annealing | 60°C - 30s | 60°C - 30s |
| DMSO | 0-3% | 0-3% | Extension | 70°C - Xs | 68/72°C - Xs |
| Primers | 0.25 µM | 0.4 µM | Final extension | 70°C - 5min | 72°C - 5min |
| Template | 1-10 ng/µl | 1-10 ng/µl | Cycle No. | 30 | 30 |
| Enzyme | 0.02 U/µl | 0.02 U/µl | (X - 30s per kb) |
2.2.6 Site-directed mutagenesis

Non-overlapping forward and reverse primers were designed to contain specific sequence modifications surrounded by at least 22bps up- and downstream. Typical conditions for site-directed mutagenesis PCR with the Phusion polymerase are described in Table 2.11. The PCR products, generated using Phusion DNA polymerase, have blunt ends. After PCR, 10 µl of the reaction was incubated with T4 Polynucleotide Kinase (PNK) to add 5’-phosphates to the blunt ends for subsequent ligation with T4 Ligase. The PCR products were then analysed by agarose gel electrophoresis. Subsequently, 1-5µl of the digested PCR reaction was transformed into bacteria as described in section 2.2.10. After restriction analysis, the plasmid DNAs were sequenced as described in section 2.2.12 to confirm the desired mutation and the integrity of the coding sequence.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
<th>PCR step</th>
<th>Temperature and duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x Phusion® HF Buffer</td>
<td>1x</td>
<td>Initial denaturation</td>
<td>98°C – 30s</td>
</tr>
<tr>
<td>dNTP’s</td>
<td>200µM</td>
<td>Denaturation</td>
<td>98°C – 10s</td>
</tr>
<tr>
<td>Primers</td>
<td>0.5µM</td>
<td>Annealing</td>
<td>60°C – 20s</td>
</tr>
<tr>
<td>Template</td>
<td>10-20mg/µl</td>
<td>Extension</td>
<td>72°C – 40s</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.02U/µl</td>
<td>Final extension</td>
<td>72°C – 5min</td>
</tr>
</tbody>
</table>

| Cycle No.       | 25                  |

2.2.7 RNA extraction

4-10ml of confluent cells were pelleted and resuspended in 1ml of TRIzol reagent. Using filter tips and DEPC treated reagents, total RNA was extracted after cell lysis according to the manufacturer’s instructions. The RNA pellets were air-dried for 5 minutes, resuspended in DEPC-treated ddH2O by pipetting and incubating at 55°C for 10 minutes. The RNA concentration was estimated by reading the 260nm absorbance using a Shimadzu spectrophotometer and samples were stored at -80°C.
2.2.8 Reverse transcription polymerase chain reaction

cDNA synthesis was performed using the High Capacity RNA-to-cDNA Kit for RT-PCR according to the manufacturer’s instructions (Life Technologies) or Superscript First-Strand Synthesis for RT-PCR kit (Invitrogen). The first-strand cDNA was synthesised using 2μg of total RNA (extracted in section 2.2.7) and 0.5μg of 12-18 Oligo(dT) primers or random decamers. The first-strand cDNA generated was then used as a template in PCR reactions for the amplification of specific gene cDNA.

2.2.9 Preparation of competent E. coli and transformation

To prepare chemically competent E. coli, a starter culture was grown overnight and used to inoculate 400ml of SOB in a 2L sterile flask. After growth of approximately 2 hours at 37°C (to an OD$_{600}$ of 0.35-0.4), the cells were chilled on ice before being pelleted by centrifugation at 3000g for 15 minutes at 4°C. The cells were resuspended in 0.1M CaCl$_2$ (50ml per 100ml of culture) and incubated on ice for 30 minutes. Subsequently the cells were pelleted (as before), resuspended gently in 0.1M CaCl2 supplemented with 15% glycerol (10ml per 100ml of culture), divided in 100μl aliquots, transferred to dry ice or snap frozen in liquid N$_2$ and stored at -80°C.

2.2.10 E.coli transformation

50μl of chemically competent bacterial cells were thawed on ice prior to the addition of half ligation mixture or ~50ng of a plasmid DNA. After mixing the cells were incubated on ice for 20 minutes, heat shocked at 42°C for 90 seconds and placed on ice for 2 minutes to recover. 1ml of LB broth was added to the mixture and the cells incubated with gentle shaking for 1 hour at 37°C. Following this, the cells were spun down at 6000g and a concentrated 50μl was spread on LB broth agar plates containing the appropriate antibiotic selection and incubated overnight at 37°C. Colonies were picked and grown overnight in LB broth cultures with antibiotic at 37°C with agitation and used for plasmid DNA preparation in the next day.

2.2.11 Agarose gel electrophoresis and purification of DNA

0.7-1.0% agarose gels were prepared using Sigma electrophoresis grade agarose in 1X TAE buffer containing 0.5 μg/ml ethidium bromide (Table 2.1). Gels were run in 1X TAE buffer in Hoefer HE33 tanks (Mini Horizontal Submarine Unit, GE Healthcare Life Sciences) according to the manufacturer’s instructions. DNA on the
gel was visualised using a Multi-Image Light Cabinet (ChemiImager 5500, AlphalImager, ProteinSimple, San Jose, CA, USA) and images were taken with a digital camera. For DNA extraction, bands of interest were excised from the agarose gel with a scalpel blade under UV light. DNA was purified using the Qiagen QIAquick Gel Extraction Kit according to the manufacturer’s instructions. 25-50 μl of milli-Q H₂O was used to elute bound DNA off the column.

2.2.12 DNA sequencing
DNA samples were sent to Source Bioscience (Dublin, Ireland) for commercial sequencing. In general, 100 ng of DNA (mini or midi prepped) and 5 – 10 pM primers were used per sequencing reaction. Sequences were analysed using Chromas software and pDRAW32 was used to construct correct vector maps with the analysed sequences (Section 2.1.4).

2.3 Protein Methods

2.3.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Protein samples for SDS-PAGE were prepared as follows: adherent cells were detached from the surface of the dish using either a cell scraper or by trypsinisation. Suspension and adherent cells were then centrifuged at 160g for 5 minutes, cells were washed in 1X PBS and cells were centrifuged again at 160g for 5 minutes. The 1X PBS was then removed and cells were resuspended in 20-50 µl lysis buffer containing protease and phosphatise inhibitors and lysed on ice for 30-60 minutes. The cells were then centrifuged at 16,100g for 20 minutes at 4°C. The supernatant was then removed and transferred to a fresh eppendorf tube and the protein concentration was determined using the Bradford method (Section 2.3.2). Appropriate concentrations of protein samples were then boiled at 95°C for 5 minutes in 3X sample buffer (Table 2.1) supplemented with 10% β-mercaptoethanol and either directly loaded onto a gel or stored at -20°C. Boiled samples were centrifuged at 16,100g for 2 minutes immediately prior to loading. Higher percentage SDS-PAGE gels were used to separate larger target proteins (composition in Table 2.12). Gels were run at 100-150V for 90 to 150 minutes in running buffer using the Hoefer mini VE equipment.
Table 2.12 Examples of SDS-polyacrylamide gels used in this study for proteins with high and low molecular weights

<table>
<thead>
<tr>
<th></th>
<th>Running gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide mix (37.5:1)</td>
<td>8-10%</td>
<td>12-15%</td>
</tr>
<tr>
<td>Tris-HCl pH 8.8</td>
<td>375mM</td>
<td>375mM</td>
</tr>
<tr>
<td>Tris-HCl pH 6.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Ammonium persulfate (APS)</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Tetramethylethylendiamine (TEMED)</td>
<td>0.04%</td>
<td>0.04%</td>
</tr>
</tbody>
</table>

### 2.3.2 Bradford Protein Assay

For the determination of protein concentration, the Bradford dye-binding protein assay was used. Briefly, 1 μl of a protein sample was diluted in 1 ml 1:1 Bradford:dH₂O. The absorbance was measured at 595nm using a BioPhotometer (Eppendorf) or Nanodrop 2000 (Nanodrop Products, Wilmington, DE, USA). The protein concentration was interpolated based on a BSA standard curve, in which absorbance was plotted against increasing concentrations of the BSA protein.

### 2.3.3 Semi-dry and wet transfers

After separation by polyacrylamide gel electrophoresis, proteins were transferred to a nitrocellulose membrane (GE Healthcare) using a semidy transfer unit (Hoefer TE 77 from GE Healthcare) or a wet transfer unit (BioRad Trans-Blot Cell). In this procedure, the SDS-PAGE gel was placed on an appropriately-sized nitrocellulose membrane between 6 or 4 sheets of Whatman paper, all previously soaked in transfer buffer. These were positioned between the plates of the transfer apparatus with the membrane closest to the cathode. The semi-dry transfer was carried out for 1 hour and 15 minutes at amperage that depended on the size of the gel (area of the gel x 0.8 \( \times XX \text{ mA} \)), while wet transfers were performed in the cold room for 2-3 hours at 350mA.

### 2.3.4 Western blot (immunoblotting)

After transfer, the membrane was washed in ddH₂O and then soaked in Ponceau S solution for 5 minutes with gentle rocking to assess transfer efficiency. An image was taken as reference for transfer quality and protein loading control. The stain was
then removed by washing 5 minutes in PBS and the membrane was incubated with 5% milk in PBS/0.05% Tween-20 solution for 30-60 minutes at room temperature on a rocking platform. After blocking, the membrane was incubated with a primary antibody in 1% milk PBS-Tween solution for 1-3 hours at room temperature or overnight at 4°C with gentle rolling. After three 5 minute washes in PBS-Tween, the membrane was incubated with a HRP-conjugated secondary antibody in 1% milk PBS-Tween solution for 45 minutes at room temperature with rocking. The membrane was washed again in PBS-Tween 3 times and once in PBS. The ECL or ECL Prime detection kit (GE Healthcare) was used according to the manufacturer’s instructions: briefly, the membrane was covered with a 1:1 mix of the ECL solutions and incubated for 1 minute followed by autoradiograph film exposure and development through a developing machine (CP 1000, AGFA, Brentford, UK).

2.4 Cell Biology Methods

2.4.1 Tissue culture techniques

Cell lines, culture media and growth conditions used in this study are outlined in table 2.7. To maintain DT40 cells growing exponentially in culture, new cultures were started by adding a minimum of 100μl of confluent cells (approximately 10⁵ cells) into 10ml of fresh media: Cell density was always maintained at less than 1x10⁶ cells/ml. For freezing and storage, 3x10⁶ confluent cells were centrifuged for 5 min at 350g, the supernatant removed and the pellet resuspended in a small volume (300-400μl) of FBS supplemented with 10% DMSO. They were then kept at -80°C for short-term storage, or in liquid nitrogen for long-term storage. To wake up the cells, the entire contents of one vial were placed into 10ml of warm media. Adherent human cells reached confluency at a cell density of 7 x 10⁶ cells/75 cm² flask (80% confluency). When approaching confluency, cells were passaged by washing in 1X PBS and trypsinizing in 1X trypsin for 2-5 minutes in the 37°C incubator. Once cells were detached, the trypsin was inactivated by adding fresh, pre-warmed medium to return the culture to its original volume. In general, a 1:10 dilution (7 x 10⁵ cells/T75 flask) of the cell suspension was made and this was returned to the incubator for continued cultivation of the cells. For the freezing of cell stocks, 2 x 10⁶ cells/vial were harvested and resuspended in 500 μl of freezing medium (70% FBS, 10%
DMSO, 20% complete medium), before transferring to cryo-vials. These were stored at -80°C for a week before being transferred to liquid nitrogen for long-term storage.

2.4.2 Transient transfections

2.4.2.1 DT40 cells

Transient transfections were generally used for a quick assessment of protein expression and were carried out using the Amaxa nucleofection system (Gaithersburg, USA) kit T (VCA-1002) as described in the manufacturer’s protocol. 2-5μg of endotoxin-free circular plasmid DNA was complexed with 100μl of Solution-T (supplied with the kit) before being used to resuspend 5x10^6 pelleted cells. This mixture was transferred into an Amaxa transfection cuvette and nucleofection was performed using the Amaxa program B-23. The cells were then pipetted into 5ml of prewarmed media, incubated for 24 hours and analysed by immunofluorescence microscopy or immunoblotting.

2.4.3 Stable transfections

2.4.3.1 DT40 cells

Electroporation was performed to generate stable DT40 cell lines overexpressing a cloned gene. In this procedure, 1x10^7 cells were harvested, washed in PBS and resuspended in 0.5ml of PBS. 20-25μg of plasmid DNA were linearised (digested overnight with a specific endonuclease), ethanol precipitated and mixed with the cells. The cell suspension was then transferred to a Bio-Rad 0.4μm cuvette and was incubated on ice for 10 minutes. Subsequently, the cells were submitted to electroporation, at 300V/600μF or 550V/25μF, using a Gene Pulser apparatus from Bio-Rad (Hemel Hempstead, UK) and incubated on ice for 10 more minutes. The cells were transferred from the cuvette to a plate containing 20ml of pre-warmed fresh medium and returned to the incubator for 16-24 hours. After this period, an equal volume of extra medium was added with an appropriate antibiotic for selection (concentrations on Table 2.7) and the culture was plated out in 4x 96-well plates where colonies were usually visible after 6-10 days. When the colonies were approximately 2mm, the content of the wells were expanded to 3ml cultures in 12-well plates and further incubated for 2-3 days. When confluent, 1.5ml of culture was frozen and 1.5ml used for further expansion or to extract proteins or genomic DNA.
Positive clones were identified by western blot, immunofluorescence and PCR based screens.

