### Table 1

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<tr>
<td><strong>Author(s)</strong></td>
<td>Conroy, Pauline Ciara</td>
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Centriolar and ciliary responses to DNA damage

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

September 2012

Supervisor: Prof. Ciaran Morrison
“The capacity to blunder slightly is the real marvel of DNA. Without this special attribute, we would still be anaerobic bacteria and there would be no music.”

Lewis Thomas
(1913 – 1993)
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<td>p53 binding protein 1</td>
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<tr>
<td>9-1-1</td>
<td>Rad9-Rad1-Hus1</td>
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<tr>
<td>AID</td>
<td>auxin-inducible degron</td>
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<td>AKAP450</td>
<td>A-kinase anchoring protein 450</td>
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<td>Aki1</td>
<td>Akt kinase-interacting protein 1</td>
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<td>ALMS1</td>
<td>Alström syndrome 1</td>
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<td>Ana2</td>
<td>anastral spindle 2</td>
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<td>APC</td>
<td>adenomatous polyposis coli</td>
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<td>APC/C</td>
<td>anaphase promoting complex/cyclosome</td>
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<td>APKD</td>
<td>Autosomal dominant polycystic kidney disease</td>
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<td>APS</td>
<td>ammonium persulphate</td>
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<td>Arl13B</td>
<td>ADP-ribosylation factor-like 13B</td>
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<td>ASPM</td>
<td>Abnormal spindle-like microcephaly-associated protein</td>
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<td>ATM</td>
<td>ataxia telangiectasia, mutated</td>
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<td>ATP</td>
<td>adenosine-5'-'triphosphate</td>
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<tr>
<td>ATR</td>
<td>ATM-Rad3 related</td>
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<td>ATRIP</td>
<td>ATR-interacting protein</td>
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<td>BB</td>
<td>basal body</td>
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<td>BBS</td>
<td>Bardet-Biedl syndrome</td>
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<td>BCC</td>
<td>Basal cell carcinoma</td>
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<tr>
<td>BLAP75</td>
<td>BLM-associated peptide 75</td>
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<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
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<td>Bld10</td>
<td>Basal body 10</td>
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<td>BLM</td>
<td>bloom syndrome protein</td>
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<tr>
<td>bp</td>
<td>base pair(s)</td>
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<tr>
<td>BRCA1</td>
<td>breast cancer associated gene 1</td>
</tr>
<tr>
<td>BRCA2</td>
<td>breast cancer associated gene 2</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BUB</td>
<td>budding uninhibited by benzimidazole</td>
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<tr>
<td>CDC</td>
<td>cell division cycle</td>
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<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CENP</td>
<td>Centromere protein</td>
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<tr>
<td>CEP</td>
<td>Centrosomal protein</td>
</tr>
<tr>
<td>Chk</td>
<td>checkpoint kinase</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>CIN</td>
<td>Chromosome instability</td>
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<td>C-NAP1</td>
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<td>CPAP</td>
<td>Centrosomal P4.1-associated Protein</td>
</tr>
<tr>
<td>CPC</td>
<td>chromosome passenger complex</td>
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<tr>
<td>C-terminus</td>
<td>carboxy terminus</td>
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<tr>
<td>DAPI</td>
<td>4&quot;, 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>Dhh</td>
<td>Desert hedgehog</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>Dna2</td>
<td>DNA replication helicase 2</td>
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<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
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<tr>
<td>DNA-PKcs</td>
<td>DNA-PK catalytic subunit</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide-5'-'triphosphate</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>DSB</td>
<td>double-strand break</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>Dvl2</td>
<td>Dishevelled 2</td>
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<tr>
<td>E2F</td>
<td>adenovirus E2 promoter binding factor</td>
</tr>
<tr>
<td>EB1</td>
<td>(microtubule) end-binding protein 1</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
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Abbreviations

EOC  epithelial ovarian cancer
ERK  extracellular signal-regulated kinase
ES   embryonic stem (cells)
Exo1 Exonuclease 1
FACS fluorescence-activated cell sorting
FANCD2 Fanconi anaemia, complementation group D2
FBS  foetal bovine serum
FITC fluorescein isothiocyanate
Fz   Frizzled
G   gravity
γ-TURC γ-tubulin ring complex
γ-TUSC γ-tubulin small complex
GCP  γ-tubulin complex protein
Gen1 Gen homologue, endonuclease 1
GFP  green fluorescent protein
Gli  Glioma
GMP  cerebellar granule precursor
GSK3 Glycogen synthase kinase 3
HEF1 Human enhancer of filamentation 1
Hh   Hedgehog
HPV  human papillomavirus
HR   homologous recombination
HSC  hematopoietic stem cell
hTERT-RPE1 human telomerase reverse transcriptase- retinal pigment epithelial
HTLV-1 human T-cell leukaemia virus type-1
Htr6 5-hydroxytryptamine (serotonin) receptor 6
HU   hydroxyurea
IF   immunofluorescence microscopy
IFT  intraflagellar transport
Ihh  Indian hedgehog
IMCD immortised kidney collecting duct
Invs Inversin
IR   ionizing radiation
IRIF IR-induced foci
kb   kilobase pair(s)
kDa  kilodaltons
Kiz  kizuna
KSHV Kaposi sarcoma herpes virus
LB   Luria-Bertani medium
LDL  Low-density Lipoprotein
MAD  mitotic arrest deficient
MAP  microtuble associated protein
MCC  mitotic checkpoint complex
MCHR1 Melanin Concentrating Hormone Receptor 1
MCPH Microcephalin
MDC1  mediator of DNA damage checkpoint protein 1
MDM2 mouse double minute 2
MEF  mouse embryonic fibroblasts
MIN  microsatellite instability
MKS  Meckel-Gruber syndrome
MRE11 meiotic recombination 11
MRN MRE11-RAD50-NBS1 complex
Mst2 Mammalian Sterile 20-like kinase 2
MTOC microtubule-organising centre
NBS1  Nijmegen breakage syndrome 1
NCS  newborn calf serum
NEDD1 Neural precursor cell expressed, developmentally down-regulated 1
NEK/NRK NIMA-related kinase
NHEJ non-homologous end joining
NIMA Never-in-mitosis A
<table>
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<td>NPHP-RC</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>SDS polyacrylamide gel electrophoresis</td>
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<td>Smoothened</td>
</tr>
<tr>
<td>SPB</td>
<td>spindle pole body</td>
</tr>
<tr>
<td>SPD-2</td>
<td>spindle defective protein 2</td>
</tr>
<tr>
<td>ss</td>
<td>serum starved</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>STAN</td>
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</tr>
<tr>
<td>STIL</td>
<td>SCL/TAL1 interrupting locus</td>
</tr>
<tr>
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</tr>
<tr>
<td>TAL1</td>
<td>T-cell acute lymphoblastic leukaemia 1</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TEMED</td>
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</tr>
<tr>
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<td>Tetracycline</td>
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<tr>
<td>TetO</td>
<td>Tet operator</td>
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<tr>
<td>Symbol</td>
<td>Abbreviation</td>
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</tr>
<tr>
<td>TG</td>
<td>tris-glycine</td>
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<tr>
<td>TIP60</td>
<td>Tat-interacting protein 60</td>
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<td>Topoisomerase II DNA binding protein 1</td>
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<td>Tumour protein 53</td>
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<tr>
<td>TRE</td>
<td>tetracycline-responsive promoter element</td>
</tr>
<tr>
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<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>tTA</td>
<td>tetracycline-controlled transactivator</td>
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<td>ultraviolet</td>
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<tr>
<td>ZYG-1</td>
<td>zygote defective protein 1</td>
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Declaration

I, Pauline C. Conroy, 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 Section 4.2.1, 4.2.2 and 4.2.5, which are clearly indicated in the figure legends, were performed as a joint project with C. Saladino and which are also presented in her thesis entitled “The impact of DNA damage on centrosomes”.
Acknowledgements

I would first like to thank my supervisor, Prof. Ciaran Morrison for giving me the opportunity to carry out a PhD in his group and for all his support, advice and encouragement over the years.

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A huge thank you to my parents, who have always been supportive and proud of me. Finally, a massive thanks to all my family and loved ones, who hadn’t a clue what I have been doing for the past four years and helped me to escape from the world of science when I needed to.

Pauline
I dedicate this thesis to my parents.
Abstract

Centrosome duplication is tightly controlled and takes place only once every cell cycle. In recent years more light has been shed on the mechanism of centrosome duplication, with key players being identified and characterised. We sought to characterise Stil, a gene mutated in autosomal recessive microcephaly and which encodes a centrosomal protein. We found that Stil localises to the centrosome in chicken DT40 and that it is evolutionarily conserved between chicken and mammals. We were unable to generate a Stil knockout cell line in DT40 and concluded that Stil is essential for viability and that precise expression levels are critical for viability.

Various centrosome abnormalities are observed in cancer cells and in cells with defective DNA damage responses. Moreover, centrosome abnormalities can arise following DNA damage. In this study, we aimed to characterise the impact of irradiation-induced DNA damage on the structure of the centrosome. We found that irradiation leads to the isolation of single centrioles, termed centriole splitting, and that this precedes DNA damage-induced centrosome amplification. We observed that DNA damage causes centriole splitting in non-transformed human cells, with the isolated centrioles carrying the mother markers Ninein and CEP170 but not Cenexin or Kizuna. Loss of centrosome cohesion through siRNA depletion of C-NAP1 or Rootletin increased radiation-induced centriole splitting, with C-NAP1-depleted isolated centrioles losing all maturation markers.

Primary cilia are sensory organelles which receive chemical and mechanical signals from the extracellular environment. Defects in primary cilia structure and signalling cause human diseases termed ciliopathies and cancer. As the mother centriole forms the primary cilium, we tested whether DNA damage or centriole splitting affected ciliogenesis. We found that irradiated cells formed apparently normal primary cilia, which arose primarily from centriole clusters and not isolated centrioles. Additionally, multiple primary cilia can form in irradiated cells. Furthermore, C-NAP1 or Rootletin knockdown reduced primary cilium formation. Therefore, the centrosome cohesion apparatus at the proximal end of centrioles may provide a target that can affect primary cilium formation as part of the DNA damage response.
Chapter 1. Introduction

1.1 The Centrosome

The centrosome was discovered at the end of the 19th century by Edouard Van Beneden and Theodor Boveri. The centrosome is the principle microtubule-organising centre (MTOC) in animal somatic cells and has evolved in multicellular organisms from the basal body/axoneme structure of the unicellular ancestor. In proliferating cells, the centrosome is responsible for the nucleating of microtubules. The centrosome regulates cell adhesion, motility, polarity, intracellular trafficking and organises the spindle poles in mitosis. The centrosome has gained increased interest in recent years with its involvement in essential cellular processes, in particular its requirement in the formation of a primary cillum, a microtubule-based antenna-like structure, present in most cells of the body (reviewed by Pazour and Witman, 2003; reviewed by Azimzadeh and Bornens, 2007).

Centrosomes are evolutionarily conserved structures but have been lost from many eukaryote phyla during evolution. Although centrosomes are important for the formation of the bipolar spindle in many organisms, they have been lost in fungi and higher plants, suggesting that centrosomes are not essential for the duplication and survival of cells (reviewed by Marshall, 2009). Yeast cells have developed what is called a spindle pole body (SPB), which functions in a manner equivalent to the centrosome and many core centrosome and SPB proteins are conserved (Carvalho-Santos et al., 2010; Hodges et al., 2010). Therefore, it is likely that the only truly vital role of centrosomes/centrioles is in the formation of cilia and flagella (reviewed by Dawe et al., 2007a). This theory has been confirmed in Drosophila melanogaster dsas4 (Spindle assembly abnormal 4) mutants, which lack centrioles but develop into morphologically normal adults. However, they die shortly after birth because their sensory neurons lack cilia (Basto et al., 2006).

1.1.1 The structure of the centrosome

Each centrosome in late G2 phase consists of two centrioles, which are each ~500 nm in length and ~200 nm in diameter and are orientated perpendicularly to each other. These two centrioles disengage and lose this perpendicular arrangement upon passage through mitosis but remain tethered by a fibrous linker (Figure 1.1). The centrosome is comprised of hundreds of proteins, including $\gamma$-tubulin and the $\gamma$-
tubulin ring complex (\(\gamma\)-TURC) and is surrounded by the pericentriolar material (PCM). The PCM, which is an amorphous mass of coiled-coil proteins, is the primary site of microtubule nucleation and increases in size throughout the cell cycle (Andersen et al., 2003; reviewed by Bettencourt-Dias and Glover, 2007; reviewed by Salisbury, 2007). Centrioles are barrel shaped, microtubule-containing structures that show complex nine-fold radial symmetry, a feature which is conserved from ciliated protists to humans. The nine sets of microtubule triplets which make up each centriole are composed of cylindrical polymers of \(\alpha/\beta\) tubulin heterodimers in mammals and taper to microtubule doublets towards the distal end of each centriole (reviewed by Kline-Smith and Walczak, 2004; reviewed by Etienne-Manneville, 2010).

![Figure 1.1. The structure of the centrosome.](image)

A. Schematic view of a G1 phase centrosome, consisting of two centrioles connected by interconnecting fibres. The mother centriole (yellow) can be distinguished by its distal and subdistal appendages. The centrosome is surrounded by the PCM (pink) and microtubules (green) are nucleated from \(\gamma\)-TURCs (red) (adapted from Doxsey, 2001). B. TEM micrograph of a centrosome, showing the mother centriole with appendages and the microtubule triplets of the daughter centriole. (TEM carried out in collaboration with T. Dantas)

Upon passage through mitosis, each cell inherits a single centrosome. The two centrioles that make up this centrosome are at different stages of maturity, with the older, mother centriole being at least a cell cycle older than the younger daughter centriole. Mother centrioles can be distinguished by two sets of appendages at their distal ends, termed the distal and sub-distal appendages. These appendages are thought to play a role in microtubule anchoring and in anchoring the centriole at the
plasma membrane during ciliogenesis (reviewed by Azimzadeh and Bornens, 2007; reviewed by Azimzadeh and Marshall, 2010). Components of these appendages include Ninein, CEP170 (Centrosomal protein 170), CEP164, Cenexin/ODF2 (Outer dense fiber protein 2) and ε-tubulin (Mogensen et al., 2000; Nakagawa et al., 2001; Bornens, 2002; Chang et al., 2003; Guarguaglini et al., 2005; Ishikawa et al., 2005; Graser et al., 2007a). Daughter centrioles mature and acquire appendages throughout the cell cycle but it takes more than one and a half cell cycles and two passages through mitosis for a newly duplicated centriole to become completely mature (reviewed by Azimzadeh and Bornens, 2007; reviewed by Azimzadeh and Marshall, 2010).

1.1.2 The centrosome as a microtubule nucleating and organising centre

Microtubules are hollow cylinders made up of α and β-tubulin heterodimers and are key components of the cell cytoskeleton, with the arrangement of microtubules around centrosomes being critical for intracellular protein trafficking, mitotic spindle assembly, cell polarity and cytokinesis. It is the organisation of the α and β tubulin proteins which gives a microtubule its intrinsic polarity, with microtubules having a slow-growing minus (α-tubulin) end and a fast-growing plus (β-tubulin) end. The minus-ends of microtubules are anchored at the centrosomes, while the plus-ends extend outwards into the cytoplasm. Microtubules can undergo rapid polymerisation and depolymerisation at their plus ends and display dynamic instability, where microtubules alternate between cycles of growth and shrinkage. This dynamic instability is particularly important during mitosis when the cytoskeleton undergoes rapid remodelling and chromosomes are segregated. Microtubules also act as tracks for dynein and kinesin motors which deliver membrane vesicles, proteins and regulatory factors to their destinations in the cell (reviewed by Kline-Smith and Walczak, 2004; reviewed by Etienne-Manneville, 2010).

The centrosome controls the nucleation and anchoring of microtubules during interphase and mitosis, with components of the PCM and certain protein kinases playing crucial roles. Pericentrin and AKAP450 (A-kinase anchoring protein 450) are PCM components involved in docking molecules that mediate the nucleation of microtubules, such as γ-tubulin, which exists in the γ-tubulin small complex (γ-
TUSC). The γ-TUSC consists of two γ-tubulin molecules and one molecule each of GCP2 and GCP3 (γ-tubulin complex protein 2 and 3) (reviewed by Bettencourt-Dias and Glover, 2007). In many eukaryotes, multiple γ-TUSCs associate with GCP4, GCP5 and GCP6 to form a γ-TURC (Guillet et al., 2011).

Microtubules are stabilised at the centrosome by γ-TURCs, which cap the minus ends of microtubules, inhibiting their depolymerisation (reviewed by Luders and Stearns, 2007). The anchoring of microtubules at the centrosome occurs at the sub-distal appendages of the mother centriole and in the PCM and depends on several components of the sub-distal appendages such as Ninein, Centriolin, Dynactin and EB1 (End binding protein 1) (reviewed by Dammermann and Merdes, 2002; Bettencourt-Dias and Glover, 2007). The C-terminus of Ninein connects to the centriole, while the N-terminus interacts with γ-TURCs. Although the exact mechanism of microtubule anchoring is not clear, it is known that in the absence of Ninein, γ-TURCs fail to anchor microtubules at the centrosome (Delgehyr et al., 2005).

The microtubule-organising activity of the centrosome reaches its maximum levels during mitosis. Plk1 (Polo-like kinase 1) phosphorylation of Pericentrin is needed for microtubule nucleation, spindle formation and the recruitment of other PCM proteins involved in centrosome maturation and microtubule nucleation, such as CEP192, Aurora A, γ-tubulin and NEDD1/GCP-WD (Neural precursor cell expressed, developmentally down-regulated 1) (Terada et al., 2003; Haren et al., 2006; Gomez-Ferreria et al., 2007; reviewed by Luders and Stearns, 2007; Guillet et al., 2011; Lee and Rhee, 2011). Non-centrosomal microtubules are thought to originate primarily at the centrosome and are released into the cytoplasm in a process which is important for cell migration and involves Katanin (Yu et al., 1993; Keating et al., 1997; Ahmad et al., 1999; Abal et al., 2002).

Cells that lack centrosomes can still organise apparently normal mitotic spindles and laser ablation of centrosomes in mammalian cells still allows for the formation of a functional bipolar spindle (reviewed by Doxsey, 2001; Hinchcliffe et al., 2001; reviewed by Luders and Stearns, 2007). However, when present, centrosomes play key roles in the organisation of the mitotic spindle. Centrosomes
are also important for the correct positioning of the mitotic spindle and in establishing the cleavage furrow site during cytokinesis. In cells where centrosomes have been ablated, there is an increased rate of failure in cytokinesis due to the inability of the spindle to properly reposition itself as the cell changes shape (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001). Centrosomal proteins such as CP110 (Centrosomal protein 110), CEP55, BBS6 (Bardet-Biedl syndrome 6) and Centriolin have been reported to localise to the midbody and are required for cell progression through cytokinesis and cleavage furrow assembly. siRNA depletion of CP110, CEP55, Centriolin and BBS6 leads to the formation of multinucleate cells due to cytokinesis failure (Fabbro et al., 2005; Gromley et al., 2005; Kim et al., 2005; Tsang et al., 2006; van der Horst et al., 2009). Furthermore, in Hela cells during cytokinesis, it was found that the mother centriole transiently leaves its central position in the cell and moves close to the intracellular bridge. Following this, cytokinesis is only completed after the mother centriole moves back to the centre of the cell, suggesting that the mother centriole may carry some sort of abscission control signal (Piel et al., 2000; Piel et al., 2001).

1.1.3 The centrosome cycle

The birth of a centrosome begins in many animals upon fertilisation, when the centrioles from the sperm unite with proteins from the egg to create the centrosome. This centrosome is then duplicated and participates in the first mitotic division of the embryo (reviewed by Schatten, 1994; reviewed by Hatch and Stearns, 2010). The centrosome cycle can be divided into four main stages: centriole disengagement, centrosome duplication, centrosome maturation and centrosome separation, as shown in Figure 1.2. In mitosis, centrosomes position themselves at opposite poles of the cell. Microtubules emanating from the centrosomes attach to the condensed chromosomes, which are then separated equally into the newly forming daughter cells (reviewed by Hinchcliffe and Sluder, 2001). In late mitosis, or early G1 phase, the mother and daughter centriole lose their orthogonal arrangement, in a process involving Plk1 and Separase and the two centrioles become disengaged but remain tethered. Each daughter cell inherits a single centrosome which will duplicate itself in the ensuing cell cycle (Tsou et al., 2009). New centrioles, termed procentrioles, assemble in late G1/early S phase adjacent to the proximal end of each mother centriole. These procentrioles lengthen and mature
throughout S and G2 phase (Kuriyama and Borisy, 1981). In late G2 phase, the
tether connecting the two newly duplicated centrosomes is cut and each centrosome
moves to opposite poles of the cell to organise the mitotic spindle (reviewed by Nigg,
2007).

**Figure 1.2. Co-ordination of the cell and centrosome cycles.**
Diagram showing the synchronised events of the cell and centrosome cycles. The four phases of the
cell cycle are shown in the centre (G1, S, G2 and M) as are the four main stages in the centrosome
cycle (centriole disengagement, procentriole formation, procentriole elongation/centrosome
maturation and centrosome separation). (Adapted from Mardin and Schiebel, 2012).

### 1.1.4 Centriole duplication

New centrioles are formed by two different mechanisms: *de novo* assembly or
semi-conservative assembly. *De novo* centriole assembly occurs naturally in multi-
ciliated cells, such as epithelial cells of the mammalian trachea, which are capable of
forming 200-300 centrioles during ciliogenesis (reviewed by Sorokin, 1962; Sorokin,
1968; Dawe et al., 2007a; Vladar and Stearns, 2007). *De novo* centriole assembly
can occur in CHO (Chinese hamster ovary) and Hela cells, where centrioles have
been removed (by laser ablation or microsurgery). Following laser ablation of
centrioles in CHO cells, new centrioles are formed during S phase, in the form of
PCM clouds containing γ-tubulin, Pericentrin and Ninein. These *de novo*-assembled
centrioles were formed in random numbers and were capable of forming multipolar
spindles during mitosis. In Hela cells, where centrioles were removed, it was found
that centrioles can regenerate, beginning with the formation of Centrin aggregates in S phase. These de novo-assembled centrioles were also capable of organising microtubules and duplicating in the next cell cycle. However, when just a single centriole was ablated, no extra centrioles were formed, indicating that centrioles, when present, suppress the de novo assembly of centrioles (Khodjakov et al., 2002; La Terra et al., 2005).

In most non-ciliated cells and in cells possessing a primary cilium, centriole duplication occurs via a semi-conservative or canonical pathway and is tightly regulated. The first step in centrosome duplication involves the disengagement of centrioles in mitosis, which is considered a key licensing step for centrosome duplication (see section 1.1.6) (reviewed by Tsou and Stearns, 2006a). The mechanisms surrounding centriole assembly have been extensively studied in Caenorhabditis elegans and from these studies five proteins essential for centriole assembly have been identified. SPD-2 (Spindle defective protein 2) is the first protein to be recruited to the parental centriole following fertilisation of the C. elegans embryo. This then allows the recruitment of ZYG-1 (Zygote defective protein 1) and in turn, this permits the SAS6-SAS5 (Spindle assembly abnormal protein 5 and 6) complex to be recruited. SAS5 and SAS6 are needed for the formation of the central tube, onto which microtubules are assembled in a SAS4-dependent manner (reviewed by Delattre et al., 2006; Pelletier et al., 2006; Azimzadeh and Bornens, 2007).

Conflicting results exist for SPD-2, whose human orthologue is CEP192 and is essential for centriole duplication, with the Drosophila orthologue appearing to be dispensable for this process (Dix and Raff, 2007; Zhu et al., 2008). PLK4/SAK (Snk/Plk-akin kinase), which has been proposed as the equivalent of ZYG-1 in C. elegans, is essential for centriole duplication in Drosophila and humans. Inhibition of Plk4 prevents centriole duplication in both human cells and Drosophila and its overexpression allows the formation of multiple daughter centrioles (Bettencourt-Dias et al., 2005; Habedanck et al., 2005).

C. elegans provides a useful model for studying centriole assembly, which occurs in two major steps. First a tubular structure, called the cartwheel, is assembled next to the mother centriole. The cartwheel is assembled at the proximal
end of the pre-existing centriole and is the scaffold for procentriole assembly. It consists of a central hub with nine symmetrical spokes, terminated by a pinhead structure onto which microtubules attach. The procentriole elongates by the addition of microtubules to the tip of the spokes, thus, retaining the nine-fold symmetry we see in centrioles. The cartwheel persists in *Chlamydomonas reinhardtii* but disappears in human cells during centriole maturation (reviewed by Strnad and Gonczy, 2008). The only component of the cartwheel to have been identified is Bld10 (Basal body 10), which is needed for centriole assembly in *C. reinhardtii*. Its human homolog is CEP135 (Matsuura et al., 2004; Mottier-Pavie and Megraw, 2009). Bld10 is also required for the formation of the central pair of microtubules in motile cilia/flagella in *Drosophila* and *Drosophila bld10* mutants are viable but male sterile, with immotile sperm which lack the central pair of microtubules (Mottier-Pavie and Megraw, 2009; Carvalho-Santos et al., 2012). However, Bld10 is not essential for cartwheel assembly/centriole formation in *Drosophila* but in the absence of Bld10, the cartwheel disassembles over time and Bld10 is thought to stabilise the connection between inner and outer centriole components (Roque et al., 2012). In mammals, CEP135 is also involved procentriole formation and is recruited to nascent procentrioles early in their formation in CHO and U2OS cells. Moreover, CEP135 serves as a platform protein, holding C-NAP1 (Centrosomal NEK2-associated protein 1) at the proximal ends of centrioles (Ohta et al., 2002; Kleylein-Sohn et al., 2007; Kim et al., 2008).

In human cells, SAS6 is essential for centriole assembly, ensuring that a single procentriole is formed adjacent to each mother centriole. Increased SAS6 levels allow the formation of multiple procentrioles next to each mother (Strnad et al., 2007). SAS6 also localises to the proximal region of ciliary axonemes, indicating a possible additional role in cilia assembly (Vladar and Stearns, 2007). Recruitment of SAS6 to procentrioles in *C. elegans* requires SAS5 and the two proteins interact with each other. Like SAS6, SAS5 is essential for centriole duplication and its likely orthologue in *Drosophila* has been identified as Ana2 (Anastral spindle 2) (Delattre et al., 2004; Leidel et al., 2005; Stevens et al., 2010). Interestingly, the human orthologue of Ana2 is STIL/SIL (SCL/TAL1 interrupting locus), which is also required for centriole duplication. STIL is recruited to procentrioles at the onset of centriole duplication and, like SAS6, it is degraded in mitosis (Strnad et al., 2007;
Stevens et al., 2010; Kitagawa et al., 2011; Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). Overexpression of STIL in human cells leads to the formation of multiple daughter centrioles around single mothers (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012).

The addition of microtubule triplets in centriole assembly is thought to occur sequentially, with single microtubules or A-tubules first attaching, followed by B-tubules and finally C-tubules. Attachment of microtubules requires SAS4, the human orthologue of which is CPAP/CENPJ (Centrosomal P4.1-associated protein/Centromere protein J) and is required for procentriole formation and elongation (Leidel and Gonczy, 2003; Basto et al., 2006; Kleylein-Sohn et al., 2007). Drosophila sas4 mutants lack centrioles but are viable. However, they die shortly after birth due to a lack of cilia (Basto et al., 2006; Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009; Tang et al., 2011). Both de novo and semi-conservative centriole assembly require SAK/PLK4, SAS6 and SAS4, therefore, these may be indispensable for both mechanisms of centriole assembly (reviewed by Rodrigues-Martins et al., 2008).

Microtubule triplets are stabilised by ε and δ-tubulin and centriole elongation begins in S phase. Proteins involved in centriole elongation include CP110, which is recruited to the centriole by CEP97, early in centriole biogenesis and is associated with the growing distal tips, suggesting a role in controlling procentriole length (Kleylein-Sohn et al., 2007; Spektor et al., 2007). Inhibition of CP110 or CEP97, leads to the formation of elongated centrioles, which also are formed following CPAP overexpression (Spektor et al., 2007; Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009). Finally, POC5 (Protein of centriole 5) is required to build the distal portion of centrioles, while OFD1 (Oral-facial-digital syndrome 1) is needed to control centriole length. In addition, POC1 is recruited to the procentriole early in assembly and also is found along the length of the centriole, therefore, it is thought to be required for both the early stages of centriole duplication and the control of centriole length (Azimzadeh et al., 2009; Keller et al., 2009; Singla et al., 2010).
1.1.5 **Centrosome maturation and separation**

Centrosome maturation occurs in late G2 phase when additional PCM proteins are recruited to the centrosome, in particular γTURCs, and this is accompanied by a dramatic increase in the microtubule nucleating capacity of the centrosome (Piehl et al., 2004). Plk1 plays a central role in centrosome maturation and inhibition of Plk1 results in monopolar spindles with reduced microtubule nucleation capacity (Lane and Nigg, 1996; Sumara et al., 2004; Lenart et al., 2007; Santamaria et al., 2011). Plk1 controls the recruitment of other PCM proteins involved in γ-tubulin attachment including CEP192, Pericentrin, CEP215 and NEDD1 (Haren et al., 2009). The phosphorylation of Pericentrin by Plk1 has also been shown to be required for the recruitment of NEDD1, γ-tubulin and Aurora A onto the centrosome during mitosis (Lee and Rhee, 2011). Activation of Plk1 requires Aurora A-dependent phosphorylation before entry into mitosis (Macurek et al., 2008).

Following centriole disengagement, the two centrioles in G1 phase are held together by a flexible linker connecting the proximal ends of centrioles, which allows partial movement of the daughter centriole around the mother (Piel et al., 2000). This movement was also observed in cells possessing a primary cilium, where the mother centriole is docked at the plasma membrane but the daughter centriole is able to change its position during ciliogenesis (Sorokin, 1962). However, before the onset of mitosis this linker is dissolved allowing the separation of centrosomes to form the bipolar spindle. Two of the structural proteins identified in this linker structure are CEP250/C-NAP1 and Rootletin. C-NAP1 localises to the proximal end of parental centrioles and acts as a docking site for Rootletin, which is thought to physically link the centrioles. Depletion of either C-NAP1 or Rootletin using siRNA depletion or antibody microinjection causes splitting of parental centrioles (Mayor et al., 2000; Bahe et al., 2005).

C-NAP1 is a cell cycle-regulated, coiled-coil protein which dissociates from the spindles in mitosis but re-accumulates at the proximal end of centrioles after cell division. It has been proposed to serve as a docking site for several other centriolar proteins (Fry et al., 1998a; Mayor et al., 2000; Bahmanyar et al., 2008). C-NAP1 recruits Rootletin to the centrosome, where Rootletin forms extensive fibres
connecting the centrosomes and also emanating from the proximal end of centrioles. Rootletin is another coiled-coil protein and is a major component of the ciliary rootlet of basal bodies in ciliated cells (Yang et al., 2002; Bahe et al., 2005; Yang et al., 2006). CEP68 and CEP215 are also required for centrosome cohesion. CEP68 appears to contribute to the linker and, like Rootletin, decorates fibres emanating from the proximal ends of centrioles and is thought to cooperate with C-NAP1 and Rootletin in maintaining centrosome cohesion. In contrast, CEP215 does not appear to form part of the linker or interact with C-NAP1 or Rootletin, but instead interacts with Pericentrin, and is discussed in more detail in Section 1.1.6 (Graser et al., 2007b).

NEK2 (NIMA (never in mitosis gene A)-related kinase 2) is a cell cycle regulated kinase, which localises to the centrosome and exists primarily as two splice variants, NEK2A and NEK2B (Fry et al., 1998b; Hames and Fry, 2002). NEK2A phosphorylates both C-NAP1 and Rootletin in late G2 phase and this leads to their dissociation from centrioles and centrosome separation (Mayor et al., 2002; Bahe et al., 2005). NEK2A has a binding site for PP1α (protein phosphatase 1α), which is able to form a complex with both NEK2A and C-NAP1 to down-regulate NEK2A activity. PP1α and NEK2A regulate centrosome cohesion by counteracting each other (Helps et al., 2000; Meraldi and Nigg, 2001; Mi et al., 2007). Another substrate of NEK2 activity is β-catenin, which is an integral part of the Wnt (Wingless-type MMTV integration site family) signalling pathway and has been linked to maintenance of centrosome integrity. β-catenin binds to and is phosphorylated by NEK2, in complex with Rootletin, and its phosphorylation is also required for centrosome separation (Dierick and Bejsovec, 1999; Bahmanyar et al., 2008).

More recently, two components of the Hippo pathway, MST2 (Mammalian Sterile 20-like kinase 2) and SAV1 (Scaffold protein Salvador 1), have been found to interact with NEK2A and regulate its ability to localise to the centrosome and phosphorylate C-NAP1 and Rootletin. SAV1-MST2-NEK2A cooperate with the kinesin-related motor protein, Eg5, to regulate centrosome separation (Mardin et al., 2010). MST2-NEK2A-PP1γ also form a complex in which PP1γ counteracts NEK2A activity. Plk1 in turn, can phosphorylate MST2 to prevent binding of PP1γ
and allow centrosome separation. Plk1 is likewise, responsible for directing Eg5 to the centrosome, where it promotes centrosome disjunction by physically separating the centrosomes, although the exact mechanism remains unclear (Mardin et al., 2011).

1.1.6 Centriole disengagement

Centrosome duplication begins in late G1 or early S phase but it is the disengagement of centrioles in mitosis or early G1 phase which licences centrioles for duplication. Wong et al. used cell fusion experiments to show that engaged G2 centrioles could not reduplicate in a cellular environment that supported duplication, while a G2 cytoplasm did not prevent re-duplication of G1 phase centrioles, indicating that centrosomes themselves, rather than the cytoplasm possess an intrinsic block to reduplication (Wong and Stearns, 2003).

Centrioles are held tightly together in an orthogonal arrangement, termed “engagement” until centriole disengagement takes place, in an action which involves Separase and Plk1. Tsou et al. used Xenopus laevis egg extract to characterise centriole disengagement. They demonstrated that centrioles which were incubated in interphase-arrested extract, failed to disengage and that new centriole growth did not occur. They went on to show that inhibition of the APC/C (anaphase promoting complex/cyclosome), a target of which is Separase, also blocked centriole disengagement, demonstrating the requirement of Separase for disengagement. When they inhibited Separase but not the APC/C, again centriole disengagement was prevented, thus proving that the APC/C is not directly responsible for centriole disengagement but that Separase is. Finally, they showed that incubation with Securin blocked both centriole disengagement and sister chromatid separation. These results eloquently demonstrated how the engaged state of centrioles after duplication is a mechanism for the centrosome-intrinsic block to reduplication (Tsou and Stearns, 2006b).