2.4.3.2 Human cells

hTERT-RPE1 cells were plated in antibiotic-free media in 10 cm dishes on day one so as to be 80-90% confluent on day two. 3-9µg of plasmid DNA was complexed with transfection reagent Lipofectamine 2000 in Opti-MEM Reduced Serum Medium (Invitrogen, Life Technologies) according to manufacturer’s instructions before addition to the prepared dish. 24h post-transfection cells were trypsinised, resuspended in antibiotic-supplemented media (see table 2.7) and serially diluted into multiple 10cm dishes to permit growth of isolated clones at 39.5°C for 10-14 days. Antibiotic supplemented media was changed every 3 days to maintain selection pressure. Colonies were picked from 10cm plates using trypsin-soaked 2mm cloning discs (Sigma, Arklow, Ireland) and transferred into 48 well adherent plates and grown for 3-6 days to expand the clones into 24 well and subsequently into 6 well plates. Positive clones were identified by western blot, immunofluorescence microscopy and PCR based screens.

2.4.4 TALEN based targeting in human cells

TALEN pair plasmids were verified by restriction enzyme diagnostic digest and sequencing. Complementary plasmid pairs were then mixed together with the targeting vector in either a 1:1:1, 2:2:1, 3:3:1, or 3:3:2 ratio (3-8µg range of total DNA). DNA was then complexed with Lipofectamine 2000 in Opti-MEM Reduced Serum Medium and added to the media of hTERT-RPE1 cells as described in stable transfection section 2.4.3.2. Clones were screened by PCR using primer combinations (Table 2.4) that amplified either a wild-type separase locus PCR product of 1.6Kb in length or a PCR product only producible from a specifically targeted separase locus of 1.1Kb in length. Positive candidates were further verified by DNA sequencing.

2.4.5 Floxing of resistance cassettes in adherent human cells

In order to recycle resistance markers, Cre recombinase was transiently expressed in the separase affinity tag knock-in heterozygote cell line to recombine the loxP sites flanking specific resistance cassettes. Transient transfections with pANMerCreMer were carried out as described for either DT40 cells or adherent human cells. 24 hours
after transfection, cells were supplemented with 100nM 4-OH-Tamoxifen to promote the nuclear localisation of the Cre recombinase and serially diluted 1:8, 1:64 and 1:512 into 10cm adherent dishes without any antibiotic selection. After 10-14 days, when colonies were visible, the colonies were picked. Each clone was then split into two new wells, one with antibiotic selection and the other without. The clones that died in selection drug-containing medium were presumed to have lost the corresponding resistance cassette. Positive candidates were verified by PCR screen and confirmed by sequencing.

2.5 Microscopy Methods

2.5.1 Methanol fixation
Adherent cells were grown on sterile coverslips for immunofluorescence microscopy, while suspension cells were adhered to poly-D-lysine slides for 15 minutes at room temperature before fixation. Medium was removed and cells were fixed and permeabilised in 95% methanol at -20°C with 5 mM EGTA, for 10 minutes at -20°C. Cells were then washed three times in 1x PBS before proceeding to staining for immunofluorescence microscopy.

2.5.2 Paraformaldehyde fixation
Cells were adhered as described in Section 2.5.1. Following this, medium was removed and cells fixed in 4% paraformaldehyde (PFA), in 1x PBS for 10 minutes at room temperature. Cells were washed three times in 1x PBS before being permeabilised with 0.15% Triton X-100 in 1x PBS for 2 minutes. Cells were washed three times in 1x PBS before proceeding to staining for immunofluorescence microscopy.

2.5.3 Immunofluorescence microscopy
Cells fixed as in section 2.5.1 or 2.5.2 were stained with the antibodies detailed in Section 2.1.2. The fixation method depended on the nature of the antibody used. Following fixation, cells were blocked in 1% BSA for 30-60 minutes at room temperature or overnight at 4°C to decrease non-specific binding of the antibody. Cells were incubated with primary antibodies, diluted in 1% BSA in a humid chamber at 37°C for 1 hour. Slides/cover slips were washed three times for 5 minutes
in 1x PBS. Cells were then incubated with secondary antibodies, diluted in 1% BSA in a dark humid chamber at 37°C for 45 minutes. Slides/cover slips were washed three times for 5 minutes in 1x PBS. Slides/cover slips were mounted in Vectashield (Vecta Labs, Burlingame, CA, USA) with DAPI (1 μg/ml). Cover slips were sealed with nail varnish and stored at 4°C in the dark. Cells were imaged using either an Olympus BX51 microscope, using 60X and 100X objectives, numerical aperture (NA) 1.35, using Openlab software (Improvision) or an Olympus IX81 microscope with a 100X oil objective, N.A. 1.30. Volocity v6.2.1 (Improvision/ Perkin Elmer) was used to obtain and process images.

2.5.4 Volumetric analysis

PCM volumes were visualized by staining with anti-CDK5RAP2 or anti-pericentrin antibodies before and 4h after 5Gy IR in cells of the indicated genotype. To measure PCM volumes, 15 fields containing G2 cells were captured, with 0.4 μm Z-steps used to capture entire centrosomes. Images were then cropped to contain G2 centrosome pairs. Crops were then deconvolved in the red channel for PCM determination by pericentrin or CDK5RAP2 signals using Iterative Restoration in Volocity with confidence limit 95% and iteration limit 10. PCM volumes were then derived using the following three steps: Remove noise from object (Input: ROIs; Filter: Fine filter); Find objects using SD intensity in red channel using lower limit of 10 and an upper limit of 100; Exclude object by size < 0.02 μm³.

2.5.5 Live cell imaging

For live cell imaging, cells were grown on glass-bottomed 35mm dishes (MatTek, Ashland, MA, USA) or Ibidi µ-slide 8 well imaging chambers (Thistle Scientific, Glasgow, UK) and analysed at 60-70% confluency. Cells were imaged at 10-20 minute intervals in an environmental chamber at 37°C in the presence of CO₂. All live cell microscopy was carried out on a Deltavision microscope with a 60X oil objective, controlled by SoftWorx software or an Ultraview VoX spinning disc confocal microscope, controlled by Volocity software. Serial Z-sections (0.5 μm) were collected and a quick projection was made using the respective softwares. Analysis of molecular sensor cleavage was determined by basic quantitation of pre- and post- anaphase intensities in the green channel relative to the red in an area of interest determined by the bound mCherry signal using Image-Pro Plus.
2.5.6 Flow cytometry

For cell cycle distribution profiling, the genomic DNA of cells was stained with propidium iodide (PI) and cells analysed by flow cytometry. Briefly, $2 \times 10^6$ cells were pelleted, washed once in 1xPBS before resuspension in ice cold buffered 2% para-formaldehyde solution. Cells were mixed gently and incubated at 4°C for 20-60 minutes to preserve fluorescence signal in the case of GFP expressing cell lines. Cells were then pelleted, washed in cold 1x PBS prior to fixation with 70% ice-cold ethanol overnight at 4°C. After washing in PBS, cells were incubated in 40μg/ml propidium iodide and 200μg/ml RNaseA in PBS for 1 hour in the dark. Cells were then analysed using a FACS Canto (Becton Dickinson, San Jose, CA) and FlowJo software version 7.6.
Chapter 3. A centrosome-localising separase activity sensor

3 A centrosome-localising separase activity sensor

3.1 Introduction

Centriole disengagement is essential for both the licensing of centriole duplication in G1/S phase, and for imposing a limit of one duplication event per cell cycle. PLK1 and separase are central effectors in centriole disengagement in vertebrates (Fig 3.1). Both chromosome segregation and centrosome biogenesis are highly regulated to ensure one segregation and duplication event per cell cycle. The shared requirement for common regulatory proteins coordinates the segregation and duplication cycles in a manner that prevents untimely disengagement of the centrioles, which could result in multi-polar spindles and chromosome mis-segregation (Tsou et al., 2009).

![Schematic of the centrosome duplication cycle.](image)

**Figure 3.1 Schematic of the centrosome duplication cycle.**
For centrosome duplication to occur, separase activity is required to cleave its substrates which promote centriolar engagement. This cleavage allows the two disengaged centrioles to each nucleate a new pro-centriole to form a complete centrosome.

One model suggests that PLK1 and separase activity at the centrosome is regulated in a manner that mirrors the regulation of sister chromatid separation, in which Plk1 first promotes dissociation of cohesin from chromosome arms in prophase before separase cleaves the remaining cohesin at centromeres in anaphase (Fig 3.2) (Gimenez-Abian et al., 2004).
Chapter 3. A centrosome-localising separase activity sensor

The role of separase and its substrates in centriole disengagement is discussed in greater detail in section 1.3.3 of the introduction. Briefly, a number of these studies were of particular interest to us, such as the demonstration that the proteolytic function of separase is required for disengagement (Tsou et al., 2006; Tsou et al., 2009) and the multiple demonstrations of cohesin as a disengagement-specific substrate. An important set of experiments produced results in support of the centriolar cohesin model including the expression of a non-cleavable SCC1 inhibiting disengagement and the engineering of independent protease cleavage sites into the cohesin subunits enabling the manipulation of centriole engagement both in vitro and in vivo (Schoeckel et al., 2011). Another significant study identified pericentrin, also known as kendrin, as a novel substrate of the protease (Matsuo et al., 2012; Lee and Rhee, 2012).

Collectively these results outline a role for separase and its substrates at the centrosome that is not yet fully understood. In this project we aimed to elucidate the role of separase in centrosome duplication. The narrow window of the protease activity is best studied in vivo such that the location and extent of separase activation can be determined in real time. Our first approach was to develop a molecular sensor that can monitor the temporal and spatial aspects of the protease’s activity in vivo.
The sensor design was similar to those later published by the Morgan and Hirota groups (Shindo et al., 2012; Yaakov et al., 2012).

### 3.2 Development of a molecular sensor for the detection of separase activity

We designed our sensor to be expressed at either the chromosomes or the centrosomes. The sensor was constructed such that a fragment (amino acids 107-268) of the hScc1 cohesin subunit (S) containing a separase cleavage site (DREIMR) was cloned into an expression vector. The fragment is flanked by a red fluorescent protein (mCherry, C) at the N-terminus (Shaner et al., 2004), and a green fluorescent protein (eGFP, G) at the C-terminus (Cormack et al., 1996). This C-S-G assembly gives the basis of the sensor which we then directed to regions of interest in the cell. To achieve specific localisation we attached either a chromosomal (H2B) or centrosome- (Chibby or Centrin4) localising protein to the N-terminus of the mCherry protein sequence (Fig 3.3). The localising tag binds the sensor via mCherry to either the histones or the centrioles. In the presence of active separase the Scc1 fragment is cleaved after Arginine (Arg) 172 (Hauf et al., 2001), leaving the tag tethering mCherry and a portion of the Scc1 fragment in situ, while the remaining portion of the Scc1 fragment attached to the eGFP is free to diffuse away. Thus activity can be measured as the relative intensity of the red signal at the region of interest, as defined by the bound mCherry signal, relative to the eGFP green signal.

![Figure 3.3 Schematic of the sensor construct](image)

*Figure 3.3 Schematic of the sensor construct*

The localising tag binds the mCherry to our target subcellular location. In the absence of active separase, the intact Scc1 fragment tethers the eGFP to anchored mCherry signal.
3.3 Optimisation of sensor expression and analysis at the chromosome

Live cell microscopy enabled the tracking of cells through entire cell cycles. We optimised expression of the sensor in vivo and monitored the location and timing of its cleavage in a series of live cell experiments. To test the ability of the sensor to detect separase activity in vivo we first used an N-terminal histone (H2B) tag to target this C-S-G construct to the chromosome where the protease activity is spatially and temporally defined (Chestukhin et al., 2003). Wild type (WT) DT40 cells were transfected with the expression vector for the sensor and positive transfectants were tracked in their progression through the cell cycle using live cell microscopy. Cells were imaged every 10 minutes for 2-4 hours. Separase activity and subsequent cleavage of the sensor was detected as the loss of the green eGFP signal relative to the red mCherry bound to the chromosomes throughout mitosis. In these preliminary control experiments we recorded n=10 anaphase events qualitatively demonstrating separase activity. Quantitation of relative signal intensities further confirmed activity seen as the loss of eGFP signal at the chromosomes, as seen in the live cell images (Fig 3.4B).

A noteworthy feature of the H2B-CSG clones generated was that expression levels of the construct were heterogeneous within clonal populations, even after subcloning was undertaken to ensure populations were derived from single clones. This was something we gave consideration to as our separase sensor was based on the expression of an Scc1 fragment driven by a CMV promoter. We were aware that we needed an expression level strong enough to image but not so strong as to completely saturate separase levels resulting in negligible change in the relative mCherry and eGFP intensity levels upon cleavage of the sensor. With this in mind we opted to track medium-level expressing cells within populations as determined arbitrarily by visual analysis.
Figure 3.4 Separase activity at the chromosome
A. Schematic of the chromosome sensor. The H2B tag binds the mCherry to the chromosomes. Prior to separase activity the intact Scc1 fragment tethers the eGFP to anchored mCherry signal.

B. Still images of the live cell data. Images obtained from cells expressing the chromosome targeted sensor show separase activity during anaphase. Scale bar 5 µm.

C. Quantitation of signal. A representative quantitation of the intensity of the green relative to the red fluorescent protein shows a decrease in the green signal corresponding to the cleavage of the sensor during anaphase.
3.4 Optimisation of sensor expression and analysis at the centrosome.
Having confirmed the ability of the control H2B-CSG sensor to detect separase activity at the chromosomes *in vivo*, we then designed a tag for localising the sensor to the centrosomes (Fig 3.5). The H2B DNA sequence at the N-terminal of the CSG sensor was exchanged for those of the chicken centrosome-localising proteins Chibby (Enjolras et al., 2012) or Centrin4 (Dantas et al., 2011). WT DT40 cells were transfected with the centrosome-localising sensors and screened by IF. Positive transfectants were counterstained with centrosome markers, γ-tubulin or Centrin2, and analysed for appropriate localisation of the mCherry and eGFP signals at the centrosome. IF on fixed cells demonstrated that both Centrin4- (data not shown) and Chibby-CSG (Fig 3.6) localised appropriately.

![Figure 3.5 Schematic of the centrosome cycle as visualised with the biosensor localised to the centrosome.](image)

During the passage through mitosis, separase cleaves its substrates at the centrosome. This cleaving process promotes separation or disengagement of the two centrioles in order for each to nucleate a new centriole to form a complete centrosome in the next cycle.

After confirming correct localisation of both the Chibby- and Centrin4- tagged sensor to the centrosome we optimised the stable cell lines for live cell microscopy. Using our Deltavision microscope the cells were tracked for 2-4h using time-lapse imaging, through prometaphase, mitosis and into G1, at 10 min intervals to minimise
bleaching. The signal to background ratio detected by the widefield microscope was of poor quality for determining relative intensity levels. Participation in an EMBO microscopy course, “Microscopy, Modeling and Biophysical Methods”, during this period of developing and optimising our sensor system, facilitated access to a confocal spinning disc microscope to get preliminary images of the centrosome-localising sensor. This microscope significantly improved the quality of the images obtained and we learned that the Chibby-CSG cell line produced sufficiently detectable signal.

The preliminary images demonstrated the detection of separase activity at the centrosome with loss of the GFP signal captured in mitosis (Fig 3.7). At this point we had identified the spinning disc confocal as the most suitable microscope for imaging the centrosome-localising sensor and began establishing collaborations with a research laboratory in Columbia University, NY, USA to obtain regular access to such microscope.
Now confident that we were detecting separase activity at the centrosome, we sought to test the sensor’s potential as an immunoblot assay of separase activity. While collaborations were being established to further the microscopy based analysis, we worked on an immunoblot approach to analysing separase activity, as detected by the sensor.

### 3.5 Immunoblot analysis of the separase sensor.

In addition to the fixed and live cell microscopy analyses, we optimised immunoblot analysis to assay for separase activity using antibodies raised against either eGFP or mCherry. DT40 cells stably expressing either H2B-CSG or Chibby-CSG were arrested in prometaphase by supplementing medium with 100ng/ml nocodazole for 4-6h (Sentein, 1977). Pro-metaphase arrest and release were confirmed by fixing treated cell population samples for microscopy analysis of the chromosomal DNA after staining with DAPI. The predicted molecular weights of the tagged sensors range from ~87-93 kDa as determined by ExPASy ProtParam.

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**Figure 3.7 Separase activity detected at the centrosome.**

Separase activity is detected as the loss of/ decrease in eGFP signal from the centrosome following mitosis. The signal from mCherry remains robust as it is tethered to the centrosome, while the cleavage product of eGFP and Scc1 remnants are released from the tether upon separase-mediated cleavage of the substrate. Scale bar, 5µm.
To maximise the chance of detecting the cleavage products, especially the smaller Scc1 fragment-GFP product, we also supplemented the medium with the proteasome inhibitor MG132 immediately after the nocodazole washout to prevent the cleavage products being rapidly degraded. The caveat of this treatment was that inhibiting the proteasome may also have affected APC/C-dependent proteolysis of securin, and Cyclin B1 (Hagting et al., 2002; Skoufias et al., 2007; Peters, 2006), early mitotic events required for mitotic progression. By adding the MG132 after the nocodazole washout we hoped that a significant number of cells would have already undergone early mitotic proteolysis events prior to the inhibiting effects of the MG132. Cells were released from nocodazole arrest by multiple washes and harvested pre- and post-washout for immunoblot analysis (Fig. 3.8).

The non-cleaved sensor can be detected at ~100 kDa in the nocodazole arrested populations. Samples harvested post-release from arrest show a diminished full length signal in addition to bands of sizes appropriate to the cleavage products expected, when immunoblotted with antibodies raised against either mCherry or eGFP. Those samples treated with MG132 showed less cleavage after release from pro-metaphase arrest than those untreated by the proteasome inhibitor, likely due to its inhibitory effect on the APC/C-mediated proteolysis. The detection of cleavage of both the H2B-CSG and the Chibby-CSG sensor in an enriched mitotic population by immunoblot analysis further confirmed the presence of active separase at the centrosome in addition to its activity at the chromosomes during mitosis. To further test that the bands we were attributing to cleavage products were specific to the tagged mCherry and eGFP fragments, we also ran a number of sensor variants alongside the full length and cleaved sensors. The variants included the addition of a super-folding GFP to the C-terminal eGFP on the sensor (H2B- or chibby- CSGG), a sensor entirely lacking in GFP (H2B-CS, and an untagged sensor (CSG).
Figure 3.8 Immunoblot analysis of separase sensor.
Cells were harvested for analysis pre- and post-release from nocodazole-induced pro-metaphase block. Ψ indicates cell populations treated with MG132 proteasome inhibitor. Asterisk indicates non-specific bands. **A. Immunoblot samples were probed with an antibody raised against GFP.** Bands of approximately the estimated size for the full length (FL) intact sensor and Scc1-GFP fragment were detected in samples harvested pre- and post-release respectively. **B. Immunoblot samples were probed with an antibody raised against mCherry.** Bands of approximately the estimated size for the full length intact sensor and Tag-mCherry-Scc1 cleavage product were detected in samples harvested pre- and post-release respectively.
3.6 Immunoblot analysis of the separase sensor in response to DNA damage.

Despite the tight control exerted by the cell cycle machinery, centrosome amplification/over-duplication can arise from a number of sources. One such is through the DNA damage response (DDR), a complex signalling mechanism that delays cell cycle progression and initiates DNA repair pathways after genotoxic stress (Kawamura et al., 2004; Dodson et al., 2004; Bourke et al., 2007). DNA damage has been shown to trigger APC/C activity which in turn stimulates centriole disengagement (Prosser et al., 2012). We hypothesised that DNA damage might elicit these responses via a pathway involving the activation of separase, activation we might be able to detect using our sensor assays.

![Immunoblot analysis of the separase sensor after DNA damage.](image)

**Figure 3.9 Immunoblot analysis of separase sensor after DNA damage.**

**A. Non-cleavable Scc1 fragment.** Point mutations rendering the Scc1 separase site non-cleavable. **B. Scc1 fragment separase site delete.** Complete removal of separase consensus site DREIMR from the Scc1 fragment. **C. Non-specific cleavage of sensor.** Cleavable and non-cleavable variants of the Chibby-CSG sensor were immunoblotted using an antibody raised against mCherry before and after exposure to 10Gy irradiation. Cell lines expressing Chibby-CSG and H2B-CSG without a separase consensus site (△Sep) exhibit non-specific cleavage/degradation after IR induced DNA damage as show in two separate blot inserts.
Chapter 3. A centrosome-localising separase activity sensor

Testing DNA damage-induced separase activity using the live cell sensor assay required a microscope facility where DNA damage could be induced in situ during live cell tracking, something we were discussing as a future progression with collaborators. In the interim we focused on testing the immunoblot assay approach. As a control, to ensure any cleavage activity we might have detected was due to separase-specific activity, we cloned a non-cleavable version of both H2B-CSG and Chibby-CSG generated by point mutating the pivotal arginine (R172A) and glutamine (E169A) amino acids in the separase consensus site in hScc1 to alanines (A) (Hauf et al., 2001). Chibby-CSG and non-cleavable Chibby-CSG-∆Sep expressing cell lines were subjected to irradiation to determine if separase activity is detectable by the sensor after DNA damage. Samples were harvested prior to and 2h post-irradiation for immunoblot analysis (Fig. 3.9).

Immunoblot analysis of separase activity in response to DNA damage revealed that both the Chibby-CSG-∆Sep and the H2B-CSG-∆Sep non-cleavable sensors were cleaved non-specifically. To rule out the possibility that the DT40 Scc1 cleavage site could still be specifically cleaved by human separase after the introduction of the point mutations we deleted the entire 18bp (DREIMR) coding sequence in the Chibby-CSG (Fig. 3.9). This separase site deletion mutant also showed non-specific cleavage after damage. A literature search revealed reports that Scc1 is specifically cleaved after an aspartic acid (Asp279) residue C-terminal of the separase cleavage site (Arg172) by a caspase in cells undergoing apoptosis, in response to multiple stimuli (Chen et al., 2002; Pati et al., 2002). This finding indicates that Scc1 is targeted for cleavage in apoptotic pathways, pathways that can be activated in response to damage. Predictive analyses of the SCC1 protein sequence identified additional potential death-box domains; however, these predicted sites require further exploration to determine their authenticity.

Thus, IR induced cleavage of the sensor was determined to be non-specific to separase and so could not be considered as a tool to study separase activation after DNA damage. The lab from Columbia that had initiated collaboration on this work with us had not progressed past the pilot experiments of live cell imaging our H2B-CSG and Chibby-CSG expressing cell lines. Communication from their side indicated that they had commitments to large experiments on other projects before
correspondence stopped completely without further explanation. In order to complete our sensor studies with the canonical centrosome cycle, we still needed access to suitable microscope facilities. Therefore, we collaborated with the May lab in Universität Konstanz, Germany to test our sensor experiments in their microscope facility. Pilot imaging was carried out successfully with the H2B-CSG cell line.

At this point our preliminary data was validated by the publication of the Schiebel group’s work in Germany who also used centrosome-localising separase sensors to demonstrate that Scc1 and additionally pericentrin are cleaved by separase in early mitosis before anaphase onset (Agircan et al., 2014). As this publication addressed and answered the temporal questions we had wished to refine in our collaboration with the May lab it was agreed that repeating Agircan’s work was not a priority for either research group.

However, our sensor work had confirmed for us, prior to collaborations or the other supporting reports including the Schiebel publication, that separase was present and active at the centrosome during mitosis. Additionally, finding that a sensor approach was unsuitable for investigating the impact of DNA damage on separase activation and centrosome duplication resulted in the design and development of an alternative approach. These findings led us to take a direct approach to look at the regulation of separase itself, an approach that is outlined in chapter 4.
Chapter 4. Separase: Targeting an essential gene

4 Separase: Targeting an essential gene

4.1 Introduction

Separase plays an essential role in cleaving cohesin to allow mitotic progression. Given this critical role, it follows that either premature or delayed activation of separase would compromise faithful execution of mitosis. Accordingly, cells have a tight regulation network controlling its activity. Vertebrate separase is maintained in inactive mode by association with either securin or cyclin B1-Cdk1. Its activation is muted until the spindle assembly checkpoint is satisfied by the appropriate alignment of the sister chromatids and microtubule attachment to kinetochores. Upon SAC alleviation, the APC/C promotes separase activation by targeting securin and cyclin B for degradation via the ubiquitin-proteasome pathway (Oliveira and Nasmyth, 2010). The activation of separase requires more than just relief from the suppressive chaperone duties of securin. Direct post translational modifications (PTMs) are made to the protease itself. Cyclin B1-Cdk1 phosphorylates separase at multiple residues as well as binding via cyclin B1 to the phosphorylated Cdc6-like domain of the protease to activate it (Boos et al., 2008; Gorr et al., 2005).

A common phenotype of DNA damage is centrosome over-duplication or amplification (Sato et al., 2000; Dodson et al., 2004). The current model for DNA damage-induced amplification theorises that DNA damage results in the misregulation of the key players involved in canonical duplication, resulting in duplication no longer being restricted to once per cell cycle. Both APC/C and Plk1 activity can promote centriole disengagement in response to damage DNA damage (Prosser et al., 2012; Inanc et al., 2010; Douthwright et al., 2014). Separase activity on centrosomal substrates SCC1 and pericentrin promotes centriole disengagement, a process essential for subsequent centrosome duplication. Our aim was to investigate the regulation and role of separase in centriole disengagement and centrosome amplification after DNA damage. We proposed to do this by analysing separase itself, before and after DNA damage, for potential alterations in PTMs or protein-protein interactions.
4.1.1 Separase function is sensitive to changes in its expression levels
The tight regulation of separase expression levels and activation is crucial to maintain genome stability (Hauf et al., 2001). Homozygous deletion of the gene is not tolerated in human cell lines and manifests as embryonic lethality in mice (Kumada et al., 2006; Wirth et al., 2006). The Stearns group engineered conditionally separase-null human somatic cells to test the requirement of separase for centriole disengagement and found cells lacking separase exited mitosis but ultimately resulted in polyploidy (Tsou et al., 2009). Experiments involving siRNA-induced separase depletion in HeLa cells and separase-deficient mouse embryonic fibroblasts (MEFs) resulted in polyploidy (Waizenegger et al., 2002; Nagao et al., 2004), while separase overexpression led to aneuploidy and tumorigenesis (Zhang et al., 2008). In summary, alterations to endogenous expression levels of separase disrupt the cell division cycle. Based on these findings, we concluded that a biochemical approach to insert an affinity tag into endogenous separase would afford us insight into its regulation in vivo without disrupting appropriate expression levels. A successful knock-in of such a tag would enable purification and subsequent analysis of separase at various cell cycle stages before and after DNA damage.

4.1.2 Genome editing of human cells
The emergence of TAL effector nuclease (TALEN) technology for targeting of human cells presented an exciting opportunity to tag human separase for analysis. TALENs enable site-specific induction of chromosomal double strand breaks (DSBs) that significantly enhance genome modification (Sun and Zhao, 2013). TALENs describe a fusion of a transcription activator-like effector (TALE) DNA binding domain to the catalytic head of the FokI endonuclease (N). The latter are naturally-occurring transcription factors first found in the plant pathogenic bacteria from the genus Xanthomonas. The TALE DNA binding domain is composed of a series of tandem repeats of typically 33-35 amino acids that recognise a single nucleotide. The specificity of DNA recognition is conferred by two variable amino acids at position 12 and 13 of these repeats that are known as the repeat-variable di-residues (RVDs). This simple code whereby one RVD recognizes one nucleotide means the DNA binding domain of each TALE can target large recognition sites with high precision.
TALENs function as a heterodimer in a tail-to-tail orientation in which the repeat units of each monomer bind target DNA sequences and create a spacer region to allow the FokI domains to dimerize and create a DSB (Sun and Zhao, 2013).