Tsou et al. demonstrated that loss of Separase prevents centriole disengagement during mitotic exit and delays the assembly of new centrioles in the subsequent S phase. However, most centrioles did eventually disengage, which led to the identification of Plk1 as an additional disengagement promoter. Inactivation of both Plk1 and Separase abolished disengagement and prevented the nucleation of
procentrioles in the next S phase. Plk1 is activated in late G2 or early M phase, before Securin degradation and Separase activation, indicating that Plk1 and Separase act at different times during mitosis to ensure that centrioles are disengaged (Tsou and Stearns, 2006b; Tsou et al., 2009).

Cohesin has recently been identified as a centriole-engagement factor. Sister chromatids are also held together during metaphase by a Cohesin ring consisting of Smc1-Smc3-Scc1-Scc3 (Structural maintenance of chromosomes 1 and 3, Sister chromatid cohesion 1 and 3). During anaphase, the APC/C targets the Separase inhibitor Securin for ubiquitin-mediated degradation. Degradation of Securin allows Separase to cleave the Cohesin subunit Scc1 and the sister chromatids begin to segregate (reviewed by Zou et al., 1999; Uhlmann et al., 2000; Haering and Nasmyth, 2003; Viadiu et al., 2005). The APC/C also targets cyclin B1, a partner of Cdk1, for degradation, thus, inactivating Cdk1 and allowing mitotic exit. Separase is held in check by its binding of both Securin and Cdk1/cyclin B1. Cdk1 binds Separase via its partner cyclin B1, which results in the inhibition of both Separase and Cdk1 (Gorr et al., 2005; Holland and Taylor, 2006). Centriole disengagement also requires the action of Separase, and this is a key licencing step for centrosome duplication in the subsequent cell cycle (Tsou and Stearns, 2006b; Tsou et al., 2009). Scc1 localises to the centrosome and is cleaved by Separase along with chromatin Scc1, suggesting it acts as a connector of engaged centrioles as well as sister chromatids (Nakamura et al., 2009; Schockel et al., 2011). Moreover, Smc1 localises to the centrosome throughout the cells cycle and with the mitotic microtubules at the spindle poles, also suggesting a centrosomal function for Smc1 (Guan et al., 2008; Wong and Blobel, 2008).

It has been shown that both premature sister-chromatid separation and premature centriole separation is induced by ectopic activation of Separase or depletion of Sgo1 (Shugosin 1), both of which can be suppressed by expression of non-cleavable Scc1 or by inhibition of the prophase pathway (by Plk1 inhibition and the depletion of the Cohesin-associated factor WAP1 (Whey acidic protein 1)). Moreover, it was found that artificially cleavable Scc1 or Smc3 triggered centriole disengagement (Schockel et al., 2011). siRNA depletion of Scc1 also causes the separation of mother and daughter centrioles in mitotic cells (Beauchene et al., 2010; Gimenez-Abian et al., 2010; Schockel et al., 2011). Wang et al. identified sSgo1, a
small splice variant of Sgo1 as a protector of centriole cohesion. sSgo1 is found at centrosomes in interphase and spindle poles during mitosis and is regulated by Plk1. Furthermore, ectopic expression of sSgo1 suppresses Sgo1-induced centriole splitting (Wang et al., 2008). Taken together, these data indicate that similar to sister chromatid cohesion, the Cohesin complex may act as a molecular glue which holds engaged centrioles together (Schockel et al., 2011). These results are consistent with those of Nakamura et al., who showed that Cohesin functions in Separase-dependent centriole disengagement through Aki1 (Akt kinase-interacting protein 1) which is required for the centrosomal recruitment of Scc1 (Nakamura et al., 2009). Astrin has also been implicated in centriole disengagement, and its down-regulation activates Separase and causes premature centriole disengagement. Therefore, the emerging picture is that Cohesin holds mother and daughter centrioles together until they disengage and the C-NAP1-Rootletin linker is established (Mayor et al., 2000; Bahe et al., 2005; Thein et al., 2007; reviewed by Mardin and Schiebel, 2012).

More recently, Separase-dependent cleavage of Pericentrin B/Kendrin has been found to be necessary for centriole disengagement at the exit of mitosis. Cleavage-resistant Pericentrin B blocked centriole disengagement and subsequent duplication (Lee and Rhee, 2012; Matsuo et al., 2012). Pericentrin B encircles the walls of mother centrioles and interacts with CEP215 (CDK5RAP2), another centrosomal protein implicated in centrosome cohesion. Furthermore, it has been suggested that these two proteins contribute to both the mother-daughter and the mother-mother centriole linkages (Graser et al., 2007b; Barrera et al., 2010; Buchman et al., 2010; Matsuo et al., 2010; Matsuo et al., 2012).

1.1.7 Co-ordination of the cell and centrosome cycles

Faithful DNA replication and centrosome duplication are crucial for the error-free transmission of genetic information to the next generation of daughter cells. These events are tightly coordinated, with both DNA replication and centrosome duplication occurring only once every cell cycle (Figure 1.2). The centrosome acts as a scaffold for anchoring cell cycle regulatory proteins, whose association with the centrosome is essential for cell cycle control (reviewed by Doxsey et al., 2005). As noted in Section 1.1.2., acentrosomal cells often fail in
cytokinesis and those which complete cytokinesis do not initiate DNA replication, clearly implicating the centrosome in DNA replication and the cell cycle (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001).

Cdk (cyclin-dependent kinase) activity regulates both the cell and centrosome cycles and ensures their co-ordination. The activation of Cdk4/6 and Cdk2, following temporal overexpression of cyclins D, E and A, triggers entry into S phase and initiation of centrosome duplication and DNA synthesis. Cyclin D, in complex with Cdk4/6, triggers DNA replication partly through the phosphorylation of Rb (Retinoblastoma protein) and the activation of the transcription factor E2F (Adenovirus E2 promoter binding factor). This then promotes synthesis of cyclins A and E, which, in association with Cdk2 drive entry into S phase and the initiation of DNA synthesis. While the centrosomal role of Cdk4/6-cyclin D remains unclear, Cdk2 in complex with cyclins A and E plays an essential role in centrosome duplication in mammalian cells (Hinchcliffe et al., 1999; Lacey et al., 1999; Meraldi et al., 1999; Matsumoto and Maller, 2004; Ferguson and Maller, 2010).

More evidence for the role of Cdns in control of centrosome duplication comes from studies on NPM/B23 (Nucleophosmin), originally identified as a nucleolar phosphoprotein. NPM is a suppressor of licencing in the centrosome cycle and suppresses centrosome amplification. It is a target of Cdk2/cyclin E during the initiation of centrosome duplication and localises to unduplicated centrosomes. Upon phosphorylation by Cdk2/cyclin E, it dissociates from the centrosome, in a licencing step necessary for centrosomes to initiate duplication (reviewed by Tokuyama et al., 2001; Harrison et al., 2011). Cdk2 also regulates other proteins which participate in centrosome duplication; for instance, CP110 is a target of Cdk2/cyclin E, Cdk2/cyclin A and also Cdc2/cyclin B (Chen et al., 2002; Harrison et al., 2011).

Recent studies, however, have demonstrated the redundancy of Cdk2 in relation to centrosome duplication. In cells that lack Cdk2, Cdk1 has been shown to be essential for DNA replication and centrosome duplication, suggesting an overlap of these two kinases in S phase control. Inhibition of Cdk1 was found not to induce re-licensing of DNA replication in G2 phase, however it does allow the re-
duplication of centrosomes and thus, the uncoupling of the cell and centrosome cycles (Hochegger et al., 2007; Steere et al., 2011).

Furthermore, the APC/C plays a role in coordinating the centrosome and cell cycles by mediating chromosome segregation and centriole disengagement in mitosis, by activating the cysteine protease, Separase as detailed in Section 1.1.6 (reviewed by Salisbury, 2007).

1.2 DNA damage responses and genome instability

Maintaining genome integrity is fundamentally important for the proper function and survival of an organism. Dividing cells are under constant assault from endogenous and exogenous sources of DNA damage. Endogenous sources of DNA damage include reactive oxygen species from cell metabolism and replication errors such as stalled and collapsed replication forks. Exogenous sources include ionising radiation (IR), ultraviolet (UV) radiation and chemical agents and can lead to many forms of DNA damage, including double strand breaks (DSBs), mispairs and base modifications, for example. DSBs are the most dangerous form of DNA damage and unrepaired DSBs can lead to chromosomal rearrangements, which in turn can cause cell senescence, cancer and other diseases, as well as cell death. Different types of genomic rearrangements can result from DSBs, such as translocations, deletions, fusions and aneuploidy. There are an estimated 10 DSBs per cell per day, therefore it is essential that these DSBs are rapidly detected and repaired (reviewed by Sancar et al., 2004; reviewed by Lieber, 2010; reviewed by Thompson, 2012). Once DNA damage has been detected, cells respond to DNA damage by arresting the cell cycle, initiating DNA repair processes and expressing genes involved in the DNA damage repair process. The coordination of this complex process depends on the DNA damage checkpoint pathway (reviewed by Liu et al., 2006).

1.2.1 The DNA damage repair checkpoint network

DNA damage checkpoints delay or arrest cell cycle progression in response to DNA damage. The DNA damage checkpoint consists of three main components; damage sensors, mediators/signal transducers and effectors. The DNA damage checkpoint response is controlled by members of the PIKK (phosphoinositide 3-kinase-related kinase) family. Following DNA damage the PIKK family kinases, ATM (Ataxia telangiectasia, mutated) and ATR (ATM and Rad3 related)
phosphorylate target proteins at serine and threonine residues, which then activates the DNA damage checkpoint (reviewed by Sancar et al., 2004; reviewed by Warmerdam and Kanaar, 2010).

Full activation of ATM is dependent on autophosphorylation at Ser376, Ser1893 and Ser1981, and interaction with the MRN complex, consisting of MRE11-RAD50-NBS1 (Meiotic recombination 11-Radiation sensitive 50-Nijmegen breakage syndrome 1) at DSB sites. MRN is thought to act as a sensor of DNA damage that tethers DNA ends together before repair and which can further increase ATM activity by unwinding the DNA ends. The MRN-ATM complex triggers two main pathways concluding in chromatin remodelling. Phosphorylation of a number of ATM targets are required for an efficient ATM-mediated DNA damage response (reviewed by Liu et al., 2006; reviewed by Bartek and Lukas, 2007; reviewed by Warmerdam and Kanaar, 2010). Mediators of DNA damage signalling through ATM include 53BP1 (p53 binding protein 1), BRCA1 (Breast cancer 1), and MDC1 (Mediator of DNA damage checkpoint protein 1). 53BP1 helps promote ATM activation and functions in the G2-M checkpoint while BRCA1 is needed for ATM-mediated phosphorylation events and checkpoint control (reviewed by Liu et al., 2006; reviewed by Cann and Hicks, 2007). ATM phosphorylates H2AX, which in turn serves as a docking site for MDC1, an adaptor/mediator protein. MDC1 binds directly to γ-H2AX and also physically interacts with NBS1. This allows the spreading of H2AX phosphorylation and provides a basis for the assembly of other DNA damage-response proteins (Lou et al., 2006). γ-H2AX is needed for the retention of BRCA1, 53BP1 and the MRN complex at DNA damage sites (reviewed by Cann and Hicks, 2007). NBS1 and Artemis are also phosphorylated by ATM. It has been suggested that NBS1 phosphorylation by ATM recruits specific substrates to DNA damage sites. Phosphorylation of Artemis by ATM is important for the trimming of damaged or non-homologous nucleotides (Lim et al., 2000; Ma et al., 2002; Chen et al., 2005).

MRN and ATM are also crucial to initiate DSB break resection and the formation of single stranded DNA (ssDNA), which is a structural intermediate for DNA repair by homologous recombination and for ATR signalling. ATR signalling is begun in response to RPA (Replication protein A) that has been loaded onto
ssDNA gaps or resected DNA. This DNA resection requires the activity of Cdns and occurs primarily in S and G2 phase of the cell cycle. ssDNA is coated and stabilised by RPA, which can then interact with the ATR-ATRIP (ATR-interacting protein) complex and recruit the complex to the site of DNA damage (Zou and Elledge, 2003; Zou et al., 2003). Independently, the Rad17-RFC (Replication factor C) complex is loaded onto the sites of DNA damage, which is made up of Rad17 and RFC2-5. The Rad17-RFC complex facilitates the loading of the 9-1-1 (Rad9-Rad1-Hus1) clamp onto DNA, where it functions in the DNA damage response and is necessary for Chk1 activation and subsequent checkpoint signalling (Zou et al., 2003; Parrilla-Castellar et al., 2004; Delacroix et al., 2007; Lee et al., 2007; Lee and Dunphy, 2010).

Another protein, TopBP1 (Topoisomerase II DNA binding protein 1), which localises to DNA damage sites, is required for ATR activity, as ATR only becomes fully active following interaction with TopBP1. TopBP1 binds Rad9 of the 9-1-1 complex, where it can then interact with ATR in the ATR-ATRIP complex (Delacroix et al., 2007; Lee et al., 2007; Lee and Dunphy, 2010; Takeishi et al., 2010). Another important interaction is the ATR-phosphorylation of Claspin, which is required for Chk1 phosphorylation and activation by ATR. DNA damage-induced phosphorylation of Claspin by ATR reduces its targeting for degradation, thus increasing Chk1 activation. The assembly of Claspin onto ssDNA requires the phosphorylation of Rad17 by ATM/ATR (Bao et al., 2001; Post et al., 2001; Wang et al., 2006).

The effector kinases Chk1 and Chk2 are critical in spreading the checkpoint signal throughout the cell. Activated Chk1 and Chk2 phosphorylate the phosphatase Cdc25A (Cell division cycle 25A) in G1-S phase and Cdc25C in G2-M phase, thus arresting the cell cycle (Discussed in detail in sections 1.2.2.2 and 1.2.2.3). In general, the DSB signal is sensed by ATM and DNA-PK (DNA-dependent protein kinase) and transduced by Chk2, while ATR is activated by ssDNA and stalled replication forks and the signal is transduced by Chk1, although there is some overlap in the functions of the two proteins (reviewed by Sancar et al., 2004; reviewed by Bartek and Lukas, 2007).
1.2.2 DNA damage-induced cell cycle checkpoints

Following DNA damage, the cell cycle is halted in order to give the cell sufficient time to repair the damage. There are four main checkpoints activated in response to DNA damage: the G1-S checkpoint, the intra-S checkpoint, the G2-M checkpoint and the spindle assembly checkpoint. During this process Chk1/Chk2 are important in distributing damage signals and arresting the cell cycle (reviewed by Sancar et al., 2004; reviewed by Liu et al., 2006).

1.2.2.1 The G1-S checkpoint response

The G1-S phase checkpoint response occurs when cells receive damage to their DNA during G1 phase and are prevented from entering S phase. DSBs caused by IR or radiomimetic agents, activate the ATM-Chk2-Cdc25A pathway, while DNA damage caused by UV light or UV-mimetic agents activates the ATR-Chk1-Cdc25A pathway. Rapid activation of either pathway is followed by p53-mediated G1/S arrest. ATM/ATR directly phosphorylates p53 at Ser15, while Chk2/Chk1 can phosphorylate p53 through Thr18 and Ser20 (Banin et al., 1998; Canman and Kastan, 1998; Canman et al., 1998; Sakaguchi et al., 2000; Shieh et al., 2000). In the meantime, MDM2 (Mouse double minute 2), the ubiquitin ligase for p53, is phosphorylated and loses its ability to ubiquitylate and degrade p53 (Craig et al., 1999; Schon et al., 2002). The increased levels of p53 allow it to induce the transcription of p21, which inhibits the cyclin A/Cdk2 complex, thereby preventing entry into S phase. p21 also binds to the cyclin D/Cdk4 complex, which prevents it from phosphorylating Rb. This in turn, prevents the transcription factor E2F (required for the transcription of S phase genes) from being released, thus preventing S phase entry (Hatakeyama and Weinberg, 1995; Brehm et al., 1999; Craig et al., 2003).

1.2.2.2 The intra-S phase checkpoint arrest

The intra-S phase arrest is activated when DNA damage occurs in S phase or if damage has escaped the G1-S phase arrest. Replication is blocked and firing of late origins of replication is prevented. Following a DSB, activation of the intra-S checkpoint requires ATM, the MRN complex and BRCA1 to both sense the damaged DNA and also initiate a signalling cascade. The intra-S checkpoint then proceeds via two alternate 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, thus inhibiting Cdk2 activation. The inhibition of Cdk2 prevents assembly of Cdc45 and DNA polymerase α into pre-replication complexes, which blocks the firing of new origins of replication (Falck et al., 2001). The second involves SMC1 phosphorylation by ATM with the aid of BRCA1, FANCD2 (Fanconi anaemia, complementation group D2) and NBS1, which initiates an arrest and also a repair process involving the Cohesins SMC1 and SMC3 (Kim et al., 2002; Nakanishi et al., 2002; Taniguchi et al., 2002; Yazdi et al., 2002). When DNA is damaged by UV light or UV-mimetic agents, the main sensor involved is ATR-ATRIP. ATR phosphorylates Chk1, which subsequently phosphorylates and down regulates Cdc25A, again inhibiting Cdk2 and the firing of origins of replication (reviewed by Sancar et al., 2004; reviewed by Liu et al., 2006).

1.2.2.3 The G2-M checkpoint

The G2-M checkpoint prevents cells from entering mitosis in the presence of damaged DNA. Again, following DNA damage, ATM/ATR phosphorylation of Chk2/Chk1 leads to phosphorylation of Cdc25C, which then binds the 14-3-3 proteins, sequestering Cdc25C in the cytoplasm (Peng et al., 1997; Kumagai and Dunphy, 1999; Capasso et al., 2002). Wee1 is also up regulated and this, along with Cdc25C downregulation maintains the Cdk1/cyclin B complex in an inactive state and blocks entry into mitosis (O’Connell et al., 1997; Raleigh and O’Connell, 2000). ATM also phosphorylates p53 which initiates an upregulation in transcription of p21. p21 can inhibit Cdk2/cyclin A and Cdk4/6/cyclin D and this results in an inhibition of the target genes of E2F. p21 can also directly inhibit the Cdk1/cyclin B complex, ensuring it remains in an inactive state (Bunz et al., 1998; DeGregori, 2002; reviewed by Obaya and Sedivy, 2002; reviewed by Sancar et al., 2004).

1.2.2.4 The spindle-assembly checkpoint

The spindle assembly checkpoint (SAC) ensures that chromosomes are properly segregated and prevents mis-segregation and aneuploidy. The SAC monitors the attachment of kinetochores to the plus-ends of microtubules. During the SAC, Cdc20 is sequestered from the APC/C, which prevents the polyubiquitylation of Cyclin B and Securin, thus, preventing their degradation. Securin inhibits Separase from cleaving the Cohesin complex and the separation of sister chromatids, as detailed in Section 1.1.6., thereby preventing entry into anaphase. In the meantime, inhibition of degradation of cyclin B prevents Cdk1
inactivation, preventing mitotic exit (reviewed by Zou et al., 1999; Uhlmann et al., 2000; Viadiu et al., 2005; Musacchio and Salmon, 2007). The SAC ensures that all chromosomes are bi-orientated between separated spindle poles on the metaphase plate and once this has been satisfied, the SAC is terminated. Studies in *Saccharomyces cerevisiae* identified a number of proteins which act downstream of this checkpoint, including BUB1/2/3 (Budding uninhibited by benzimidazole), MAD1 (Mitotic arrest deficient 1), MAD2, MAD3/BUBR1 (BUB1-related), and MSP1 (Multipolar spindle protein 1), many of which have homologues in higher eukaryotes. It is thought that unattached kinetochores cause the formation of a mitotic checkpoint complex (MCC), consisting of MAD2, MAD3/BUBR1, BUB3, as well as Cdc20 and this complex prevents Cdc20 from activating the APC/C until all chromosomes are aligned. The MCC associates with the Cdc20 binding site on the APC/C, holding it in a locked state, to prevent the binding of APC/C substrates (reviewed by Musacchio and Salmon, 2007; Herzog et al., 2009; Chao et al., 2012).

1.2.3 DNA double-strand break repair

DSBs are the most cytotoxic form of DNA lesion and can result from external sources such as IR and radio-mimetic agents. DSBs can be particularly difficult to repair as they occur on both strands of the double helix. Failure to successfully repair both strands of DNA, without the loss or alteration of genetic information can contribute to genome instability. In eukaryotes, there are two major pathways for the repair of DSBs, which are non-homologous end joining (NHEJ) and homologous recombination (HR) (reviewed by Shrivastav et al., 2008; reviewed by Hartlerode and Scully, 2009).

1.2.3.1 Non-homologous end-joining

NHEJ is the major pathway for repair in G1/G0, however, it often results in the loss of genetic information, due to nucleotide deletions (reviewed by Jackson et al., 2002; reviewed by Mahaney et al., 2009). NHEJ does not need template DNA to repair the damage, instead it modifies two free DNA ends before joining them together (Figure 1.3). The first step in NHEJ is the detection of the DSB by Ku, which binds to the DSB. Ku is a heterodimer, consisting of a subunit of Ku70 and Ku80 and forms a ring structure, which on one side protects the DNA and on the other open side, allows other repair proteins to bind. The N-terminus of Ku recruits
DNA-PKcs ([DNA-dependent protein kinase catalytic subunit]). Other NHEJ proteins recruited to the DSB in the NHEJ pathway include, XRCC4 (X-ray repair complementing defective repair in Chinese hamster cells 4), DNA ligase IV, XLF (XRCC4-like factor) and Artemis (reviewed by Jackson et al., 2002; reviewed by Mahaney et al., 2009). The loading of XRCC4, in complex with DNA ligase IV stimulates DNA end-ligation. XLF also interacts with XRCC4 and its addition may stimulate DNA ligase activity towards ligation of non-compatible DNA ends, which may help prevent the loss of nucleotides and preserve the DNA sequence (Ahnesorg et al., 2006; Tsai et al., 2007; reviewed by Mahaney et al., 2009; reviewed by Lieber, 2010). Artemis directly interacts with DNA-PKcs to clean up DNA ends by trimming non-homologous or damaged nucleotides (Ma et al., 2002). Additional proteins involved in NHEJ are PNK (Poly[ribo)nucleotide kinase), WRN (Werner syndrome) and DNA polymerases μ and λ. PNK removes non-ligatable end groups from DNA ends, while WRN plays a role in processing the DNA ends prior to rejoining. DNA polymerases μ and λ carry out gap-filling functions (reviewed by Mahaney et al., 2009).

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Figure 1.3. DSB repair via the NHEJ pathway.
Schematic representation of DSB repair by NHEJ and the key proteins involved in the process. The DSB is recognised by Ku, which in turn recruits repair factors, such as DNA-PKcs and Artemis to the DSB for end-processing. The final end-joining is carried out by the XLF-XRCC4-DNA ligase IV complex and involves DNA pol μ and λ (adapted from Jackson, 2002).
1.2.3.2 Homologous Recombination

HR is a highly important mode of DNA repair which is utilised predominantly in S and G2 phase of the cell cycle. This pathway uses a homologous template from either the sister chromatid or the homologous chromosome to repair the damage, therefore it is less error-prone than NHEJ (reviewed by Liang et al., 1998; Bernstein and Rothstein, 2009). The first event in HR involves processing of the DSB to form an extended region of ssDNA, which is then bound by RPA (Figure 1.4). This step requires the recruitment of the MRN complex, EXO1 (Exonuclease 1), DNA2 (DNA replication helicase 2), CtIP/SAE1 (Sumo activating enzyme subunit 1) and SGS1 (Salivary gland secretion 1) to the damaged site. Rad51 subsequently binds to the damaged site, with the aid of mediator proteins, such as BRCA2 and several Rad51 paralogues for instance, Rad51B, Rad51C, Rad51D, XRCC2, XRCC3 (Tarsounas et al., 2004; reviewed by Bernstein and Rothstein, 2009; reviewed by Heyer et al., 2010). Once recruited to the site of damage, Rad51 searches for the homologous sequence and a joint DNA structure is formed, in which the damaged molecule invades the other DNA strand. The 3’ end of the damaged DNA is extended and information is copied from the undamaged strand. The resulting Holliday junction is resolved in a complex mechanism, involving Topoisomerase 3, BLM (Bloom syndrome protein), BLAP75 (BLM-associated peptide 75) complex/ RMI1 (RecQ mediated genome instability 1), GEN1 (Gen homologue, endonuclease 1) and other co-factors. Holliday junction resolution can lead to crossover or non-crossover products (reviewed by Jackson, 2002; reviewed by Bernstein and Rothstein, 2009; reviewed by Heyer et al., 2010).
Figure 1.4. DSB repair via the HR pathway.
Schematic representation of DSB repair carried out by the HR pathway. The MRN complex and Sae1 are recruited to the DSB, for the initial resection of the DNA, resulting in ssDNA overhangs. These overhangs serve as templates for long-range DNA resection, using either Sgs1-Dna2 or Exo1. The exposed ssDNA is then coated with RPA, which recruits the Rad52 group of proteins, to enable Rad51 filament formation. After finding a homologous sequence, a joint DNA structure and Holliday junction is formed, which is resolved by the BLAP75 complex and involves Gen1 (adapted from Bernstein and Rothstein, 2009).

1.2.4 Aneuploidy, chromosome instability and tumourigenesis

Accurate repair of damaged DNA and cell cycle checkpoints ensure that genetic information is accurately relayed to daughter cells without the loss of any genetic information. Many cancer cells display chromosome instability (CIN) and aneuploidy. CIN is defined as an increased rate in the gain or loss of whole chromosomes during cell division. Aneuploidy refers to a cell having abnormal chromosome number, therefore, some cancer cells may be stably aneuploidy, due to an early alteration in chromosome number, followed by clonal expansion. However, cancer cells also display a vast array of chromosome abnormalities, such as base
substitutions, partial insertions or deletions, amplifications, translocations, which may not alter the overall chromosome number but may lead to loss or gain of large amounts of genetic information and lead to increased tumourigenesis (reviewed by Lengauer et al., 1998; reviewed by Yuen, 2001; reviewed by Holland and Cleveland, 2009).

The SAC is an overseer of chromosome segregation, which ensures that chromosomes are bi-orientated between the two spindle poles and all kinetochores are attached to microtubules and are under tension before mitosis can proceed. For instance, MAD2 and BUBR1 are essential to restrain anaphase onset while kinetochores are still attaching microtubules (Meraldi et al., 2004; reviewed by Tanaka, 2008). A compromised SAC can cause both CIN and aneuploidy, with mutation in BUBR1, for example, leading to CIN and microsatellite instability (MIN) and either reduced levels or overexpression of MAD2 leading to tumourigenesis in mice (Cahill et al., 1998; Michel et al., 2001; Sotillo et al., 2007; Sotillo et al., 2010). However, in certain cancer cells, depletion of MAD2 or BUBR1 led to cell death due to massive chromosome loss (Dobles et al., 2000; Kops et al., 2004). Thus, a complete loss of the SAC could lead to cell death and embryonic lethality but a weakened SAC could allow anaphase to proceed before all chromosomes are aligned and correctly attached, leading to chromosome mis-segregation. Mutations in SAC components have been found in aneuploid human leukaemias, breast and ovarian cancer, to name but a few (reviewed by Yuen, 2001; reviewed by Holland and Cleveland, 2009).

Improper attachment of kinetochores to microtubules can cause chromosome mis-segregation. CENPs (Centromere proteins) play a central role in the assembly of the kinetochore and some CENPs are overexpressed in cancer. For example, CENPA and CENPH are both overexpressed in colorectal cancers (Tomonaga et al., 2003; Tomonaga et al., 2005). Improper attachment can occur when two microtubules from the same pole attach both sister kinetochores (syntelic attachments), allowing both sister chromatids to be segregated into the same daughter cell. As the kinetochores are under tension, this may not be detected by the SAC. Alternatively, merotelic attachments occur when microtubules from opposite poles attach a single kinetochore and can lead to lagging chromosomes, which are lost from both daughter cells. The chromosome passenger complex (CPC)
destabilises syntelic and merotelic attachments and corrects kinetochore-microtubule attachment errors (Cimini et al., 2004; reviewed by Tanaka, 2008; Thompson and Compton, 2008; reviewed by Holland and Cleveland, 2009).

Extra centrosomes have been reported in numerous cancer types and have been thought to lead to increased CIN. Numerous studies, however, have reported that cells with extra centrosomes rarely underwent multipolar divisions. Instead, cells went through a multipolar intermediate before clustering their centrosomes and undergoing an apparently bipolar division. There were, however, increased occurrences of merotelic attachments in cells with extra centrosomes (Section 1.3.3) (Ring et al., 1982; Guidotti et al., 2003; Ganem et al., 2009; Silkworth et al., 2009).

Loss of sister chromatid cohesion may also lead to chromosome mis-segregation. If sister chromatids separate too early, this can prevent the bi-orientation of chromosomes and allow syntelic attachments (Hoque and Ishikawa, 2002; reviewed by Tanaka, 2008). However, if sister chromatids fail to separate properly, this again can cause chromosome mis-segregation. Mammalian cells without Securin lose chromosomes at a high frequency, due to repeated failed attempts to separate sister chromatids. In these cells Separase is also not activated and therefore, is not able to cleave Scc1 (Jallepalli et al., 2001). However, overexpression of both Securin and Separase is seen in many cancers also (reviewed by Jallepalli and Lengauer, 2001; Yu et al., 2003b; Zhang et al., 2008).

1.3 Centrosome abnormalities and cancer

Aneuploidy, polyploidy and CIN are common characteristics of cancer cells and cancer cells often display centrosome abnormalities, such as structural abnormalities and centrosome amplification (reviewed by Lingle and Salisbury, 1999; Duensing et al., 2000; Chan, 2011). Nearly a century ago, Theodor Boveri was the first to suggest that aneuploidy was due to extra centrosomes. However, whether centrosome amplification is a cause of consequence of cancer progression has been the source of much debate ever since (Boveri, 2008; reviewed by Zyss and Gergely, 2009). Other than numerical abnormalities of the centrosome, cancer cells can display several structural abnormalities including, excess PCM, unincorporated microtubule complexes, abnormal centriole barrel structures, centrioles of abnormal
length, aberrant protein phosphorylation and centriole splitting (Lingle et al., 1998; Lingle and Salisbury, 1999).

1.3.1 **Centriole splitting and centrosome fragmentation**

A potential cause of centrosome amplification is the improper splitting of paired centrioles or the fragmentation of pericentriolar material (Hut et al., 2003; Ehrhardt and Sluder, 2005; Date et al., 2006). In a normal cell cycle, centriole engagement is proposed to be an intrinsic block to reduplication. Centriole disengagement is considered a licensing step which allows for the duplication of centrioles (reviewed by Wong and Stearns, 2003; Tsou and Stearns, 2006b; Tsou and Stearns, 2006a). Centriole splitting, also termed centrosome fragmentation is when a single centriole becomes separated from its centriole partner. If premature centriole splitting occurs in duplicated centrosomes, this can lead to multipolar mitotic cells, as single centrioles can nucleate microtubules (Hut et al., 2003; Peloponese et al., 2005). Alternatively, if centrioles split in G2 phase and then are able to reduplicate during a cell cycle arrest, this could result in a cell with supernumerary centrosomes, which again may result in a multipolar mitosis (reviewed by Tsou and Stearns, 2006b; Tsou and Stearns, 2006a).

Centriole splitting can occur by different means. For example, centriole splitting occurs in human colon cancer cell line, HCT116, following RAD51B deletion and siRNA depletion of RAD51B expression in the human fibrosarcoma cell line, HT1080 (Date et al., 2006). Hut *et al.* demonstrated in CHO cells, that in the presence of incompletely replicated or damaged DNA, centriole splitting can occur in mitosis, leading to the formation of multipolar spindles (Hut et al., 2003). HTLV-1 (Human T-cell leukaemia virus type-1), an oncogenic retrovirus which encodes the oncoprotein Tax, can also induce centriole splitting in mitotic cells. Additionally, fragmentation of the pericentriolar material may also lead to the formation of multipolar spindles as the PCM without centrioles can nucleate microtubules, as is the case with RNAi depletion of Kizuna in Hela cells, which causes PCM fragmentation and dissociation from centrosomes and results in the generation of multipolar spindles (Oshimori et al., 2006). Such spindle abnormalities can lead to abnormal chromosome segregation and aneuploidy (Ganem et al., 2009).
1.3.2 Centrosome amplification

Control of structural integrity and correct centrosome number is essential to prevent the formation of multipolar spindles, which may in turn lead to CIN and aneuploidy. Centrosome amplification can occur in many different circumstances, following DNA damage, overexpression/depletion of proteins involved in centrosome duplication, virally-induced centrosome amplification, cytokinesis failure, cell fusion and de novo centriole assembly, all of which can have serious implications for genomic stability of the cell.

1.3.2.1 DNA damage-induced centrosome amplification

Centrosome duplication and DNA replication are two tightly coordinated cycles in the cell, ensuring that like DNA replication, centrosome duplication occurs only once per cell cycle (reviewed by Tsou and Stearns, 2006a; reviewed by Strnad and Gonczy, 2008). DNA damaging agents such as IR and UV irradiation cause both DNA damage and centrosome amplification. When DNA replication is stalled to allow time to repair the damaged DNA, the centrosome cycle can become uncoupled from DNA replication and proceeds, leading to centrosome amplification (Balczon et al., 1995; Dodson et al., 2004). Further evidence that the cell and centrosome cycles are intimately linked lies in the fact that several DNA damage checkpoint proteins localise to the centrosome for at least part of the cell cycle, for example, NBS1, BRCA1, ATM, ATR, ATRIP, CHK1, CHK2, p53, CDC25, cyclin B/CDK1 and MCPH1 (Microcephalin 1) (Jackman et al., 2003; Takada et al., 2003; Dutertre et al., 2004; Kramer et al., 2004; Tsvetkov and Stern, 2005; Oricchio et al., 2006; Zhang et al., 2007; Jeffers et al., 2008). Dysfunction of many of these proteins can lead to centrosome amplification (reviewed by Shimada and Komatsu, 2009). Centrosome amplification has also been reported in cell lines deficient in BRCA1 (Xu et al., 1999), BRCA2 (Tutt et al., 1999), RAD51 (Dodson et al., 2004), MRE11 (Yamaguchi-Iwai et al., 1999), XRCC2, XRCC3 (Griffin et al., 2000) and PARP-1 (Poly(ADP-Ribose) polymerase 1) (Kanai et al., 2003). In addition, although Mcph1−/−DT40 cells (chicken B-lymphocyte cell line) have normal centrosome numbers, treatment with IR induces massive levels of centrosome amplification compared to IR-treated control cells. Mcph1+/−DT40 cells show a normal G2-M cell cycle arrest but a slower return to the cell cycle, suggesting a deficiency in checkpoint recovery (Brown et al., 2010).
Centrosome amplification has been reported after IR-induced DNA damage, when cells arrest in G2 phase (Sato et al., 2000b; Dodson et al., 2007). Following IR, ATM/ATR activation leads to the G2-M checkpoint arrest, which has both p53 dependent and independent components (Section 1.2.2.3). The ensuing signalling cascade results in Chk1/Chk2 activation and inhibition of Plk1. This results in Cdc25 inhibition, which in turn, prevents dephosphorylation and activation of Cdk1. Mitotic entry is thereby prevented, as activation of Cdk1 is needed for mitotic entry (reviewed by Sancar et al., 2004). The requirement for Cdk1 for mitotic entry has also been shown in Cdk1-inactivated cells arrested at the G2-M phase border. DNA replication is stalled, although centrosomes continue to cycle, leading to high levels of centrosome amplification (Hochegger et al., 2007; Steere et al., 2011).

p53 has been proposed to coordinate both centrosome duplication and DNA replication, as well as preventing centrosome reduplication. Loss/mutation of p53 and centrosome hyperamplification have been seen in certain cancer types, including breast and prostate cancers (Ouyang et al., 2001; Lingle et al., 2002). However, other studies have shown that inactivation of p53 in human diploid cell lines did not induce centrosome amplification (Bunz et al., 2002). Interestingly, in mouse embryonic fibroblasts (MEFs), when p53 was inactivated and Cdk2/cyclin E was constitutively active, centrosome amplification occurred, implying that strict control of Cdk2/cyclin E may also restrict centrosome amplification in human cells (Mussman et al., 2000). IR-induced centrosome amplification is reduced in Chk1−/− DT40 cells compared to wild-type cells, indicating that Chk1 activity is needed for centrosome amplification (Bourke et al., 2007). Furthermore, following IR, Cdk2 activity is upregulated in wild-type but not Chk1−/− DT40 cells, indicating that after IR, Chk1 signalling can cause centrosome amplification by upregulating Cdk2 activity (Bourke et al., 2010).