Figure 4.1 TALEN specificity code. TALENs recognise and bind specific DNA sequences based on a simple protein–DNA code. The TALE components comprising the repeat domain are repeat units of 33–35 aa that each recognize a single nucleotide. The highly variable amino acids at positions 12 and 13 in the repeat units correspond to specific DNA bases. Two TALEN monomers can be constructed to have recognition sites flanking the cut site that upon binding enable dimerization of the FokI endonuclease (Cellectis, 2013).

Our strategy to insert an affinity tag into the N-terminus of hSeparase required the design of a TALEN that would induce DSBs proximal to the starting methionine codon (ATG) in the hESPL1 (separase) locus. This induction should promote the targeted genomic incorporation of our affinity tag sequence by homologous recombination based on the homology in the 3’ and 5’ arms of the targeting vector.

Figure 4.2 Tagged hSeparase. Schematic of the separase protein indicating the location of the affinity-purification tag. Not to scale.
4.2 TALEN design and generation

TALENs were at this stage an untested technology in our research centre, so we enlisted the assistance of Cellectis Bioresearch in the generation of separase specific TALENs. We had selected the karyotypically stable, untransformed hTERT-RPE1 cells as our target cell line for the project, thus, our first step was to verify the genomic sequence of the *hESPL1* locus surrounding the starting ATG codon. Genomic DNA was isolated from hTERT-RPE1 cells and primers were designed to amplify an 800bp fragment flanking the ATG codon. The 800bp fragment was sequenced and verified, before 100 nucleotides (nt) upstream and downstream of the start ATG were analysed for potential TALEN binding sites. Potential sites were identified and the generation of TALENs specific for those sites was initiated. Specificity for these sites was achieved by assembling repeat unit with RDVs coding for the corresponding binding sites flanking the *ESPL1/separase* ATG (Fig. 4.3). Each specific central repeat domain was flanked by the nuclear localising signal in the N term coding region and endonuclease C term coding region of the TALEN. The assembled sequences code for monomeric TALENs that were cloned into individual expression vectors for transfection into human cell lines.

Our potential TALENs were tested by Cellectis Bioresearch for the ability to cleave our target sequence using a yeast single-strand annealing (SSA) assay to measure the cleavage activity of the nuclease on an episome containing the target sequence flanked by a disrupted *lacZ* gene. The disruption is a short internal duplication which resection following DSB induction will result in the repair and restoration of *lacZ* activity via a SSA process. TALEN induced cleavage can be quantified by the detection of β-galactosidase expression from the restored *lacZ* gene. This assay identified two TALEN pairs: A and B that were capable of cleaving the target separase sequence. These two pairs were further analysed for their ability to induce insertion/deletion (indel) events in HCT116 cells. $10^4$ cells were transfected with 150ng of either TALEN pair A or B. Three days post-transfection, genomic DNA was harvested and between 1787 and 1827 sequences were analysed by deep sequencing. TALEN pairs A and B were found to induce indels with 30% and 7% efficiency respectively. Both mutagenesis rates were sufficiently high to proceed to our targeted knock-in of an affinity tag to the *hESPL1* locus of hTERT-RPE1 cells.
4.3 Separase targeting strategy

Upon release from its suppressive binding partner securin, the auto-catalytic activity of separase results in cleavage of its C-terminal region. For this reason we opted to target the N-terminus for tagging. To knock an affinity tag into the separase locus we designed the DNA sequence for a 3xFLAG tag and a strepII tag connected to each other and the downstream separase sequence for exon 2 and intron 2 by DNA sequence encoding a hydrophilic linker (Fig 4.2). This Gly-Ser-Thr rich linker was codon optimised for human cells and its length was extended to avoid restrictions a tag might impose on the in vivo folding of hSeparase. At the time of design there was no published correlation between homology arm length and efficiency of donor matrix incorporation via TALEN-induced HR. We opted to generate 5’ and 3’ homology arms of 1.0 and 1.2 Kb respectively as a conservative homology basis for HR promotion. More recent studies in hiPSCs and zebrafish indicate that optimal homology arm lengths are between 1-2 Kb, with this degree of length being of particular importance in the downstream (3’ arm) homology arm (Byrne et al., 2015; Shin et al., 2014). Exon 1 was contained within the 5’ arm and was not altered in the targeting design. Exon 2 contained the start ATG, the target site for integration of the tag-linker-resistance cassette sequence. The 3’ arm spanned the 1.2 Kb of genomic sequence that codes for exon 3 (Fig 4.4).
4.4 Separase targeting vector construction

To generate this vector, genomic sequences were amplified from hTERT-RPE1 cells for the *ESPL1* homology arms by PCR. The primer sets used are listed in Table 2.4. The amplified fragments were then cloned into individual pGEM-T Easy vectors following the manufacturer’s instructions and then sequenced. A DNA tag sequence was designed to include the exon containing the starting codon and two affinity tags in the downstream intron (Appendix. 1). This sequence was then synthesised with appropriate restriction enzyme (RE) sites for insertion between the resistance cassette and the 5’ arm in the final targeting vector. Puromycin or blasticidin resistance cassettes were inserted into the intronic sequence of intron 2 bridging the tag and the 3’ arm.

The availability of suitable RE sites dictated the order of assembly of the targeting vector. The 3’ arm was first inserted into a pGEM-T Easy backbone using ClaI and BamHI sticky end cloning. The tag-exon2-intron2 fragment was then added using BamHI and MluI sticky end cloning. Next, either a puromycin or blasticidin resistance cassette was inserted between the 3’ arm and tag fragment using sticky end

Figure 4.4 Schematic depicting targeting strategy for *hESPL1*.Successful targeting will integrate an affinity tag comprised of 3xFLAG and strepII immediately 5’ of the start Met codon. The endogenous start codon occurs in exon 2. A resistance cassette for selection is also included after the affinity tag and exon 2.
BamHI cloning. The final cloning step used KpnI and NsiI to stick the 5’arm to the tag fragment (Fig 4.5). The final targeting vector was combined with one of the two verified TALEN pairs for transfection into hTERT-RPE1 cells (Section 2.4.4). Multiple ratios of targeting vector to TALEN concentrations were trialled for optimisation of the transfections. Transfected cells were kept under selection for two weeks prior to selection of resistant clones. Isolated clones were expanded to be frozen and screened for specific integration of the tag.

A PCR based screen was used to detect candidates that had the insertion tag at the specific target site. To distinguish a targeted allele from a WT allele, specific combinations of primers were designed to amplify products that bridged endogenous and unique tag insert sequence (Fig 4.6).
PCR screen

PCR products spanning the endogenous DNA and the integration site were analysed by agarose gel, then sequencing to confirm that the tag had been successfully integrated immediately downstream of the start ATG in exon 2 in a number of clones (Fig 4.7). A total of 106 clones derived from TALEN-pair A transfections and 57 clones from TALEN-pair B were screened. TALEN-pair A transfections proved to have a targeting efficiency of 6.3% with 4 heterogenous mutants identified by PCR screening, while TALEN-pair B transfections achieved a 5.4% targeting efficiency with 2 heterogeneous mutants identified (Fig 4.8a). The resistance cassettes were inserted into intron 2 via a BamHI site that had required only one base change to be engineered into the endogenous intronic sequence. This insertion point was chosen to facilitate the cell machinery in splicing out the intron-flanked resistance cassette so that floxing steps to remove the cassette might not be required. Heterozygous clones were analysed by immunoblot for expression of tagged endogenous separase from the targeted allele.
Separase is a 2120 aa protein that has a predicted molecular weight of 230 KDa. However commercial separase antibodies specifically detect bands ranging from ~170 KDa to 250 KDa. The insertion of the tag sequence would add 83 aa of unique sequence to endogenous separase, a 4% increase in protein sequence. Heterozygous clones prepped for immunoblot analysis were probed with antibodies against the streptavidin tag. No signal was detected in the clones, indicating that the targeted allele was not being expressed, or at least not to detectable levels (Fig 4.8b).

Figure 4.7 PCR screen-based identification of heterozygous mutants.
A. First round targeting analysis primers. Combinations of primers 1-3 were used to identify heterozygous targeting clones containing a targeted allele. B. Initial identification of heterozygotes. Primers 1,2 amplified the WT allele only as the GC regions of the resistance cassette sequence in a targeted allele cannot be traversed by the KOD polymerase. Primers 1b,3 bind within the targeting vector sequence and so indicate only that the clone has integrated the targeting vector into the genome. The 1,3 primers amplified a product that bridges the endogenous and targeted sequence and identified heterozygous mutants with the tag inserted in the specific target site. C. Primer combinations and product sizes. Primer combinations are calculated to amplify fragments of the indicated sizes, depending on the genotype of the allele.
Next, we sought to use transfection of the heterozygote clones with cre recombinase to excise the resistance cassette via the loxP sites flanking it, thus preventing its potential for impeding transcription of the targeted genomic allele. Cre recombinase transfectants were incubated until individual clones were expanded for screening. Initial screening involved duplicating culture dishes of the clones, half of which were then put under appropriate selection for 2-4 days. Clones that could not survive under selection were deemed to have had their resistance cassette floxed out in the transfection. Removal of the resistance cassette was further confirmed by a PCR screen and sequencing of PCR products (Fig 4.9).
Successfully floxed heterozygotes were analysed by immunoblot to test for expression of tagged endogenous separase from the targeted allele. These candidates for tagged separase expression were probed with antibodies against both separase itself and streptavidin (Fig 4.10).

Figure 4.9 PCR screen-based identification of floxed heterogeneous mutants. 
A. First round targeting analysis primers. Combinations of primers 1–4 were used to identify heterozygous clones with no resistance cassette. 
B. PCR confirmation of floxed heterozygotes. Primers 1,2 amplified the WT allele as well as the floxed targeted allele. The 1,3 primers amplified targeted alleles regardless of flox status. Primers 4,2 can only amplify a fragment from alleles that are both targeted and floxed. Inset gel image shows the clearer separation of the WT allele from the floxed heterozygote allele. 
C. Primer combinations and product sizes. Primer combinations are calculated to amplify fragments of the indicated sizes, depending on the genotype of the allele. Band sizes in brackets indicate products containing the resistance cassette which will not be amplified by the polymerase due to the GC rich regions of sequence. 
D. Floxing efficiencies. 3 heterozygote clones were transfected with cre recombinase. Floxing efficiencies of each clone as determined by PCR screen and sequencing are indicated in the table.
Neither blot indicated that the floxed heterozygote clones were expressing tagged separase. We hypothesised that haploinsufficiency was being tolerated by the cells and they were perhaps expressing separase solely from the unaltered WT allele.

**Figure 4.10 Immunoblot analysis of floxed heterozygote clones.**

A. Separase immunoblot. No detectable change in expression levels or protein size was detected in either unfloxed or floxed heterozygote clones. B. Streptavidin immunoblot. Prolonged exposure of the streptavidin blot failed to reveal any specific bands that could be attributed to tagged separase. Strep-tagged ATR was used as a positive control for the antibody.

**4.6 Second round targeting.**

Neither blot indicated that the floxed heterozygote clones were expressing tagged separase. We hypothesised that haploinsufficiency was being tolerated by the cells and they were perhaps expressing separase solely from the unaltered WT allele.
Second round targeting was undertaken to try and obtain a homozygote clone by imposing selective pressure on the clones to express tagged separase to survive. Floxed heterozygotes were transfected as before with the more efficient TALEN-pair A and the targeting vector in a 3:3:2 ratio (Fig 4.8). Potential homozygous clones were expanded and screened by PCR as before. Homozygote clones would have no WT allele to amplify, hence using primer combinations 1,2 should amplify only bands of the floxed, targeted alleles (1.85 Kb) and the WT product of 1.65 Kb would be not be detected (Fig 4.11).

**Figure 4.11 Analysis of homozygote candidates.**

A. **PCR screen primers.** Primers 1,2 are sufficient to detect a product if both alleles are targeted based on the size of the bands amplified. B. **PCR screen products.** In the case of only one allele being targeted a WT band of 1.6 Kb and a targeted allele band of 1.85 Kb will be amplified. If both alleles are targeted only the 1.85 Kb will be amplified. C. **Homozygote candidate screen.** A WT, untargeted allele remains in each of the homozygote clone candidates. The persistent presence of the WT band confirms that the candidates are not homozygous for tagged separase. The asterisk denotes the DNA ladder lane. D. **Total homozygote candidate screen results.** A total of 50 candidate clones were screened, with no positive candidates identified.
A total of 50 candidate clones were screened for second round targeting. However, all were confirmed as negative due to the presence of a WT allele still being present. At this point we decided to investigate whether the impediment to translation of our heterozygote was at the transcriptional level. To do this, we isolated RNA from the heterozygote clones and generated cDNA. cDNA was generated using either oligo dT or random decamer primers to ensure we were detecting mature mRNA transcripts as well as pre-mRNAs. We then designed a PCR screen, again using specific primer combinations to amplify either WT cDNA or cDNA potentially transcribed from the targeted heterozygote that contained the affinity tag (Fig 4.12).