1.3.2.2 Overexpression of centrosome duplication proteins and centrosome amplification
The number of procentrioles which can form around a mother centriole is tightly controlled in a process called copy number control, and this ensures that each mother centriole can nucleate only a single procentriole (reviewed by Nigg and Stearns, 2011). However, overexpression of certain proteins involved in procentriole formation can override this control. For instance, overexpression of Plk4, SAS6 or
STIL leads to the formation of multiple daughter centrioles around single mother centrioles. In addition, the inhibition of Plk4, SAS6 or STIL prevents centriole duplication, and leads to abnormal spindle formation and the formation of monopolar spindles (Bettencourt-Dias et al., 2005; Habedanck et al., 2005; Strnad et al., 2007; Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). Interestingly, overexpression of Cdk2/cyclin E also allows for the formation of multiple daughter centrioles surrounding a single mother centriole. How Cdk2/cyclin E cooperates with Plk4, SAS6 and/or STIL remains to be determined (Duensing et al., 2007).

1.3.2.3 Viruses and centrosome amplification

Another possible cause of centrosome amplification comes from viral infection. For instance, the human papillomavirus (HPV) type 16 E7 oncoprotein can cause centrosome amplification by uncoupling centrosome duplication from the cell cycle. This, in turn, can induce abnormal centrosome number, mitotic defects and genome instability (Duensing et al., 2000). Furthermore, this oncoprotein induces the formation of multiple daughter centrioles around each mother centriole, in a mechanism involving the degradation of Rb, inactivation of cyclin-dependent inhibitors such as p21, which causes deregulated expression of Cdk2/cyclin E (Duensing et al., 2007). Moreover, the Kaposi sarcoma herpes virus (KSHV) encodes a K-cyclin, which is a homologue of cyclin D. K-cyclins can phosphorylate NPM, which is necessary for initiation of centrosome duplication and expression of this K-cyclin in primary cells is associated with failure in cytokinesis, multinucleation and centrosome amplification (Verschuren et al., 2002; Cuomo et al., 2008).

1.3.2.4 Cytokinesis failure, cell fusions and de novo assembly

Cytokinesis failure may result from several causes, such as unrepaired DNA damage, mitotic slippage, aberrant SAC or overexpression of oncoproteins. The resulting cell will have double the correct chromosome complement but likewise, double the correct number of centrosomes. Failure to undergo cytokinesis triggers a p53 checkpoint response and cells arrested in the presence of p53 will eventually undergo cell death. However, p53 deficient cells may continue to cycle and undergo repeated cytokinesis failures, resulting in multinucleated cells with amplified centrosomes (reviewed by Nigg, 2002; reviewed by Fukasawa, 2008). In the absence of functional p53, overexpression of Aurora A, Aurora B and Plk1 have been shown
to lead to tetraploidisation and centrosome amplification (Meraldi et al., 2002). Furthermore, inactivation of the tumour suppressor, BRCA2 in MEFs and Hela cells delays and prevents cell cleavage. Therefore, centrosome amplification and micronuclei in BRCA2 deficient cells may be due to cytokinesis failures (Tutt et al., 1999; Daniels et al., 2004).

Cell fusion is not a common mechanism for centrosome amplification, although it can occur naturally during development in the placenta, muscle and bone. Cell fusion can also be virally induced, as is the case with the Sendai virus, or with overexpression of the ubiquitin-conjugating enzyme Rad6, resulting in multinucleated cells, increased aneuploidy and centrosome amplification (Shekhar et al., 2002; reviewed by Ogle et al., 2005; reviewed by Duelli and Lazebnik, 2007).

An additional mechanism for centrosome amplification is de novo assembly of centrioles. De novo assembly of centrioles is normally restricted to multiciliated cells. However, recent studies in CHO and Hela cells ablating pre-existing centrioles established that centrioles in non-ciliated cells can be formed de novo, and in random numbers, suggesting that numerical control of centriole number had been lost (Section1.1.4) (Sorokin, 1968; Khodjakov et al., 2002; La Terra et al., 2005).

1.3.3 **Mechanisms to prevent multipolar divisions**

For a long time, it was thought that cancer cells with extra centrosomes carried out multipolar divisions, leading to daughter cells with gross chromosome missegregation. However, many studies in recent years have shown that cancer cells can often overcome potentially lethal mitotic cell divisions. There are two main mechanisms whereby cells can form a bipolar spindle in the presence of extra centrosomes, centrosome clustering and centrosome inactivation. Evidence is emerging that many cancer cell types cluster their centrosomes and carry out bipolar divisions, avoiding multipolar divisions (Ring et al., 1982; Guidotti et al., 2003; Ganem et al., 2009; Silkworth et al., 2009). A screen to identify centrosome clustering proteins was performed in *Drosophila* S2 cells and identified components of the SAC, microtubule associated proteins (MAPs) and regulators of acto-myosin contractibility. The authors of this study also showed that the interphase cell shape and adhesion pattern played a role in the successful clustering of extra centrosomes in mitosis (Kwon et al., 2008).
Recent studies have shown that cells with multiple centrosomes undergo a transient multipolar state in mitosis before clustering their centrosomes into a bipolar spindle. However, during this multipolar spindle intermediate, merotelic attachment errors accumulate. Therefore, when centrosomes are then clustered and the cell divides, there may be high frequencies of lagging chromosomes which may not be incorporated into either cell, or are incorporated into the wrong daughter cell. Although mechanisms exist to correct attachment errors, merotelic attachments are not detected by the SAC and many of these merotelic attachment errors are not resolved before anaphase (Cimini et al., 2003; Cimini et al., 2006). Thus, unresolved merotelic attachments can lead to lagging chromosomes and chromosome missegregation errors (Ganem et al., 2009; Silkworth et al., 2009).

There are no examples of partial centrosome inactivation to allow cancer cell division in the presence of multiple centrosomes. However, in early Drosophila embryogenesis, mutations in DNA checkpoint genes lead to chromosome segregation failures. These segregation failures were associated with loss of centrosome function in mitosis and the dissociation of components of the γ-tubulin ring complex. The authors in this study proposed centrosome inactivation to be a damage control mechanism when the DNA damage checkpoint fails and would ultimately lead to cell death (Sibon et al., 2000). Therefore, in mammalian cells it could be a possible mechanism to rid an organism of cells with extra centrosomes and prevent possible CIN and aneuploidy. Basto et al. observed that in Drosophila where Plk4/SAK had been inactivated, brain cells with extra centrosomes still divided in a bipolar manner 93% of the time. Both centrosome clustering and partial centrosome inactivation were reported in mitotic cells, where extra centrosomes were clustered at spindle poles, but in addition, there were centrosomes not associated with any microtubule asters and contained less PCM than centrosomes at spindle poles (Basto et al., 2006). Centrosome clustering and partial centrosome inactivation together could be a possible mechanism for cancer cells to escape the cell death associated with multipolar divisions (Basto et al., 2006; Ganem et al., 2009) (reviewed by Marthiens et al., 2012).
1.4 Cilia

Cilia and flagella are organelles which project from the cell surface and are conserved in eukaryotes. Cilia exist as either motile cilia or immotile primary cilia and are found on most cells of the human body. Motile cilia usually occur in groups and emanate from centrioles which are formed via the de novo centriole assembly pathway (Sorokin, 1968). Motile cilia generally consist of a ring structure of nine pairs of microtubule doublets and a central microtubule pair and have important functions in the body. For example, they exist on the epithelial cells lining the lung where they move mucus up the respiratory tract and in the oviduct, where they move the ovum to the uterus (reviewed by Afzelius, 2004). Dysfunction in cilia-related proteins lead to a range of human diseases, termed ciliopathies. Though motile cilia are essential for the functioning of an organism, the focus of this study will be on immotile primary cilia.

The primary cilium is a sensory organelle, which exists as a single projection on the cell surface, receiving mechanical and chemical signals from the extracellular environment, which it then transmits to the nucleus to elicit a response. Once thought to be an evolutionary vestige in vertebrates, there has recently been increased interest in the primary cilium and in its involvement in human diseases. The primary cilium is involved in signalling pathways such as Hedgehog (Huangfu et al., 2003; Corbit et al., 2005; Huangfu and Anderson, 2005; Ocbina and Anderson, 2008), Wnt (Gerdes et al., 2007; Corbit et al., 2008) and platelet-derived growth factor (PDGF) signalling pathways (Schneider et al., 2005). Therefore, mutations in genes which disrupt signalling through the primary cilium result in a broad array of disorders, such as obesity, skeletal malformations, polycystic kidneys, abnormalities of the hepatic and pancreatic systems and developmental defects (reviewed by Baker and Beales, 2009).

1.4.1 The structure and cell cycle regulation of the primary cilium

Primary cilia, like motile cilia, are composed of a ring of nine microtubule doublets. However, they lack the central pair of microtubules (reviewed by Afzelius, 2004). The primary cilium grows from a basal body, a term used to define the mother centriole once it has docked close to the plasma membrane (Figure 1.5). Adjacent to the distal end of the basal body is the transition zone, the region where
the microtubule triplets taper to doublets (Sorokin, 1962; Gilula and Satir, 1972). The transition zone is composed of transition fibres (distal appendages), Y-linkers and the ciliary necklace. The transition zone is thought to be a site of regulation of ciliary function and may act as a gating mechanism for trafficking of proteins (reviewed by Czarnecki and Shah, 2012). The axoneme grows from the transition zone and extends into the extracellular environment. Primary cilia vary in length depending on the tissue type/cell line. For example, primary cilia in hTERT-RPE1 cells are 2.6 ± 0.8 μm in length, while in IMCD cells (immortalised kidney collecting duct) primary cilia can grow up to 6μm in length, which is up to twelve times the length of the mother centriole from which they grow (Yoshimura et al., 2007; Besschetnova et al., 2010). As no protein synthesis takes place in the primary cilium, intraflagellar transport (IFT) must deliver axoneme building blocks to the ciliary tip for assembly (reviewed by Rosenbaum and Child, 1967; Garcia-Gonzalo and Reiter, 2012). The axoneme is covered by a ciliary membrane, which is formed in epithelial cells by the fusion of a ciliary vesicle with secondary vesicles into a ciliary sheath. The ciliary sheath then fuses with the plasma membrane and the primary cilium emerges from the interior of the cell. The primary cilium is anchored and stabilised by the basal feet (subdistal appendages) and a striated rootlet, of which Rootletin is a major component (Sorokin, 1962; Yang et al., 2002). Depletion of the distal appendage components, CEP164 or Cenexin, prevents primary cilium assembly, suggesting that the distal appendages are indispensable for ciliogenesis (Ishikawa et al., 2005; Graser et al., 2007a).
Figure 1.5. The structure of the primary cilium.  
Schematic representation of a generic mammalian primary cilium. The basal body (BB) or mother centriole nucleates the axoneme of the primary cilium, which is contained within the ciliary membrane. The BB ends with the transition zone (TZ), where the transition fibres and Y-linkers are located. The primary cilium is stabilised by the basal foot and striated rootlet. (Diagram not to scale). (adapted from Garcia-Gonzalo and Reiter, 2012).

1.4.2 Primary cilium assembly and disassembly

Primary cilia are formed in G0/G1 phase of the cell cycle and are resorbed prior to mitosis. The exact point of ciliary resorption varies between different cell types, with certain cells resorbing cilia in S phase and others not until the G2/M transition (reviewed by Plotnikova et al., 2009). In recent times, more light has been shed on primary cilium assembly, although the exact molecular mechanisms remain unclear. Two proteins implicated in centriole biogenesis and control of centriole length, CP110 and CEP97, are also known to regulate ciliogenesis with siRNA depletion of either CP110 or CEP97 leading to increased levels of ciliation in (ciliated) 3T3 cells. Proteins which localise to the primary cilium, acetylated tubulin, polyglutamylated tubulin and IFT88/Polaris all localised to these structures, while Centrin did not, indicating that these are true cilia. However, when either CEP97 or CP110 are depleted in non-ciliated U2OS cells, elongated centrioles are formed.
which contain Centrin and γ-tubulin. Therefore, both proteins appear to have specialised roles in ciliated cells. Interestingly, overexpression of CP110, and to a lesser extent CEP97, suppressed ciliation in 3T3 cells also, clearly implicating both proteins in primary cillum assembly (Spektor et al., 2007). Overexpression of CPAP/CENPJ has also been reported to cause the formation of elongated centrioles in U2OS cells (Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009). More recently, CPAP has been shown to be necessary for primary cillum formation in neuronal cells, with shRNA depletion of CPAP preventing ciliation and overexpression causing increased levels of primary cillum formation (Wu and Tang, 2012).

Other centriolar components reported to play a role in the assembly and/or maintenance of the primary cillum include ALMS1 (Alström syndrome 1) and POC1. ALMS1 localises to the proximal end of centrioles and plays a role in ciliogenesis, although its precise ciliary and centrosomal roles remain unclear. siRNA depletion of ALMS1 leads to the formation of stunted primary cilia and mutation of ALMS1 leads to the human ciliopathy, Alström syndrome, characterised by neuronal defects, childhood obesity and diabetes (Hearn et al., 2002; Collin et al., 2005; Graser et al., 2007a; Li et al., 2007; Knorz et al., 2010). POC1, likewise, localises to the proximal ends of centrioles and has two human paralogues, POC1A and POC1B. Depletion of POC1A and POC1B revealed that POC1A did not affect primary cillum formation, whereas depletion of POC1B led to reduced levels of primary cilia and shorter primary cilia. However, a more recent study has found that truncation of POC1A causes primordial dwarfism, which has recently been characterised as a ciliopathy. When fibroblasts from an affected individual were examined, it was found that cells had reduced levels of ciliation and again, cilia were shorter than in control fibroblasts (Keller et al., 2009; Pearson et al., 2009; Shaheen et al., 2012). Hence, although the exact mechanisms of primary cillum assembly remain unclear, it is clear that proteins which play key roles in procentriole formation and elongation are also intimately involved in ciliogenesis.

Primary cillum disassembly is again a relatively undefined process. Aurora A is reported to be required for primary cillum resorption, although exactly how it mediates disassembly remains to be clarified. When microinjected into cells, Aurora A promotes cillum disassembly and siRNA depletion led to higher levels of ciliation.
and/or prevented cillum disassembly (Pan et al., 2004; Pugacheva et al., 2007; Inoko et al., 2012; Lee et al., 2012). Plk1 has recently been reported to play a role in primary cillum disassembly. Plk1 in complex with Dishevelled 2 (Dvl2), promotes primary cillum disassembly by stabilising HEF1 (Human enhancer of filamentation 1) and allowing activation of the HEF1-Aurora A complex (Lee et al., 2012). Finally, IFT, the process which transports structural building blocks between the ciliary base and tip, has also been implicated in primary cillum disassembly. IFT particles are multi-protein complexes, which associate with ciliary proteins to transport them to the ciliary tip. In this way, axoneme subunits are transported to the ciliary tip by anterograde movement, while cargo-less IFT particles or ones carrying turnover products are transported by retrograde movement. Consequently, it is thought that cillum disassembly occurs when the rate of anterograde movement of particles to the ciliary tip decreases and the rate of retrograde movement of disassembled ciliary components from the ciliary tip to the cell body increases (Pan and Snell, 2005; Pugacheva et al., 2007; reviewed by Seeley and Nachury, 2010).

### 1.4.3 Ciliopathies

Defects in the structure and function of primary cilia can lead to a wide range of developmental and degenerative diseases such as Polycystic Kidney Disease, Retinitis Pigmentosa, Nephronophthisis, Bardet-Biedl syndrome, Meckel-Gruber syndrome and Joubert syndrome (reviewed by Hildebrandt et al., 2011). Autosomal dominant polycystic kidney disease (ADPKD) was the first disease to be linked to primary cillum dysfunction. Mutations in *PKD1* and *PKD2* cause ADPKD, which is characterised by renal cysts and cardiovascular complications. *PKD1* and *PKD2*, which encode polycystin-1 and polycystin-2 respectively, localise to the primary cillum in kidney cells. Studies have shown that *Pkd1⁻/⁻* endothelial cells and *Pkd2⁻/⁻* mice have supernumerary centrosomes, which leads to the formation of multiple primary cilia. These *Pkd1⁻/⁻* and *Pkd2⁻/⁻* cells also showed chromosome segregation errors in mitosis which led to CIN and polyploidy. Interestingly, Survivin, a chromosomal passenger protein, involved in coordinating chromosomal events in mitosis, was also down-regulated in these cells (Yoder et al., 2002; AbouAlaiwi et al., 2011).
Meckel-Gruber syndrome (MKS) is an autosomal recessive disorder, characterised by renal cystic dysplasia, encephalocele, polydactyly and biliary dysgenesis. Mutations in nine different genes have been implicated in this disorder, including \textit{MKS1} and \textit{MKS3}, which encode MKS1 and Meckelin respectively. MKS1 localises to the mother centriole, while Meckelin localises to the primary cilium and plasma membrane in ciliated cells. Conflicting reports exist as to the impact of MKS1 and MKS3 on primary cilium formation. It has been reported that kidney cells from MKS1 and MKS3 patients showed centrosome amplification and multiple primary cilia, which were abnormally long. However, other studies have shown that siRNA depletion of MKS1 and Meckelin prevented centrosome migration and anchoring at the plasma membrane, thus impeding primary cilium formation. The cilia that were present were shorter than those in control cells (Dawe et al., 2007b; Tammachote et al., 2009; Cui et al., 2011).

Bardet-Biedl syndrome (BBS) is characterised by obesity, retinal dystrophy, renal anomalies, polydactyly and cognitive defects. Sixteen causative genes (\textit{BBS1-12, 15, 16, MKS1 and CEP290}) have been implicated in BBS, all of which localise to the centrosome or primary cilium. Seven of these genes encode proteins which interact to form the BBSome, a complex involved in trafficking of proteins to and inside the primary cilium. Mutations in BBS genes show different ciliary phenotypes for example, knockdown of \textit{BBS9}, leads to reduced numbers of cilia which were shorter than in control cells. Whereas, cilia in \textit{BBS2, BBS3} and \textit{BBS4} knockout mice are of normal length and number, although, they lack ciliary localisation of MCHR1 (Melanin Concentrating Hormone Receptor 1). MCHR1 is involved in feeding behaviour, therefore aberrant ciliary localisation is the likely cause of the obesity associated with BBS (Berbari et al., 2008; Zhang et al., 2011; Veleri et al., 2012). Furthermore, mutations in \textit{BBS7} and \textit{BBS8} in \textit{C. elegans} leads to dissociation of IFT particles and cilia are of abnormal length and structure (Blacque et al., 2004).

Although there are conflicting data on the phenotypes associated with mutation of \textit{MKS1} or \textit{MSK3}, it is clear from studies into ADPKD and MKS that disruption of centrosome or primary cilium formation in ciliated cells can lead to severe ciliopathies, whether due to a loss of cilia or multiple primary cilia formation. Additionally, although not all of \textit{BBS} gene mutations affect primary cilium
formation, alteration in protein trafficking to the primary cilium can lead to severe human disorders like BBS.

1.4.4 Cilia and Cancer

The primary cilium is central to the activities of a number of signalling pathways, including Hedgehog (Hh), Wnt and PDGF signalling. There is increasing evidence that inappropriate activation of these pathways can affect cell migration, differentiation, proliferation and can potentially lead to the development of cancer. The increased attention being paid to these signalling pathways and the primary cilium may eventually represent new cancer therapies which specifically target ciliary proteins involved in aberrant signalling (reviewed by Michaud and Yoder, 2006).

1.4.4.1 Hedgehog signalling and cancer

In mammals, there are three secreted ligands in the Hedgehog (Hh) family: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). Hh signalling is normally restrained by the tumour suppressor, Patched 1 (Ptch1). Ptch1 inhibits the activity of Smoothened (Smo), a signal transducer of the pathway, in part by preventing its trafficking to the primary cilium. When Hh binds to Ptch1, inhibition of Smo is relieved, allowing Smo to move to the primary cilium and activate the signalling pathway through the Glioma (Gli) family of activators (Figure 1.6). Gli1 and Gli2 then translocate to the nucleus, where they activate target genes, provide feedback control of the pathway and control cell proliferation. On the other hand, Gli3 can act as both activator and repressor and Hh signalling prevents the processing of Gli3 into a short repressor form (Corbit et al., 2005; Huangfu and Anderson, 2006; Rohatgi et al., 2007; Wang et al., 2007).
Figure 1.6. Hedgehog signalling through the primary cilium.

Binding of the Hh ligand to Ptc1 relieves Smo inhibition. This allows Smo to translocate to the primary cilium, where it activates Gli family members. Gli then translocate to the nucleus where they activate target genes (adapted from Li et al., 2011).

Abnormal activation of Hh signalling is associated with a diverse array of tumour types, including Basal cell carcinoma (BCC) and medulloblastoma. BCC cells are often ciliated, while cerebellar granule precursors (GMPs) require the primary cilium and Hh pathway for proliferation, and can give rise to medulloblastomas. Ciliary ablation inhibited both BCC-like tumours and the formation of medulloblastomas in mouse models, which were induced by a constitutively-active form of Smo. However, in contrast, ciliary ablation accelerated tumour growth in BCC-like tumours and medulloblastoma growth in tumours induced by activated Gli2. Therefore, depending on the nature of the oncogenic initiating event, the primary cilium can either activate or repress tumourigenesis, playing a dual role in activating and repressing Hh signalling (Han et al., 2009; Wong et al., 2009).

1.4.4.2 PDGF signalling and cancer

PDGF signalling is required for embryogenesis, inflammation and wound healing. Aberrant PDGF signalling has also been implicated in cancer. The PDGF pathway consists of four ligands (PDGF-A, B, C, D) and two receptors (PDGFα and PDGFβ). PDGFα has been shown to localise to the primacy cilium in MEFs. The ligands form homodimers and AB heterodimers and bind to two receptors
simultaneously, thereby activating the receptors. This activation allows the binding of intracellular signalling molecules to autophosphorylated tyrosine residues on the receptors and initiates signalling cascades such as the Akt and ERK (extracellular signal-regulated kinase) pathways (reviewed by Heldin and Westermark, 1999; reviewed by Yu et al., 2003a).

Aberrant PDGF signalling has been detected in a number of tumour types such as gliomas, neurofibromas and osteosarcomas, with PDGFα expression prominent in gliomas. It is thought that perturbation in PDGFα signalling can occur both early in tumour progression and also in later stages where PDGFα can promote tumour growth (reviewed by Yu et al., 2003a). Expression of PDGFα in breast cancer is coupled with poor prognosis and PDGF signalling promotes metastasis in animal breast cancer models (Carvalho et al., 2005; Jechlinger et al., 2006). PDGF plays a crucial role in embryonic angiogenesis and similarly in tumour angiogenesis, a key step in metastasis. PDGF ligands released by tumour cells induce the migration of endothelial cells and vascular smooth muscle cells, which are major components of blood vessels and stimulate their proliferation (Thommen et al., 1997; reviewed by Yu et al., 2003a).

1.4.4.3 Wnt signalling and cancer

Wnt signalling can be divided into two categories, non-canonical signalling and canonical/β-catenin signalling. The canonical Wnt pathway functions in cell proliferation, differentiation, adhesion and survival. This pathway begins when Wnt ligands bind Fz (Frazzled) or the LDL (Low-density lipoprotein) receptor-related proteins 5/6, which in turn, activates Dvl (Dishevelled). Dvl inhibits GSK3 (Glycogen synthase kinase 3) and prevents phosphorylation and destruction of β-catenin, thus, allowing β-catenin to accumulate in the cytoplasm before translocating to the nucleus, where it activates transcription of target genes. In the absence of Wnt ligands, β-catenin is recruited to a destruction box, whose scaffold consists of APC (Adenomatous polyposis coli) and Axin, and where β-catenin is phosphorylated and destroyed. Many of the studies on canonical Wnt signalling and cancer involve mutations in the APC gene, which is mutated in more than 80% of sporadic colorectal tumours. Mutations in APC could allow accumulation of β-catenin and lead to changes in gene transcription (Kinzler and Vogelstein, 1996). Additionally
mutations in the β-catenin gene \((CTNNb1)\) and \(Axin1\) and 2 have been found in a range of human cancers such as colorectal and hepatic cancers. Therefore, it is likely that most cancers originating from aberrant canonical Wnt signalling arise from inappropriate gene activation by β-catenin (reviewed by Polakis, 2012).

There are several non-canonical Wnt signalling pathways, for example, the Wnt-calcium pathway and the planar cell polarity (PCP) pathway (reviewed by Lai et al., 2009). The Wnt-calcium pathway functions in embryonic dorsal-ventral patterning and in regulating cell migration and heart development (Kuhl, 2004). PCP regulates cell polarity, migration and orientation during embryogenesis. Recent reports suggest that the primary cilium can act as a switch between canonical and non-canonical signalling, with overexpression of the ciliary protein, Invs (Inversin) inhibiting canonical Wnt signalling and inhibition of Invs resulting in loss of PCP signalling (reviewed by Simons et al., 2005; Lai et al., 2009). Components of non-canonical Wnt signalling have also been found to be overexpressed in many cancers and are correlated with high metastatic potential. WNT5A has been best studied in melanomas, where it has been found to promote metastasis (Weeraratna et al., 2002). VANGL1 (Van Gogh-like 1) is another component of the non-canonical Wnt signalling pathway, which is overexpressed in colon cancers with high metastatic potential (reviewed by Jessen, 2009).

In conclusion, many signalling pathways converge on the primary cilium, where it is crucial for cell migration, differentiation and proliferation. Therefore deregulation of any of these pathways can lead to uncontrolled cell growth and diversity of cancers.
1.5 Aims of this study

In the first part of this project, we aimed to characterise the role of STIL in centrosome function and centriole biogenesis. Mutations in STIL were known to cause microcephaly and it was known that STIL localised to the centrosome but the precise centrosomal functions of STIL were unknown when this project was undertaken. We sought to overexpress chicken STIL and characterise its centrosomal location and cell cycle expression. We attempt to generate a Stil<sup>−/−</sup> cell line in chicken DT40, which would allow us to study the effect Stil deletion on the structure and function of the centrosome.

In the second part of this project, we investigate the impact of IR on centrosome structure in human cancer and non-transformed cells. We pay particular attention to single isolated centrioles which are observed after IR in order to determine if their structure varies from that of untreated centrioles which are connected to their centriole partner. We also investigate if centriole splitting is a specific response to DNA damage, or if centrioles can split following a G2/M cell cycle arrest without DNA damage. We go on to test if proteins involved in centrosome cohesion play a role in DNA-damage induced centriole splitting and if depletion of any of these centrosome cohesion components affects the composition of the single isolated centriole.

In the final part of this study, we investigate the impact of IR on the formation and structure of the primary cilium. We seek to determine if ciliated cells might respond differently to DNA damage compared to non-ciliated cells. Finally, we study the impact that loss of centrosome cohesion may have on ciliogenesis.
Chapter 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), BDH (Hertfordshire, UK), Fisher (Leicestershire, UK) or GE Healthcare Life Sciences (Buckinghamshire, UK). All solutions were prepared using ddH₂O or Milli-Q purified water, Millipore (Billerica, USA) and where appropriate, autoclaved prior to use. Organic solvents, alcohols and acids were supplied by Sigma (Arklow, Ireland), VWR (Bridgeport, USA) or Fisher (Leicestershire, UK). Oligodeoxynucleotide primers were purchased from Sigma-Aldrich. All common reagents and buffers used throughout this study are presented in Table 2.1 (listed in alphabetical order).

<table>
<thead>
<tr>
<th>Name</th>
<th>Composition</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking solution 1</td>
<td>1X PBS, 0.05% Tween-20, 5% skimmed milk</td>
<td>To reduce antibody non-specific binding in immunoblotting</td>
</tr>
<tr>
<td>Blocking solution 2</td>
<td>1X PBS, 1% BSA</td>
<td>To reduce antibody non-specific binding in immunofluorescence</td>
</tr>
<tr>
<td>Detection buffer</td>
<td>100 mM Tris, 100 mM NaCl, pH 9.5</td>
<td>For detection of DIG labelled probes</td>
</tr>
<tr>
<td>DNA loading dye</td>
<td>20% Sucrose, 0.1 M EDTA pH 8.0, 1% SDS, 0.25%</td>
<td>Added to DNA sample prior to running agarose gel to allow visual tracking of DNA migration during electrophoresis</td>
</tr>
<tr>
<td></td>
<td>Bromophenol blue, 0.25% Xylene cyanol.</td>
<td></td>
</tr>
<tr>
<td>Fixing solution</td>
<td>Chilled Methanol with 5 mM EGTA</td>
<td>To fix cells for immunofluorescence microscopy</td>
</tr>
<tr>
<td>High stringency</td>
<td>0.5X SSC with 0.1% SDS</td>
<td>For membrane washes in non-radioactive Southern blot</td>
</tr>
<tr>
<td>wash buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation buffer 1</td>
<td>1X PBS, 0.05% Tween-20, 1% skimmed milk</td>
<td>To dilute antibodies used in immunoblotting</td>
</tr>
<tr>
<td>Incubation buffer 2</td>
<td>1X PBS, 1% BSA</td>
<td>To dilute antibodies used in immunofluorescence</td>
</tr>
<tr>
<td>Low stringency</td>
<td>2X SSC with 0.1% SDS</td>
<td>For membrane washes in non-radioactive Southern blot</td>
</tr>
<tr>
<td>wash buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LB (Luria-Bertani)</td>
<td>1% tryptone, 0.5% yeast extract, 1% NaCl, pH</td>
<td>Bacterial culture medium</td>
</tr>
<tr>
<td>Broth</td>
<td>adjusted to 7.0 with 4 M NaOH</td>
<td></td>
</tr>
<tr>
<td>Lysis Buffer</td>
<td>50 mM Hepes, 150 mM NaCl, 1 mM EDTA, 2.5 mM</td>
<td>For whole cell lysate preparation and analysis by SDS-PAGE</td>
</tr>
<tr>
<td></td>
<td>EGTA, 10% glycerol, 0.1% Tween-20</td>
<td></td>
</tr>
<tr>
<td>Maleic acid buffer</td>
<td>100 mM Maleic acid pH 7.5, 150 mM NaCl, pH</td>
<td>For the pre-incubation of the membrane before blocking in non-radioactive Southern blot</td>
</tr>
<tr>
<td>(MAB)</td>
<td>adjusted to 7.5 with NaOH</td>
<td></td>
</tr>
<tr>
<td><strong>MAB washing buffer</strong></td>
<td>MAB supplemented with 0.3% Tween 20</td>
<td>For membrane washes in non-radioactive Southern blot</td>
</tr>
<tr>
<td>------------------------</td>
<td>-------------------------------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Non-Radioactive Southern Blot Blocking Solution</strong></td>
<td>10% Caseine in Maleic acid wash buffer</td>
<td>For blocking of Southern blot membranes</td>
</tr>
<tr>
<td><strong>PBS (Phosphate buffered saline)</strong></td>
<td>2.68 mM KCl, 1.47 mM KH$_2$PO$_4$, 136.9 mM NaCl, 8.1 mM Na$_2$HPO$_4$</td>
<td>Solution is made up to 10X by dissolving Tablets in distilled water. Solutions were autoclaved. Working dilution 1X.</td>
</tr>
<tr>
<td><strong>Phosphatase inhibitors (50X)</strong></td>
<td>2.5 mM NaF, 1.8 mM β-glycerophosphate, 0.5 mM Na$_3$VO$_4$, 2.4 mM EGTA, 12.5 mM sodium pyrophosphate</td>
<td>To inhibit phosphatase enzymes while immunoblotting for phosphorylation</td>
</tr>
<tr>
<td><strong>Ponceau S. solution</strong></td>
<td>0.5% Ponceau S., 5% acetic acid</td>
<td>To stain proteins on the nitrocellulose membrane</td>
</tr>
<tr>
<td><strong>Protease inhibitors (100X)</strong></td>
<td>3 μM leupeptin, 10 μM pepstatin A, 4.9 mM PMSF, 10.5 mM benzamidine, 10 μM antipain, 6.6 μM chymostatin (dissolved in DMSO) in ethanol</td>
<td>To inhibit protease enzymes during sample preparation for immunoblot</td>
</tr>
<tr>
<td><strong>RIPA Buffer</strong></td>
<td>50mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 0.25% Na-Deoxycholate, 1 mM EDTA (protease and phosphatase inhibitors added before use)</td>
<td>For the lysis of cells and extraction of proteins from total cell extracts</td>
</tr>
<tr>
<td><strong>Running Buffer</strong></td>
<td>25 mM Tris, 250 mM glycine, 0.1% SDS</td>
<td>For running SDS-PAGE gels</td>
</tr>
<tr>
<td><strong>Salt-sodium citrate (SSC)</strong></td>
<td>1.5 M NaCl, 0.15 M sodium citrate, pH adjusted to 7.0 with citric acid</td>
<td>10x stock, for transfer of genomic DNA to nylon membrane in Southern blot analysis</td>
</tr>
<tr>
<td><strong>SDS-PAGE Sample buffer (3X)</strong></td>
<td>150 mM Tris pH 6.8, 45% sucrose, 6mM K-EDTA pH 7.4, 9% SDS, 0.03% bromophenol blue.</td>
<td>For denaturation and loading of proteins prior to SDS-PAGE</td>
</tr>
<tr>
<td><strong>Sodium cacodylate buffer</strong></td>
<td>0.2 M, diluted in milli-Q H$_2$O, adjusted to pH 7.2 with 1 M HCl</td>
<td>Buffering agent for preparation and fixation of cells for transmission electron microscopy</td>
</tr>
<tr>
<td><strong>TAE (Tris-acetate EDTA)</strong></td>
<td>40 mM Tris-acetate pH 8.0, 1 mM EDTA</td>
<td>For agarose gel electrophoresis</td>
</tr>
<tr>
<td><strong>Tail Buffer</strong></td>
<td>50 mM Tris pH 8.8, 100 mM EDTA, 100 mM NaCl, 1% SDS</td>
<td>For the preparation of genomic DNA. 0.5 mg/ml of proteinase K is added before use</td>
</tr>
<tr>
<td><strong>TfbI (transformation buffer I)</strong></td>
<td>30 mM Potassium Acetate, 100 mM RbCl$_2$, 10 mM CaCl$_2$, 50 mM MnCl$_2$, 15% glycerol; pH adjusted to 5.8 with 50% HCl, filter sterilised and stored at 4°C.</td>
<td>For the preparation of chemically competent <em>E. coli</em></td>
</tr>
<tr>
<td><strong>TfbII (transformation buffer II)</strong></td>
<td>10 mM MOPS, 75 mM CaCl$_2$, 10 mM RbCl$_2$, 15% glycerol, pH adjusted to 6.5 with KOH, filter sterilised and stored at 4°C.</td>
<td>For the preparation of chemically competent <em>E. coli</em></td>
</tr>
</tbody>
</table>
(Semi-dry) Transfer buffer

1X Tris-Glycine (TG), 20% Methanol, 0.037% SDS

For the semi-dry transfer of SDS-polyacrylamide gels onto nitrocellulose membranes

(Wet) Transfer buffer

72 mM Tris, 58.5 mM glycine, 15% methanol, 0.1% SDS

For the wet transfer of SDS-polyacrylamide gels onto nitrocellulose membranes

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), DNA ligase, were purchased from New England Biolabs (Hertfordshire, 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, USA). DNA and protein size markers were supplied by New England Biolabs, Fermentas (Glen Burnie, USA) or BioRad (Hercules, 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) 80lacZΔM15 ΔlacX74deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(StrR) endA1 nupG. Plasmid DNA preparations were carried out using GenElute™ 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.