Figure 4.12 Analysis of heterozygote transcriptional status. 
A. cDNA PCR screen primers. Primer pairs 5,6 and 5,7 were designed to distinguish between transcribed WT separase mRNAs and targeted tagged-separate mRNAs. Separase schematic not to scale.  
B. PCR screen products. Primers 5,6 amplify a WT band of 1.4 Kb and a targeted allele band of 1.65 Kb. Primers 5,7 amplify a WT band of 216 bp and a targeted allele band of 516 bp. 
C. Heterozygote mRNA screen. In primer pair 5,6 screens, the WT amplicon of 1.4Kb gave a very strong signal and the unfloxed targeted allele failed to amplify a product. Both the WT and the expected targeted allele band were amplified from the floxed heterozygote cDNA. 
D. In the 5,7 primer pair screen, the WT control band was approximately 200 bp and the targeted, floxed allele product was the expected band size of ~500 bp. Rd (random decamer) and odT (oligo dT) primers refer to the RNA primers that were used to generate the cDNA from RNA via reverse transcription.
Primers 5,6 amplified a 1.4 Kb region of ESPL1 mRNA sequence spanning sequence from the 5’ UTR to exon 5. The WT-derived cDNA provided a template for only this 1.4 Kb amplicon. The heterozygote appears to not be transcribed prior to floxing, although it is not clear why a WT allele transcript was not detected in the sample. In the case of the targeted allele being transcribed, there would be an additional 249 bp added by the tag sequence, a total product size of 1.65 Kb. This product was amplified in the floxed heterozygote but only by the random decamer-primed cDNA (Fig 4.12C). This indicates that the separase transcripts might be in pre-mRNA stages of processing, having not yet become polyA tailed. To further test for heterozygote transcripts we used primers spanning a smaller region around the target integration site. This screen was designed to amplify products in a size range more accurately defined on the DNA size marker, meaning that we could discern more precisely whether the expected increase in size due to targeting was achieved. This primer pair resulted in a larger relative increase in the size of the WT product (~200 bp) to the targeted allele product (~500 bp), giving a very clear readout in the case of targeted allele transcripts being made (Fig 4.12D). This experiment indicated that both the untargeted and targeted hESPL1 alleles were being transcribed in the hTERT-RPE1 cells.

4.7 Discussion

Given that we now had some evidence of the targeted separase allele being transcribed, we sought to identify reasons why the transcripts were not being translated into detectable protein. We had earlier verified the genomic sequence of every heterozygote clone from the start ATG codon through the inserted tag sequence and subsequent exons and introns. We decided to extend the sequencing verification further upstream of the start ATG to check for any unexpected alterations to the sequence that might be affecting upstream regulatory elements of the hESPL1 gene. All six heterozygote clones were re-sequenced to determine the fidelity of the upstream sequence. The results showed that each clone had at least one unexpected indel 5’ to the start ATG codon (Fig 4.13).

Heterozygotes 1 and 2 had the same 15 bp deletion occurring 205 bp upstream from the start codon ATG. Heterozygote 3 had a 28 bp duplicated fragment inserted between the A and TG bases of the start codon, as well as a C to G point mutation and a point deletion. The sequence of heterozygote 4 showed the same duplication
event as Heterozygote 3. Heterozygote 5 had a 79 bp duplicated fragment inserted between the A and TG bases of the start codon. The sixth heterozygote showed a larger duplicated fragment insertion of 120 bp occurring just 2 bp upstream of the start ATG codon, as well as a point deletion. The deletions in heterozygotes 1 and 2 are upstream of the 5’UTR but could potentially be disrupting regulatory elements such as promoters or enhancers, resulting in alterations to the transcription process that could either prevent transcription or produce mutant transcripts.

**Figure 4.13 Heterozygote unexpected indels.**

**A. Schematic of indels on a targeted allele.** Large inserts of duplicated sequence were detected proximal to the start ATG codon, point mutations and deletions were detected in the 5’UTR and deletions occurred upstream of the 5’UTR. **B. Indels at sequence level.** The WT with tag insert sequence represents the expected sequence of a targeted allele with no indels. **Colour Key:** Black – wild-type sequence. **Highlighted** – consensus sequence omitted for presentation view. **Red** – codon coding for endogenous start methionine in separase. **Purple** – sequence of integrated affinity tag. **Blue** – unexpected insertion. **Green** – unexpected deletions. **Pink** – unexpected point mutations/deletions.

/CGAGAGGGAAGATCTCTTGTTTTCACCGATGATTGCATTGCAGGATGTTCCTCCTTCAGTAG/
The insertions proximal to the start ATG in heterozygotes 3-6 are all duplications of the WT sequence, meaning the regulatory and splicing machinery may be acting on unexpected duplications in erratic positions on the 5’ UTR that could impact on the designation of a start codon or splice site (Moore and Proudfoot, 2009). At the transcript level, the integrity of the UTR sequence is important because it contains a ribosome-binding site. Human mRNA may contain regulatory sequences, including binding sites for proteins, that can affect the stability of the mRNA or the efficiency of its translation. The insertion events proximal to the start codon could potentially inhibit the ability of the initiator tRNA molecule to bind the AUG codon (Kozak, 2000).

To ensure that these indels were not a result of errors in the homologous ‘donor template’, we re-sequenced the 5’ arm and tag fragment of the targeting vector that span the regions in which the indels occurred. The sequence was verified and proved to be correct. The TALENs were designed to induce DSBs proximal to the ATG codon where many of the indels occurred. It is clear that the cells did employ homologous recombination in response to the DSBs as the exogenous tag sequence was incorporated from the targeting vector. However, it is not clear how the indels were introduced to the genomic sequence. The TALEN binding sites should only recognise WT separase sequence. The incorporation of the tag sequence immediately 3’ of the ATG codon was designed in the knowledge that it would alter the local sequence to the extent that it would no longer be a substrate for the TALENs. The additional sequence insert prevents the ability of the right TALEN monomers to bind their recognition site within sufficient proximity to form a FokI dimer with the left TALEN (Fig 4.3). Thus, a targeted allele should no longer be an accessible substrate for the TALENs to cleave.

Given the prevalence and variety of indels occurring in all of the heterozygotes obtained we hypothesise that the introduction of exogenous sequence to the 5’ end of the hESPL1 was poorly tolerated by hTERT-RPE1 cells. Further clarification of whether the indels are due to cell or species-specific intolerance, non-specific TALEN binding and activity, or exogenous sequence incompatibility would require extensive exploration beyond this study. It is not clear that identifying the reasons for the occurrence of the indels would lead to successful insertion and expression of an affinity tag into human separase.
In conclusion, we generated a number of tagged separase heterozygotes, none of which expressed detectable levels of the protein. We were unable to obtain a homozygote clone and hypothesise that this may be due to indels in the 5’UTR and further upstream in the hESPL1 locus that are representative of an intolerance of the manipulation to this essential gene.
5 Impacts of DNA damage on the centrosome

5.1 Introduction
Centrosome duplication is normally tightly controlled by the cell cycle machinery. Despite this, centrosome amplification or over-duplication can still arise from a number of sources (Loffler et al., 2006). One such source is via the DNA damage response (DDR), an intricate signalling network that delays cell cycle progression and initiates repair of DNA lesions after genotoxic stresses (Ciccia and Elledge, 2010). Given that DNA damage phenotypes such as centriole splitting and centrosome amplification are believed to be due to the misregulation of the normal regulating proteins and duplication factors, we hypothesised that key proteins required for duplication licensing such as separase and PLK1 are likely to be involved in over-duplication. Similarly, the expansion of the PCM that facilitates mitosis might occur at aberrant cell cycle stages.

As another approach to investigating the role of separase at the centrosome after DNA damage we sought to test the contributions of the protease’s activity on its centrosomal substrates. Whether there exists a distinct isoform or regulation of cohesin that localises it specifically to the centrosome has yet to be determined. Any potential impacts arising from manipulation of the SCC1 protein would be very difficult to attribute accurately to cohesin’s role at the centrosome over its essential role in sister chromatid cohesion. Instead we focused on pericentrin, a centrosomal substrate of both PLK1 and separase. The recent identification of the large coiled-coiled protein that is a major component of the PCM as substrate of separase led us to hypothesise that it also might play a role downstream of separase in the molecular events that result in centrosome amplification after damage (Li et al., 2001; Matsuo et al., 2012; Lee and Rhee, 2012).

In addition to its multiple roles as a major PCM component, pericentrin recruits and interacts with a number of ATM network components such as CHK1, MCPH1, PKA, and DISC1, as discussed in more detail in the Introduction. CHK1 is a major effector of the DDR (Bartek and Lucas, 2003). A requirement for CHK1 has been demonstrated in centrosome amplification after a range of different genotoxic insults (Loffler et al., 2007; Robinson et al., 2007; Bourke et al, 2007). How CHK1 activation in the DDR leads to centrosome amplification is not clear. Current models
include the activation of the centrosome-regulatory Cdk2 kinase (Bourke et al., 2010), and the excessive assembly of centriolar satellites (Löffler et al., 2013), a process that also occurs during extended S-phase delays that permit centrosome overduplication (Prosser et al., 2009).

Research in our own group demonstrated that pericentrin is involved in the centrosomal responses to DNA damage (Wang et al., 2013). In addition to pericentrin, the tumour suppressor, MCPH1/microcephalin/Brit1 (hereafter MCPH1), is a CHK1 interactor and regulator that also localises to the centrosome and contributes to the regulation of centrosome number (Alderton et al., 2006; Griffith et al., 2008; Brown et al., 2010). Overexpression of pericentrin can distort the PCM and cause centrosome overduplication in S-phase-arrested cells (Lawo et al., 2012; Loncarek et al., 2008). However, the impact of DNA damage on the PCM is not known. In this study we aimed to elucidate the regulatory links between separase, pericentrin and the DDR that result in aberrant centrosome numbers and structure.
5.2 The impact of DNA damage on the PCM.

To further investigate the convergence of both cell cycle and DDR regulators on pericentrin, we sought to monitor the protein itself after DNA damage. Tagging pericentrin with a fluorescent protein would allow us to visualise the protein and potential alterations that might be induced by the DDR. Overexpression of pericentrin leads to genetic instability arising from atypical and supernumerary centrosomes (Chen et al., 2004; Kim et al., 2008; Loncarek et al., 2008), meaning the introduction of tagged pericentrin cDNA under the control of an exogenous promoter into cells would likely confound any structural analyses of the PCM.

![Schematic of pericentrin-GFP](image)

### Figure 5.1 Centrosomal localisation of pericentrin-GFP.

**A. Schematic of pericentrin-GFP.** The coding sequence for sfGFP was knocked into the chicken PCNT locus immediately 5’ of the stop codon in exon 40. The Pcnt-GFP cell line was generated by Dr. Alicja Antonczak.

**B. IF micrograph.** Pcnt-GFP localises to the centrosome with markers α-Tubulin, Centrin3, γ-Tubulin, PCM1 and PCNT. Scale bar, 5 µm. The IF gallery was created by Lisa Mullee.

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Therefore, we opted to knock the coding sequence for superfolding GFP (sfGFP) into the endogenous pericentrin (PCNT) locus (Fig 5.1A). Due to the range of relevant mutant DT40 cell lines we had available for this study we chose to also create the knockin in the DT40 cell line. Homozygote clones were identified by Southern blot (data not shown) and verified for correct localisation by immunofluorescence (IF) microscopy counterstaining with centrosome markers (Fig 5.1B). Three-dimensional structured-illumination microscopy (3D-SIM) was used to obtain a detailed visual analysis of the Pcnt-GFP localising to discrete toroidal structures surrounding the centrioles throughout the cell cycle (Fig 5.2A). The volume of these Pcnt-GFP structures increased, while maintaining the toroidal shape during mitosis, as previously described in earlier SIM analyses (Lawo et al., 2012; Menella et al., 2012; Fu et al., 2012; Sonnen et al., 2012).

**Figure 5.2 3D-SIM microscopy of DT40 centrosomes.**

A. **Toroidal PCM structure.** Anti-GFP was used to visualise Pcnt-GFP; green around the centrioles and shows co-localisation with Cep135, B glutamylated tubulin, C centrin3; red, at all cell cycle stages examined. An increase in Pcnt-GFP volume was seen in mitosis. Scale bar, 0.6 μm. D. 3D-SIM of pericentrin in DT40 cells using antibodies to the N-terminal region (PCNT-N; green) or to the C terminus (red) confirms the exterior positioning of the N-terminus of pericentrin. Scale bar, 0.6 μm. SIM experiments were conducted by Dr. Alicja Antonczak.
We analysed our C-terminal GFP tag in combination with an antibody to the N-terminus of pericentrin and found that the C-terminus GFP toroidal signal lies within the toroidal signal from the N-terminal antibody (Fig 5.2D). This orientation of pericentrin within the PCM was consistent with that reported for both human pericentrin and the *Drosophila* orthologue, PLP (Lawo et al., 2012; Mennella et al., 2012). Thus, 3D-SIM visualisation of the PCM in chicken DT40 cells revealed that pericentrin is anchored with its C terminus at the centriole and its N terminus extending outwards to the centrosomal periphery, as is seen in other metazoan centrosomes.

**Figure 5.3 IR-induced changes in the PCM.**

**A. 3D-SIM of a G2 cell.** Antibodies to Cep135 (red) and GFP (green) were used to visualise G2 cells 24 h after exposure to 5 Gy. Both centrosomes are shown. Scale bar, 0.6 μm. SIM experiments were conducted by Dr. Alicja Antonczak. **B, C. Light microscopy analyses.** Individual G2 centrosomes from cells of the indicated genotype were imaged, before and 4 h after 5 Gy IR, using antibodies to pericentrin (scale bar, 0.6 μm) or CDK5RAP2 (scale bar, 1 μm). Generation of CHK1, PCNT, MCPH1 and PCNT/MCPH1 mutant DT40 cell lines has been described previously, as have the CHK1 rescue lines (Bourke et al., 2007; Brown et al. 2010; Wang et al., 2013; Zachos et al., 2003).
5.3 Volumetric analyses of DNA damage-induced PCM expansion.

Next, we examined the effect of irradiation (IR) on the PCM. As shown in Figure 5.3A, IR treatment led to a marked dispersion and loss of discrete PCM signal that was clearly distinct from the alterations that accompany PCM maturation during the onset of mitosis, showing that one impact of the DDR is the disruption and expansion of the PCM. We then used indirect immunofluorescence with antibodies to pericentrin and CDK5RAP2, another PCM component (Barr et al., 2010), to image the PCM (Fig 5.3B and C). To ensure that the effects on the PCM were DDR-dependent and did not include mitotic effects on the PCM, (Lee and Rhee, 2011) we restricted our analysis to G2 cells by examining the PCM only in cells with two centrosomes and non-condensed chromosomes. To measure changes in the PCM, three-dimensional volumes of the PCM were determined using the Volocity software. After IR, the mean volume of the PCM increased significantly when measured using antibodies to either pericentrin or CDK5RAP2, plateauing by 3–4 h post-treatment (Fig 5.3 and 5.4).

- **Figure 5.4 IR-induced changes in the PCM require CHK1 and pericentrin.**
  - The PCM of G2 centrosomes was visualised by staining with anti-CDK5RAP2 antibodies before and 4 h after 5 Gy IR in cells of the indicated genotype. Data show individual (black) and mean (red) volumes ± s.e.m. calculated from 90 G2 centrosomes in three separate experiments. Statistical comparisons indicated in red are with the untreated wild-type sample, and other comparisons are as indicated. ***P<0.001; **P<0.01; NS, not significant by t-test.

Given the effects of MCPH1 and CHK1 on centrosome amplification, and their reported interactions with pericentrin we sought to determine if they contributed to the dynamics and dependencies of this expansion and dispersion of the PCM. As
shown in Figure 5.4, the mean CDK5RAP2-defined PCM volume in wild-type cells increased by 28% after IR. Mean PCM volumes in CHK1-deficient cells were 21% lower compared with those seen in wild-type cells before IR and showed no increase in volume after exposure.

The PCM volumes in untreated pericentrin-deficient cells showed no difference from wild-type cells but exhibited no increase after IR. MCPH1 deficiency caused a significant 15% increase over wild-type cells in the mean PCM volume before IR, which was further increased by 15% after IR treatment. Interestingly, deletion of pericentrin from MCPH1-deficient cells restored the PCM volume to wild-type levels and blocked any increase in PCM volume after IR (Fig 5.4). The pericentrin-defined PCM volumes showed similar trends after IR, with wild-type cells expanding by 27%, MCPH1 null by 56% and CHK1 by a nonsignificant 1% (data not shown). Taken together, these results indicate that the expansion of the PCM after DNA damage requires CHK1 and pericentrin, and is restrained by MCPH1.

5.4 Effects of non-cleavable pericentrin on DNA-damage induced phenotypes.

Given the reported role of separase-mediated cleavage of pericentrin promoting centriole disengagement and thus permitting centriole duplication, we hypothesised that this pericentrin cleavage might also promote the expansion of the PCM (Lee and Rhee et al., 2012; Matsuo et al., 2012; Agircan et al., 2014). Separase is activated by DNA damage (Prosser et al., 2012), therefore pericentrin might be a relevant centrosomal target of the DDR. We cloned full-length chicken pericentrin and generated a mutant without the consensus sequence for separase cleavage (ΔSep) (Fig 5.5A and B). The transient expression of wild-type pericentrin had no significant impact on PCM expansion in wild-type cells or MCPH1 knockouts. Although wild-type pericentrin expression did not significantly rescue PCM expansion in PCNT knockouts, which we attribute to the low level of expression attained in transient experiments, the expression of the ΔSep pericentrin mutant led to a reduced PCM expansion in wild-type and MCPH1-deficient cells (Fig 5.5D), suggesting that the cleavage of pericentrin is required for PCM expansion. We saw an accumulation of cells with 2C DNA content in ΔSep transfectants but not in cells that overexpressed wild-type pericentrin (Figure 5.5C), consistent with previous data indicating a role for pericentrin cleavage in cell cycle progression (Lee and Rhee et al., 2012; Matsuo et al., 2012).
Figure 5.5 Separate-mediated cleavage of pericentrin promotes PCM expansion.

Chicken pericentrin was cloned into pGEM-T Easy, sequenced and assembled by restriction ligation into pEGFP-C1, yielding pEGFP-cPCNT. The chicken PCNT sequence so generated has been deposited in GenBank as KM262664. cPCNT was cloned by Dr. Yifan Wang (Antonczak et al., 2015). 

A. Pericentrin ΔSep. WT DNA coding sequence for the separase consensus site SPE2493IMR was point mutated to code for the non-cleavable SPAIMA protein sequence. Sequencing results from PCR-generated ΔSep candidates confirmed that the site-directed point mutations had been introduced to the cPCNT sequence.

B. Pericentrin WT and ΔSep. Schematic depicting the position of the key amino acids required for separase directed cleavage on the pericentrin protein. cDNAs for both WT and ΔSep were generated and verified by sequencing.

C. Flow cytometry analysis. PCNT-deficient DT40 cells were analysed before and 24 h after transfection with the indicated pericentrin expression constructs.

D. Volumetric analysis. PCM volumes were visualised by staining with anti-CDK5RAP2 antibodies before and 4 h after 5 Gy IR in cells of the indicated genotype. Data show individual (black) and mean (red) volumes ± s.e.m. calculated from 90 G2 centrosomes in three separate experiments. Statistical comparisons indicated in red are with the untreated wild-type sample, and other comparisons are as indicated. ***P<0.001; **P<0.01; NS, not significant by t-test.
5.5 CHK1 requirements in DNA damage-induced centrosome abnormalities.

The requirement for CHK1 in PCM expansion suggested that the PCM might be a target of the DDR. We therefore tested the requirement for CHK1 kinase activity and the highly conserved phosphorylation site at Ser-345 by stably expressing defined CHK1 mutants on a CHK1-deficient background (Bourke et al., 2007). Ser-345 mutation normally ablates CHK1 functions (Bourke et al., 2007; Lee and Rhee et al., 2011) but, interestingly, it has been reported that CHK1 deficiency can be complemented by targeting a non-phosphorylatable Ser-317Ala/Ser-345Ala mutant form to the centrosome by a PACT domain fusion (Niida et al., 2007). As shown in Figure 5.6, expression of wild-type CHK1 fully restored the PCM response to IR. Unexpectedly, both the kinase-dead and the Ser-345-Ala mutants of CHK1 supported a DNA damage-induced expansion in PCM volume, even though this response was greatly reduced in cells that expressed the kinase-dead version of CHK1. These results demonstrate that CHK1, but not necessarily its activity, is required for PCM expansion.

Where in the cell CHK1 acts to allow centrosome amplification is controversial. Both centrosomal and nuclear locations for the key Cdk1-regulatory activities of CHK1 have been indicated, with conflicting results regarding its precise localisation (Kramer et al., 2004; Tibelius et al., 2009; Matsuyama et al., 2011; Gruber et al., 2011). As shown in Figure 5.6B, GFP-tagged CHK1 localised to both the nucleus and the cytoplasm before IR. After DNA damage, however, a significant fraction localised to the centrosome, as was seen after bleomycin treatment (Niida et al., 2007). To examine whether PCM expansion might require CHK1 outside the nucleus, we used site-directed mutagenesis to ablate the reported nuclear export signal (NES) and nuclear localisation signal (NLS) of CHK1 (Wang et al., 2012) and then expressed these mutant forms of CHK1 in CHK1-null DT40 cells (Zachos et al., 2003). Consistent with the impact of similar mutations within the human CHK1 sequence (Bekker-Jensen et al., 2006), the ΔNES form of chicken CHK1 was retained within the nucleus and the ΔNLS mutant excluded (Fig. 5.6B). Notably, neither the NLS nor the NES mutant was observed at the centrosome, regardless of radiation treatment. However, the volume of the PCM increased after IR in cells expressing either the NLS or the NES mutant, albeit to a nonsignificant level in cells that expressed the NLS mutant (Figures 5.6B and C).
Figure 5.6 PCM expansion requires CHK1 but not its activity.

**A and C. Volumetric analysis.** PCM volumes visualised by staining with anti-pericentrin (A) or transiently (C) transfected with CHK1 rescue constructs. Data show individual (black) and mean (red) volumes ± s.e.m. calculated from 90 G2 centrosomes in three separate experiments. ***P<0.001; **P<0.01; *P<0.05; NS, not significant by t-test. **B. Micrographs with localisation of WT and mutant CHK1 rescues.** Micrographs show CHK1-deficient cells 24 h after transfection with expression plasmids for the indicated CHK1 mutant. Where indicated, cells were treated with 5 Gy. GFP-CHK1 signals are shown in green and indirect immunofluorescence detection of γ-tubulin in red. DNA was labelled with DAPI (4',6-diamidino-2-phenylindole) (blue). Scale bar, 6 μm. Mutants were generated by Dr. Alicja Antonczak (Antonczak et al., 2015). **D. Immunoblot of immunoprecipitates.** Cells transfected with the indicated GFP-CHK1 expression constructs were treated with 5 Gy and harvested 2 h later. Immunoprecipitation was then performed using GFP-Trap M beads, before washing and analysis by SDS-PAGE.
When we examined the extent to which CHK1 became activated in response to DNA damage, we found that the radiation-induced increase in CHK1 Ser-345 phosphorylation seen in the wild-type form was abrogated by the deletion of the NLS sequence (Figure 5.6D). The NES deletion mutant showed a higher level of basal activation, but was not further stimulated upon IR. Taken together, these data show that PCM expansion can occur without proper CHK1 activation, but that CHK1 activation requires its appropriate, dynamic localisation between the nucleus and the cytoplasm.

5.6 Contributions of MCPH1 to the CHK1 response to DNA damage.

Given the altered IR responses of the PCM in cells that lack pericentrin or MCPH1, we next tested how CHK1 responded to DNA damage in these mutants. In total cellular lysates, we saw no effect of pericentrin or MCPH1 deficiency on CHK1 phosphorylation after IR (data not shown). However, as this analysis would not reveal the intracellular dynamics of checkpoint activation, we performed cell fractionation experiments to separate nuclear and cytoplasmic cell components.

Actin was used as a control for the cytosolic fraction and Scc1 cohesin for the nuclear components (Fig 5.7A). A number of different protein species were detected with the anti-phospho-CHK1 antibody that we used, particularly in the nucleus (Fig 5.7B). Although we do not know precisely what they represent, we consider these to be specific, because they were absent from equivalent fractions from CHK1−/− cells. A time-course analysis after 5 Gy IR revealed a weaker induction of nuclear CHK1 phosphorylation in pericentrin-deficient cells compared with that in wild-type cells, with MCPH1−/− cells showing notably more robust CHK1 activation compared with wild-type cells (Fig 5.7C). A less pronounced difference was seen in cytoplasmic CHK1 phosphorylation. Interestingly, the double PCNTΔ/ΔMCPH1−/− mutants showed approximately wild-type levels of nuclear CHK1 phosphorylation, indicating that the two genes can act as suppressors of one another with respect to CHK1 activation (Fig 5.7C), which correlates with their effects in DNA damage-induced centrosome amplification (Wang et al., 2013).
We next tested the localisation of phosphorylated CHK1 after IR treatment. As shown in Figure 5.8, antibodies to Ser-345-phosphorylated CHK1 detected robust induction of nuclear foci in irradiated cells. We did not see any signal at centrosomes under these conditions. These foci colocalised with γ-H2AX (data not shown) and, while some background signal was detected in CHK1−/− DT40 cells, this did not increase after IR (Fig 5.8). Although these data contrast with a previous analysis in bromodeoxyuridine-sensitised, microirradiated U2OS cells, which found that CHK1 did not localise to breaks (Bekker-Jensen et al., 2006), we conclude that IR-induced foci (IRIF) of phosphorylated CHK1 at the sites of DNA damage are still being detected. Quantitating the cellular intensity of the phospho-CHK1 IRIF, we found...
that pericentrin deficiency greatly attenuated the activation of CHK1 over time after 5 Gy IR. This activation was restored by the deletion of MCPH1 (Fig 5.8). Taken together with the centrosome amplification data and our previous analysis that demonstrated a sustained CHK1 activation in the absence of MCPH1 (Brown et al., 2010), these observations suggest that pericentrin is necessary for full CHK1 activity and that MCPH1 normally restricts CHK1 activation. With the impact that pericentrin deficiency has on the PCM, it is possible that CHK1 activation occurs, at least in part, at the centrosome in a pericentrin-dependent manner.

Figure 5.8 Pericentrin is necessary for complete CHK1 activation that is normally restricted by MCPH1.
A. Immunofluorescence microscopy of phospho-CHK1. Foci in cells of the indicated genotype were analysed before and 30 min after 5 Gy IR. Nuclear IRIF identified as being above background are highlighted. Scale bar, 10 μm. B. Quantitation of phospho-CHK1 IRIF. Cells of the indicated genotype were analysed after 5 Gy IR. Data points are the mean ± s.d. of three experiments in which at least 4000 cells were scored per experiment. Experiment conducted by Dr. Yifan Wang (Antonczak et al., 2015).
5.7 Requirements for centrosome amplification in response to DNA damage.
We next analysed the levels of DNA damage-induced centrosome amplification as an additional readout of CHK1 activity. We have previously shown that CHK1 kinase, its activity and the phosphorylation site at Ser-345 are required to allow centrosome amplification after DNA damage (Bourke et al., 2007; Bourke et al., 2010). We observed no significant centrosome amplification in cells that expressed the CHK1 NLS mutant, in keeping with the lack of CHK1 activation (Fig 5.9B). Interestingly, expression of the NES mutant significantly reduced cells’ capacity for centrosome amplification after IR, suggesting that the extranuclear movement of CHK1 is required to allow control levels of centrosome amplification in response to IR treatment. These data indicate a separation of CHK1-dependent functions at the centrosome: PCM expansion, which requires CHK1 but not its activity, is not sufficient to allow centrosome amplification in the absence of CHK1 activation.