A number of commercially available cloning and expression plasmids were used during the course of this project, as shown in Table 2.2.

<table>
<thead>
<tr>
<th>Plasmid name</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, USA)</td>
</tr>
<tr>
<td>pEGFP-C1/N1</td>
<td>Expression in mammalian cells</td>
<td>Clontech (Palo Alto, USA)</td>
</tr>
<tr>
<td>pCMV-3TAG-2A/B/C</td>
<td>Mammalian expression vector carrying 3 myc tags</td>
<td>Stratagene (La Jolla, USA)</td>
</tr>
<tr>
<td>ptTA 2/3/4</td>
<td>Regulator plasmids, encoding tTA, used in the Tet-inducible system</td>
<td>Stratagene (La Jolla, USA)</td>
</tr>
<tr>
<td>pUHG10.3</td>
<td>Tet-controlled expression vector</td>
<td>(Gossen and Bujard, 1992)</td>
</tr>
</tbody>
</table>
2.1.3 Antibodies

Primary and secondary antibodies (Table 2.3 and 2.4) used throughout this study were used in immunoblotting (IB) detection, immunofluorescence (IF) microscopy and fluorescence-activated cell sorting (FACS) analysis. Tables 2.3 and 2.4 show the clone/reference number, host species, working dilutions and source of these antibodies.

Table 2.3 Primary antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</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>Actin</td>
<td>A2066</td>
<td>Rabbit polyclonal</td>
<td>1:5000</td>
<td>-</td>
<td>Abcam</td>
</tr>
<tr>
<td>Acetylated-tubulin</td>
<td>T6793</td>
<td>Mouse monoclonal</td>
<td>-</td>
<td>1:2000</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>B512</td>
<td>Mouse monoclonal</td>
<td>1:10000</td>
<td>1:2000</td>
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</tr>
<tr>
<td>Aurora A</td>
<td>35C1</td>
<td>Mouse polyclonal</td>
<td>1:1000</td>
<td>1:250</td>
<td>Abcam</td>
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<tr>
<td>C-Nap1</td>
<td>42</td>
<td>Mouse monoclonal</td>
<td>1:500</td>
<td>1:250</td>
<td>BD transduction</td>
</tr>
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<td>Centrin2</td>
<td>sc-27793R (N17R)</td>
<td>Rabbit polyclonal</td>
<td>1:1000</td>
<td>1:500</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Centrin2</td>
<td>poly6288</td>
<td>Rabbit polyclonal</td>
<td>-</td>
<td>1:500</td>
<td>Biolegend</td>
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<tr>
<td>Centrin3</td>
<td>3E6</td>
<td>Mouse monoclonal</td>
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<td>1:1000</td>
<td>Abnova</td>
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<td>Centrobin</td>
<td>Ab70448</td>
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<tr>
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<tr>
<td>Cep170</td>
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<td>1:500</td>
<td>Giulia Guarguaglini (Guarguaglini et al., 2005)</td>
</tr>
<tr>
<td>Cep170</td>
<td>41-3200</td>
<td>Mouse monoclonal</td>
<td>-</td>
<td>1:500</td>
<td>Invitrogen</td>
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<tr>
<td>Cep76</td>
<td></td>
<td>Rabbit polyclonal</td>
<td>-</td>
<td>1:500</td>
<td>Brian Dynlacht (Tsang et al., 2009)</td>
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<tr>
<td>Cep164</td>
<td>NBP1-77006</td>
<td>Rabbit polyclonal</td>
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<td>Novus biologicals</td>
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<td>Mouse monoclonal</td>
<td>1:1000</td>
<td>-</td>
<td>Sigma-Aldrich</td>
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<td>Mouse monoclonal</td>
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<td>1:1000</td>
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<td>Protein</td>
<td>Code</td>
<td>Source</td>
<td>Dilution</td>
<td>Company</td>
<td></td>
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<tr>
<td>Glutamylated tubulin</td>
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<td>Mouse monoclonal</td>
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<td>Carsten Janke (Wolff et al., 1992)</td>
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<td>Naoki Oshimori (Oshimori et al., 2006)</td>
<td></td>
</tr>
<tr>
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<td>1:1000</td>
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<tr>
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<td>Rabbit polyclonal</td>
<td>1:1000</td>
<td>Andreas Merdes (Haren et al., 2006)</td>
<td></td>
</tr>
<tr>
<td>Nek2</td>
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<td>Mouse monoclonal</td>
<td>1:250</td>
<td>BD transduction</td>
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</tr>
<tr>
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<td>Rabbit polyclonal</td>
<td>1:200</td>
<td>Andreas Merdes (Mogensen et al., 2000)</td>
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</tr>
<tr>
<td>PCM-1</td>
<td>817</td>
<td>Rabbit polyclonal</td>
<td>1:10000</td>
<td>Andreas Merdes (Dammermann and Merdes, 2002)</td>
<td></td>
</tr>
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<td>Ab4448</td>
<td>Rabbit polyclonal</td>
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<tr>
<td>STIL</td>
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<td>Leonard Zon (Pfaff et al., 2007)</td>
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<tr>
<td>53BP1</td>
<td>NB100-904</td>
<td>Rabbit polyclonal</td>
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<td>Novus Biologicals</td>
<td></td>
</tr>
<tr>
<td>Reactivity</td>
<td>Conjugation</td>
<td>Host Species</td>
<td>Working dilution for IB</td>
<td>Working dilution for IF</td>
<td>Source</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>--------------------------</td>
<td>--------------</td>
<td>-------------------------</td>
<td>-------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Mouse IgG (H &amp; L)</td>
<td>Texas red</td>
<td>Goat</td>
<td>-</td>
<td>1:200</td>
<td>Jackson Labs</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 Labs</td>
</tr>
<tr>
<td>Mouse IgG (H &amp; L)</td>
<td>FITC</td>
<td>Donkey</td>
<td>-</td>
<td>1:200</td>
<td>Jackson Labs</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 peroxidase)</td>
<td>Goat</td>
<td>1:10000</td>
<td>-</td>
<td>Jackson Labs</td>
</tr>
<tr>
<td>Rabbit IgG (H &amp; L)</td>
<td>FITC</td>
<td>Goat</td>
<td>-</td>
<td>1:1000</td>
<td>Jackson Labs</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>Molecular Probes</td>
</tr>
<tr>
<td>Rabbit IgG (H &amp; L)</td>
<td>HRP</td>
<td>Goat</td>
<td>1:10000</td>
<td>-</td>
<td>Jackson Labs</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>

2.1.4 Tissue culture reagents and cell lines

All sterile plastic ware used for tissue culturing was obtained from Sarstedt (Numbrecht, Germany), Corning (Riverfront Plaza, NY) and Sigma-Aldrich. The following media were used in cell culture: Dulbecco’s modified eagle medium (DMEM), Dulbecco’s modified eagle medium with Ham’s F12 nutrient mix (DMEM-F12) and Roswell Park Memorial Institute media (RPMI) 1640 were from Lonza (Cambridge, UK). Iscove’s modified eagle medium (IMDM) and Leibovitz’s medium (L-15) were from Sigma-Aldrich.

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 (San Diego). Transfections for the generation of stable chicken cell lines were carried out with the Gene pulser apparatus from Bio-Rad (Hercules, CA).
Table 2.5 shows the cell lines used during this study, along with their culture medium and growth conditions.

<table>
<thead>
<tr>
<th><strong>Table 2.5 Cell lines and growth conditions used during this study</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell line</strong></td>
</tr>
<tr>
<td>DT40</td>
</tr>
<tr>
<td>hTERT-RPE1</td>
</tr>
<tr>
<td>U2OS</td>
</tr>
<tr>
<td>U2OS::H2B-RFP</td>
</tr>
<tr>
<td>HCC1937</td>
</tr>
<tr>
<td>MDA-MB-436</td>
</tr>
<tr>
<td>CAPAN-1</td>
</tr>
</tbody>
</table>

*Medium was enriched with 15 mM HEPES, 12. g/l sodium bicarbonate, 200 mM L-glutamine and 7.5% sodium bicarbonate (unless already added to medium)

**Medium was enriched with 10 mM HEPES, 1 mM sodium pyruvate, 4.5g/l glucose

***Medium was enriched with 10 μg/ml insulin, 16 μg/ml glutathione

Table 2.6 shows the drugs used for the selection of positive clones following stable transfection into DT40 cells.

<table>
<thead>
<tr>
<th><strong>Table 2.6 Drugs used for stable cell line selection</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Name of the drug</strong></td>
</tr>
<tr>
<td>Blasticidin</td>
</tr>
<tr>
<td>Geneticin</td>
</tr>
<tr>
<td>G418</td>
</tr>
<tr>
<td>Histidinol</td>
</tr>
<tr>
<td>Hygromycin</td>
</tr>
<tr>
<td>Puromycin</td>
</tr>
</tbody>
</table>
RO-3306 is a CDK1 inhibitor, used to block cells at the G2/M phase border and induce centrosome amplification at the concentration shown in Table 2.7.

| Table 2.7 Drugs used during this study |
|----------------|----------------|----------------|
| Drug          | Final Concentration | Source                  |
| RO-3306       | 6 µM             | Merck Millipore (Darmstadt, Germany) |

siRNA was used to deplete expression of a protein of interest. The genes targeted with siRNA and the target sequences are shown in Table 2.8.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>siRNA sequence</th>
<th>Final Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nek2</strong></td>
<td>CGAUCUGGCUAGUGUAUU</td>
<td>50 nmol</td>
<td>Dharmacon</td>
</tr>
<tr>
<td></td>
<td>GCAGACAGAUCCUGGGCAU</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GGCAUAUCUUGAGUAGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCUGAUAUAUAUAACCAUG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CEP250</strong></td>
<td>GAGCAGAGCUACAGCGAAU</td>
<td>50 nmol</td>
<td>Dharmacon</td>
</tr>
<tr>
<td></td>
<td>GGACCUCUGUGAAACACUU</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AACUGACUGUGGUGAAUAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAGAAUUGAUCCAAGAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CROCC</strong></td>
<td>AGGAGGAGGGUGCAGCGUU</td>
<td>50 nmol</td>
<td>Dharmacon</td>
</tr>
<tr>
<td></td>
<td>GGAGAUAUGUCACCCGCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAGCGACUCGCAGGUGAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Kizuna</strong></td>
<td>UGUCCAAGCUAGUGCUA</td>
<td>50 nmol</td>
<td>Dharmacon</td>
</tr>
<tr>
<td></td>
<td>GAAAUAUCUGACGCGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCAAAGAUUUAUAAGCGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAUAUUGGGACCGGUGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chk1</strong></td>
<td>CAAGAGUGUGUGGUACUUUA</td>
<td>50 nmol</td>
<td>Dharmacon</td>
</tr>
<tr>
<td></td>
<td>GAGAAGGCAUAUAUCAAUA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCACACUGGCUAGUACUAUU</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GAAGUUGGGCUAUAUGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CEP250</strong></td>
<td>CUGGAAGAGCGUCAUCGUA</td>
<td>50 nmol</td>
<td>Qiagen</td>
</tr>
<tr>
<td><strong>CROCC</strong></td>
<td>AAAAGCCAGUCUAGACAAGGA</td>
<td>50 nmol</td>
<td>Qiagen</td>
</tr>
<tr>
<td><strong>CEP164</strong></td>
<td>CAGGUGACAUUUACUUAUCUA</td>
<td>50 nmol</td>
<td>Qiagen</td>
</tr>
</tbody>
</table>

### 2.1.5 Computer Software

DNA plasmid maps were created using pDRAW32 software (Acacleone, [www.acaclone.com](http://www.acaclone.com)) or DNA strider software (version 1.4f2). Sequenced DNA samples were viewed using Chromas software (version 2.31, Digital River GmbH, Shannon, Ireland). For bioinformatic analyses, BlastN or BlastP ([http://www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)), ClustalW ([www.ebi.ac.uk/clustalw](http://www.ebi.ac.uk/clustalw)) and dbEST ([http://www.ncbi.nlm.nih.gov/dbEST/](http://www.ncbi.nlm.nih.gov/dbEST/)) were used. Microscopy imaging was performed using an Olympus BX-51 microscope, driven by OpenLab software
(version 5, Improvison, Emeryville, USA). Deconvolved images were saved as Adobe Photoshop images (version 7, San Jose, USA). Analysis of flow cytometry samples was carried out using CELLQuest (version 3.3, Becton Dickinson and Company, Oxford, UK) or BD FACS Diva Software (version 6.1.2, Becton Dickinson, Oxford, UK). All live cell microscopy was carried out on a Deltavision microscope, controlled by Softworx software (Applied Precision, Issaquah, WA). Statistical analysis of microscopy data was carried out on Prism 5 (Graphpad, La Jolla, CA).

2.2 Nucleic Acid methods

2.2.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was carried out using either, KOD or Takara polymerases, depending on the experiment performed. PCR experiments were carried out on a TGradient (Biometra, Göttingen, Germany). Table 2.9 gives an example of the PCR conditions and programmes used for sequence amplification.

Table 2.9 Example of typical PCR reaction conditions

<table>
<thead>
<tr>
<th>Reagents</th>
<th>TaKaRa LA Taq Polymerase</th>
<th>KOD Polymerase</th>
<th>PCR steps</th>
<th>TaKaRa LA Taq</th>
<th>KOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer (10x)</td>
<td>1x</td>
<td>1x</td>
<td>PCR steps</td>
<td>94°C – 1 min</td>
<td>94°C – 2 min</td>
</tr>
<tr>
<td>Primers</td>
<td>0.2 µM</td>
<td>0.2 µM</td>
<td>Denaturation</td>
<td>98°C – 10 sec</td>
<td>94°C – 1 min</td>
</tr>
<tr>
<td>dNTP's</td>
<td>200 µM</td>
<td>200 µM</td>
<td>Annealing</td>
<td>58-64°C– 30 sec</td>
<td>58-64°C– 30 sec</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>2.5 mM</td>
<td>2 mM</td>
<td>Extension</td>
<td>68°C – 3 min</td>
<td>72°C – 2 min</td>
</tr>
<tr>
<td>Enzyme</td>
<td>0.5 µl (5 U/µl)</td>
<td>0.5 µl (5 U/µl)</td>
<td>Final extension</td>
<td>72°C – 10 min</td>
<td>72°C – 10 min</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. cycles</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

2.2.2 Digoxygenin (DIG) Labelling of probes for Southern blot analysis

For labelling of probes with digoxigenin in non-radioactive (cold) Southern hybridizations, the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany) was used. Table 2.10 shows an example of conditions and programmes used for DIG labelling of probes. Table shows the PCR conditions for DIG labelling of probes.
Table 2.10 Conditions for DIG labelling of probes

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
<th>Typical Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template DNA</td>
<td>100 ng</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Primers</td>
<td>0.2 μM</td>
<td>1 μl of 1:10 dilution of each</td>
</tr>
<tr>
<td>PCR DIG probe synthesis mix - containing dNTPs and DIG-11-dUTP</td>
<td>dNTPs – 200 μM each DIG-11-dUTP- 70 μM</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>Enzyme mix</td>
<td>0.5 μl (3.5 U/μl)</td>
<td>0.75 μl</td>
</tr>
<tr>
<td>DIG buffer</td>
<td>10X</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>H2O</td>
<td>-</td>
<td>16 μl</td>
</tr>
<tr>
<td>PCR steps</td>
<td>‘Hot start’</td>
<td>95°C – 3 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td></td>
<td>95°C – 30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td></td>
<td>58°C – 30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72°C – 1 min</td>
</tr>
<tr>
<td>Final extension</td>
<td></td>
<td>72°C – 5 min</td>
</tr>
<tr>
<td>cool</td>
<td></td>
<td>10°C – ∞</td>
</tr>
<tr>
<td>No. of cycles</td>
<td>-</td>
<td>30</td>
</tr>
</tbody>
</table>

2.2.3 Plasmid DNA preparation

Mini and midi plasmid DNA preparations were carried out using GenElute™ Plasmid Miniprep Kit and Midiprep Kit according to manufacturer’s instructions. Briefly, bacterial cell cultures were grown overnight at 37°C on a shaking platform, in the presence of selective antibiotics. For mini preps, DNA was isolated from 2 ml of bacterial culture and resuspended in 50-100 μl of deionised water. For midi preps, 100 ml of bacterial culture was resuspended in 100-200 μl of deionised water.

2.2.4 Restriction digest of DNA

All restriction endonucleases used for the digestion of plasmid or genomic DNA were purchased from New England Biolabs. 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 manufacturers indicated temperature 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.

2.2.5 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. In order to reduce self-ligation, digested plasmid DNA was dephosphorylated on the 5’ ends using shrimp alkaline phosphatase ((SAP) 1U, pmol of DNA ends). The reaction was carried out in SAP buffer at 37°C for 1 hour.
Following this, SAP was 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).

### 2.2.6 Preparation of competent *E. coli* and transformation

*E. coli* cells were grown in 500 ml of LB broth at 37°C with shaking. When culture reached an $A_{600nm}$ of 0.5, cells were transferred to ice for 5 minutes and pelleted by centrifugation at 5000 $g$ for 15 minutes. The cell pellet was re-suspended in ice cold Tfb I (40 ml per 100 ml culture, Table 2.1). Next, cells were spun and re-suspended in ice cold Tfb II (4 ml per initial 100 ml culture, Table 2.1). Subsequently cells were incubated on ice for 15 minutes, snap frozen in liquid nitrogen and stored at -80°C.

For transformations, 50 μl of competent *E. coli* cells were first thawed on ice. They were then mixed with plasmid DNA/ligation mix and incubated on ice for 20 minutes. The transformation mixture was next transferred to 42°C for 90 seconds before being chilled on ice for 90 seconds. 1 ml of LB broth was then added to transformation mixture followed by gentle shaking for 30 minutes at 37°C. For ligations, cells were pelleted by centrifugation at 16,100 $g$ for 1 minute, resuspended in 100 μl of LB broth and plated onto agar plates containing appropriate antibiotics. In the case of pure plasmid DNA transformations only 50 – 100 μl of culture was plated onto agar plates. Plates were inserted and incubated at 37°C overnight.

### 2.2.7 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 (ChemIlmager 5500, Alpha Innotech, Medical Supply Company, Dublin, Ireland) 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. 20-50 µl of milli-Q H₂O was used to elute bound DNA off the column.

2.2.8 DNA sequencing

DNA samples were sent to either Cogenics (Takeley, UK) or Agowa GmbH (Berlin, Germany) for commercial sequencing. In general, 250 ng of DNA (mini or midi prepped) and 5 – 10 pM primers were used per sequencing reaction. Sequences were analysed using Chromas software (Digital River GmbH, Shannon, Ireland) and pDRAW32 was used were used to construct correct vector maps with the analysed sequences (Acaclone, www.acaclone.com) or DNA strider software.

2.2.9 Preparation of genomic DNA from tissue culture cells

Genomic DNA was prepared from chicken DT40 cells to screen clones for potential targeting events by digoxigenin-based Southern hybridisation (Roche, Mannheim, Germany) (Section 2.2.2). Following stable transfection (Section 2.4.2), the cells were plated into 96 well plates and incubated at 39.5°C for 7-10 days. Colonies were picked from 96 well plates and transferred into 24 well plates and grown for 3-4 days to expand the clones. 1.5 ml of confluent cells was frozen down and 1.5 ml was taken for DNA preparation. The cells were pelleted at 160 g for 5 minutes and re-suspended 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, 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. The supernatant was removed and 1 volume of isopropanol was added to precipitate the DNA by inverting the mixture. DNA was spun at 16,100 g for 10 minutes, the pellet was washed in 1 volume, 70% ethanol and again spun at 16,100 g for 10 minutes. DNA was air dried for 5-15 minutes and re-suspended in 70 µl of milli-Q H₂O and incubated at 37°C to aid resuspension of DNA.

2.2.10 Cold Southern blot

Genomic DNA digestion was performed as described in section 2.2.4. Each digest consisted of a final volume of 45 µl with the addition of RNase (10 µg/ml),
BSA and the appropriate endonuclease were incubated overnight at 37°C. Digested DNA was separated on a 0.8% agarose gel as described in section 2.2.7. DNA was nicked and residual proteins removed by treatment with 0.25 M HCl for 20 minutes, followed by rinsing in water and DNA was then denatured with 0.5 M NaOH/1.5 M NaCl for 20 minutes. The gel was rinsed again in water and DNA fragments were transferred by capillary transfer onto a positively charged nylon membrane (GE Healthcare, Bucks, UK) overnight in 0.2 x SSC (Table 2.1). DNA was cross-linked to the membrane with UV 3000 J/m² at 50 J/sec, using a UV Cross-linker (Hoefer UVC500, GE Healthcare, Bucks, UK). Digoxigenin labelling and amplification of probes was performed, as described in Section 2.2.2. 5-7 µl of the PCR reaction was then denatured in 50 µl of MiliQ water at 95°C for 10 minutes, then cooled on ice. The membrane was pre-treated with Hybe Buffer for 30 minutes (Roche, Mannheim, Germany). The denatured probe was then added to the pre-hybridised membrane and incubated overnight at the appropriate hybridization temperature (T_m was calculated according to manufacturer’s instructions). The next day, the membrane was washed twice with Low Stringency Buffer (Table 2.1) for 5 minutes at room temperature, followed by two washes in High Stringency Buffer (Table 2.1) at 65°C for 15 minutes each. The membrane was pre-incubated in maleic acid buffer for 3 minutes and then blocked with non-radioactive Southern blot blocking solution (Table 2.1) for 30 minutes at 25°C, followed by incubation with anti-digoxigenin antibody in blocking solution for 30 minutes at 25°C. Non-specifically bound antibody was removed with two 15 minute washes in maleic acid washing buffer at 25°C and the membrane incubated with detection buffer for 2 minutes at 25°C to bring the membrane to pH required for probe detection (Table 2.1). The membrane was then incubated with CSPD substrate (diluted 1:1 with detection buffer) for 5 minutes at room temperature. Excess CSPD substrate was removed and the membrane was sealed in the plastic film and incubated at 37°C for 10 minutes to enhance the signal. The membrane was exposed to film for 4-18 hours depending on the signal strength.

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 160 g for 5 minutes, cells were washed in 1X PBS and cells were centrifuged again at 160 g for 5 minutes. The 1X PBS was then removed and cells were resuspended in 20-50 μl lysis buffer containing protease and phosphatase 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 found using the Bradford method (Section 2.3.2). 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. In general, 30-40 μg of protein was loaded per sample lane.

Table 2.11 gives examples of different percentages of gel mixes used for protein detection, depending on molecular weight.

<table>
<thead>
<tr>
<th>Table 2.11 Example of upper gel mixes and stacking gel for SDS-PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel Percentage</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Acrylamide/bis-acrylamide ratio</td>
</tr>
<tr>
<td>Acrylamide</td>
</tr>
<tr>
<td>Bis-acrylamide</td>
</tr>
<tr>
<td>Tris-HCl pH 8.8</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
</tr>
<tr>
<td>Ammonium persulfate (APS)</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED)</td>
</tr>
</tbody>
</table>

2.3.2 Bradford Protein Assay

For the determination of protein concentration, the Bradford dye-binding protein assay was used as described (Bradford, 1976). Briefly, 1 μl of a protein sample was diluted in 1 ml 1:1 Bradford : dH₂O. The absorbance was measured at 595nm. The protein concentration was calculated based on a BSA standard curve, in which absorbance was plotted against increasing concentrations of the BSA protein.

2.3.3 Western blotting

Proteins were separated by SDS-PAGE and then transferred to nitrocellulose membrane (GE Healthcare, Bucks, UK). Small proteins, up to 100 kDa were transferred for 90 minutes at room temperature and constant amperage, in semi-dry
Chapter 2

2.1 Transfer of Proteins

Transfer buffer, using a Hoefer TE 77 Semi Dry Transfer Unit (GE Healthcare, Bucks, UK) (Table 2.1). The amperage was dictated by the size of the membrane following the formula \( mA = 0.8 \times \text{membrane area} \left( \text{cm}^2 \right) \). Transfer of proteins larger than 100 kDa was performed using a Hoefer TE 22 Mighty Small Transfer apparatus (GE Healthcare, Bucks, UK). Transfer was carried out at 4°C in wet transfer buffer (Table 2.1) for 3 h at 100 V, 350 mA or overnight at 15 V. The membrane was then rinsed briefly in \( \text{dH}_2\text{O} \) and Ponceau S. solution (Table 2.1) was used to visualise the quality of protein transfer. To decrease non-specific antibody binding, the membrane was blocked with a 5% milk solution in 1X PBS-0.05% Tween-20 for 1 hour on a rocking platform at room temperature (Table 2.1). The blocked membrane was then incubated overnight in the primary antibody solution (in 1% milk in 1X PBS-0.05% Tween-20) at 4°C at the concentrations shown in Table 2.3. The next day, the membrane was washed three times for 7 minutes in 1X PBS-0.05% Tween-20 and transferred to the secondary antibody solution (Table 2.4) in 1% milk for 60 minutes at room temperature. Again, 3 x 7 minute washes in 1X PBS-0.05% Tween-20 were performed, and the specific proteins detected with ECL detection kit (GE Healthcare or Milipore) and autoradiograph film exposure (Hartenstein, Germany). The exposed film was then fixed and developed by passing it through a developing machine (CP 1000, AGFA, Brentford, UK).

2.4 Cell Biology Methods

2.4.1 Tissue culture techniques

The cells used for this study were cultured as described in Table 2.5. Adherent human cells reached confluency at a cell density of \( 7 \times 10^6 \text{ cells/75 cm}^2 \) 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 (\( 6 \times 10^5 \text{ cells/75 flask} \)) 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, \( 2 \times 10^6 \text{ cells/vial} \) were harvested and resuspended in 500 \( \mu \text{l} \) of freezing medium (60% FBS, 10% DMSO, 30% 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.
DT40 cells reached confluency at a density of $1 \times 10^6$ cells/ml. Cells were counted using a haemocytometer and maintained at densities between $1 \times 10^5$ cells/ml and $8 \times 10^5$ cells/ml. For freezing of cell stocks, $1 \times 10^6$ cells/ml were harvested by centrifugation at 1000 g for 5 minutes, cells were resuspended in 500 µl of freezing medium (90% FBS, 10% DMSO) and transferred to cryo-vials and stored in the same manner as adherent cells. For the resuscitation of adherent and suspension cells, cells were thawed in a 37°C water bath then added to 10 ml of prewarmed media and returned to the incubator.

Cells were irradiated using a $^{137}$Cs irradiator (Mainance Engineering, Hampshire, UK), at a dose rate of 23.5 Gy/minute.

Cells were serum starved by washing twice in warm 1X PBS before medium with 0.2% newborn calf serum (NCS) was added (unless otherwise indicated).

### 2.4.2 Stable transfections and gene targeting

Electroporation was used to either generate stably expressing DT40 cell lines or for a gene targeting, as previously described (Sonoda et al., 1998; Takata et al., 1998; Morrison et al., 2000). $1 \times 10^7$ of cells were pelleted for 5 minutes at 160 g, washed once in sterile 1X PBS and re-suspended in 0.5 ml of 1 x sterile PBS. 20-25 µg of linearised DNA was added to the cells and gently resuspended before the mixture was transferred to an electroporation cuvette (BioRad, 0.4 cm gap) and incubated on ice for 10 minutes. Electroporation was carried out in BioRad Gene Pulser (Hercules, USA) at conditions of 300 V/600 µF for targeted integration of DNA and 550 V/25 µF for random integration of DNA into the DT40 genome. The electroporated cells were again incubated on ice for 10 minutes prior to being transferred to 20 ml of fresh, pre-warmed medium. After 18 to 24 hours, 20 ml of fresh media was added to the transfected cells. The appropriate selective antibiotics (Table 2.6) were added to the culture and cells were plated out into four 96 well plates (100 µl/well). Plates were incubated at 39.5°C until colonies were visible through the bottom of the plate. Single colonies were transferred to 24 well plates containing 3 ml of medium and incubated under non-selective conditions at 39.5°C until confluent. Following this, 1.5 ml was frozen down and 1.5 ml was used for screening by IF, IB or cold Southern blot analysis.
2.4.3 RNA mediated interference

RNA mediated interference was carried out on hTERT-RPE1 cells using either ON-TARGETplus smartpools of RNA duplexes (Dharmacon, Lafayette, CA) or custom siRNA target sequences from Qiagen to knock down messenger RNA transcripts for proteins of interest as detailed in Table 2.8. As a negative control, a non-targeting short interfering pool was used, also from Dharmacon. Cells were seeded the day prior to transfection in antibiotic free medium, so that a density of 20-30% would be obtained at the time of transfection (generally a 1:10 dilution from an 80-90% confluent T75 flask would yield this concentration). siRNAs were resuspended in siRNA buffer to yield a concentration of 20 μM. The siRNA protocol involved the preparation of two separate solutions which were mixed prior to adding to cells. The transfection of cells seeded in a 60 mm dish was carried out as follows: 2.5 μl siRNA (to yield a final concentration 50 nmol) was mixed with 177.5 μl of serum-free OptiMEM and separately, 6 μl of Oligofectamine was mixed with 14 μl of OptiMEM. Both mixtures were incubated at room temperature for 5 minutes. These were then gently mixed together and incubated at room temperature for a further 20 minutes. In the meantime, cells were removed from the incubator and washed three times in 1X PBS in order to remove all traces of serum from cells. 800 μl of OptiMEM was added to cells and the siRNA-Oligofectamine complex was then added to the cells. The dishes were rocked gently by hand to disperse the siRNA-Oligofectamine complex and cells were returned to the incubator. After 4 hours, 1000 μl of medium containing 30% FBS was added to the cells, to return serum levels to normal and cells were returned to the incubator. The following day, the medium was removed and medium containing 10% FBS but no antibiotics was added to the cells. The cells were fixed and analysed at the required times. Where necessary, cells were irradiated 24 hours after transfection.

2.4.4 Flow Cytometry

For cell cycle analysis, adherent cells were detached by trypsinization as described in Section 2.4.1. Cells were centrifuged at 160 g for 5 minutes, washed in 5 ml of 1X PBS and centrifuged again at 160 g for 5 minutes. PBS was removed and cells were fixed by addition of 1 ml ethanol (pre-chilled to -20°C) and gentle resuspension, before being stored at 4°C prior to flow cytometry. Before analysis, cells were washed in medium (pre-warmed to 37°C) to remove precipitated salt and
resuspended in 1X PBS supplemented with 100 μg/ml RNase A and 40 μg/ml propidium iodide (PI, Sigma). After 20 minutes incubation at room temperature or, optionally overnight (in the dark), cells were analysed using a FACS Calibur or FACS Canto (Becton Dickinson, San Jose, CA) and Cell Quest (version 3.3, Becton Dickinson) or BD FACS Diva Software (version 6.1.2, Becton Dickinson), respectively.

2.5 Microscopy Methods

2.5.1 Methanol fixation

Adherent cells were grown on sterile coverslips for immunofluorescence microscopy (IF), 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 -20°C, 95% methanol with 5 mM EGTA, for 10 minutes at -20°C. Cells were then washed three times in 1X PBS before proceeding to immunofluorescence microscopy.

2.5.2 Paraformaldehyde fixation

Cells were adhered as described in Section 2.5.1. Following this media 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 immunofluorescence microscopy.

2.5.3 Immunofluorescence microscopy

Cells were fixed using either methanol or PFA fixation as described in the previous sections before being stained with the antibodies detailed in Section 2.1.3. The fixation method depended on the nature of the antibody used. Following fixation, cells were blocked in 1% BSA for 30 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) with DAPI (1 µg/ml). Coverslips were sealed with nail varnish and stored at 4°C in the dark. Images were taken on an Olympus BX51 microscope, using 40X, 60X and 100X objectives, numerical aperture (NA) 1.35, using Openlab software (improvision). Serial Z-sections (0.15 µm) were taken, deconvolved, merged and saved as photoshop TIFF files.

In order to visualise primary cilia using an acetylated tubulin antibody, all other microtubules needed to be first deacetylated (Piperno et al., 1987). To do this, cells were incubated on ice for 20 minutes prior to methanol fixation. This would deacetylate microtubules and allow the visualisation of highly acetylated primary cilia without other microtubules being visible.

2.5.4 Live cell imaging

For live cell imaging, cells were grown on glass-bottomed 35mm dishes (MatTek, Ashland, MA) and analysed at 60-70% confluency. Cells were imaged at hourly 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 objective, controlled by SoftWorx software. Serial Z-sections (0.5 µm) were collected and a quick projection was made using SoftWorx software.

2.5.5 Transmission electron microscopy

Cells were prepared for TEM using an adapted version of the protocol as described (Liptrot and Gull, 1992). hTERT-RPE1 cells were grown on 10 cm dishes. Before fixation, they were washed in PBS and then scraped into eppendorf tubes before being pelleted at 250 g for 5 minutes. The cell pellet was washed twice in 0.1 M sodium cacodylate buffer, pH 7.2 (Section 2.1.1). The cell pellet was centrifuged again at 250 g for 5 minutes. The cell pellet was incubated in 2% osmium tetroxide/0.1 M sodium cacodylate buffer, pH 7.2, for at least an hour or until the cell pellets turned dark brown. Cell pellets were then dehydrated through a graded series of ethanol (30, 60, 90, and 100%) before propylene oxide was added to the pellet. Next, the cell pellets were embedded as follows: cell pellets were incubated in 50:50 propylene oxide: resin solution for 1 hour, in 25:75 propylene oxide: resin solution for 1 hour, in pure resin for an hour and replaced again with pure resin for a day. This was replaced again with fresh resin and the pellet was transferred to a 60 °C
incubator for 2 days to solidify the resin and improve the quality of the sections. Sections were cut on a microtome (Reichert-Jung Ultracut E; Leica), stained with uranyl acetate and lead citrate, and then viewed on an electron microscope (H-7000; Hitachi). Images were taken with a camera (ORCA-HRL; Hamamatsu Photonics) and processed using AMT version 6 (AMT Imaging).
Chapter 3. Cloning, characterisation and knockout of chicken \textit{Stil}

3.1 Introduction

3.1.1 Gene Targeting

Gene targeting is the introduction of site-specific modifications into the genome by homologous recombination (HR) (reviewed by Hudson et al., 2002). Gene targeting is regularly used in mouse embryonic stem (ES) cells to generate knockout mice. This is where one copy of the gene is targeted by HR in ES cells. These targeted cells are then injected into blastocysts to generate germ line chimeras. The resulting heterozygote mice are then interbred to create homozygous mutant mice (Capecchi, 1989). In general, however, the application of targeted HR for genetic manipulation is limited in mammalian cells due to low integration efficiency of foreign DNA into chromosomes. The chicken DT40 cell line is an ideal model for gene targeting due to its high ratio of targeted to random integration of transfected DNA (Buerstedde and Takeda, 1991). The DT40 cell line was originally derived from an avian leukosis virus (ALV)-induced bursal lymphoma (Baba and Humphries, 1984; Baba et al., 1985). These cells continuously undergo gene conversion of the light chain immunoglobulin variable region and it is thought that this is why these cells have such high levels of HR (Buerstedde and Takeda, 1991; Arakawa and Buerstedde, 2006).

Disruption of essential genes can prove problematic and targeting of these genes is not achievable using standard gene targeting approaches as the cells cannot proliferate in the absence of such genes. In these cases, conditional knockout strategies have been developed in order to study the function of essential genes. These methods include: transgene removal by Cre/loxP and FLP/frt mediated recombination or auxin-mediated protein degradation (Gu et al., 1993; Nishimura et al., 2009). Additionally, essential genes can be expressed using a tetracycline regulated transgene. This prevents the death of the targeted cells by expressing the cDNA of the essential gene under the control of a tetracycline operator. After gene targeting, the essential transgene or activity of the protein it encodes can be inactivated and the null phenotype can then be analysed (Gossen and Bujard, 1992; reviewed by Hudson et al., 2002).
In the Tet-Off system, a tetracycline-controlled transactivator (tTA) protein, which is composed of the Tet DNA binding protein (TetR, from the Tc resistance operon of *E. coli* transposon Tn10) and the VP16 activation domain (AD, from the Herpes Simplex virus), regulates expression of a gene which is under transcriptional control of a tetracycline-responsive promoter element (TRE). The TRE consists of a Tet operator (TetO) repetitive sequence fused to a minimal promoter. The tetracycline (Tc) or doxycycline (Dox) deactivates the tTA protein which renders it incapable of binding the TRE element, therefore preventing transactivation of target genes (Gossen and Bujard, 1992; reviewed by Hudson et al., 2002). In order to obtain controllable protein expression at a range of different levels of induction, some modifications have been introduced into the VP16 activation domain of the tTA vectors (ptTA2/3/4), which causes different efficiencies of binding to the target (Baron et al., 1997). The tetracycline-controlled activation system behaves as an efficient genetic switch in many eukaryotic cell types and it has certain advantages over Cre/loxP and FLP/frt systems, such as, its reversibility and the tight control of expression (Resnitzky et al., 1994; Weinmann et al., 1994; Kistner et al., 1996).

### 3.1.2 STIL

The *STIL* gene (SCL/TAL1 interrupting locus, also called *SIL* and *MCPH7*) was initially cloned from a common chromosomal rearrangement in T-cell acute lymphoblastic leukaemia 1 (TAL1), which is also known as stem cell leukaemia (SCL) (Aplan et al., 1991). STIL localises to the centrosome in interphase and mitotic cells (Pfaff et al., 2007; Kumar et al., 2009; Tang et al., 2011; Vulprecht et al., 2012). Recently, several mutations in *STIL* have been identified in autosomal recessive primary microcephaly (Kumar et al., 2009). All of the genes known to be mutated in primary microcephaly encode proteins which localise to the centrosome or mitotic spindle; *Microcephalin/BRIT1* (*MCPH1*), *WDR62* (*MCPH2*) (WD repeat domain 62), *CDK5RAP2/CEP215* (*MCPH3*), *CEP152* (*MCPH4*), *ASPM* (*MCPH5*) (*Abnormal spindle*-like microcephaly-associated protein) and *CPAP/CENPJ* (*MCPH6*) (Bond et al., 2002; Jackson et al., 2002; Bond et al., 2005; Zhong et al., 2005; Jeffers et al., 2008; Guernsey et al., 2010; Nicholas et al., 2010). More recently, mutations in *CEP135* and *CEP63* have also been identified as microcephaly-causing mutations, the products of which also localise to the centrosome (Sir et al., 2011; Hussain et al., 2012). It is thought that microcephaly is
caused by a reduced number of neurons due to defective mitosis of foetal neuronal precursor cells (reviewed by Thornton and Woods, 2009).

Mice lacking Stil die at mid gestation and show growth retardation, defects in the developing neural fold and randomization of the left-right asymmetry (Izraeli et al., 1999). In zebrafish, mutations in Stil lead to neuronal death and metaphase arrest with disorganised mitotic spindles that often lack one or both centrosomes (Pfaff et al., 2007). In mouse embryonic fibroblasts and 3T3 mouse fibroblasts, loss of STIL also leads to the absence of identifiable centrosomes in interphase (Castiel et al., 2011). Mouse Stil\textsuperscript{-}\textsuperscript{c} embryonic fibroblasts also lack primary cilia and siRNA-mediated depletion of STIL in U2OS and Hela cells leads to asymmetrical spindle poles and the loss of centrioles (Kitagawa et al., 2011; Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012)

STIL is a regulator of mitotic entry and cell survival. In multiple cancer types, increased STIL expression is correlated with expression of mitotic spindle checkpoint genes and increased metastatic potential (Ramaswamy et al., 2003; Erez et al., 2004; Erez et al., 2007). STIL is required for centriole duplication and is recruited to the nascent daughter centrioles at the onset of duplication, before being degraded in late mitosis (Kitagawa et al., 2011; Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). Interestingly, overexpression of STIL causes overduplication of daughter centrioles in human cells. STIL localises asymmetrically to the daughter centriole and can be detected at the site of procentriole formation even earlier than markers for the distal end of centrioles, such as CP110, which is detected shortly after daughter centrioles begin to form (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012).

When this project was initiated, little was known about STIL. It was known to localise to the centrosome in mammalian cells but its exact centrosomal location was not yet elucidated. The involvement of STIL in microcephaly had been established but its centrosomal function was yet to be unravelled. No information on chicken Stil had been published and only a predicted sequence was available on the NCBI database. The work presented in this chapter will show bioinformatic analysis of chicken Stil (cStil), the cloning of cStil cDNA, the overexpression of STIL in DT40, analysis of its centrosomal localisation and the targeting of its genomic locus.
3.2 Results

3.2.1 Cloning and analysis of cStil cDNA

To generate full-length cStil cDNA, reverse transcriptase-PCR was carried out using genomic DNA isolated from chicken DT40 cells (Section 2.2.9). The target cDNA was amplified using primers designed from the predicted cStil sequence found in the NCBI database (NCBI accession number XM_422457). Agarose gel electrophoresis was then carried out to confirm that the PCR product was the expected size of 3.9 Kb, based on the predicted sequence (Figure 3.1). The cStil cDNA was then cloned into pGEM-T Easy and a number of clones were sequenced to confirm whether the cDNA matched that of the predicted sequence from the NCBI database.

![Figure 3.1. Generation of full-length chicken Stil.](image)

A. cStil was amplified by RT-PCR using primers based on a predicted NCBI database sequence and was found to be the expected size of 3.9 Kb. B. cStil was cloned into pGEM-T Easy and a restriction digest was carried out using NotI to confirm that full-length cStil was successfully cloned.

Of the four sequences analysed, all had six identical codon changes encoding amino acids which differed to the predicted sequence, leading us to suspect that these were the correct codons for these positions. All of these changes resulted from a single nucleotide change, with the exception of the first, which resulted from two nucleotide changes. We performed sequence alignment of the predicted cStil sequence using BLAST against the chicken genome and found that there was a partial sequence for the chicken Scl gene locus (NCBI accession number AJ131018) that contained all of these codon changes, confirming that these were in fact the correct codons at these positions. A seventh codon change occurred in three out of the four sequences, again resulting from a single nucleotide change. The fourth sequence and the partial chicken Scl gene coded for the predicted asparagine so we could not be fully confident as to which was the correct codon for this position. However, as the Scl gene sequence only partially aligned with our cStil sequence and as there were other codon variations between our cStil sequence and the partial Scl gene sequence, we could not be certain of its accuracy. We therefore, decided to proceed with the cloning of the cStil sequence we had generated. We cloned the
sequence into a GFP vector and stably transfected it into DT40 cells to determine if STIL localised to the centrosome in chicken DT40 and if the possible codon/amino acid variations would affect the centrosomal localisation of STIL. As STIL-GFP is being constitutively overexpressed, it would also allow us to determine the effect of STIL overexpression in DT40 cells.

CStil encodes a protein of 1303 amino acids and has a predicted size of 142 kDa. Orthologues of Stil have been identified in several eukaryotes from human to Xenopus laevis. It has also been suggested, based on weak sequence analysis, that the Caenorhabditis elegans orthologue of Stil is sas-5 and that the Drosophila melanogaster orthologue is Ana-2 (Stevens et al., 2010). Human and mouse Stil share 75% sequence identity, while human and chicken and human and X. laevis share 50% and 41%, respectively (Figure 3.2).
weaker group fully conserved.

ClustalW analysis of vertebrate STIL showing conserved coiled coil and STAN domains.

Figure

- Xenopus
- Human
- Mouse
- Chicken

Mouse

PhnqMtatAncfLVKCFKPVPIIFIIIFATNLXSLNLSSLKVQGVTQYLYLOMEDTRKLLEDLEPDFVSPLVLLMSLGNTHSIYSQPCWACLRYPF 285

Chicken

FsnfWavxLAVTfNLCFVPIIFIIIFATNLXSLNLSSLKVQGVTQYLYLOMEDTRKLLEDLEPDFVSPLVLLMSLGNTHSIYSQPCWACLRYPF 266

Mouse

StixqervxQGLTIFILVVTITKEFPPFCGCGIPFIFPPQGGLIT1SFLTVKLLNSLKVQGVTQYLYLOMEDTRKLLEDLEPDFVSPLVLLMSLGNTHSIYSQPCWACLRYPF 374

Mouse

StixqervxQGLTIFILVVTITKEFPPFCGCGIPFIFPPQGGLIT1SFLTVKLLNSLKVQGVTQYLYLOMEDTRKLLEDLEPDFVSPLVLLMSLGNTHSIYSQPCWACLRYPF 369

Mouse

StixqervxQGLTIFILVVTITKEFPPFCGCGIPFIFPPQGGLIT1SFLTVKLLNSLKVQGVTQYLYLOMEDTRKLLEDLEPDFVSPLVLLMSLGNTHSIYSQPCWACLRYPF 366

Mouse

StixqervxQGLTIFILVVTITKEFPPFCGCGIPFIFPPQGGLIT1SFLTVKLLNSLKVQGVTQYLYLOMEDTRKLLEDLEPDFVSPLVLLMSLGNTHSIYSQPCWACLRYPF 298

****:********:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:**:****
Using the COILS algorithm, we found that there is a coiled coil domain at amino acid position 730-760 in chicken STIL (Figure 3.3). This thirty amino acid sequence is very well conserved between human, mouse, chicken and *X. laevis*, and is also partially conserved in *D. melanogaster* and *C. elegans* (Figure 3.2). There is also a STAN (*STIL/ANA-2*) domain at position 1060-1147 in human STIL and again this is well conserved in mouse, chicken and *X. laevis* (Lupas et al., 1991; Stevens et al., 2010). STIL interacts with CPAP but this interaction is dependent on neither the coiled coil nor STAN domain, but instead on the N-terminal half of STIL. However, both the coiled coil and STAN domain are required for the centrosomal localisation of STIL in human cells (Vulprecht et al., 2012).

![Figure 3.3. Prediction of coiled coil domain in chicken Stil.](image)

Figure 3.3. Prediction of coiled coil domain in chicken Stil. Coiled coil domain located at amino acid position 730-760 in chicken STIL based on the COILS algorithm.

We further analysed cStil by comparing the exons to those in human STIL (hSTIL). Table 3.1 shows the comparison of synteny between the two species. hSTIL contains seventeen exons, whereas cStil contains only fifteen. However the first two exons of hSTIL are non-coding. The other fifteen exons are roughly the same size, with the hSTIL coding sequence being 3867 bp (isoform 1) or 3864 bp (isoform 2) in length (1288/1287 aa), compared to cStil, which is 3912 bp and which encodes a 1303 amino acid protein. There are two isoforms of human STIL, caused by alternative splicing, which vary by a single codon (NCBI accession numbers: NP_001041631.1 and NP_003026.2) (reviewed by Kaindl et al., 2010).
Table 3.1. Analysis of exon number and length (in base pairs) in human and chicken Stil.

*hcStIL* contains two non-coding exons and the remaining fifteen exons are of similar size to *cStil*. Red and purple shading shows exons containing the coiled-coil and STAN domains, respectively.

<table>
<thead>
<tr>
<th>Human Exon</th>
<th>Exon length</th>
<th>Chicken Exon</th>
<th>Exon length</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>112</td>
<td>-</td>
<td></td>
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<tr>
<td>2</td>
<td>87</td>
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<td>969</td>
<td>X</td>
<td>1056</td>
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<td>166</td>
<td>XI</td>
<td>167</td>
</tr>
<tr>
<td>14</td>
<td>232</td>
<td>XII</td>
<td>228</td>
</tr>
<tr>
<td>15</td>
<td>214/211</td>
<td>XIII</td>
<td>192</td>
</tr>
<tr>
<td>16</td>
<td>251</td>
<td>XIV</td>
<td>252</td>
</tr>
<tr>
<td>17</td>
<td>831</td>
<td>XV</td>
<td>832</td>
</tr>
</tbody>
</table>

3.2.2 Microscopy analysis of GFP-tagged cStil

To confirm the centrosomal localisation of STIL, we stably transfected chicken DT40 cells with a cStil-GFP construct. Using light microscopy, we confirmed that STIL localises to the centrosome in DT40 cells. Only a low percentage of cells expressed STIL-GFP, due either to low transfection efficiency or, more likely, to the detrimental effect of STIL overexpression on cells, as has been described (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). In cells expressing STIL-GFP, we were able to see it throughout the cell cycle (by quantifying $\gamma$-tubulin foci) (Figure 3.4). In G1-S phase cells, 81% out of twenty six cells had two STIL-GFP foci that co-localised with $\gamma$-tubulin, with the remainder having one STIL-GFP focus. In late S phase or early G2 phase (eight cells), over 50% of the cells had two STIL-GFP foci, with the rest having four foci. In late G2 phase, 92% out of thirteen cells contained two STIL-GFP foci, one at each centrosome. Of the few mitotic cells imaged, all had one focus at each centrosome.

![Figure 3.4. STIL-GFP is detectable throughout the cell cycle in DT40 cells.](image-url)

Micrographs of DT40 cells expressing cSTIL-GFP (green) and stained for $\gamma$-tubulin (red). cSTIL-GFP could be seen at the centrosome throughout the cell cycle. 50 cells were counted in total. Boxed insets of centrosomes are shown magnified at right of micrographs. Scale bar, 10 µm.
Next, we stained the STIL-GFP expressing DT40 cell lines for other centrosomal markers to see where at the centrosome STIL localises. We first stained for Centrin3 and Cep76, which localise to both centrioles (Middendorp et al., 2000; Tsang et al., 2009). As shown in Figure 3.5, STIL localises to both centrioles but does not completely overlap with either Centrin3 or Cep76 staining, suggesting that STIL does not localise to the entire centriole but instead might localise to one end of the centriole. We then stained for Aurora A, which localises to the pericentriolar material (Lukasiewicz and Lingle, 2009). Aurora A localises between the STIL-GFP dots in G1 phase cells, indicating that STIL is not a component of the pericentriolar material (Figure 3.5). In G2 phase cells, Aurora A surrounds the STIL-GFP dots (data not shown). This increase in Aurora A is expected, as it reaches its peak levels in late G2 and its surrounding of STIL-GFP shows that STIL is a centriolar, rather than a pericentriolar component. Next, we stained with the mother centriole marker Ninein (Mogensen et al., 2000). From our IF analysis (Figure 3.5), one dot of STIL-GFP can be detected close to Ninein, without overlapping with it, suggesting that STIL is not a distal or subdistal appendage component. As we see a single Ninein focus when we see two STIL-GFP foci, we conclude that STIL localises to both centrioles in G1 phase cells. Based on this analysis, we believe that STIL localises to the proximal portion of both centrioles.

**Figure 3.5. Characterisation of cSTIL-GFP.**
Micrographs of DT40 cells expressing cSTIL-GFP and stained for centrosomal markers in order to determine its exact centrosomal localisation. cSTIL-GFP (green), marker (red). Boxed insets of centrosomes are shown magnified at right of micrographs. Scale bar, 10 μm.

During our analysis we also noted the presence of multiple centrosomes in cells stably transfected with STIL-GFP, although at a low frequency (Figure 3.6A and 3.6B). These amplified centrosomes were detected in both interphase and mitotic cells. This suggests that STIL overexpression can lead to the overduplication
of centrosomes and could also lead to multipolar mitotic cells, as seen in Figure 3.6. These amplified centrioles were also detected by others studying the effect of STIL overexpression in human cell lines (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012).

![Figure 3.6. Overexpression of STIL-GFP causes centriole amplification.](image)

Micrographs of DT40 cells expressing cSTIL-GFP and stained for centrin3 (A) and γ-tubulin (B). Amplified centrioles were seen during interphase (A) and mitosis (B). cSTIL-GFP is shown in green and marker in red. Boxed insets of centrosomes are shown magnified at right of micrographs. Scale bar, 10 μm.

To confirm our analysis of chicken STIL, we stained U2OS cells with an antibody against human STIL and also against γ-tubulin, as shown in Figure 3.7. We did not observe any early G1 phase cells with STIL localised at the centrosome. This is consistent with published observations that describe STIL at the centrosome from late G1 phase to metaphase (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). In G2 and mitotic cells, STIL is surrounded by γ-tubulin, again indicating that STIL is not a pericentriolar marker but instead localises to a very specific region of both centrioles. The apparent decrease from four STIL foci to two, as shown in Figure 3.7, suggests that STIL is lost from either the mother or daughter centriole in late S or early G2 phase, while being retained at the other centriole.

![Figure 3.7. STIL localises to the centrosome in human U2OS cells.](image)

Micrographs of U2OS cells stained with antibodies against hSTIL and γ-tubulin. STIL could be seen at the centrosome in interphase (A) and mitosis (B). STIL (green), γ-tubulin (red). Boxed insets of centrosomes are shown magnified at right of micrographs. Scale bar, 10 μm.

Based on this IF analysis, we determined that STIL localised to the proximal portion of both centrioles. This would also explain why we see either one or, two dots at each centrosome; if the two centrioles are still engaged or very close together, it may not be possible to see two separate STIL dots. Then, while the procentriole is
elongating in S phase, STIL localises throughout the procentriole and this may allow us to see two foci at each centrosome in a manner similar to SAS-6 (Dammermann et al., 2004; Gopalakrishnan et al., 2010). Figure 3.8 shows the hypothetical centrosomal localisation of STIL at each cell cycle stage based on our immunofluorescence microscopy analysis and on published work.

![Diagram](image)

**Figure 3.8. Hypothetical centrosomal localisation of STIL throughout the cell cycle.**
STIL is shown in green. Disengagement of centrioles upon passage through mitosis allows STIL to be seen as two separate foci at both centrioles in G1 phase. Then as the procentrioles elongate, STIL is seen at the proximal end of the mother centriole and throughout the procentriole. Once the procentrioles have fully elongated, STIL once again is seen only at the proximal ends of centrioles which are engaged and seen as a single focus.
3.2.3 Targeting of the cStil genomic locus

As mentioned in Section 3.2.1., chicken Stil is over 13 Kb in length. Previously published studies have shown that knockout strategies worked more efficiently when no more than ~5 Kb of a gene was disrupted (Arakawa and Buerstedde, 2006). It was, therefore, decided to delete 5.13 Kb of cStil, which would disrupt exons I (including the start codon) to VI and most of exon VII (111 bp out of 132 bp) as shown in Figure 3.9. Primers were designed using the predicted sequence described in Section 3.2.1 to allow the amplification of the 5’ and 3’ arms, which were 3 Kb and 5.5 Kb, respectively. These were then cloned into pBluescript, flanking both sides of the resistance cassettes; histidinol, blasticidin, puromycin and neomycin. The newly cloned vectors were named; pHis-StilK/O, pBSR-StilK/O, pPuro-StilK/O and pNeo-StilK/O, respectively. Also shown in Figure 3.9 and Table 3.2, are the AflII restriction sites and change in expected sequence size in wild-type and targeted constructs.

**Figure 3.9. Knockout strategy for the disruption of the cStil genomic locus.**
Strategy for the partial disruption of the cStil locus with restriction sites for AflII and distances between restriction sites indicated. Grey boxes indicate the exons, which are numbered in Roman numerals. 5’ and 3’ arms are shown flanking the resistance cassettes as are the 5’ and 3’ external probes. Diagram not to scale.

**Table 3.2. Expected band sizes following AflII digest on wild-type and targeted DT40 genomic DNA.**
Table representing the size of wild-type genomic DNA using the 5’ and 3’ external probe and the expected size following successful targeting using each of the resistance constructs.

<table>
<thead>
<tr>
<th>Resistance construct</th>
<th>5' external probe wild-type</th>
<th>3' external probe wild-type</th>
<th>5' and 3' probes targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHis-StilK/O</td>
<td>9.9 Kb</td>
<td>6.4, 1.2 Kb</td>
<td>14.5 Kb</td>
</tr>
<tr>
<td>pHygro-StilK/O</td>
<td></td>
<td></td>
<td>14.5 Kb</td>
</tr>
<tr>
<td>pPuro-StilK/O</td>
<td></td>
<td></td>
<td>14 Kb</td>
</tr>
<tr>
<td>pNeo-StilK/O</td>
<td></td>
<td></td>
<td>13.7 Kb</td>
</tr>
<tr>
<td>pBSR-StilK/O</td>
<td></td>
<td></td>
<td>13.9 Kb</td>
</tr>
</tbody>
</table>
Primers were also designed in order to generate external probes for detection of successfully targeted clones. Both 5’ and 3’ probes were generated using reverse transcriptase PCR. The 5’ and 3’ external probes were then tested with wild-type DT40 genomic DNA, using a number of different restriction endonucleases, which did not cut in the resistance cassettes, using the cold Southern approach as described in Section 2.2.10 (Figure 3.10, Table 3.3). AflII gave fragments of the expected sizes, although using the 3’ probe an unspecific band of ~4 Kb was obtained. PciI also gave fragments of the expected sizes but with an additional band for the 3’ probe. PsiI and SspI gave a number of unspecific bands of similar size when tested using the 5’ probe. It was decided that using AflII to cut the 5’ probe was the best option, as a targeted clone would show a shift upwards in size of ~4 Kb and this shift would be easily detected as shown in Figure 3.11 and Table 3.2, although PciI was a usable option also.

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Expected size 5’ probe</th>
<th>Expected size 3’ probe</th>
<th>Actual size 5’ probe</th>
<th>Actual size 3’ probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>AflII</td>
<td>9.9 Kb</td>
<td>6.4, 1.2 Kb</td>
<td>~9.9 Kb</td>
<td>~6.4, 4, 1.2 Kb</td>
</tr>
<tr>
<td>PciI</td>
<td>5.8 Kb</td>
<td>10.9 Kb</td>
<td>~5.8 Kb</td>
<td>~10.9, 3.5 Kb</td>
</tr>
<tr>
<td>PsiI</td>
<td>5.2 Kb</td>
<td>does not cut</td>
<td>~ 5.6, 5.2 Kb</td>
<td>does not cut</td>
</tr>
<tr>
<td>SspI</td>
<td>5.7 Kb</td>
<td>7.6 Kb</td>
<td>~ 5.2, 5, 3.5 Kb</td>
<td>~7.6 Kb</td>
</tr>
</tbody>
</table>

Figure 3.10. Digestion of genomic DNA to determine efficacy of external probes. Southern blot analysis of DT40 genomic DNA digested with the indicated restriction endonucleases and probed using the 5’ and 3’ external probes.

Prior to transfection, the targeting vectors were linearised to facilitate a more efficient incorporation of the vectors into the chicken DT40 genome. Transfection was carried out using electroporation as described in Section 2.4.2 and the colonies
obtained were screened using the 5’ external probe and AflII digest. The results of the targeting of the first allele of \(cStil\) are shown in Table 3.4.

![Southern blot analysis](image)

**Figure 3.11. Targeting of the first allele of \(cStil\).** Southern blot analysis of wild-type (WT) and \(Stil^{+/-}\) genomic DNA digested with AflII and probed with the 5’ external probe.

Chicken \(Stil\) is located on the reverse strand of chromosome 8 and therefore there are two copies of the gene in DT40 cells. We obtained targeted clones for the first allele with histidinol, puromycin and blasticidin resistances (Figure 3.11, Table 3.4) and proceeded with targeting of the second allele. Although targeting with a histidinol resistance cassette had given a very good targeting efficiency for the first allele, no positive clones were obtained when targeting the second allele. Similarly, targeting using a puromycin resistance cassette yielded no homologous targeted clones (Table 3.4). Therefore, all the negative clones obtained had integrated the targeting constructs randomly into the DT40 genome, thus conferring resistance to the selective antibiotics on the clones. The failure to obtain clones with the second allele of \(cStil\) targeted indicated that this gene may be essential for viability and that complete loss of \(cStil\) is lethal to DT40 cells. With this in mind, we attempted to generate a conditional knockout of \(Stil\) in chicken DT40 cells.

<table>
<thead>
<tr>
<th>Background</th>
<th>Resistance</th>
<th>No. Clones Screened</th>
<th>No. of targeted Clones</th>
<th>Targeting efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>Histidinol</td>
<td>60</td>
<td>26</td>
<td>43.3%</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Puromycin</td>
<td>50</td>
<td>4</td>
<td>8%</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Blasticidin</td>
<td>56</td>
<td>1</td>
<td>1.8%</td>
</tr>
<tr>
<td>(Stil^{+/-}) His</td>
<td>Puromycin</td>
<td>67</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>(Stil^{+/-}) Puro</td>
<td>Histidinol</td>
<td>66</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>(Stil^{+/-}) Bsr</td>
<td>Histidinol</td>
<td>147</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

### 3.2.4 Generation of a conditional expression system for \(cStil\)

After screening 280 clones in the targeting of the second allele of \(cStil\) and obtaining no positive clones, we concluded that \(Stil\) was essential for viability. We then aimed to generate a conditionally null DT40 cell line with a tet-regulated
transgene (Gossen and Bujard, 1992). With the cStil expression under the control of the tetracycline promoter, this could be switched off once the gene was disrupted.

To generate the transgene, cStil was first tagged with three myc tags at its N terminal end, as there was no working antibody available to chicken STIL. Myc-tagged cStil was then cloned into pUHG10.3, an expression vector containing the tetracycline-repressible promoter (Gossen and Bujard, 1992). This cStil expression vector was then co-transfected into Stil+/− DT40 cells using pBsrStilK/O (Bsr#49), along with a tet transactivator protein-expressing plasmid (pTA-2/3/4) containing a neomycin resistance cassette. Clones were expanded and doxycycline was added in order to repress myc-STIL expression. The potentially positive clones were then screened by immunoblotting for myc and the level of myc-STIL expression and repression was examined. Six out of the nineteen clones screened both expressed myc-STIL and repressed it after doxycycline addition. Figure 3.12 shows three clones which had varying levels of myc-STIL expression, but complete repression when doxycycline was added.

![Figure 3.12. Expression and repression of myc-STIL in cStil+/− DT40 cells. Immunoblot analysis of myc-STIL expression levels in transgenic cStil transcripts before (-) and after (+) doxycycline addition. Clones with different levels of expression and complete repression are shown. α-tubulin was used as a loading control.](image)

Clones which had low, medium and high expression of myc-STIL and also complete repression after doxycycline were selected for a further round of targeting to disrupt the second endogenous allele of cStil using the pHisStilK/O targeting vector described previously. In total, 219 clones were screened and none were successfully targeted (Table 3.5). As all three clones targeted had different levels of myc-STIL expression and none returned a positive clone with the second allele of cStil targeted, it is possible that a different, optimal level of myc-Stil is crucial for the survival of a successfully targeted cell line. Alternatively, it is possibly that the three myc tags at the N terminus of STIL affected its folding and conformation, which in turn could affect its centrosomal localisation and/or interaction with other proteins.
Table 3.5. Targeting of cStil second allele in cells conditionally expressing myc-STIL.
cStil \textsuperscript{+/–} DT40 cells which conditionally expressed repressible myc-STIL were transfected with a histidinol resistance construct and the resulting clones were screened by Southern blot. The targeting efficiency is shown in the above Table, as are the number of clones screened and the number of targeted clones.

<table>
<thead>
<tr>
<th>Background</th>
<th>Resistance</th>
<th>No. Clones Screened</th>
<th>No. of targeted Clones</th>
<th>Targeting efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>#49Stil \textsuperscript{+/–} Bsr pUHG10.3-myc-cStil</td>
<td>Histidinol</td>
<td>219</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

As we obtained no positive clones in the targeting of the second allele of cStil we decided to not invest any more time in the knockout of cStil in chicken DT40 cells. All of the clones screened represent random integration of the cStil resistance constructs into the chicken DT40 genome. We concluded that either, a minimum level of expression of STIL is crucial to maintain cell viability and we had not generated cells expressing myc-STIL over the minimum threshold level or, myc-STIL could not function in the same manner as endogenous STIL. As we did not attempt to tag STIL at the C terminus with myc, we could not confirm if STIL-myc would be functional in DT40 cells

3.3 Discussion

3.3.1 Cloning and analysis of cStil cDNA

Our main aim when undertaking this project was to define the function of STIL by knocking out the Stil gene in chicken DT40 cells. Before we undertook this, we needed to perform a detailed bioinformatic analysis of Stil to confirm that the predicted cStil sequence on the NCBI database was correct. Full-length chicken Stil was successfully cloned using the primers designed from the predicted sequence. There were a number of errors in the cDNA sequence of cStil generated by PCR, which were most likely due to the long length of cStil cDNA. However, most of these errors could be removed by excising and ligating correct parts of the sequence to generate one full-length sequence. The newly generated sequence contained a number of differences from the predicted sequence. However, as six of these codon changes were in all the sequences analysed and also in a partial Scl sequence on the NCBI database, we concluded that these are in fact the correct codons for those positions. A seventh codon change was contained in three out of the four sequences analysed, therefore this may indeed be the correct codon for that position.
Importantly, these variations from the predicted sequence did not affect the centrosomal localisation of STIL-GFP.

We found that the cStil coding sequence was the expected size of 3.9 Kb. Human STIL contains a coiled coil domain and using the COILS algorithm we were able to confirm that chicken STIL also contains a coiled coil domain in the same region. This coiled coil domain is well conserved between human, mouse, chicken and X. laevis, as is the STAN domain, which is found close to the C-terminal end (Arquint et al., 2012). Both the coiled coil and STAN domain are required for centrosomal localisation of STIL (Vulprecht et al., 2012). We also found that the exon positions and sizes were very similar between human and chicken Stil, suggesting that the gene itself is also evolutionarily conserved between chicken and human. This analysis confirmed that we had cloned the chicken homologue of Stil gene.

3.3.2 Microscopy analysis of cSTIL

After generating full-length cStil, we tagged it with GFP and stably transfected it into DT40 cells. Once stable cell lines had been generated, we proceeded with immunofluorescence microscopy analysis of STIL-GFP. We confirmed that STIL localised to the centrosome in chicken cells and that STIL co-localised with γ-tubulin throughout the cell cycle. We detected STIL-GFP in G1-S phase, throughout G2 phase and in mitosis. It has recently been published that STIL localises to the centrosome from late G1 and is abolished from the centrosome in late anaphase and telophase (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). These publications showed the cell cycle centrosomal expression of STIL and confirmed that the cStil we had cloned, localised to the centrosome at the expected cell cycle stages. This again showed that Stil is conserved between mammals and chicken.

We next wished to determine the exact centrosomal location of STIL in DT40 cells using immunofluorescence microscopy. We stained the STIL-GFP expressing cells with centrosomal antibodies known to work in chicken DT40 cells. Having first stained these cells for Centrin3, which localises to the entire centriole (Middendorp et al., 2000), we detected STIL-GFP at each centriole. However, it did not overlap entirely with Centrin3, suggesting that STIL did not localise to the entire
length of the centriole. We confirmed this observation by staining for Cep76 which also localises to centrioles and co-localises with Centrin3 (Tsang et al., 2009). Again, although we could clearly see two centrioles, STIL did not completely co-localise with Cep76 but appeared to be present at both centrioles in G1 or early S phase cells. It has been reported that STIL localises to the pericentriolar material around the parental centriole (Vulprecht et al., 2012). We had also stained the STIL-GFP cell line for the pericentriolar marker Aurora A and found that in G1 or early S phase cells, when Aurora A expression is very low, Aurora A localised between the two STIL-GFP foci (Lukasiewicz and Lingle, 2009). Later, when cells appeared to be in late G2 phase, we found that the STIL-GFP foci were enveloped by a large accumulation of Aurora A. This indicated to us that STIL was not a pericentriolar protein. If the report of Vulprecht et al. is accurate, STIL would have to localise to a very discrete position in the pericentriolar material, directly adjacent to the centrioles (Vulprecht et al., 2012). Finally, in order to determine if STIL localised to the distal or proximal end of a centriole or if it localised specifically to either the mother or daughter centriole, we stained the STIL-GFP expressing cells for the mother marker Ninein. Ninein localises to the subdistal appendages of the mother centriole (Delgehyr et al., 2005). We found that, although both Ninein and STIL localised to the centrosome, the two centrosomal components showed no co-localisation. This confirmed that STIL did not localise to the distal end of centrioles. In G1 or early S phase cells, there is only one Ninein focus present at the mother centriole; however, there usually appeared to be two STIL foci. From this, we concluded that STIL localises to both centrioles in G1 and early S phase cells. One of these STIL foci was quite close to the Ninein localisation but did not overlap and the other was well separated from Ninein. Based on this analysis, we concluded that STIL localises to the proximal ends of both centrioles. It has recently been published that STIL localises to the site of procentriole formation in G1/early S phase, which fits well with our observations (Tang et al., 2011; Arquint et al., 2012).