Similar to PCM expansion (Fig 5.4), centrosome amplification was potentiated by MCPH1 deficiency and suppressed by loss of pericentrin (Wang et al., 2015). Pericentrin overexpression reduced DNA damage-induced centrosome amplification in wild-type and MCPH1-deficient cells, but had no impact on amplification in a pericentrin-deficient cell background (Fig 5.9C-E). However, overexpression of the mutant that lacked the separase cleavage site caused a significant reduction in centrosome amplification on all three backgrounds (Fig 5.8C-E). These data implicate the PCM in controlling centrosomal amplification after DNA damage. Although centrosome amplification occurred normally in pericentrin-deficient cells (Wang et al., 2013), we suggest that the cleavage of existing pericentrin, and thus the dynamic alteration of the PCM, may potentiate amplification in a manner not seen where cells have none to begin with.
Figure 5.9 IR-induced centrosome amplification requires CHK1 mobility and pericentrin cleavage.

**A. IF analysis of amplification.** Micrograph shows centrosomes in a wild-type cell before and 24 h after 5 Gy IR treatment, stained with antibodies to centrin3 (red) and Cep135 (green), with DNA shown in blue. Scale bar, 5 μm. **B-E. Quantitation of amplification.** Centrosome numbers were quantitated using antibodies to centrin3 in wild-type, and CHK1-, MCPH1- and PCNT-null cells transfected with the indicated GFP-CHK1 or GFP-pericentrin expression constructs. Histograms show mean ± s.d. of three experiments in which at least 100 cells were quantitated. ***P<0.001; **P<0.01; NS, not significant by t-test.
Our findings in this study indicate a significant role for separase-regulated pericentrin cleavage in promoting PCM expansion and sustaining centrosomal CHK1 activity. The impact of removing MCPH1 on the PCM implicates MCPH1 as a negative regulator of CHK1 activity at the centrosome. Collectively, these data suggest that pericentrin and MCPH1 function as opposing regulators of CHK1 activity.
6 Discussion and future perspectives

Throughout this project we aimed to elucidate how the centrosome was affected by the DDR, with particular interest in the potential contributions from separase. The separase activity sensor confirmed the presence of active separase at the centrosome at mitosis, encouraging us to investigate the direct regulation of separase itself. Our additional approaches analysed the impact of DNA damage on centrosomal substrates of separase and the DDR regulators CHK1 and MCPH1.

The separase activity sensor was developed at the beginning of this research project. The initial aim was to confirm the proposed centrosomal activity of separase (Gorr et al., 2005; Tsou et al., 2006; Tsou et al., 2009) in our experimental system, the chicken DT40 cell line. The sensor’s ability to detect separase in vivo was tested and optimised using a chromosome-localising (H2B-CSG) version of the sensor. Live cell microscopy enabled real-time monitoring of separase activity at the chromosomes. Activity was detected as fluorescent signal changes at the chromosomes induced by cleavage of the SCC1 fragment linking two fluorescent proteins. Basic quantitation of the relative intensities of fluorescent signals at the region of interest confirmed that ability of the sensor to detect separase activity during mitosis. This result confirmed the feasibility of our aim to detect separase in vivo at the centrosome.

We next redesigned the sensor to probe for separase activity at the centrosome. To achieve this we trialled centrosome-localising protein tags that would direct and anchor the sensor to the centrosome throughout the cell cycle. The human AKAP450 domain failed to localise the sensor to the centrosomes so we next tried chicken Centrin4 and Chibby, both small centrosomal proteins (171 aa and 126 aa respectively), and confirmed co-localisation of both Centrin4- and Chibby- tagged sensors with multiple centrosome markers. Analysis of the stable cell lines by live cell microscopy allowed us to track the centrosome and monitor for separase activity. The centrosome sensor signal was expectedly smaller and of a lower intensity than that of the chromosome sensor. This weaker signal proved difficult to detect robustly using the centre’s microscope facility, but accessing a spinning-disc confocal microscope improved the quality of our live cell analyses. Cleavage of the centrosome-localising sensor was detected in early mitosis. These results were also
seen in immunoblot assays that compared the levels of intact full length sensors to cleavage products detected after cell populations concentrated in prometaphase were released into anaphase after release from a nocodazole block. This detection of separase activity in DT40 mitosis validated further investigation of the protease and its centrosomal substrates in the DT40 experimental system.

Given reports in the literature of APC/C, PLK1 and separase activity in response to DNA damage (Inanc et al., 2010; Prosser et al., 2012; Douthwright et al., 2014), we next investigated the effect of DNA damage on separase activity as detected by the sensor. Preliminary immunoblot assay results, in both the chromosome-localising- and centrosome-localising sensors, indicated that the sensors were being cleaved in response to DNA damage in asynchronous cell populations. We next considered expressing the centrosome-localising sensor in DT40 cell lines deficient in various DDR proteins to investigate if they contribute to separase activation after DNA damage. However, control experiments with chromosome-localising- and centrosome-localising sensors expressing non-cleavable SCC1 indicated that the cleavage detected after IR was not specific to separase. There are reports of SCC1 being specifically cleaved after an aspartic acid (Asp279) residue C-terminal to the separase cleavage site (Arg172) by a caspase in cells undergoing apoptosis, in response to multiple stimuli (Chen et al., 2002; Pati et al., 2002). This finding indicates that Scc1 is targeted for cleavage in apoptotic pathways, that can be activated in response to damage, thus the cleavage we see may instead be due to caspase activity in an early apoptotic response to the IR dose administered. Predictive analyses of the SCC1 protein sequence identified additional potential death-box domains; however, these predicted sites require further exploration to determine their authenticity.

While the sensor proved unsuitable for investigating DNA damage impacts on separase activation, confirmation of separase activity at the centrosome in DT40 cells was valuable to the future direction of our research presented in Chapter 5.
Chapter 6. Discussion and future perspectives

Our decision to affinity-tag separase in the human cell line hTERT-RPE1 was influenced by a number of factors. Our main motivation to tag endogenous separase was to investigate any potential post-translational alterations to the protein in response to DNA damage that might account for the activation reported in the study by the Fry lab (Prosser et al., 2012). The chicken DT40 system afforded no major advantage over other available systems for this study. While we did consider proceeding in this system, the incompleteness of the chicken genomic database, with regard to the 5’ region of the separase locus, presented an obstacle in designing homology arms for promotion of HR-based genetic recombination. In addition, the emergence of TALENs as an accessible tool for genome editing in human cells (Miller et al., 2011; Hockemeyer et al., 2011), afforded an exciting opportunity to target the hESPL1 locus. hTERT-RPE1 cells were selected based on their non-transformed status and, most importantly, their karyotypic stability (ATCC, 2015).

Although CRISPR technology was also emerging at the design stages of this study, it was still in its infancy and had only been reported as tool for gene disruption rather than precise incorporation of donor sequence, with unresolved concerns regarding off-target effects (Jinek et al., 2012). TALENs, on the other hand, had already been used to introduce specific insertions in human somatic and pluripotent stem cells using double-stranded donor templates (Miller et al., 2011; Hockemeyer et al., 2011). TALEN off-target effects can be countered by choosing unique target sequences that differ by at least 7 nucleotides from any other site in the genome (Kim et al., 2013). Unlike TALENs, early CRISPR editing nucleases functioned as monomers, inducing unwanted mutations at off-target sites that differ by up to several nucleotides from on-target sites (Cradick et al., 2013; Fu et al., 2013; Hsu et al., 2013; Cho et al., 2014), risking thousands of potential off-target sites per CRISPR in the human genome.

The PCR-based targeting screen identified clones heterozygous for the unique affinity tag sequence introduced from our exogenous donor matrix to the 5’ end of the hESPL1 locus. The edited coding sequence was verified when the heterozygotes were initially obtained. However, no protein expression was detected, either before or after heterozygote floxing, despite clones screening positive for both wildtype and targeted allele transcripts. Nor were any homozygotes obtained, despite multiple second round targeting and screening experiments. In search of clues to the lack of
expression, we re-examined the upstream, non-coding sequence of the heterozygote clones and discovered indels and/or duplication events in the 5’ UTR or further upstream in each clone.

The reasons for these divergences from the expected sequence were unclear. Re-sequencing of our donor sequence ruled out potential design or cloning error in the homology arms or insert sequence. We also reviewed the the double-stranded donor matrix, designed to introduce minimal unique sequence while still incorporating two affinity tag options with a hydrophilic linker. There were no obvious features of the design that would logically induce indels or prevent translation. While one could reduce the linker length, it had been deliberately designed and codon-optimised to prevent any interference with protein folding after translation. The length of our homology arms fell within the recently recommended range for recombination efficiency (Shin et al 2014; Byrne et al., 2015), suggesting that increasing homology length would be unlikely to increase targeting efficiency or fidelity.

TALENs’ requirement to form a dimer for function requires independent monomers to recognise and bind DNA sequence in sufficient proximity to allow the FokI nuclease domain to dimerise (Bitinaite et al., 1998). While this requirement increases the specificity of TALENs for their programmed target, off-target events are still a concern (Koo and Kim, 2015). Improper repair of both on- and off-target DSBs can give rise to deletions (Lee et al., 2010), inversions (Lee et al., 2012; Park et al., 2014), in addition to local point and larger mutations. We used two different TALENs to target two proximal but distinct sites flanking the start ATG codon in hESPLI. With both pairs, the TALEN binding sites should only recognise wild-type separase sequence. The incorporation of the unique affinity tag sequence immediately 3’ of the ATG codon was designed to alter the local sequence to the extent that it would no longer be a substrate for the TALENs. The additional sequence insert prevents the right TALEN monomers from binding their recognition site within sufficient proximity to form a FokI dimer with the left TALEN. Thus, a targeted allele should no longer be an accessible substrate for the TALENs to cleave.

Heterozygote clones were obtained from separate targeting rounds using each pair, thus homologous recombination occurred. For the TALENs to be responsible for the indels and duplications, it would mean that both pairs were able to separately cleave
the genome at off-target sites. This being the case, it would most likely not be a worthwhile investment to design alternative TALENs to target the limited amount of appropriate target sequence remaining untested. Advances in CRISPR technology now offer an improved alternative tool for inducing DSBs (Kleinstiver et al., 2015; Tsai et al., 2014; Guilinger et al., 2014).

One could also consider switching experimental system to a different human cell line, or indeed a different species/model organism for further attempts at this targeting project. It is possible that other human cell lines, such as U2OS, might be more genetically tractable than hTERT-RPE1 cells, although such traits would have to be weighed up against the advantages of the non-transformed diploid, karyotypically stable hTERT-RPE1 cells which also possess a normal DDR. Ultimately, the 5′ indels and lack of translation might be simply due to an intolerance of manipulation to this essential gene. Separase knockout mice are embryonically lethal and flies and mice with hypomorphic or conditional separase alleles exhibit severe cell proliferation defects (Pandey et al., 2005; Kumada et al., 2006; Wirth et al., 2006). Notably, these manipulations were introduced further downstream in the murine separase locus than our targeted integration site immediately 3′ of the start ATG codon. The Kumada study introduced loxP sites into the intronic sequence flanking exon 6, while Wirth and colleagues incorporated loxP sites into the intronic sequence flanking exons 24-31, enabling conditional deletion of the eight C-terminal exons (Kumada et al., 2006; Wirth et al., 2006). Manipulations of the hESPL1 have also been reported, with Tsou and colleagues generating human somatic (HCT116) cells conditionally null for separase. This manipulation involved the introduction of loxP sites flanking exon 21 (Tsou et al., 2009). Given the position of these manipulations in the separase locus, it is possible that these targeted alleles may permit the translation of fragments of separase protein that retain some cell-cycle regulatory functions. Retention of such function might increase the tolerance of manipulations to the separase gene, despite loss of proteolytic activity.

In summary, there are many factors to consider and test for to understand the occurrence of indels, duplications and lack of translation. However, it is not certain that understanding the origin of these obstacles would enable us to successfully generate tagged endogenous separase. This level of technical investigation was not a
direction we endeavoured to pursue in the scope of this study. Instead we approached our investigations into the impacts of DNA damage on the PCM and centriole disengagement by analysing key substrates at the centrosome, such as pericentrin.

To explore the impacts of DNA damage on the PCM, we analysed the impacts of IR on pericentrin, a major scaffold component of the PCM. We returned to the chicken DT40 experimental system as we had many reagents and cell lines in this system that were of particular relevance to the experimental questions we aimed to answer. We used three-dimensional structured-illumination (3D-SIM) and light microscopy to analyse pericentrin structure and centrosome amplification in response to IR. The pericentrin knockin cell line was generated to enable analysis of the protein without risking the confounding effects of altering its expression levels (Chen et al., 2004; Kim et al., 2008; Loncarek et al., 2008). These microscopy-based analyses found that IR treatment caused a loss of PCM organisation and an expansion in its volume (Fig 6.1). This effect was seen for both pericentrin and CDK5RAP2, with PCM expansion plateauing approximately 4 h post-IR treatment. Notably, we also saw this phenotype in hTERT-RPE1 cells in response to IR, but as outlined above, the DT40 experimental system offered us more scope for further exploration with regard to reagents and cell lines.