We also noted the presence of multiple centrioles in a small number of cells. These multiple STIL foci always localised closely with either Centrin3 or γ-tubulin. We detected these amplified centrioles in both interphase and mitosis, where multipolar mitotic cells were observed. This led us to conclude that overexpression of STIL can lead to the amplification of centrioles and could have detrimental
consequences for cells, possibly leading to asymmetrical cell divisions. Our observations were again confirmed by a number of other studies on STIL overexpression in mammalian cells, where it was shown that STIL overexpression allows the formation of multiple daughter centrioles, often in a flower formation around single mother centrioles (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012).

To confirm that our immunofluorescence microscopy analysis of STIL-GFP was not due to off-target effects of overexpression of STIL-GFP or simply a characteristic of chicken STIL, we obtained an antibody against human STIL. We stained human U2OS cells for STIL and γ-tubulin. Again, in late S or early G2 phase cells, we could clearly see four STIL foci surrounded by γ-tubulin. We concluded that, in late S or early G2 phase, STIL localises to the proximal ends of mother centrioles and also throughout the growing procentriole, likely playing a role in the elongation of the growing centriole as with human STIL. This could allow us to see four STIL foci in late S or early G2 phase. This procentriole localisation may then be lost when the newly formed centriole has reached its full length. Then, as the cell progresses through G2 phase, the STIL localisation can only be detected as a single focus at each centrosome as the two centrioles are engaged and very close together. The STIL signal again can be seen at both centrioles after they disengage upon passage through mitosis, at the site of procentriole formation, in a manner similar to SAS-6 localisation (Dammermann et al., 2004; Gopalakrishnan et al., 2010). The analysis by Arquint et al. fits our observations best. They report, using IF and EM, that STIL is present at the mother centriole at the site of procentriole formation from late G1 phase. Then, as the procentriole begins to elongate, STIL is also seen throughout the newly forming centriole (Arquint et al., 2012).

Although the observation of four STIL foci in S or early G2 phase has not been reported by others studying STIL localisation, we were able to confirm our DT40 cell findings, using an antibody to STIL in human cells. It has been shown that STIL is necessary for SAS-6 recruitment to centrioles and that the two resemble one another both in terms of localisation and cell cycle expression (Arquint et al., 2012; Vulprecht et al., 2012). STIL interacts directly with CPAP and forms a complex with SAS-6, and SAS-6 and STIL also co-localise with each other.
Depletion of SAS-6, like STIL, leads to monopolar spindles, as it is also required for centriole assembly. Dammermann et al. showed that in human cells expressing YFP-SAS-6, which appeared to be in S or early G2 phase, there also appeared to be four SAS-6 foci and later in mitosis only two SAS-6 foci could be detected, which is the same localisation pattern we observed with STIL (Dammermann et al., 2004). It has not been shown conclusively that STIL and SAS-6 directly interact with each other. However, it has been shown that STIL and SAS-6 are mutually dependent for their centriolar localisation (Tang et al., 2011; Vulprecht et al., 2012).

### 3.3.3 Targeting of the cStil genomic locus

The role of Stil has been well established in other organisms. In mice, it is required for embryonic axial development and left-right specification. Mice Stil heterozygotes appear normal but Stil−/− embryos die after embryonic day 10.5 showing many defects including reduced size, neural tube defects and abnormal left-right development (Izraeli et al., 1999). In the zebrafish cassiopeia mutant, embryos show increased mitotic index, disorganised mitotic spindles and often lack centrosomes (Pfaff et al., 2007). These loss-of-function mutations demonstrate the important role Stil plays in the development of organisms and in particular its role in mitosis. It also led us to believe that although Stil is essential for the viability of an organism, Stil−/− cell lines can be generated, at least in mice and zebrafish embryonic cells. With this in mind, we aimed to delete Stil in chicken DT40 cells and study the effects of its deletion on chicken cells.

Using the information we had gathered on chicken Stil, we designed a strategy to disrupt 5.13 Kb of cStil. We designed arms both upstream of the ATG sequence and downstream of the region we wished to disrupt. We then cloned resistance cassettes in between these arms and transfected the linearised; histidinol, blasticidin and puromycin resistance constructs into wild-type chicken DT40 cells. We successfully targeted the first allele of cStil. The histidinol resistance construct gave an excellent targeting efficiency of over 43%, while puromycin and blasticidin had a targeting efficiency of 8% and almost 2%, respectively. As all the transfections were carried out in precisely the same manner, the only explanation for these targeting efficiency differences was the orientation of the resistance cassettes. Histidinol was in the 3’-5’ orientation, while puromycin and blasticidin were in the...
5’-3’ orientation. There is no clear evidence that the orientation of the resistance cassettes affects targeting efficiency and we did not verify whether this was the case in this situation. It is, however, the only way we can explain the large variation in targeting efficiency for the first allele of cStil.

Once we had obtained cStil<sup>lt</sup> clones with three different resistances, we proceeded to target the second allele of cStil. In total, we screened 280 clones, but unfortunately obtained no cStil knockout clones. It is expected that the targeting efficiency will drop somewhat when going from the first to second allele (Buerstedde and Takeda, 1991). However, our targeting efficiency had dropped to 0% and this was not expected. We concluded from this, that cStil may be essential for viability in the chicken DT40 cells and its deletion would be lethal for cells, thus prevention the generation of a cStil<sup>lt</sup> cell line in DT40.

It was surprising that we could not generate a knockout cell line in chicken DT40 cells, since it has been shown to be possible in mouse and zebrafish embryos (Izraeli et al., 1999; Pfaff et al., 2007). However, it has also been shown that STIL is essential for mitotic entry and survival of various human cancer cells (Erez et al., 2007). As the DT40 chicken B cell line is derived from an avian leukosis virus (ALV)-induced bursal lymphoma, this might go some way to explain our failure to obtain viable DT40 cells with cStil deleted (Baba and Humphries, 1984; Baba et al., 1985). Although mitosis is a general physiologic process, cancer cells are unusually sensitive to mitotic inhibitors (reviewed by Jackson et al., 2007). STIL is essential for the growth of tumour explants in mice. Also, inducible knockdown of STIL in cancer cells delayed entrance into mitosis, decreased activation of the CDK1-cyclin B complex and induced apoptosis in a p53-independent manner (Erez et al., 2004; Erez et al., 2007).

As previous published work showed the fundamental role Stil plays in mitosis and that it is essential in cancer cells, this led us to believe that Stil was essential in chicken DT40 cells and we proceeded with the generation of a conditional knockout (Erez et al., 2007; Pfaff et al., 2007).

### 3.3.4 Generation of a conditional expression system for cStil

We attempted to generate a conditional knockout of cStil in DT40 cells. We myc-tagged cStil, cloned it into a vector containing a tetracycline-repressible
promoter and transfected it into $cStil^{+/−}$ DT40 cells. This would allow us to conditionally knock down the expression of $cStil$ using a tetracycline-repressible promoter (Gossen and Bujard, 1992). We obtained clones that expressed myc-STIL and repressed it when doxycycline was added. We then targeted the second allele of $cStil$ with the histidinol resistance construct. Again, we did not obtain any successfully targeted clones. Although we had targeted clones with varying expression levels of myc-STIL, we think that a crucial threshold level of expression of STIL is necessary for cell viability in DT40. Levels too high or too low would have detrimental effects on cells, leading to either monopolar or multipolar mitotic cells (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012).

Izraeli et al. examined the cell cycle regulation of Stil mRNA in proliferating EL4 mouse lymphoblast cells. Stil mRNA levels are constant in proliferating cells, although STIL protein expression varies depending on the cell cycle phase and STIL is degraded at the end of the cell cycle (Izraeli et al., 1997). Thus, as our transgene is under control of the CMV promoter instead of the endogenous $cStil$ promoter, $cStil$ mRNA is constantly being expressed, but this should not affect the cell cycle-regulated expression of STIL.

Unfortunately, as we did not have an antibody which recognises chicken STIL, we could not determine the optimal level of expression of STIL in DT40 cells. However an alternate approach could have been to measure the mRNA levels using RT-PCR. STIL protein expression levels vary dramatically in different cancer cells lines and lymphomas have abundant expression of STIL (Erez et al., 2004). Therefore, it is possible that the CMV promoter was not strong enough to drive the optimal expression of myc-STIL and when the second allele of $cStil$ was deleted, the low levels of myc-STIL being expressed were not sufficient to prevent the DT40 cells from entering apoptosis.

However, given that Stil$^{+/−}$ DT40 cells are viable, perhaps the addition of the three myc tags to the N-terminus of STIL affected its conformation, functionality or localisation. Human STIL has been tagged with GFP at its N-terminus and given that GFP is much larger than the myc tag, this indicates that attaching a tag to the N-terminus of human STIL does not affect its conformation, functionality or localisation (Tang et al., 2011; Arquint et al., 2012). However, there is considerable
variation between the N-termini of human and chicken STIL (Figure 3.2). Interestingly, we were unable to obtain stable clones in DT40 cells transfected with pEGFP-STIL, i.e. where STIL was tagged with GFP at the N-terminus, adding further support to theory that an N-terminal tag may adversely affect some aspect of chicken STIL function. Additionally, STIL interacts with CPAP via its N-terminus (Vulprecht et al., 2012). Therefore, it is also possible that a tag at the N-terminal end of STIL may affect this interaction in DT40 cells. A failure to localise CPAP to the centrosome would also lead to a loss of centrosomes, as CPAP is necessary for procentriole formation and elongation (Leidel and Gonczy, 2003; Basto et al., 2006; Kleylein-Sohn et al., 2007). Therefore, myc-STIL may successfully localise to the centrosome in DT40, as its centrosomal localisation requires the coil-coiled and STAN domains, which are located in the C-terminus of STIL (Stevens et al., 2010; Vulprecht et al., 2012). However, the STIL-CPAP interaction may be prevented, leading to a failure to generate procentrioles. Unfortunately, we did not attempt to tag STIL with myc at its C-terminus to test this theory.

In conclusion, although we were unable to knock out CSTIL in DT40 cells, we concluded that STIL is essential for viability in the chicken DT40 cells and that a critical level of expression may be necessary to ensure the viability of DT40 cells.
Chapter 4. The impact of DNA damage and loss of centrosome cohesion on centrosome structure

4.1 Introduction

Centrosome duplication is a semi-conservative process, where new centrioles are formed at right angles to pre-existing centrioles. Centrioles disengage upon passage through mitosis, prior to the next round of duplication, an action which involves Plk1 and Separase (reviewed by Tsou and Stearns, 2006b; Tsou et al., 2009). Then in late G1 or early S phase, each disengaged centriole acts as a mother which is capable of nucleating a single procentriole which elongates throughout S and G2 phase (Kochanski and Borisy, 1990). The mother centriole can be distinguished by its subdistal and distal appendages, which are involved in microtubule anchoring and primary cilium formation (Bornens, 2002; Graser et al., 2007a). At the subdistal appendages are the maturity markers Ninein and CEP170, while Cenexin/ODF2 is found at both the distal and subdistal appendages (Mogensen et al., 2000; Nakagawa et al., 2001; Bornens, 2002; Guarguaglini et al., 2005; Graser et al., 2007a). The pericentriolar material (PCM) surrounding the mother centriole differs to that of the daughter centriole and the daughter centriole is mobile in the cytoplasm until centriole duplication is well underway in S phase (Piel et al., 2000). The PCM component Kizuna associates mainly with the mother centriole and stabilises mitotic centrosomes to ensure spindle bipolarity (Oshimori et al., 2006).

The newly duplicated centrosomes separate prior to entry into mitosis, when a physical linker that connects the two mother centrioles together is removed. This linker contains Rootletin and C-NAP1, which interact with one another. Both are coiled coil proteins and are phosphorylated by NEK2, which leads to their dissociation from centrioles and separation of the centrosomes in late G2 phase (Fry et al., 1998a; Fry et al., 1998b; Mayor et al., 2000; Mayor et al., 2002; Faragher and Fry, 2003; Bahe et al., 2005). Furthermore, it has been shown that siRNA depletion of either Rootletin or C-NAP1 cause separation of paired centrioles (Bahe et al., 2005). Another target of NEK2 is β-catenin, a mitotically-stabilised form of which mediates centrosome separation (Bahmanyar et al., 2008). NEK2 overexpression or protein phosphatase 1α (PP1α) inhibition drives centrosome splitting (the separation of parental centrioles) (Meraldi and Nigg, 2001), while ATM-mediated activation of
PP1\(\alpha\) to oppose NEK2 activity inhibits centrosome separation after IR (Fletcher et al., 2004; Mi et al., 2007).

Centrosome duplication is a tightly regulated process that occurs once and only once per cell cycle (Hinchcliffe and Sluder, 2001; Tsou and Stearns, 2006b; Nigg, 2007). Centrosome amplification and structural abnormalities are a phenotype of cancer cells and abnormal centrosome number can lead to abnormal chromosome segregation and aneuploidy (Lingle et al., 1998; Pihan et al., 2001; D'Assoro et al., 2002; Ganem et al., 2009). The centrosome and chromosome cycles are closely linked and are both controlled through cyclin-Cdk activities (Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). Cyclin B1-Cdk1 can be detected at the centrosome in late G2 phase and its activation is involved in the separation of centrosomes in late G2 phase (Crasta et al., 2006; Crasta and Surana, 2006). Inhibition or loss of Cdk1 has been shown to prevent cell entry into mitosis and also allows for multiple rounds of centrosome duplication (Laronne et al., 2003; Steere et al., 2011).

Another cause of centrosome amplification is DNA damage (Sato et al., 2000a; Dodson et al., 2004). DNA damage induces Chk1-dependent centrosome amplification which occurs during a prolonged G2 phase and involves ATM (Dodson et al., 2004; Bourke et al., 2007). Mutations in BRCA1, BRCA2 and TP53 (Tumour protein 53) have also been shown to lead to centrosome amplification and BRCA1 inhibition causes premature separation of centrioles followed by reduplication (reviewed by Deng, 2002; reviewed by Tutt and Ashworth, 2002; Ko et al., 2006; D'Assoro et al., 2008). It has also been shown that in the presence of incompletely replicated or damaged DNA, centrosomes can split into fractions containing single centrioles during mitosis. This can, in turn lead to the formation of multipolar spindles (Hut et al., 2003).

This work presented in this chapter shows the impact of IR on the centrosomes of cells with mutations in BRCA1, BRCA2 and TP53. We determine the impact of IR on centrosomes in non-transformed human cells and study the structure and maturity of split centrioles following IR in these cells. We use CDK1 inhibition to determine if centrioles can split after a G2/M arrest without DNA damage. We investigate the structure of amplified centrosomes following CDK1 inhibition and
explore whether centrosome amplification can lead to multipolar divisions. Finally, we test if proteins involved in centrosome cohesion are involved in DNA damage-induced centriole splitting and we determine if loss of centrosome cohesion proteins affects the composition of the split centriole.

4.2 Results

4.2.1 Centriole splitting is a precursor to DNA damage-induced centrosome amplification

The involvement of centrosome amplification in chromosome instability (CIN) in cancer cells was first suggested by Boveri nearly a century ago and it has long been debated as to whether amplified centrosomes are a cause or consequence of CIN (reviewed by D’Assoro et al., 2002; Boveri, 2008). It has been shown by our group that centrosome amplification can occur after a prolonged G2 arrest following DNA damage (Dodson et al., 2004; Bourke et al., 2007). We wished to investigate the impact of DNA damage on the centrosome, with particular attention being paid to the lesser studied phenotype of centriole splitting, which has been shown to occur in the presence of impaired DNA integrity (Hut et al., 2003).

BRCA1 and BRCA2 are key mediators in the DNA damage response and have also been shown to regulate centrosome duplication (reviewed by Deng, 2002; reviewed by Tutt and Ashworth, 2002). Impaired p53 function has also been shown to lead to centrosome amplification in breast cancer cells (D’Assoro et al., 2008). We wished to investigate how deletions or mutations in BRCA1, BRCA2 and TP53, and thus, a defective DNA damage response, would impact on centrosomes in wild-type and irradiated cells. The human cancer cell lines we used for this study were HCC1937, MDA-MB-436, CAPAN-1 and U2OS (Figure 4.1). HCC1937 is homozygous for the BRCA1 5382insC mutation, as well as multiple additional genetic changes, including a TP53 mutation (Tomlinson et al., 1998). MDA-MB-436 has a 5396 + 1G>A mutation in the splice donor site of exon 20 resulting in two transcripts of BRCA1; one of which had an in-frame deletion and the other encoding for a truncated protein. This cell line also has biallelic mutations in the TP53 gene (Elstrodt et al., 2006). CAPAN-1 cells carry a deletion of BRCA2 on one homolog and codes for a protein truncated at amino acid 1981 on the other homolog (Goggins et al., 1996). As a control for this study we used the human osteosarcoma cell line;
U2OS, which is wild-type for BRCA1, BRCA2 and TP53. (Ponten and Saksela, 1967; Florenes et al., 1994; Martin and Ouchi, 2005). As an additional control, we used the non-transformed, telomerase-immortalised, human cell line hTERT-RPE1 (Bodnar et al., 1998).

In our initial IF analysis of non-transformed and cancer cells, after IR, we noted two types of centrosome abnormalities; split and amplified centrioles (Figure 4.1). In this study, we describe centriole splitting as occurring when cohesion and/or tethering are lost between centrioles and a single, or occasionally two individual centrioles become isolated and separated from their centriole partner (Mayor et al., 2000; Bahmanyar et al., 2008). This is not the normal cell cycle event which occurs when centrioles disengage upon passage through mitosis but remain tethered. Based on analysis carried out in our group (thesis: Saladino, 2010) and that of published work, we define a centriole as being split, when it is separated from another centriole by more than 2 μm (Figure 4.1.C and D) (Mayor et al., 2000; Faragher and Fry, 2003). These split centrioles may then be licenced for reduplication and lead to centrosome amplification (reviewed by Nigg, 2007). Centrosome amplification is also shown in Figure 4.1 E and F, where there are more than four centrioles in a cell, either clustered together or separated in the cell.

**Figure 4.1. Interphase centrosomes and centrosome aberrations in hTERT-RPE1 cells.** Micrograph showing wild-type cells with normal centrosomes; A. a G1 phase centrosome, B. G2 phase centrosomes and centrosome abnormalities after IR; C. and D. split G2 phase centrosomes, E. and F. amplified centrioles. hTERT-RPE1 cells were treated with 5Gy IR and fixed after 48 hours. Cells were stained for γ-tubulin (green) and Centrin2 (red). Boxed insets of centrosomes are shown magnified at right of micrographs. Scale bar, 10 μm.

From our immunofluorescence analysis (Figure 4.2), we saw that cell lines which had mutations in BRCA1 and TP53 or BRCA2 had high levels of centriole splitting in unirradiated cells, with HCC1937 showing 35% splitting and MDA-MB-436 and CAPAN-1 showing 25% and 18%, respectively. In contrast, U2OS and hTERT-RPE1 cells, which have fully functioning TP53, BRCA1 and BRCA2 showed
very low levels of centriole splitting. \textit{BRCA1/TP53} deficient cell lines also showed higher levels of centrosome amplification, with HCC1937 and MDA-MB-436 showing around 6\% amplification, compared to 1-2\% seen in CAPAN-1 and U2OS cells. These results demonstrate that loss of \textit{BRCA1}, \textit{TP53} and to a lesser extent \textit{BRCA2} deregulates the DNA damage response and allows the premature splitting of centrioles, which may then allow for over-duplication of centrioles. A similar result was found in the non-tumorigenic epithelial cell line MCF10A, following BRCA1 inhibition, which found that BRCA1 function is critical to prevent premature centriole separation and reduplication prior to mitosis (Ko et al., 2006).

![Figure 4.2. \textit{BRCA1/TP53} and \textit{BRCA2} deficient cell lines show high levels of centrosome abnormalities.](image)

We next wished to investigate the impact of DNA damage on \textit{BRCA1/TP53} and \textit{BRCA2} deficient cell lines. We treated cells with 10 Gy IR, then cells were fixed 72 hours after IR and analysed by immunofluorescence microscopy. After IR, we saw marked increases in centrosome amplification in cell lines with \textit{TP53} and \textit{BRCA1} mutations, with roughly 45\% of HCC1937, and 25\% of MDA-MB-436 cells having amplified centrosomes. CAPAN-1, U2OS and hTERT-RPE1 cells all had similar levels of centrosome amplification of approximately 10-15\%. As seen in figure 4.2, HCC1937 and MDA-MB-436 have high levels of centriole splitting in untreated cells. However, 72 hours after IR, we see that these cell lines now have much lower levels of centriole splitting (HCC1937, \~12\% and MDA-MB-36, 20\%). Conversely, we now see these cell lines with high levels of centrosome amplification. CAPAN-1 cells also show increased levels of centrosome amplification after IR. These findings indicate that, in cell lines deficient in \textit{BRCA1}
and TP53, and to a lesser extent BRCA2, centriole splitting precedes and potentiates DNA damage-induced centrosome amplification.

We then sought to establish if this observation of centriole splitting preceding centrosome amplification is exclusively a characteristic of BRCA1/TP53 and BRCA2 deficient cancer cells or if non-transformed cells with fully functioning DNA damage response genes showed the same pattern. To do this, we again used the non-transformed human cell line, hTERT-RPE1, which had fully functioning BRCA1, BRCA2 and TP53 to study the levels of centriole splitting and amplification after IR.

![Figure 4.3. Dose and time dependency of centrosome abnormalities after IR.](image)

As shown in Figure 4.3, γ-irradiation caused a dose-dependent increase in centriole splitting 48 hours post-treatment, with 17% of cells having split centrioles after 5 Gy IR. The highest level of centrosome amplification was seen with 2 Gy IR. Time course analysis showed that centriole splitting increases until 48 hours after 5 Gy IR and then declines, while centrosome amplification increases steadily until 48 hours after IR, after which, it shows a more dramatic increase. These observations indicate that after IR, centrioles first split and then reduplicate, with the level of splitting dependent on the level of DNA damage. They also fit well with the observations of centriole splitting preceding amplification in BRCA1 and TP53 deficient cells and show that this response to DNA damage is not dependent on mutations in DNA damage response genes. However, mutations in these genes allow centriole splitting without IR-induced DNA damage and amplify this centrosomal response after DNA damage. The lower levels of centriole splitting and
amplification in irradiated hTERT-RPE1 cells compared to BRCA1/TP53 and BRCA2 mutant cell lines demonstrates the crucial roles these genes in controlling centrosome number. BRCA1 suppresses premature separation of centrioles and centrosome reduplication in mammalian cells (Deng, 2002; Ko et al., 2006). Impaired p53 function causes centrosome amplification in breast cancer cells (D'Assoro et al., 2008). BRCA2 localises to the centrosome in S and M phase, where it may regulate centrosome duplication and separation. Forced displacement of BRCA2 from the centrosome leads to abnormal centrosome duplication and positioning (Nakanishi et al., 2007). Therefore cells with fully functioning DNA damage responses and regulation of centrosome duplication will be more capable of suppressing centriole splitting and centrosome amplification following IR.

4.2.2 Microscopy analysis of split centrioles after irradiation

To further understand the phenotype of centriole splitting as a consequence of IR, we went on to examine the composition of centrioles before and after IR by immunofluorescence microscopy on fixed hTERT-RPE1 cells. We first examined the centriolar components, Centrin2 and glutamylated tubulin, which localise to the entire centriole throughout the cell cycle (Figure 4.4 and 4.5). After IR, these markers can be detected at both engaged and split centrioles, indicating that split centrioles are single, intact centrioles. We used γ-tubulin as our standard marker of engaged and split centrioles because of the reliable absence of background staining and also because it was consistently detected at the split centriole along with Centrin2, even though γ-tubulin is actually a component of the pericentriolar material (Figure 4.4) (Stearns et al., 1991).

Having concluded that the split centrioles we see after IR treatment are intact centrioles, we wanted to determine the maturity of the split centrioles. First in interphase cells, we found that the marker of immature centrioles, Centrobin, and the distal and sub-distal appendage marker, Cenexin/ODF2, were present at some of the interphase centrioles, as was expected giving the varying ages of centrioles (Nakagawa et al., 2001; Zou et al., 2005). The sub-distal appendage markers, CEP170 and Ninein, again could be seen at interphase centrioles, as was Kizuna, which is normally associated with mature PCM (Figure 4.4) (Mogensen et al., 2000; Guarguaglini et al., 2005; Oshimori et al., 2006; Graser et al., 2007a). Following IR,
CEP170 and Ninein could be detected at both the engaged and split centrioles, indicating that split centrioles carry markers diagnostic of maturity. However, as CEP170 and Ninein are also found at the proximal ends of centrioles (Mogensen et al., 2000; Guarguaglini et al., 2005; Graser et al., 2007a), and given the presence of Centrobin at the split centrioles, this indicates that split centrioles are not fully mature centrioles (Zou et al., 2005). Cenexin was only found at 10% of split centrioles, again indicating that these split centrioles are not fully mature. Finally, split centrioles also lack Kizuna, with only 4% of split centrioles carrying Kizuna staining. This suggests that split centrioles do not carry fully mature PCM (Oshimori et al., 2006). The complete percentages of localisation in interphase and IR-split centrioles can be found in Table 4.1.
Chapter 4

Figure 4.4. Maturity of interphase and IR-split centrioles. Immunofluorescence microscopy analysis of untreated and IR-split centrioles. hTERT-RPE1 cells were fixed 48 hours after 5 Gy IR, where indicated, then stained with the indicated antibodies (green) and γ-tubulin (red). Cells were counterstained with DAPI to visualise the DNA. Inserts show blow-ups of split and engaged centrioles, magnified at right of micrographs. Scale bar, 10 μm. (Work carried out in collaboration with C. Saladino).

We next looked at components which localise to the pericentriolar material, Pericentrin and NEDD1. As shown in Figure 4.5, these components of the PCM are detected at interphase centrosomes in unirradiated cells and following IR, they can be detected at both engaged and split centrioles. This shows that split centrioles carry apparently normal PCM and therefore, may be able to form functioning spindle poles (Haren et al., 2006). Following this, we investigated components involved in centrosome cohesion, NEK2, C-NAP1 and Rootletin (Fry et al., 1998a; Fry et al., 1998b; Bahe et al., 2005). These three components are detected at interphase centrosomes in unirradiated cells. After IR, NEK2 was entirely absent from all...
centrioles, as previously reported (Fletcher et al., 2004). C-NAP1 and Rootletin appeared unaffected after IR, with both being detected at engaged and split centrioles, indicating that components which oppose separation are unaffected after IR (Figure 4.5).

**Figure 4.5. Composition of interphase and IR-split centrioles.** Immunofluorescence microscopy analysis of untreated and IR-split centrioles. hTERT-RPE1 cells were fixed 48 hours after 5 Gy IR, where indicated, then stained with the indicated antibodies (green) and γ-tubulin (red). Cells were counterstained with DAPI to visualise the DNA. Inserts show blow-ups of split and engaged centrioles, magnified at right of micrographs. Scale bar, 10 μm. (Work carried out in collaboration with C. Saladino).
**Table 4.1. Composition of engaged and split centrioles.**

hTERT-RPE1 cells were treated with indicated siRNAs for 24 hours, then either fixed after a further 24 hours or treated with 5 Gy and fixed after 24 hours and stained for γ-tubulin and the indicated markers. Engaged and split G2 phase centrioles were scored as to the presence of the indicated marker. The table shows the percentage of centrioles that carry the indicated marker. At least 50 cells were counted in each case. WT=wild-type, Scr=scrambled, CROCC=Rootletin, Kiz=Kizuna, CEP250=C-NAP1. (Work carried out in collaboration with C. Saladino).

<table>
<thead>
<tr>
<th>Marker</th>
<th>siRNA (+IR)</th>
<th>siRNA (-IR)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Scr</td>
</tr>
<tr>
<td><strong>Split</strong></td>
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<td></td>
</tr>
<tr>
<td>centrioles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cenexin</td>
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<tr>
<td>CEP170</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Ninein</td>
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</tr>
<tr>
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<td>100</td>
</tr>
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<tr>
<td>Centrobin,</td>
<td></td>
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<tr>
<td>C-NAP1, Glut. tubulin, NEDD1, Pericentrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Engaged</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>centrioles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cenexin, Centrin, Centrobin, CEP170, C-NAP1, Glut. tubulin, Kizuna, NEDD1, Ninein, Pericentrin</td>
<td>78-100</td>
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</table>

To further analyse the maturity of the split centriole, we carried out analysis using antibodies against Centrin (Centrin2 and Centrin3), along with antibodies against Cenexin/ODF2 and Centrobin. As shown in Table 4.2 and Figure 4.6 A and B, Centrin can always be detected at three clustered centrioles and the single split centriole in IR-split cells, again confirming that these are intact single centrioles, without amplification. When we looked at Cenexin localisation in untreated G2 phase cells, we only saw a single focus in 94% of G2 phase cells, indicative of the “grandmother” centriole. In split centrioles following IR, again in 88% of cells only a single Cenexin focus at the clustered centrioles (Table 4.2, Figure 4.6 A). Generally three Centrobin foci are seen in untreated and irradiated G2 phase cells, at the three younger centrioles, with an occasional fourth faint focus visible (Table 4.2, Figure 4.6 B). In 84 % of cells with split centrioles, Centrobin was readily detected at two clustered centrioles and at the split centriole (Table 4.2).
Figure 4.6. Cenexin and Centrobin localisation in engaged and split centrioles.
Immunofluorescence microscopy analysis of untreated and IR-split centrioles. hTERT-RPE1 cells were fixed 24 hours after 5 Gy IR, where indicated, then stained with the indicated antibodies. Cells were counterstained with DAPI to visualise the DNA. Inserts show blow-ups of split and engaged centrioles. Scale bar, 10 μm.

We investigated Cenexin and Centrobin localisation together in untreated cells, and saw that in 81% of cells, Centrobin was seen at the three younger centrioles, which were devoid of Cenexin staining. Occasionally a very faint fourth Centrobin focus was seen at the oldest centriole, which was positive for Cenexin. In cells with split centrioles, the single Cenexin focus was at a centriole in the cluster, while Centrobin was at two clustered centrioles and the split centriole. (Table 4.2, Figure 4.6 C). This shows that the split centriole is not fully mature. Although this study does not conclusively determine which centriole becomes split, it does show that the oldest “grandmother” centriole rarely becomes split after IR. This indicates that daughter centrioles are more likely to become split and mobile, as has been shown by Piel et al. (Piel et al., 2000).

Table 4.2. Cenexin and Centrobin localisation at engaged and split centrioles.
hTERT-RPE1 cells were fixed 24 hours after 5 Gy IR, where indicated, then stained with the indicated antibodies. Engaged and split centrioles were scored as to the presence of Cenexin and/or Centrobin. The table shows the % of centrioles that carry the indicated markers. At least 50 cells were counted in each case.

<table>
<thead>
<tr>
<th>IR</th>
<th>Splitting</th>
<th>Centrin No. foci</th>
<th>Cenexin No. foci</th>
<th>Centrobin No. foci</th>
<th>Cenexin/Centrobin location</th>
<th>% cells</th>
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<td>-</td>
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<td>84</td>
</tr>
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4.2.3 Centriole splitting as a response to DNA damage

It has been established that a cell cycle arrest can lead to multiple rounds of centrosome duplication, when the cell and centrosome cycle become uncoupled (Dodson et al., 2004; Kuriyama et al., 2007). After showing that centriole splitting precedes amplification following DNA damage, we wished to determine if premature centriole splitting in hTERT-RPE1 cells was due to the DNA damage response or if a cell cycle arrest in G2 without DNA damage could cause centriole splitting. To do this, we used a CDK1 inhibitor, RO-3306, which blocks cells at the G2/M phase border as seen in Figure 4.7 (Vassilev et al., 2006). Using this inhibitor at a concentration of 6 μM for 24 hours, we could successfully arrest up to 60% of hTERT-RPE1 cells at the G2/M phase border and by washing out RO-3306, the majority of cells were able to enter the next cell cycle within 3-6 hours post release (Figure 4.7A). Following release from longer durations of cell cycle arrest (30 and 48 hours), fewer cells were seen to return to the cell cycle (Figure 4.7 B and C).

![Figure 4.7](image)

**Figure 4.7. RO-3306 arrests cells at the G2/M border and washout allows cells to re-enter cycle.**

hTERT-RPE1 cells were treated with 6 μM RO-3306 for the indicated times before being washed out, allowing cells to re-enter cell cycle. Samples were taken at the indicated times, PI staining was carried out and samples were analysed by FACS. After a short cell cycle arrest, the vast majority of cells re-enter G1 (A), after a longer arrest, most cells enter G1 (B), after an extended arrest, few cells are able to enter G1 and an increase in the 8N population is seen after release (C).

Using this method, we aimed to determine if centrioles split in response to a cell cycle arrest, without DNA damage. First, we needed to confirm that RO-3306 did not induce a DNA damage response in hTERT-RPE1 cells. To do this, we carried out immunoblot and immunofluorescence analysis using an antibody against γ-H2AX, which is detected rapidly at the sites of DNA double strand breaks after IR (reviewed by Srivastava et al., 2009). As an additional control, we also carried out immunoblot analysis on HCC1937 cells, which are hypersensitive to IR, due to a BRCA1 mutation (Scully et al., 1999). As shown in Figure 4.8 A and B, treatment with RO-3306 did not lead to an increase in γ-H2AX levels in hTERT-RPE1 cells, compared with cells treated with 10 Gy IR, as determined by IB and IF analysis.
Similarly, in HCC1937 cells, following IR, there is a large increase in \(\gamma\)-H2AX, whereas in RO-3306 treated cells, levels of \(\gamma\)-H2AX do not exceed that of untreated cells (Figure 4.8 A). This clearly shows that RO-3306 treatment does not induce a DNA response in both non-transformed and cancer cells.

**Figure 4.8. RO-3306 does not induce a DNA damage response.**

A. hTERT-RPE1 and HCC1937 cells treated with 6 \(\mu\)M RO-3306 for the indicated times or with 10 Gy IR for 2 hours, where indicated, before immunoblot analysis was carried out using an antibody against \(\gamma\)-H2AX. 30 \(\mu\)g of protein was loaded for each sample. B. hTERT-RPE1 cells were treated as in A. and fixed and stained for \(\gamma\)-H2AX (green) and DAPI to visualise the DNA. Scale bar 10 \(\mu\)m.

We next compared centrosome abnormalities in irradiated cells with cells arrested with RO-3306. First, we examined centriole splitting in hTERT-RPE1 cells and found, as shown in Figure 4.9 A, that roughly 12% of cells had split centrioles when fixed 30 and 48 hours after 5 Gy IR. When we compared this to cells treated with RO-3306, we saw that less than 5% of cells had split centrioles 30 hours after RO-3306 addition, which remained unchanged after 48 hours treatment. When we scored the levels of centrosome amplification in irradiated cells, we saw a maximum of 5% centrosome amplification in irradiated cells (Figure 4.9 B). In RO-3306-treated cells, we saw very high levels of centrosome amplification in cells, 23% after 24 hours treatment, increasing to 65% after 48 hours. These results indicate that cells arrested at G2/M phase border reduplicate their centrosomes in the absence of a DNA damage response and without an initial DNA damage-induced centriole splitting. These data also show how uncoupling of the cell and centrosome cycles can lead to centrosome amplification, as we see a low 3C population of cells by FACS analysis but high levels of centrosome amplification (Figure 4.7 and 4.9).

These data suggest that centriole splitting in hTERT-RPE1 cells is a specific response to genotoxic stress and does not occur as a result of a G2/M phase arrest alone. If centrioles in cells arrested with RO-3306, were to split before reduplication, it would have to occur within 24 hours of treatment. With the low levels of centriole splitting and high levels of centrosome amplification seen in RO-3306 treated cells, this appears to be unlikely, in non-transformed cells at least.
Conversely, centrioles may still disengage (but remain tethered) and reduplicate during this cell cycle arrest, as centrosomes are continuing to cycle. Our findings were confirmed in other studies, which found that CHO and DT40 cells arrested at the G2/M phase border reduplicated their centrosomes without the endoreplication of DNA. In CHO cells, Cdk1 was inhibited using RO-3306 and in DT40 cells, Cdk1 was knocked out by chemical genetics (Hochegger et al., 2007; Steere et al., 2011).

**Figure 4.9. G2/M arrest leads to centrosome amplification but not centriole splitting.**

hTERT-RPE1 cells were treated with, either, 6 µM RO-3306 or 5 Gy IR for the indicated times before being fixed and stained for Centrin2 and γ-tubulin. Cells were scored as to the presence of A, split or B, amplified centrioles. Data show the mean ± S.E.M of three experiments in which at least 50 cells were counted.

### 4.2.4 Characterisation of amplified centrosome following CDK1 inhibition

The amplified centrosomes seen after prolonged CDK1 inhibition were either clustered or separated around the nucleus of the cell (Figure 4.10). These amplified centrosomes were fully formed centrioles, as confirmed by Centrin2 and CEP76 staining, which were always present at all centrioles (White et al., 2000; Tsang et al., 2009). They also contained the centrosome cohesion component C-NAP1, which was present at clustered and separated amplified centrosomes (Fry et al., 1998a). Some but not all of the centrioles contained the maturity markers CEP170, Ninein and Kizuna, indicating these centrioles were at various stages of maturity (Mogensen et al., 2000; Guarguaglini et al., 2005; Oshimori et al., 2006; Graser et al., 2007a). When we looked at the pericentriolar material component Aurora A, we found that when centrosomes were separated, Aurora A was always detected, however, when the centrosomes were clustered, Aurora A was absent (Figure 4.10). Aurora A levels are normally low in G1 and peak in late G2, when centrosomes separate to opposite poles of the cell (reviewed by Lukasiewicz and Lingle, 2009). These data indicate that the centrosome cycle was still proceeding and centrosomes were being duplicated in a regulated manner, while the DNA cycle was arrested (Figure 4.7 and
Our observation of amplified centrosomes being either clustered or separated contradicts the findings of Steere et al., which found that in human cells, including hTERT-RPE1 cells, RO-3306 did not induce amplified centrosomes that could separate (Steere et al., 2011). However, many factors could have affected their findings, including; drug concentration and duration of inhibition.

**Figure 4.10. Characterisation of amplified centrosomes after prolonged CDK1 inhibition.**
Micrographs of hTERT-RPE1 cells treated with RO-3306 for 48 hours before being fixed and stained for γ-tubulin (red) and the indicated centrosomal markers (red). Boxed insets of centrosomes are shown magnified at right of micrographs. Scale bar, 10 μm.

We also noted the presence of pseudo-bipolar and multipolar mitotic cells following release from cell cycle block (Figure 4.11). With the high levels of centrosome amplification seen in RO-3306-treated cells, we used this method to investigate the outcome of centrosome amplification in cells entering mitosis.

**Figure 4.11. Mitotic cells after release from a prolonged G2/M phase cell cycle arrest.**
Micrographs of mitotic hTERT-RPE1 cells which were released after 30 hours of G2/M phase cell cycle arrest with 6 μM RO-3306. Cells were stained for α-tubulin (red) and γ-tubulin (green). Blow-ups show centrosomes, magnified at right of micrographs. Scale bar 10 μm.
We treated U2OS cells that stably expressed H2B-RFP (generated by Dr. Helen Dodson) with RO-3306 to induce centrosome amplification, then washed out the inhibitor, allowing cells to enter mitosis. We then used time-lapse microscopy to follow cells through mitosis (Figure 4.12). As these cells did not express any centriolar marker, we were unable to confirm which cells had amplified centrosomes. However, with the high levels of centrosome amplification seen in all cell lines analysed (data not shown), it was expected that a significant number of mitotic cells imaged had amplified centrosomes. 29% of cells imaged were found to complete an apparently normal, bipolar division. As shown in Figure 4.12, we observed just a single example of a cell dividing into three daughter cells, although it could also be a multinucleated cell which failed to divide during cytokinesis. From these data, we concluded that even with high levels of centrosome amplification, cells rarely undergo a multipolar division to form multiple daughter cells. This question has been extensively studied by others, who found that cells cluster their centrioles to divide in a bipolar manner. However, this often leads to lagging chromosomes, which contributes to genome instability (Ring et al., 1982; Guidotti et al., 2003; Ganem et al., 2009; Silkworth et al., 2009).

![Figure 4.12. Centrosome amplification is unlikely to lead to multipolar cell divisions.](image)

Micrographs of time-lapse microscopy showing a multipolar division in H2B-RFP expressing U2OS cells, treated with 6 μm RO-3306 for 30 hours. RO-3306 was then washed out, allowing cells to enter mitosis. Imaging began immediately and cells were imaged at one hour intervals over the course of eleven hours. In total, 145 cells were imaged, 42 of which entered mitosis.
4.2.5 The involvement of centrosome cohesion proteins in regulation of centriole splitting

We hypothesised that centrosome cohesion proteins may control centriole splitting after IR. To test this hypothesis, we used RNAi to deplete NEK2, CROCC (Rootletin), CEP250 (C-NAP1) and PLK1S1 (Kizuna) in hTERT-RPE1 cells. Transcripts were depleted using specific RNA duplexes, shown in Table 2.8, Section 2.1.4. We found that 50 nM of siRNA was sufficient to deplete protein levels of NEK2, C-NAP1 and Kizuna, 24 hours after transfection with the siRNAs (Figure 4.13). As a control experiment, we performed siRNA knockdown of CHK1, a key transducer of the DNA damage response, which has been shown to be involved in regulating centrosome overduplication after IR (Bourke et al., 2007). With Rootletin depletion, as we were unable to achieve more than roughly 50% knockdown even with higher concentration of siRNA, we decided to use a concentration of 50 nM siRNA as with the other transfections (Figure 4.13). Using non-targeting siRNA, we analysed the effect of transfection on each protein, to confirm that the decrease in protein levels was not an off-target effect of the transfection. As shown in Figure 4.13, C-NAP1, Rootletin, Kizuna and CHK1 protein levels were unaffected following transfection of non-targeting siRNA. NEK2 protein levels increased 24 hours after transfection, possibly a result of the stress caused by transfection, but these levels returned to normal after 48 hours. Also shown in Figure 4.13, IR did not affect the knockdown efficiency of any of the siRNAs.

Figure 4.13. Analysis of protein levels of centrosome cohesion components after RNAi.
Immunoblot analysis if RNAi efficiency over time. Cells were treated with the indicated siRNAs for 24 hours prior to 5 Gy IR, where indicated, so that irradiated and unirradiated cells were subjected to siRNA treatment for up to 72 hours in both cases. Actin was used as a loading control. (Work carried out in collaboration with C. Saladino).
We next performed flow cytometry analysis on hTERT-RPE1 cells following siRNA treatment to investigate the integrity of the DNA damage response following depletion of C-NAP1, NEK2, Rootletin and Kizuna (Figure 4.14). We observed no alteration in the cell cycle profile following NEK2, Rootletin and Kizuna depletion, compared to non-targeting siRNA, although we did see an increase in the 2C fraction in unirradiated C-NAP1 knockdown population. This indicated there are slight difficulties in cell cycle progression in the absence of C-NAP1 (Figure 4.14) (Faragher and Fry, 2003). Following IR, all siRNA treated cells showed a G2/M arrest similar to that of untreated or control siRNA treated cells, demonstrating that the DNA damage responses are intact after RNAi treatment.

Next, we investigated the involvement of NEK2, C-NAP1, Rootletin and Kizuna in centriole splitting. hTERT-RPE1 cells were transfected with siRNA, with and without IR, which was performed 24 hours after siRNA treatment. Cells were analysed by immunofluorescence microscopy and levels of centriole splitting were quantified 24, 48 and 72 hours after transfection, before and after IR. As shown in Figure 4.15, cells treated with control siRNA showed no significant alteration in levels of centriole splitting with and without IR, compared to untreated cells. NEK2 depletion had no significant impact on centriole splitting, consistent with the literature, which reports that overexpression of NEK2 leads to increased levels of centriole splitting (Fry et al., 1998b). These data show that NEK2 is not required for
DNA damage-induced centriole splitting. Kizuna depletion suppressed centriole splitting, although this was at the limit of statistical significance \( (P=0.055) \), which suggests that Kizuna may be a positive regulator of centriole splitting. As a control experiment, we found that knockdown of CHK1 had no statistically significant effect on centriole splitting, only showing a modest reduction in centriole splitting (Figure 4.15).

However, knockdown of C-NAP1 and Rootletin caused marked increases in centriole splitting, indicating that siRNA depletion of Rootletin or C-NAP1 causes disengaged G1 centrioles to split (Figure 4.15), consistent with high levels of splitting seen in U2OS cells after depletion of either protein \( (\text{Bahe et al., 2005}) \). After treatment with either C-NAP1 and Rootletin siRNA, followed by irradiation, 25% of cells showed split centrioles in both cases, compared to 11% seen in irradiated wild-type cells. As IR allows for the splitting of centrioles in G2 phase, these data suggest that C-NAP1 and Rootletin depletion enhances the splitting of centrioles in irradiated cells.

![Figure 4.15. Levels of centriole splitting in RNAi treated cells.](image)

Quantitation of centriole splitting in siRNA-treated hTERT-RPE1 cells at the indicated times after siRNA treatment. Where indicated, cells were treated with 5 Gy 24 hours after siRNA treatment. Cells were fixed and stained for Centrin2 and \( \gamma \)-tubulin at the time points shown and the level of centriole splitting quantitated. Data points are mean of + s.e.m of 3 separate experiments in which at least 100 cells were counted. Statistical significance was determined by comparison with irradiated and unirradiated scrambled siRNA control, as appropriate. *** \( P \leq 0.01 \); ** \( P \leq 0.05 \); * \( P \leq 0.055 \). (Work carried out in collaboration with C. Saladino).
We then looked at the cell cycle distribution of centriole splitting in Rootletin-deficient cells. We found that with siRNA depletion of Rootletin without IR, the vast majority of split centrioles were in G1 phase, with almost no split G2 centrioles observed (1.3% and 0% split G2 centrioles after 48 and 72 hours siRNA treatment, respectively) (Figure 4.16). When cells were treated with 5 Gy IR, 24 hours after siRNA treatment, this lead to a decrease in split G1 centrioles and an increase in split G2 centrioles at both 24 and 48 hours after IR (Figure 4.16). In wild-type cells treated with 5 Gy IR for 24 and 48 hours, we see roughly 4% and 11%, respectively, of cells with split centrioles, all of which are in G2 phase (due to the G2 arrest following IR). The percentage of split G2 centrioles is even lower in cells treated with Scrambled siRNA and IR for 24 and 48 hours (3.3% and 6.7% respectively) (Figure 4.15). Although we see mainly split G1 centrioles with Rootletin siRNA alone, the increase in split G2 centrioles with Rootletin siRNA and IR compared to IR alone indicates that Rootletin plays a role in opposing IR-induced centriole splitting. The decrease in split G1 centrioles may be due to split G1 centrioles reduplicating and then arresting in G2 after IR. Together, these data indicate that centrosome tethering by C-NAP1 and Rootletin, which links G1 phase centrioles and keeps duplicated centrosomes together, also opposes the splitting off of individual centrioles that is facilitated by IR-induced centriole disengagement as illustrated in Figure 4.17 (Bahe et al., 2005; Prosser et al., 2012).

![Figure 4.16](Image)

**Figure 4.16. G1 and G1 distribution of split centrioles in Rootletin deficient cells.**
Quantitation of G1 and G2 centriole splitting in Rootletin-depleted hTERT-RPE1 cells after siRNA treatment. Where indicated, cells were treated with 5 Gy 24 hours after siRNA treatment. Cells were fixed and stained for Cenrin2 and γ-tubulin at the time points shown and the level of centriole splitting quantitated. Data points are mean of + s.e.m of 3 separate experiments in which at least 100 cells were counted.
C-NAP1 or Rootletin depletion primarily leads to the separation of disengaged G1 centrioles, while IR causes the splitting of engaged daughter centrioles from mother centrioles. Our data indicates that C-NAP1 and Rootletin also oppose the splitting of daughter centrioles, which is facilitated by IR-induced centriole disengagement.

We then examined, by immunoblot analysis, what happens to the levels of these proteins after IR. As shown in Figure 4.18, levels of NEK2, CHK1 and Kizuna declined, all of which showed little impact on centriole splitting following siRNA depletion. On the other hand, C-NAP1 and Rootletin, whose loss has a dramatic impact on centriole splitting, remained relatively constant. These data show that hTERT-RPE1 cells retain cohesion components that oppose premature centriole splitting, and lose positive regulators of such splitting after IR.

**Figure 4.17. C-NAP1 and Rootletin restrain splitting off of individual centrioles following IR.**
C-NAP1 or Rootletin depletion primarily leads to the separation of disengaged G1 centrioles, while IR causes the splitting of engaged daughter centrioles from mother centrioles. Our data indicates that C-NAP1 and Rootletin also oppose the splitting of daughter centrioles, which is facilitated by IR-induced centriole disengagement.

**Figure 4.18. Expression levels of centrosome cohesion components after IR.**
Immunoblot analysis of hTERT-RPE1 cells after being treated with 5 Gy IR and fixed at the indicated time points. Protein levels of centrosomal proteins were measured at the indicated time points. Actin was used at a loading control. (Work carried out in collaboration with C. Saladino).
Having described a role for C-NAP1 and Rootletin in regulating centriole splitting, we wished to investigate the impact of their depletion and also NEK2 depletion on centriole structure. When we knocked down Kizuna, very few split centrioles were seen, therefore, we were unable to analyse the impact of Kizuna depletion on split centrioles. As shown in Table 4.1, Section 4.2.2, centriolar and centrosomal markers were unaffected following depletion of NEK2. However, there was a decline in the number of split centrioles positive for the mother marker, Ninein in NEK2-depleted cells. We found that in C-NAP1-depleted cells, split centrioles were devoid of all maturation markers, with CEP170 and Ninein being absent from the split centrioles (Figure 4.19, Table 4.1). A reduction in the percentage of split centrioles positive for Rootletin was also seen with 36% of split centrioles containing Rootletin, consistent with what has been described in other studies, where C-NAP1 was depleted (Bahe et al., 2005). A less pronounced phenotype was seen in Rootletin-deficient cells. Rootletin-depleted cells showed a 50% decrease in split centrioles positive for Ninein, but CEP170 was unaffected. Split centrioles in all cells under all conditions, remained devoid of Kizuna and Cenexin. As C-NAP1 and Rootletin and NEK2 depleted cells still contained CEP170 and Ninein at the engaged centrioles, and given that CEP170 and Ninein also localise to the proximal ends of centrioles, we concluded that loss of C-NAP1, and to a lesser extent, NEK2 and Rootletin, may alter the composition of the proximal end of split centrioles.

**Figure 4.19. Split centrioles lose mother markers after C-NAP1 depletion.**
Micrograph showing the impact of C-NAP1 depletion on the composition of the split centrioles. hTERT-RPE1 cells were treated with siRNA against C-NAP1 for 24 hours, then either fixed after a further 24 hours or treated with 5 Gy and fixed after 24 hours and stained with the indicated markers. Insets show blow-ups of split and engaged centrioles. Scale bar, 10 µm. (Work carried out in collaboration with C. Saladino).
4.3 Discussion

4.3.1 Centriole splitting is a precursor to DNA damage-induced centrosome amplification

The high levels of centriole splitting and subsequent amplification after IR, which we observe in BRCA1/TP53 and BRCA2 mutant cells lines fit well with the model for the control of centrosome duplication, in which Separase-controlled centriole disengagement is necessary for new centriole growth (Tsou and Stearns, 2006b; Tsou et al., 2009). Ko et al. demonstrated that inhibition of BRCA1 allowed for premature centriole separation, followed by reduplication, without a passage through mitosis (Ko et al., 2006). From our analysis, we found that BRCA1 and TP53 mutations alone were not sufficient to drive centrosome amplification, indicating that there are additional controls preventing the reduplication of centrioles. However, after IR a high level of amplification was seen, as the split centrioles are already licensed for reduplication. This indicates that centriole splitting potentiates DNA damage-induced centrosome amplification. It has been shown that mutations in BRCA2 can also lead to centrosome amplification in MEF’s (Tutt et al., 1999). However, the low levels of centrosome amplification seen in CAPAN-1 cells following IR, even with the high levels of split centrioles seen before IR, is consistent with additional roles for BRCA1 and TP53 in controlling centrosome number (Starita et al., 2004; D’Assoro et al., 2008).

In non-transformed, hTERT-RPE1 cells, treated with IR, we saw a dose dependent increase in centriole splitting. We also saw that centriole splitting increased overtime, reaching a maximum level 48 hours after IR. After this time, centriole splitting decreased and amplification increased more dramatically. This again illustrates how centriole splitting is a controlled response to DNA damage in non-transformed cells and that centriole splitting may allow for the reduplication of centrioles.

Our current model suggests that centriole splitting is a key DNA damage-induced step in potentiating centrosome amplification. More light has been shed on the mechanism in a recent study by Prosser et al. which demonstrated that an arrest in G2 following DNA damage can cause the untimely activation of the APC/C, which leads to Securin degradation and the release of Separase. Plk1 was also shown
to have APC/C dependent and independent roles in promoting disengagement which can in turn lead to centrosome amplification. Although Plk1 declines after IR, residual Plk1 activity appears to promote disengagement after IR (Bassermann et al., 2008; Prosser et al., 2012). However, a mother centriole with a pre-existing daughter centriole is not sufficient to limit centriole duplication, as has been shown with both STIL and PLK4 overexpression, where multiple daughter centrioles form around a single mother (Kleylein-Sohn et al., 2007; Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). Therefore, although centriole engagement prevents the reduplication of centrioles, the precise molecular mechanisms of different types of centrosome amplification have yet to be defined.

### 4.3.2 Microscopy analysis of split centrioles after irradiation

We found that IR leads to the splitting off of fully-formed centrioles in G2 phase, as confirmed by Centrin2 and glutamylated tubulin staining and therefore not arising as a result of centriole fragmentation or amplification (Bobinnec et al., 1998; White et al., 2000). This was further confirmed in cells with split centrioles, which only had four Centrin foci and were counterstained for Cenexin and Centrobin, where we do not see extra foci indicative of mature or immature centrioles (Nakagawa et al., 2001; Zou et al., 2005). When we investigated the maturity of split centrioles, we found that split centrioles carried the maturity markers CEP170 and Ninein. However, they were devoid of Cenexin and Kizuna. As CEP170 and Ninein can also be detected at the proximal ends of centrioles, we concluded that the centriole that becomes split and mobile after IR, was usually a daughter centriole (Mogensen et al., 2000; Piel et al., 2000; Guarguaglini et al., 2005; Oshimori et al., 2006; Graser et al., 2007a). The presence of the immaturity marker Centrobin at the split centriole, again suggests that split centrioles are not fully mature. Split centrioles carried the pericentriolar material components NEDD1 and Pericentrin, indicating that they could be capable of nucleating microtubules and forming organised microtubule arrays (Doxsey et al., 1994; Haren et al., 2006). However, the absence of Kizuna suggests that split centrioles that form spindle poles may be less likely to endure the pulling forces on the spindle during mitosis (Oshimori et al., 2006). We found that the cohesion component NEK2 was absent from all centrioles after IR, consistent with published data, while C-NAP1 and Rootletin levels appeared to be unaffected at all centrioles (Fry et al., 1998a; Fry et al., 1998b; Fletcher et al.,...
DNA damage has previously been shown to block G2 phase centrosome separation by inhibiting NEK2, thus blocking the phosphorylation of C-NAP1 and Rootletin, a mechanism which prevents the centrosomes from moving to opposite poles and entering mitosis with damaged DNA (Mayor et al., 2000; Fletcher et al., 2004; Bahe et al., 2005).

### 4.3.3 Centriole splitting as a response to DNA damage

We went on to induce centrosome amplification using a CDK1 inhibitor, which blocks cells at the G2/M phase border, without causing a DNA damage response (Vassilev et al., 2006). Although we saw increasing levels of centrosome amplification with longer periods of cell cycle arrest, as has been shown in other studies (Dodson et al., 2004; Kuriyama et al., 2007), we saw virtually no centriole splitting. Based on these data, we determined that centriole splitting is a response to DNA damage and not as a result of a cell cycle arrest alone. This is consistent with the work of Hut et al. who showed that during mitosis, in the presence of damaged DNA, centrosomes can split into fractions containing single centrioles (Hut et al., 2003). We also concluded that uncoupling of the DNA and centrosome cycles in human cells can lead to centrosome amplification when DNA replication is stopped but centrosomes continue to cycle. This has been shown to occur in CHO and DT40 cells, where Cdk1 has been inhibited either using RO-3306 or using chemical genetics (Hochegger et al., 2007; Steere et al., 2011). Given that centrosomes appear to cycle normally in the absence of Cdk1, however, indicates that centriole disengagement is still occurring following treatment with RO-3306 as has been shown by Prosser et al. (Prosser et al., 2012).

### 4.3.4 Characterisation of amplified centrosomes following CDK1 inhibition

When we looked more closely at the structure of these amplified centrosomes, we found that they were either clustered or separated out around the cell. When separated, each centrosome generally contained two fully formed centrioles, as confirmed by Centrin2 and CEP76 staining (White et al., 2000; Tsang et al., 2009). The cohesion component, C-NAP1, was also present at both clustered and separated centrosomes (Fry et al., 1998a). The maturity markers CEP170, Ninein and Kizuna were present at some but not all centrioles, indicating that the centrioles were at different stages of maturity due to the continuation of the
centrosome cycle (Mogensen et al., 2000; Guarguaglini et al., 2005; Oshimori et al., 2006; Graser et al., 2007a). Interestingly, we found that Aurora A was absent when centrosomes were clustered together but present at all centrosomes when they were separated around the periphery of the nucleus. As levels of Aurora A are low in G1 and peak in late G2, this indicates that centrosomes may be continuing to cycle, with protein levels fluctuating depending on the stage the centrosome cycle was at (reviewed by Lukasiewicz and Lingle, 2009). The phenotype of centrosome migration around the nucleus has been seen in HT2-19 cells, with a conditional CDK1 mutation, although, in this case centrosome reduplication was found to be coupled with DNA endoreplication (Laronne et al., 2003). Centrosome migration was not found to occur in any of the human cells studied by Steere et al., who found that centrosomes only migrated in CHO cells using RO-3306 (Steere et al., 2011).

After washing out RO-3306, cells were able to enter mitosis, often in the presence of amplified centrosomes. We found that the majority of cells which entered mitosis completed an apparently normal mitotic division, with only a single example of a multipolar division being found. These results fit well with those of Ganem et al., who found that the majority of cells that enter mitosis with amplified centrosomes often cluster their centrosomes and complete a bipolar division. In these cells, however, amplified centrosomes still contribute to genome instability, as there are increased frequencies of lagging chromosomes (Ganem et al., 2009).

4.3.5 The involvement of centrosome cohesion proteins in regulation of centriole splitting

Using RNAi, we knocked down expression of NEK2, CEP250 (C-NAP1), CROCC (Rootletin), PLK1S1 (Kizuna) and CHK1. DNA damage responses were intact after treatment with all of the siRNAs, with cells arresting after IR in G2, in a similar manner to wild-type cells. Kizuna depletion was found to slightly decrease IR-induced centriole splitting, consistent with its minimal effect on interphase centrosomes (Oshimori et al., 2006), while both CHK1 and NEK2 depletion had a minor impact on centriole splitting after IR. C-NAP1 and Rootletin depletion led to high levels of centriole splitting both before and after IR, consistent with studies by Bahe et al. showing that loss of either of these proteins leads to premature splitting (Bahe et al., 2005). When we looked at the G1 and G2 distribution of Rootletin-
depleted cells, we found that without IR, the majority of splitting occurs in G1 phase centrosomes. However after IR, we see a higher percentage of split G2 phase centrioles in Rootletin-depleted cells compared to cells treated with IR alone or with scrambled siRNA. Together, these findings suggest that C-NAP1 and Rootletin repress centriole splitting and that their phosphorylation by NEK2 is not required for centriole splitting. If daughter centrioles only split when allowed to do so by centriole disengagement, then tethering/centrosome cohesion components may regulate the extent to which disengaged centrioles move apart (Fry et al., 1998a; Fry et al., 1998b; Mayor et al., 2000; Faragher and Fry, 2003; Fletcher et al., 2004; Bahe et al., 2005; Prosser et al., 2012).

When we analyse the levels of these proteins after IR, we found that NEK2, Kizuna and CHK1 all declined, while Rootletin and C-NAP1 remained relatively constant. This illustrates how cells retain cohesion components that oppose the separation of centrosomes following a G2 arrest after IR, while they lose positive regulators of separation and reduplication after IR (Fry et al., 1998a; Fletcher et al., 2004; Bahe et al., 2005; Bourke et al., 2007).

We then investigated the effect of NEK2, C-NAP1 and Rootletin depletion on the composition of the split centriole. NEK2 only had a minor effect, with less split centrioles carrying the mother marker Ninein. Split centrioles in Rootletin-depleted cells carried less CEP170 but Ninein was unaffected. However, when C-NAP1 was depleted we found a more dramatic phenotype, as split centrioles we devoid of both CPE170 and Ninein. As we have suggested, it is normally a daughter centriole that becomes isolated. As both CEP170 and Ninein can also be detected at the proximal ends of centrioles (Mogensen et al., 2000; Guarguaglini et al., 2005; Graser et al., 2007a), we conclude that loss of C-NAP1, and to a lesser extent Rootletin and NEK2, may affect the composition of the proximal end of centrioles. This would explain why CEP170 and Ninein are still present at the engaged centrioles, as the subdistal appendages of mother centrioles are presumably unaffected following loss of centrosome cohesion components.
Chapter 5. The impact of DNA damage and loss of centrosome cohesion on ciliogenesis

5.1 Introduction

The primary cilium is a solitary microtubule-based organelle, which emanates from the surface of most animal cells. The primary cilium receives mechanical and chemical signals from neighbouring cells and the extracellular environment, which it then transmits to the nucleus to initiate an response (reviewed by Pazour and Witman, 2003) and it is central to a number of signalling pathways such as Wnt, PDGF and Hedgehog signalling (Huangfu et al., 2003; Corbit et al., 2005; Huangfu and Anderson, 2005; Schneider et al., 2005; Corbit et al., 2008; Han et al., 2008; Spassky et al., 2008). The basal body or mother centriole nucleates a primary cilium after it docks close to the plasma membrane in G0/G1 phase and primary cilia are resorbed prior to mitosis, releasing the mother centrioles, thereby allowing the centrosome to participate in formation of the mitotic spindle (Sorokin, 1962; reviewed by Plotnikova et al., 2009). The precise molecular mechanisms underlying primary cilium assembly and disassembly have not yet been fully elucidated. However, it is known that proteins which play a role in procentriole elongation, such as CP110, CEP97 and CPAP, also participate in primary cilium formation (Spektor et al., 2007; Kohlmaier et al., 2009; Schmidt et al., 2009; Tang et al., 2009). Primary cilium resorption is again a relatively unknown process which involves Plk1 and Aurora A (Pan and Snell, 2005; Pugacheva et al., 2007; Inoko et al., 2012; Lee et al., 2012).

Defects in cilia formation, maintenance or function are responsible for an ever increasing array of diseases termed ciliopathies and include polycystic kidney disease (PKD) and Bardet-Biedl syndrome (BBS) (reviewed by Badano et al., 2006). Recently, there has been increased interest in the role, if any, primary cilia play in the development and progression of cancer. For example, oncogenic Hedgehog signalling can result in basal cell carcinomas (BCCs) and medulloblastomas. Primary cilia appear to play a role in both mediating and repressing tumourogenesis following aberrant Hedgehog signalling depending on the initiating oncogenic event (Han et al., 2009; Wong et al., 2009). Aberrant Wnt and PDGF signalling through the primary cilium has also been linked to many other forms of cancers with
increased metastatic potential, from colorectal cancer and melanomas resulting from aberrant Wnt signalling to breast cancers with aberrant PDGF signalling (Kinzler and Vogelstein, 1996; Weeraratna et al., 2002; Carvalho et al., 2005).

The primary cilium may also be implicated in the way cells respond to DNA damage, with proteins involved in primary cilium formation also playing roles in the DNA damage response (DDR). For instance, overexpression of NEK1, a member of the NIMA-related kinase (NEK/NRK) family, inhibits ciliogenesis, while mutations in Nek1 lead to PKD in mice. Knockdown or deletion of NEK1 reduces DNA repair following different types of DNA damage, including UV and IR. These data indicate that NEK1 is important for both ciliogenesis and efficient DNA damage repair (Upadhya et al., 2000; Polci et al., 2004; Chen et al., 2008; Han et al., 2008; Shalom et al., 2008; White and Quarmby, 2008; Pelegrini et al., 2010).

CEP164, which localises to the distal appendages of the mature centriole and is indispensable in primary cilium formation, has been identified as a mediator of DNA damage signalling and is phosphorylated by ATM/ATR following IR and UV-induced DNA damage. siRNA depletion of CEP164 reduced phosphorylation of Chk1/2, H2AX, RPA and MDC1 and demonstrated a crucial role for CEP164 in the G2/M checkpoint (Graser et al., 2007a; Sivasubramaniam et al., 2008; Pan and Lee, 2009). Using whole-exome resequencing, Chaki et al. found that mutations in CEP164, ZNF423 (Zinc finger protein 423) and MRE11, caused Nephronophthisis-related ciliopathies (NPHP-RC). Moreover, CEP164, ZNF423 and MRE11 all function in the DDR, with DNA damage causing their co-localisation with DNA damage-induced TIP60 (Tat-interacting protein 60) foci, a protein known to activate ATM (Chaki et al., 2012).

In this chapter we study the impact of ionising radiation and thus, DNA damage, on the formation and the structure of the primary cilium. We investigate whether ciliated cells respond differently to DNA damage than do non-ciliated cells. Finally, we test if centrosome cohesion plays a role in ciliogenesis and determine if loss of centrosome cohesion impacts on primary cilium formation.
5.2 Results

5.2.1 Characterisation of primary cilia in irradiated cells

Following IR-induced DNA damage, cells can arrest in G2 phase, during which time damaged DNA is repaired before cells enter mitosis (reviewed by Sancar et al., 2004). It has also been established that centriole splitting and centrosome amplification can occur during this cell cycle delay (Dodson et al., 2004; Bourke et al., 2007; Saladino et al., 2009). We wished to investigate whether irradiation might also impact primary cilium formation or structure. To first determine if IR impedes primary cilium formation, wild-type and irradiated hTERT-RPE1 cells were depleted of serum to induce high levels of primary cilia formation, these were then fixed at the times indicated in figure 5.1. We found comparable levels of primary cilia in untreated and irradiated cells, with irradiated cells having slightly higher levels of primary cilia compared to untreated cells. These results indicate that IR does not directly impact on ciliation.

![Figure 5.1. The impact of irradiation on ciliogenesis.](image)

hTERT-RPE1 cells were depleted of serum for the indicated times and where indicated, treated with 5 Gy IR immediately prior to serum depletion. Cells were then fixed and stained for acetylated tubulin and γ-tubulin and scored for the presence or absence of a primary cilium. Data points are mean of ± s.e.m of 3 separate experiments in which at least 100 cells were counted.

It is possible that the increased level of primary cilia in irradiated cells is due to a failure to resorb the primary cilium as opposed to higher levels of ciliation. A failure to resorb a primary cilium could prevent the release of the centrosome prior to mitosis and may contribute to the G2 arrest which occurs after IR treatment, as there are almost no examples of cells entering mitosis with a primary cilium (Archer and Wheatley, 1971; Rieder et al., 1979; reviewed by Plotnikova et al., 2008). Interestingly, Plk1 is inhibited after DNA damage (Smits et al., 2000), and recently
Dvl2 and Plk1 have recently been implicated in primary cilium resorption. shRNA depletion of either Dvl2 and Plk1 leads to an increase in the G2 population, perhaps in due to a failure to absorb the primary cilium and preventing cells from entering mitosis (Lee et al., 2012). Thus, primary cilia may play a role, albeit not centrally, in the DDR. Whether a failure to resorb a primary cilium after IR is a cause or consequence of the DDR remains unclear, although it does implicate the primary cilium in the timely execution of mitosis. It has also been demonstrated that Aurora A is inhibited after DNA damage (Cazales et al., 2005; Krystyniak et al., 2006). Therefore, the higher levels of cilia in irradiated cells could be due to the decline in the activation of Plk1 and Aurora A after IR, as Aurora A is also implicated in primary cilium resorption, as well as in timely mitotic entry (Smits et al., 2000; Marumoto et al., 2002; Pan et al., 2004; Pugacheva et al., 2007; Lee et al., 2012).