To better understand the controlling factors in centrosome abnormalities after DNA damage, the analysis of these features was extended to a number of DDR mutant cell lines. CHK1 is required for DNA damage-induced amplification (Dodson et al., 2004; Bourke et al., 2007; Loffler et al., 2007), whereas MCPH1 deficiency greatly increases the amplification seen after DNA damage (Brown et al., 2010). This effect was dependent on CHK1 and pericentrin, and was exacerbated in the absence of the CHK1 interactor and regulator, MCPH1 (Gruber et al, 2011; Tibelius et al, 2009). The extent to which PCM expansion arose reflected both the level of centrosome amplification after DNA damage, which is notably elevated in MCPH1-deficient cells and absent in CHK1 nulls (Alderton et al, 2006; Bourke et al, 2007; Brown et al, 2010). Following CHK1 activation after IR, by immunoblot and immunofluorescence microscopy, showed that the extent to which CHK1 activation persisted after genotoxic insult also influenced the extent to which the PCM expanded.
CHK1 has two highly conserved motifs, CM1 and CM2, which function as a nuclear export signal (NES) and nuclear localisation signal (NLS), respectively (Wang et al., 2012). We investigated the activation responses of NES- and NLS- CHK1 mutants to IR. We found that the IR-induced increase in CHK1 Ser-345 phosphorylation seen in the wild-type form was abrogated by the deletion of the NLS sequence. The NES deletion mutant showed a higher level of basal activation, but was not further stimulated upon IR. Collectively, these data show that PCM expansion can occur without proper CHK1 activation, but that CHK1 activation requires its appropriate, dynamic localisation between the nucleus and the cytoplasm (Kramer et al., 2004; Niida et al., 2007; Matsuyama et al., 2011; Pabla et al., 2012).

Figure 6.1 Signalling between the nucleus and the centrosome in response to DNA damage.
Schematic outlines the known signalling pathways from the nucleus that impact on the centrosome, as well as the potential centrosome-mediated signalling effects on nuclear responses. The mechanistic details of centrosomal effects on the nuclear DDR remain to be explored in detail.
Non-cleavable pericentrin was generated to investigate the requirement for separase-mediated cleavage in IR-induced effects on the PCM and centrosome duplication. The ΔSep pericentrin mutant led to a reduced PCM expansion in wild-type and MCPH1-deficient cells, indicating that the cleavage of pericentrin is required for PCM expansion. The accumulation of cells with 2C DNA content that we saw in ΔSep transfectants, but not in cells that overexpressed wild-type pericentrin was consistent with previous data indicating a role for pericentrin cleavage in cell cycle progression (Lee and Rhee et al., 2012; Matsuo et al., 2012). Non-cleavable pericentrin also caused a significant reduction in centrosome amplification, indicating that the PCM contributes to the control of centrosomal amplification after DNA damage. While centrosome amplification occurred normally in pericentrin-deficient cells (Wang et al., 2013), our recent data suggests that centrosomes are better able duplicate with no pericentrin rather than with the restriction of non-cleavable pericentrin. Thus, we hypothesise that the cleavage of existing pericentrin, and thus the dynamic alteration of the PCM, may potentiate duplication/amplification in a manner not seen where cells have none to begin with.

PCM disruption has been reported in Drosophila embryos and in mammalian cells as a consequence of replication stress (Hut et al, 2003; Sibon et al, 2000) so that the dynamics of the PCM are clearly responsive to the integrity of the genome and contribute to the control of centrosome number. How PCM changes influence the nuclear DNA damage response is not fully understood. With the data available to date, we propose a model in which PCM expansion, through separase-regulated pericentrin cleavage, stabilises an activated, centrosomal CHK1 signal that is normally downregulated through MCPH1. Our results support the existence of a feedback loop between PCM expansion and centrosomal CHK1 activity that ensures a checkpoint signal within the nucleus (Fig 6.2).
While there is evidence to support this model, the relationship between the centrosome and the DDR remains enigmatic, with many questions still to be answered. The role of the numerous DDR components at the centrosome is one such open question. A detailed review of the literature on a range of organisms concluded that there is no DNA at centrosomes (Marshall and Rosenbaum, 2000). Although there is evidence for some specifically-associated centrosomal RNA (Alliegro and Alliegro, 2008), this would not be expected to require the localisation of the DDR apparatus to centrosomes.

Current evidence suggests that the centrioles are not critical in cell cycle checkpoint control, despite being crucial for carrying out efficient mitosis and allowing normal development (Basto et al, 2006). The loss of centrioles caused by Sas4 deletion in murine fibroblasts (Bazzi and Anderson, 2014) or by Cep152 knockout in chicken DT40 cells (Sir et al, 2013) did not lead to radical changes in the ability of cells to respond to DNA damage, as determined by clonogenic assay or gamma-H2AX focus formation. The removal of centrioles by laser ablation or microsurgery (La Terra et al, 2005; Uetake et al, 2007) did not abrogate cell cycle progression, even without the de novo centriole assembly pathway re-establishing centrosomes. Notably, recent studies that used inhibition of PLK4 to ablate centrosomes found that

**Figure 6.2 PCM expansion working model.**
Schematic depicts the varying extents of expansion in response to DNA damage on the indicated genetic backgrounds. These results indicate a relationship between PCM expansion and centrosomal CHK1 activity that promotes complete CHK1 activation and amplifies a checkpoint signal within the nucleus. Not to scale.
centrosome loss imposed an indefinite checkpoint delay in non-transformed cells through a poorly-understood p53-dependent mechanism, even though cancer cells could continue proliferation in the absence of centrioles (Lambrus et al, 2015; Wong et al, 2015). It should be noted that the PCM in these examples was still present, although these components were no longer organised around the centrioles, and no detailed analysis was performed of the centriolar satellites. What happens with these structures in cells without centrioles will be informative in further integrating the centrosome into the DDR. How PCM changes influence the nuclear DNA damage response is not fully understood. We have proposed a feedback loop that involves the centrosome as a mechanism by which nuclear CHK1 activation is regulated (Antonczak et al, 2015). Thus, our working model is that the centrosome contributes to the overall timing and regulation of the DDR. The proximity of centrosomes to the nuclear pore, at the centre of a microtubule network, makes it feasible for them to serve as a nexus for DNA damage signals emanating from the nucleus. However, this renders it experimentally difficult to separate the centrosome from the nucleus and to dissect the role of centrosomes in modulating the DDR.

As discussed in section 1.7, it is clear that several signals- ATM/ ATR/ CHK1/ CHK2 kinase activity, BRCA1 ubiquitin ligase functions and polyADP-ribosylation by members of the PARP family- are involved in the regulation of centrosome duplication and function after DNA damage. Centrosome amplification is a general response to genotoxic stress. While there is excellent evidence to show that centrosome abnormalities strongly impact the cell cycle, centrosome amplification does not cause inevitable lethality (Ganem et al, 2009). Therefore, it appears that centrosome amplification is not simply an addition to the apoptotic machinery in ensuring the elimination of cells with excessive DNA damage. What the marked changes in centrosome organisation seen after DNA damage and in cells with defective DNA repair mean for the cell remains to be defined. In summary, a key question for the future is whether the centrosomal effects of DNA damage are stochastic effects of the dysregulation of the cell cycle, or a component of a concerted response to genotoxic stress.
The data presented in this thesis support the hypothesis that the centrioles and the PCM are involved in distinct regulatory activities initiated by DNA damage. Specifically, the findings shape our current working model in which PCM expansion, through separase-regulated pericentrin cleavage, stabilises an activated, centrosomal CHK1 signal that is normally downregulated through MCPH1. To test and develop this model, a number of outstanding questions should be addressed.

1. Can PCM expansion in the DT40 system be monitored and measured in real-time after DNA damage?

The GFP-PCNT cell line is an excellent reagent for visualising how the PCM responds to DNA damage. Live cell experiments with the fluorescently tagged sensor in Chapter 3, proved technically challenging in the DT40 system. However, as with the sensor, a spinning disc confocal microscope could overcome a number of these challenges. Live cell imaging of the GFP-PCNT cell line in response to DNA damage would provide more information about the rate and extent of dynamic PCM expansion/distortion.

2. Does endogenous CHK1 localise to the centrosome during the canonical cycle and/or in response to DNA damage?

The controversial localisation of CHK1 to the centrosome, as discussed in detail in section 1.7.3, remains contentious in the field. While our data shows overexpressed GFP-CHK1 localising to the centrosome after DNA damage, a more faithful and informative investigation would be achieved by tagging the endogenous protein (Antonczak et al., 2015). Testing whether endogenous CHK1 exhibits the same localisation behaviour would validate the overexpression findings. Targeting CHK1 with a visible tag for microscopy analyses, under different conditions and cell cycle stages, would allow us to determine its localisation pattern in our experimental system. Expanding these analyses into a live cell system where one could monitor any dynamic alterations in CHK1 localisation would provide insight into the nuclear-cytoplasmic shuttling, and centrosome-localisation, of this kinase (Kramer et al., 2004; Enomoto et al., 2009). Furthermore, the inclusion of an auxin-inducible
degron on the knockin tag would afford refined control over loss of CHK1 via the rapid, auxin-induced depletion of the tagged protein (Nishimura et al., 2009), thus allowing finer dissection of both where and when CHK1 contributes to centrosome phenotypes after DNA damage.

3. Is the presence of CHK1, active or inactive, at the centrosome sufficient to induce PCM expansion and/or centrosome amplification?

Our data on the impact of various CHK1 mutants on the centrosomes after DNA damage suggest that PCM expansion and to a certain extent centrosome amplification can occur without proper CHK1 activation. CHK1 localisation to the centrosome may be exclusive to post-DNA damage cellular responses. Forcing CHK1, active or otherwise, to the centrosome would indicate whether the presence of the kinase alone is sufficient for PCM expansion and subsequent centrosome amplification. Tagging CHK1 with a centrosome-localising domain such as AKAP450, either endogenous CHK1 or expressing tagged CHK1 cDNA in the null mutant, would allow us to test whether other DNA damage response factors, either in concert or parallel, are required to initiate these phenotypes after DNA damage.

4. How does MCPH1 negatively regulate CHK1 activity and where does this regulation occur?

CHK1 centrosome localisation, reported to couple the centrosome cycle with mitosis via the CDC25-CDK1 pathway, is dependent on MCPH1 (Gruber et al., 2011). Centrosomal targeting of chicken MCPH1 was shown to be independent of ATM, BRCA1 or CHK1 (Jeffers et al., 2008). How MCPH1 defects cause loss of CHK1 at the centrosome is not fully understood. Abolishing centrosome localisation of MCPH1 would indicate whether its negative regulation of CHK1 activity requires its localisation to the centrosome. Identifying which domains of MCPH1 are required for the negative regulation of CHK1 might give insight into how and where the regulation occurs. As with question 2, increasing our library of endogenously-tagged proteins of interest would also provide useful tools for interaction experiments to establish if CHK1 interacts, even if only transiently, with pericentrin and MCPH1 after damage. If direct interactions are detected, mapping the regions responsible would help us better understand the nature of those interactions.
Together these experiments will define how pericentrin, MCPH1, and CHK1 contribute to PCM and centrosome activity, as well as cell cycle control, in both the presence and absence of DNA damage. Understanding these mechanisms will provide insight into a molecular relationship that is significant for the DNA damage response, the importance of which is underlined by its dysregulation/disruption leading to microcephaly and dwarfism.
7 References


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Appendix 1. hSeparase N-terminal tag

Sequence of affinity tag designed and synthesised for hESPL1 knockin targeting strategy.

CTACGCGTGAAGTCGACGCAGGTACC GCCGCGACGCGGCCGCGAGACTACAAGGACCAC
GACGGGCACAAGGACACATCACTACTACAAGGATGACGATGACAAAGAGCGCTGCTGCCCT
TGGCTGGTCGCCCCACTGGTTGGAGGGGCGTTGTGGCCTGGCGCGCGACGCGCTGCTGCG
GCAGCTGGAGCCATTTGGAGAAAGACGCAGCGGCGGTGGCGGGTGGCGGCAGCGGTGGCGGC
GGTGGCGGCAGCTGGAGCCATCCACAGTTTGAGAAAG

Breakdown of unique tag sequence, exonic and intronic hESPL1 sequence, and restriction sites included for assembly into final targeting vector

[ MluI - SaII - KpnI - Lick - 3x Flag - link - StrepII - link - Ndel ]

CT **ACGCGT** GA **GTGCAC** GCA **GTTACC** GCC GCC AGC GCC GCC GCC GCA GAC TAC
AAG GAC CAC GAC GCC GAC AAG GAC CAC GAC ATC GAC TAC AAG GAT GAC GAT
GAC AAG AGGAGCTGGCCCTGGCTGGGCGGACGCGCCGCCTGGCTGGCTTGGG

(Exon 2, 16-93 bases) AGGAGCTCTC AAAAGAGTCA ACTTTGGGAG TCTGCTAAG C
AGCCAGA AGGAGGCTG AAGA GTTGCTGCCC GACTTGAAAG

Intron 2 (1-27 of 177 bases-BamHI)

GTGGGGGTTGCT GCCTGGCTCG **GGATCC** GAC [BamHI]

The GAC insertion is just to allow digestion of the fragment without the necessity for insertion into a plasmid.
Appendix 2. Scientific communications

Publications

**Centrosomes in the DNA damage response- the hub outside the centre.**

**Opposing effects of pericentrin and microcephalin on the pericentriolar material regulate CHK1 activation in the DNA damage response.**

Scientific Presentations

**Editing of an essential gene: manipulation of the Separase locus in human cells.**
Lisa Mullee, Yifan Wang, and Ciaran G. Morrison.
FASEB Conference on Genome Engineering – Cutting Edge Research and Applications, Nassau, Bahamas, June 2014.

**A centrosome-localizing biosensor for Separase activity after DNA damage.**
Lisa Mullee and C.G. Morrison
Poster presentation at ASCB Annual Meeting in New Orleans, LA, December 2013. ASCB TV Q&A: https://www.youtube.com/watch?v=nYaK2UM_J0k (0:00-0:08 min and 1.41-1.58 min)

**The role of Separase in the DNA damage response.**
Lisa Mullee and Ciaran G. Morrison

**A molecular sensor to investigate the spatial and temporal activation of Separase in vivo.**
Lisa Mullee and Ciaran G. Morrison.
EMBO Practical Course on Microscopy, Modeling and Biophysical Methods, Heidelberg, Germany, August 2012.