During our analysis we also noted the presence of multiple primary cilia in cells which had been irradiated, usually emerging side by side, possibly from a single ciliary pocket (Figure 5.2). Compound cilia have been reported in multi-ciliated cells, where multiple axonemes are surrounded by a common ciliary sheath and can cause ciliated cysts and other ciliopathies (reviewed by McAuley and Anand, 1998; Hagiwara et al., 2000; Ho et al., 2003). However, using IF microscopy we were unable to determine if this was the case with the multiple primary cilia we observed. 17% of ciliated cells were found to have multiple primary cilia after IR, which only ever grew from fully mature centrioles, as confirmed by Cenexin staining (Figure 5.2) (Nakagawa et al., 2001; Anderson and Stearns, 2009). We found similar results in hTERT-RPE1 cells arrested with the CDK1 inhibitor, RO-3306, which induces a cell cycle arrest at the G2/M border (data not shown). These data demonstrate how an arrest in G2 phase allows the younger mother centriole to fully mature and be capable of primary cilium formation without passage through mitosis. This also suggests that hTERT-RPE1 cells can form a primary cilium in G2, in addition to G0/G1 phase. Similar results were found by Anderson et al., who reported that in mouse 3T3 cells arrested at the G2/M border, when given sufficient time, the younger mother centrioles can acquire competence for ciliogenesis (Anderson and Stearns, 2009). Multiple primary cilia can also form in hTERT-RPE1 cells with amplified centrioles due to Plk4 overexpression. In these cells, centrioles only
nucleated extra primary cilia after centrioles matured upon passage through mitosis (Mahjoub and Stearns, 2012).

Figure 5.2. IR can lead to the formation of multiple primary cilia.
Micrographs of primary cilia in hTERT-RPE1 cells, serum starved for 24 hours and, where indicated treated with 5 Gy IR, 48 hours prior to serum depletion. Cells were fixed and stained for acetylated tubulin (red) and γ-tubulin or Cenexin (green). Boxed insets are shown magnified at right of micrographs. Scale bar, 10 μm.

We next carried out ultra-structural analysis on primary cilia in hTERT-RPE1 cells with and without IR treatment, to further explore if IR has an impact on the primary cilium structure, which we could not establish by IF. We first examined normal primary cilia in unirradiated cells and saw that primary cilium formation in hTERT-RPE1 cells follows the intracellular pathway as described by Sorokin, where the mother centriole docks with a primary cilium vesicle, into which the axoneme elongates, forming a ciliary sheath. The ciliary sheath then fuses with the plasma membrane, allowing the primary cilium to emerge into the extracellular environment. Therefore, a significant proportion of the primary cilium remains within the cell during its formation (Sorokin, 1962; Sorokin, 1968; Molla-Herman et al., 2010). Figure 5.3 shows primary cilia at different stages of elongation, with A and B showing an often asymmetrical ciliary sheath during the early stages of ciliogenesis, while C and D show fully formed primary cilia with a symmetrical ciliary pocket.
Figure 5.3. Primary cilium formation in hTERT-RPE1 cells.
Electron micrographs of primary cilia in hTERT-RPE1 cells, which were serum starved for 24 hours before being fixed and processed for TEM. A and B show early ciliogenesis where the ciliary sheath is visible. C and D show later stages of ciliogenesis where the ciliary pocket is visible. E is a highlighted version of D, showing the mother centriole (red), the daughter centriole (blue), the ciliary pocket (yellow) and the axoneme (green). Scale bar, 500 nm.

When we viewed the ultrastructure of primary cilia in cells which had been irradiated prior to serum starvation, we were unable to see any obvious structural abnormalities. Figure 5.4 shows the early stages of ciliogenesis in irradiated cells, where again the ciliary vesicle/sheath is visible. Figure 5.4 shows the later stages of ciliogenesis, where the ciliary pocket and axoneme appear to have formed normally in all cases. This indicates that IR does not impede the formation of primary cilia or impact on its structure.
Figure 5.4. Primary cillum formation in irradiated hTERT-RPE1 cells.
Electron micrographs of primary cilia in irradiated hTERT-RPE1 cells, which were treated with 5 Gy IR, 48 hours prior to being serum starved for 24 hours. Cells were then fixed and processed for TEM. A to D show early ciliogenesis where the ciliary vesicle/sheath is visible. E to H show later stages of ciliogenesis where cilia have elongated. I is a highlighted version of H, showing the mother centriole (red), the ciliary pocket (yellow) and the axoneme (green). Scale bar, 500 nm.

Due to the close proximity of the multiple primary cilia which we see in irradiated cells, we suspected that these cilia were emerging from a single ciliary pocket or were surrounded by a single ciliary membrane. However, using TEM we were unable to find any clear examples of cells possessing multiple primary cilia from a single ciliary pocket. We did find one example of a cell which appears to have two primary cilia (Figure 5.5 A, B). These two cilia were formed in very close proximity to each other, but as we could not see the base of the cilia, we were not able to confirm if these cilia emerged from a single ciliary pocket. Also, in Figure 5.5 C and D, we can clearly see four centrioles and possibly what appear to be two ciliary vesicles, adjacent to two of the centrioles. This may indicate that two ciliary vesicles initially form adjacent to the older centrioles and as these enlarge, they fuse together to form a single ciliary sheath and then a single ciliary pocket. Sorokin noted the presence of secondary ciliary vesicles which fuse with the primary ciliary vesicle to form the ciliary sheath as the axoneme grows (Sorokin, 1962). This would
explain why the majority of multiple primary cilia seen by IF appear side by side. It may also allow these multiple primary cilia to function as a single organelle and reduce any possible detrimental effects which multiple primary cilia may have on protein trafficking which takes place through the primary cilium and ciliary membrane (reviewed by Pazour and Bloodgood, 2008).

Recently, Mahjoub and Stearns made similar observations in hTERT-RPE1 cells which possessed amplified centrosomes following Plk4 overexpression. Using TEM, they were able to confirm that multiple primary cilia which formed from supernumerary centrosomes emerged from a single ciliary pocket. Interestingly they also found that ciliary levels of proteins such as Smoothened (Smo), Htr6 (5-hydroxytryptamine (serotonin) receptor 6), PKHD1 (Polycystic kidney and hepatic disease 1) and Arl13B (ADP-ribosylation factor-like 13B), all of which localise to the primary cilium, were diluted in cells with extra primary cilia, depending on the number of cilia. However, overall cellular levels of these proteins remained comparable to those in cells with a single primary cilium. The authors, therefore, suggested that the ciliary pocket is the rate limiting structure for the trafficking of ciliary proteins (Mahjoub and Stearns, 2012).

Figure 5.5. Abnormal primary cilium formation in irradiated hTERT-RPE1 cells.
Electron micrographs of primary cilia in hTERT-RPE1 cells, which were treated with 5 Gy IR, 48 hours prior to being serum starved for 24 hours. Cells were then fixed and processed for TEM. A. Two primary cilia emerging from a single cell. B. is a highlighted version of A, showing the mother centriole (red), the daughter centriole (blue) and the axoneme(s) (green). C. Two ciliary vesicles forming close to two centrioles. D. is a highlighted version of C showing the ciliary vesicle (yellow). Scale bars, 500 nm.

5.2.2 DNA repair pathways of ciliated cells

As the primary cilium is a sensory organelle which receives mechanical and chemical signals from other cells and the extracellular environment, we wished to test if the presence of a primary cilium was related to cellular responses to DNA damage. hTERT-RPE1 cells were serum-starved for 24 hours, then treated with 5Gy IR. We then fixed and stained cells for acetylated tubulin, to distinguish ciliated and
non-ciliated cells, and for Rad51 recombinase, which forms IR-induced foci (IRIF) during DNA repair (Figure 5.6 A) (Haaf et al., 1995). As shown in Figure 5.6 B, ciliated cells formed fewer Rad51 foci than non-ciliated cells during DNA repair, suggesting that homologous recombination (HR) may be limited in cells with primary cilia.

**Figure 5.6. Fewer RAD51 foci in ciliated cells.**
A. Micrographs of hTERT-RPE1 cells, serum starved (ss) for 24 hours prior to treatment with 5 Gy IR. Cells were fixed and stained for acetylated tubulin and RAD51. Scale bar, 10 μm. B. Cells were treated as in A and fixed at the indicated times. Cells with > 5 RAD51 foci were counted as positive. Data points are mean of ± s.e.m of 3 separate experiments in which at least 100 cells were counted.

When we quantified the IRIF formed by 53BP1, a component of the DDR involved in non-homologous end joining (NHEJ) (Schultz et al., 2000), we found no difference between ciliated and non-ciliated cells (Figure 5.7). These data indicate that ciliated cells respond differently to genotoxic stress compared to non-ciliated cells in the same population, possibly implicating ciliogenesis in the outcome of DNA damage.
Figure 5.7. Non-HR responses are unaffected in ciliated cells.

A. Micrographs of hTERT-RPE1 cells, serum starved (ss) for 24 hours before treatment with 5 Gy IR. Cells were fixed and stained for acetylated tubulin and 53BP1. Scale bar, 10 μm. B. Cells were treated as in A and fixed at the indicated time points. Cells with > 5 53BP1 foci were counted as positive. Data points are mean of ± s.e.m of 3 separate experiments in which at least 100 cells were counted.

As HR occurs mainly in late S/G2 phase, we checked whether the proportion of cells in late S/G2 phase varied between ciliated and non-ciliated cells (Rothkamm et al., 2003). As shown in Figure 5.8 A, we found no difference between ciliated and non-ciliated cells in terms of cell cycle distribution, as determined by centriole quantitation. This suggests that the populations of ciliated and non-ciliated cells have the same cell cycle distribution and it is unlikely that ciliated cells in hTERT-RPE1 cells are in a quiescent state and therefore would be less likely to use HR as a repair mechanism, while non-ciliated cells are still cycling, as has been shown in quiescent hematopoietic stem cells (HSCs), (Mohrin et al., 2010). However, also shown in Figure 5.8 B are the levels of 53BP1 and RAD51 in asynchronous cells with and without IR, where we see higher levels of RAD51 foci in irradiated cells with normal serum levels. This may indicate that serum-depleted cells have a higher proportion of cells in G0/G1 and fewer cells in late S/G2 phase, which we were unable to determine by centriole quantification.
Figure 5.8. Cell cycle distribution and DNA damage response in hTERT-RPE1 cells.
A. hTERT-RPE1 cells were, where indicated, serum starved for 24 hours and treated with 5 Gy IR, where indicated, four hours before fixation. Cells were stained for centrin2 and acetylated tubulin. Data points are mean of ± s.e.m of 3 separate experiments in which at least 100 cells were counted, except for asynchronous ciliated cells where 50 cells were counted. B. Asynchronous hTERT-RPE1 cells were treated with 5 Gy IR and fixed after 4 hours to determine the number of 53BP1 and RAD51 positive cells in an asynchronous population, with normal serum levels. Cells were stained for either 53BP1 or RAD51. Data points are mean of ± s.e.m of 3 separate experiments in which at least 100 cells were counted.

To determine if the initial DDR varied between ciliated and non-ciliated cells, we investigated γ-H2AX levels (reviewed by Srivastava et al., 2009). In our preliminary analysis, we found comparable levels of γ-H2AX positive cells between serum-starved and asynchronous populations, with the serum-starved population having marginally fewer γ-H2AX positive cells. This suggests that the initial detection of DNA damage was comparable between ciliated and non-ciliated cells, although it may indicate slightly slower responses to DNA damage in ciliated cells.

Figure 5.9. γ-H2AX levels in asynchronous and serum-depleted cells.
Asynchronous and serum-starved hTERT-RPE1 cells were treated with 5 Gy IR and fixed at indicated times to determine the number of γ-H2AX positive cells in the population. Cells with >5 foci were considered positive. Data points are mean of ± s.e.m of 3 separate experiments in which at least 100 cells were counted.
5.2.3 The role of centrosome cohesion proteins in ciliogenesis

We next asked if centriole splitting affected ciliogenesis or if centrosome cohesion proteins played additional roles in ciliogenesis. To test this question, we used siRNA to deplete expression of NEK2, Rootletin, C-NAP1 and Kizuna with and without IR. As a control, we depleted CHK1, a key transducer in the DNA damage response and its role in DNA damage-induced centrosome amplification has been described in our group (Bourke et al., 2007). Cells treated with scrambled siRNA showed levels of ciliogenesis comparable to untreated hTERT-RPE1 cells before and after IR (Figure 5.10). We found that depletion of Rootletin and C-NAP1 significantly reduced cilia formation, with only ~25% of cells being ciliated, compared to cells treated with Scrambled siRNA, where at least 50% were ciliated. Depletion of both of these centrosome cohesion components was shown in Section 4.2.5 to cause high levels of centriole splitting, thus implicating centrosome cohesion proteins in the control of ciliogenesis. Up to 80-90% of hTERT-RPE1 cells can be ciliated after serum starvation when cells almost fully confluent (data not shown). However in our studies, cells were plated at low densities for optimal transfection efficiencies.

When we depleted Rootletin and C-NAP1 and then irradiated cells, again we observed reduced levels of ciliogenesis (Figure 5.10). Rootletin is required for the formation of the ciliary rootlet which provides structural support to the primary cilium, with its deletion in mice leading to fragility of the ciliary base (Yang et al., 2005). However, it has not yet been implicated in primary cilium formation. C-NAP1 also has not yet been implicated in primary cilium formation. However, C-NAP1 does co-localise with proteins known to play roles in primary cilium formation and/or maintenance such as ALMS1 and POC1 (Hearn et al., 2002; Collin et al., 2005; Graser et al., 2007a; Li et al., 2007; Pearson et al., 2009; Knorz et al., 2010). Depletion of NEK2, which has been previously implicated in reducing primary cilium formation (Graser et al., 2007a; Mikule et al., 2007), also reduced levels of ciliation in our studies, although only significantly after IR. Cells depleted of Kizuna or CHK1 showed increased levels of ciliogenesis. As shown in Section 4.2.5, depletion of both Kizuna and CHK1 slightly decreased levels of centriole splitting. These data indicate that loss of centrosome cohesion affects primary cilium formation.
Figure 5.10. The impact of centrosome cohesion on ciliogenesis.

hTERT-RPE1 cells were treated with indicated siRNA for 24 hours prior to being serum starved for 24 hours then fixed and stained for acetylated tubulin and γ-tubulin. Where indicated, cells were treated with 5 Gy IR 24 hours after siRNA treatment and cells were serum starved 24 hours after irradiation. Data points are mean of ± s.e.m of 3 separate experiments in which at least 100 cells were counted. Statistical significance was determined by comparison with irradiated and unirradiated scrambled siRNA control, as appropriate. **P ≤0.001; * P ≤0.05.

Previous experiments depleting C-NAP1 and Rootletin reported no significant impact on primary cilium formation (Graser et al., 2007a). To explore why we saw a significant impact while Graser et al. did not, we repeated our experiments under the other authors’ conditions, which had involved using different siRNAs, longer siRNA incubation times and longer serum starvation times with serum-free medium, as opposed to medium containing 0.2% newborn calf serum (NCS). Using the previously-published siRNAs, we found no significant effect of C-NAP1 or Rootletin depletion under either our conditions or those of the previous study (Figure 5.11 C and D). Using the siRNAs featured in this study under the previously-published conditions, we saw a reduction of ciliogenesis only with Rootletin depletion (Figure 5.11 D). As a control, we depleted CEP164, which had been shown by Graser et al. to reduce ciliation to ~25%. We found that CEP164 depletion reduced ciliogenesis to 17% in a single control experiment (Figure 5.11 B and D), so that our data are consistent with those that have been previously published, under those conditions (Graser et al., 2007a). As we see efficient knockdown of our targets with the previously-published siRNAs (Figure 5.14 A), we concluded that our serum starvation conditions, where we add 0.2% NCS to alleviate the impact of irradiation and siRNA knockdown, allow detection of the effects of C-
NAP1 or Rootletin depletion that were not seen in the previous work. NEK2 depletion has also proven controversial where two studies saw a decline in ciliogenesis with NEK2 depletion (Graser et al., 2007a; Mikule et al., 2007), but not in another (Spalluto et al., 2012).

Figure 5.11. Comparison of different siRNA and culture conditions. 
A. Immunoblot analysis of RNAi efficiency. Cells were treated with 50nM siRNA for the indicated times. α-tubulin was used as a loading control. B. Immunofluorescence analysis of Cep164 RNAi efficiency. Where indicated cells were treated with 50nM of Ce164 siRNA for 48 hours. Cells were fixed and stained for γ-tubulin and CEP164. Scale bar, 10µm. C. Ciliogenesis in RPE1 cells that had been siRNA treated for 48 hours before being serum starved in medium containing 0.2% NCS for 48 hours, then fixed and stained for acetylated tubulin and γ-tubulin. Data show the mean ± s.e.m of 3 separate experiments in which at least 100 cells were counted. ***P ≤0.001 compared with scrambled siRNA control. D. Ciliogenesis in RPE1 cells that had been siRNA treated for 48 hours before being serum starved in serum-free medium (SFM) for 48 hours, then fixed and stained for acetylated tubulin and γ-tubulin. Data show the mean ± s.e.m of 3 separate experiments in which at least 100 cells were counted, except for CEP164 siRNA, where data show a single experiment where at least 100 cells were counted. * P ≤0.05 compared with scrambled siRNA control.
We went on to investigate ciliation in cells with split centrioles, to directly study the impact of loss of centrosome cohesion of primary cilium formation. To do this, we depleted cells of NEK2, Rootletin or C-NAP1 and after 24 hours we treated cells with 5 Gy IR. As shown in Figure 5.12A, we made three distinct classifications of cells using IF microscopy, 1. no ciliation, 2. ciliation from centriole cluster and 3. ciliation from the split centriole. When we depleted NEK2, Rootletin and C-NAP1, we found virtually no difference compared to untreated and scrambled siRNA treated cells, with 65-76% of cells lacking a primary cilium in all cases (Figure 5.12B). Of the cells with split centrioles which did form a primary cilium, cilia generally emerged from the centriole cluster and rarely from the split centriole. However, as we have indicated in Section 4.2.2, it is normally a daughter centriole which becomes split following IR. Therefore, in irradiated cells, it is unlikely that a split centriole will be capable of forming a primary cilium. However, given that a small percentage of isolated centrioles did form a primary cilium and given that irradiated cells do not have decreased levels of primary cilia, these results indicate the splitting of centrioles after IR does not impact on ciliogenesis.

Figure 5.12. The impact of centrosome cohesion on ciliogenesis
A. Micrographs of irradiated hTERT-RPE1 cells with split centrioles with and without a primary cilium. Cells were fixed and stained for acetylated tubulin and \( \gamma \)-tubulin. Boxed insets are shown magnified at right of micrographs. Scale bar, 10 \( \mu \)m. B. hTERT-RPE1 cells were treated with the indicated siRNAs. Cells were then treated with 5 Gy IR, 24 hours after siRNA treatment and cells were serum starved 24 hours after irradiation then fixed and stained as in A. Cells with split centrioles were scored for the presence or absence of a primary cilium. The location of a primary cilium was also scored. Data points are mean of \( \pm \) s.e.m of 3 separate experiments in which at least 50 cells were counted.
We then measured the length of primary cilia in cells depleted of C-NAP1, Rootletin, NEK2 and Kizuna, as shown in Figure 5.13. In untreated cells, primary cilia varied in length and were found to be between 1.5 and 3.4 μm in length and having an average length of 2.19 ± 0.10 μm (scrambled siRNA, 2.34 ± 0.13 μm). These results are consistent with published data, which showed primary cilia in hTERT-RPE1 cells to be 2.6 ± 0.8 μm in length (Yoshimura et al., 2007). Cells depleted of Rootletin, NEK2 and Kizuna all possessed cilia of similar size to untreated or cells treated with scrambled siRNA. Cells depleted of C-NAP1 had an average cilium length of 1.81 ± 0.11 μm, which was significantly shorter that cells treated with scrambled siRNA. These results indicate that although depletion of Rootletin reduces ciliation, it does not impact on primary cilium length. C-NAP1, on the other hand, appears to play a role in both ciliation and control of primary cilium length.

![Graph](image)

**Figure 5.13. The impact of centrosome cohesion proteins on primary cilium length.**
hTERT-RPE1 cells were treated with indicated siRNA for 24 hours prior to being serum starved for 24 hours then fixed and stained for acetylated tubulin and γ-tubulin. At least 20 primary cilia were measured for each treatment. Statistical significance was determined by comparison with scrambled siRNA control, as appropriate. **P ≤0.005.

### 5.3 Discussion

#### 5.3.1 Characterisation of primary cilia in irradiated cells

As primary cilia are normally formed in G0/G1 and resorbed prior to mitosis, we wished to investigate how IR and thus, a cell cycle arrest would impact on primary cilium formation. We compared the level of primary cilium formation in
serum starved hTERT-RPE1 cells and cells which were irradiated directly prior to serum depletion. We found that IR does not reduce ciliation, with irradiated cells having slightly more primary cilia than non-irradiated cells. The marginally increased levels of ciliation in irradiated cells may be due to decreased levels of Plk1 and Aurora A, which have been reported to be needed for primary cilium resorption (Smits et al., 2000; Pan et al., 2004; Pugacheva et al., 2007; Lee et al., 2012). Recently Inoko et al. proposed that siRNA depletion of Aurora A also induces primary cilium formation (Inoko et al., 2012). While these studies provide evidence that Aurora A plays a role in either ciliation or cilium disassembly, the precise role of Aurora A in ciliation needs to be investigated further.

Ciliation may also aid in the G2/M cell cycle arrest following IR, ensuring that the centrosome is not released to form the bipolar spindle, although this may be more a consequence of the DDR than a contributor. hTERT-RPE1 cells treated with Aurora A siRNA have higher levels of primary cilia but do not show an extended G2 phase arrest, instead cells arrest in G1 phase. On the other hand, epithelial ovarian cancer (EOC) stem cells treated with an Aurora A inhibitor show a G2/M arrest and Hela cells treated with Aurora A siRNA also show an increased G2/M population (Chefetz et al., 2011; Inoko et al., 2012). However, given that cancer cells usually lack cilia, Aurora A may play slightly different roles in ciliated and non-ciliated cells (reviewed by Plotnikova et al., 2008). Lee et al. recently reported a non-mitotic role for Plk1 in primary cilium disassembly and showed that Plk1 in complex with Dvl2 mediated cilia disassembly by stabilising HEF1 and activating Aurora A. It was also found that depletion of Plk1 or Dvl2 by shRNA led to an increased G2/M population in hTERT-RPE1 cells, which the authors suggest may be due to a delay in mitotic entry until cells can resorb the primary cilium (Lee et al., 2012).

When we studied primary cilia after IR by IF microscopy, we often saw two primary cilia emerging from a single cell. These primary cilia were normally in close proximity to each other as only the base of each cilium could be seen separately. As these multiple primary cilia only emerged from mature centrioles, we concluded that the cell cycle arrest after IR allowed the younger mother centriole to mature sufficiently to form a primary cilium without passing through mitosis (Nakagawa et al., 2001). This also suggests that hTERT-RPE1 cells can form a
primary cilium in G2 phase as well as G1, as we and others have found similar results following CDK1 inhibition (Anderson and Stearns, 2009).

We went on to use TEM to determine if the multiple primary cilia were surrounded by a single ciliary sheath, as is seen in compound cilia of multi-ciliated cells (McAuley and Anand, 1998; Hagiwara et al., 2000; Ho et al., 2003), or if they shared a single ciliary pocket, which could also explain why they remained in such close proximity (Mahjoub and Stearns, 2012). We saw that ciliation in hTERT-RPE1 cells follows the intracellular pathway where the basal body does not directly dock with the cell membrane but instead docks with a ciliary vesicle which enlarges and eventually docks with the cell membrane allowing the primary cilium to emerge (Sorokin, 1962; Sorokin, 1968; Molla-Herman et al., 2010). We were unable to find any obvious structural abnormalities in primary cilia from irradiated cells, indicating that IR does not impact on ciliary structure and we were also unable to find any clear examples of multiple primary cilia. We found one example of what appears to be two primary cilia emerging from a cell, in close proximity to one another, possibly from a single ciliary pocket. In another example, we see the early stages of ciliation where two ciliary vesicles appear to have docked with two centrioles. If these vesicles were to enlarge into ciliary sheaths and fuse as in normal ciliation (Sorokin, 1962), this would allow the two cilia to emerge from the same ciliary pocket. Mahjoub et al. recently reported that following Plk4 overexpression, multiple primary cilia emerge from a single ciliary pocket in super-ciliated hTERT-RPE1 cells. It was found that the fraction of cells with multiple cilia increased with passage through the cell cycle allowing the extra centrioles to mature. Therefore, the number of primary cilium in a cell is controlled by the presence of only one fully mature mother centriole and this control can be perturbed after IR, as with Plk4 overexpression. However, the ciliary pocket may act as a rate limiting structure for the trafficking of ciliary proteins, with overall cellular concentrations of ciliary proteins remaining stable regardless of the number of cilia. Having extra cilia may impact negatively on the epithelial organisation and architecture of tissues, as monociliated IMCD-3 cells were able to form organised spheroid structures resembling in vivo epithelium architecture, while super-ciliated cells did not (Mahjoub and Stearns, 2012).
Having more than a single primary cilium could have dire consequences for humans, as is the case with tuberous sclerosis, where mutations in *TSC1* or *TSC2* genes (Tuberous sclerosis complex 1 and 2) cause an autosomal dominant disorder, characterised by benign tumours of the brain, kidneys, retina, heart and skin and in which severe renal cystic disease can occur. There are severe neurological manifestations with this disorder which include: infantile spasms, intractable epilepsy and autism (Hartman et al., 2009; Franz et al., 2010; Orlova and Crino, 2010). *Tsc1* and *Tsc2* null mice show increased cilia formation and *Tsc2*−/− MEFs can contain extra centrosomes and cilia (Hartman et al., 2009).

Mahjoub *et al.* also found that super-ciliated cells had defective activation of the Sonic hedgehog (Shh) pathway, as determined by Gli-GFP activation. Shh signalling plays crucial roles in development, such as establishing left-right asymmetry, neural tube closure and patterning and formation of limbs, teeth, pancreas, lungs and hair follicles (reviewed by Michaud and Yoder, 2006). Therefore, if cells form extra cilia during development, the effect of aberrant Shh signalling could be detrimental to the developing embryo.

### 5.3.2 DNA repair pathways of ciliated cells

We wished to determine if ciliated cells respond in the same manner to IR as non-ciliated cells. We found no significant difference in γ-H2AX levels between serum-depleted cells and cells grown in normal serum conditions. As γ-H2AX is detected rapidly at the sites of DNA double strand breaks after IR (reviewed by Srivastava et al., 2009), this indicates that the initial DNA damage response is unaffected in serum-depleted populations which would therefore, have a higher number of ciliated cells. We found that ciliated cells had lower levels of RAD51 foci induction than non-ciliated cells, an indication that HR may be impaired in ciliated cells. When we studied levels of 53BP1, a protein involved in NHEJ, although not exclusively (Schultz et al., 2000), we observed no difference in 53BP1 levels in ciliated versus non-ciliated cells. We concluded that DNA repair involving NHEJ was unaltered in ciliated cells. This suggests that ciliated cells may be more likely to use the error-prone method of NHEJ to repair DNA damage instead of the more reliable HR repair mechanism.
Why a ciliated cell would respond differently to DNA damage compared to non-ciliated cells remains unclear. However, a small number of proteins, including NEK1 and CEP164 play roles in both ciliation and DNA damage responses. NEK1 has been implicated in both ciliogenesis and the repair of damaged DNA. It has been shown that overexpression of NEK1 inhibits ciliogenesis and knockdown or deletion of NEK1 reduced DNA damage repair following IR (Polci et al., 2004; Shalom et al., 2008; White and Quarmby, 2008; Pelegrini et al., 2010). Other proteins involved in HR, such as MDC1, MRE11 and ATRX have been shown to interact with NEK1, indicating that proteins involved in control of ciliogenesis may also play a role in the DNA damage response (Surpili et al., 2003; Polci et al., 2004). CEP164 has also been implicated in both ciliogenesis and the DDR. CEP164 is an appendage protein required for ciliogenesis which interacts with ATM and ATR and is phosphorylated following IR and UV damage (Graser et al., 2007a; Sivasubramaniam et al., 2008). CEP164 is recruited to UV-damaged sites and reduced expression of CEP164 sensitises cells to UV irradiation (Pan and Lee, 2009). More recently, Chaki et al. reported that mutations in MRE11, ZNF423 and CEP164, all of which function in the DDR, caused Nephronophthisis-related ciliopathies (NPHP-RC), a degenerative disease which affects the kidney, retina and brain. MRE11 and ZNF423 also localise to nuclear foci following UV-induced DNA damage (Chaki et al., 2012).

ATM has been shown to be required for HR but not NHEJ and inhibition of ATM in serum-starved cells using caffeine, reduced HR by 70%, suggesting that ATM is important for HR outside S and G2 phase (Golding et al., 2004; reviewed by Cann and Hicks, 2007). As ATM localises to the centrosome for part of the cell cycle (Oricchio et al., 2006), perhaps expression levels of ATM in ciliated cells affect the likelihood a cell can use HR to repair DNA damage and the exact molecular mechanisms are still unclear. Further studies investigating the interaction of ciliary proteins and proteins involved in DNA damage responses will enhance our understanding of this complex process.

5.3.3 The role of centrosome cohesion proteins in ciliogenesis

We next asked if proteins that are involved in centrosome cohesion also play a role in primary cilium formation. We found that cells depleted of C-NAP1 or Rootletin which therefore had high levels of centriole splitting, had decreased levels
of ciliation both before and after IR. Although neither protein has yet been implicated in ciliation, depletion of Rootletin has been shown to cause fragility of the ciliary base in mice (Yang et al., 2005). C-NAP1 has not been implicated in ciliation, however it does co-localise with ALMS1 and POC1, both of which play roles in primary cillum formation and/or maintenance (Hearn et al., 2002; Collin et al., 2005; Graser et al., 2007a; Li et al., 2007; Pearson et al., 2009; Knorz et al., 2010). Interestingly, ALMS1 localises to the proximal ends of centrioles and its depletion leads to decreased levels of C-NAP1 and increased levels of centriole splitting (Knorz et al., 2010). Depletion of NEK2 also decreased ciliation in our studies, although it has been reported to both decrease ciliation and compromise the cell’s ability to resorb the primary cillum, therefore its role in ciliation is not completely clear (Graser et al., 2007a; Mikule et al., 2007; Spalluto et al., 2012). We also found that depletion of Kizuna and CHK1, which caused decreased levels of centriole splitting, led to increased levels of primary cillum formation. These results indicate that depletion of centrosome cohesion proteins impacts on primary cillum formation.

We then went on to investigate ciliation in irradiated cells which had split centrioles, to directly study the impact of loss of centrosome cohesion on ciliogenesis. Again we depleted C-NAP1 and Rootletin, which leads to centriole splitting (Bahe et al., 2005) and NEK2, depletion of which does not impact splitting, followed by treatment with IR. We found that regardless of whether a centrosome cohesion component is depleted or not, cells with split centrioles are unlikely to form a primary cillum. When a primary cillum was formed in cells with split centrioles, it was usually from the cluster of centrioles and rarely the split centriole. As previously noted, a daughter centriole is more likely to become mobile and split from the cluster of centrioles (Piel et al., 2000), therefore it is also less likely that the split centriole would be capable of forming a primary cillum. However, we do observe some single centrioles form a primary cillum, albeit at low levels. Thus, it is possible that an isolated mother centriole is capable of forming a primary cillum and centriole splitting may not have significant biological implications for ciliogenesis if a single centriole can still form a primary cillum. Multi-ciliated cell can form hundreds of cilia from single, de novo assembled centrioles (Sorokin, 1968; Vladar and Stearns, 2007), therefore, it is likely that in cells possessing a primary cillum, a
mother centriole does not need to engaged or tethered to a daughter centriole in order to form a primary cilium.

Finally, we measured the length of primary cilia in cells depleted of C-NAP1, Rootletin, NEK2 or Kizuna. We saw that although Rootletin decreased ciliation, it did not affect the length of primary cilia, consistent with published data, involving Rootletin in the long term stability of cilia (Yang et al., 2005). When we depleted C-NAP1 we found that both the levels of ciliation and primary cilium length were decreased, implicating C-NAP1 both in primary cilium formation and elongation. This implies that C-NAP1 and Rootletin play similar but separate roles in ciliogenesis.

It is established that the appendages at the distal end of the centrioles are critical for ciliogenesis and provide anchoring and support to the primary cilium, with the distal appendage proteins CEP164 and Cenexin being indispensable for primary cilium formation (Ishikawa et al., 2005; Graser et al., 2007a; reviewed by Kobayashi and Dynlacht, 2011). However, little is known if the proximal end of the centriole is implicated in ciliation. The reduced levels of ciliation in C-NAP1, Rootletin and to a lesser extent NEK2 may be due to an alteration of the composition of the proximal end of centrioles following their depletion, as was discussed in Sections 4.2.5 and 4.3.5, and which may impact ciliation in a manner similar to ALMS1 depletion, where centriole splitting occurs and primary cilia are stunted. Mutations in ALMS1 cause Alström syndrome, which is characterised by neurosensory degeneration and metabolic defects. This suggests that loss of centrosome cohesion components leading to alteration in the composition of proximal end of centrioles could have serious implications for primary cilium formation (Hearn et al., 2002; Collin et al., 2005; Graser et al., 2007a; Li et al., 2007; Knorz et al., 2010; Purvis et al., 2010). It remains to be tested precisely what is the involvement of centrosome cohesion components in ciliogenesis. Perhaps they help anchor the centrosome at the plasma membrane in a manner similar to the Rootletin-containing ciliary rootlet (Yang et al., 2005). Centrosome cohesion components, in particular C-NAP1, could possibly play a more direct role in controlling the elongation of the primary cilium from the basal body once it has docked close to the plasma membrane. We see shorter primary cilia as well as a reduction in number,
and this may indicate that C-NAP1 acts in a manner similar to ALMS1, with which it co-localises (Knorz et al., 2010).
Chapter 6. Conclusions and future perspectives

In the first part of this study, we wished to characterise Stil, a gene which is mutated in autosomal recessive primary microcephaly and encodes a protein which localises to the centrosome in mammalian cells (Aplan et al., 1991; Pfaff et al., 2007; Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). All of the genes known to be mutated in primary microcephaly encode proteins which localise to the centrosome or pericentriolar material for at least part of the cell cycle, some of which are involved in procentriole formation and centrosome cohesion (Bond et al., 2002; Jackson et al., 2002; Bond et al., 2005; Zhong et al., 2005; Graser et al., 2007b; Jeffers et al., 2008; Schmidt et al., 2009; Tang et al., 2009; Guernsey et al., 2010; Hatch et al., 2010; Nicholas et al., 2010; Sir et al., 2011; Steere et al., 2011; Hussain et al., 2012). Therefore, it was of great interest to determine the centrosomal localisation and function of STIL in chicken DT40 cells and what impact its loss would have on centrosome structure and cell viability. We found that chicken STIL is a centriolar protein, which localises to the proximal end of centrioles, indicating that Stil is evolutionarily conserved between chicken and mammals, where it shows similar localisation and was recently found to function in procentriole formation (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012).

We were unable to generate a Stil$^{-/}$ DT40 cell line, suggesting that STIL is essential for viability in chicken DT40 cells and it was later reported that siRNA knockdown of Stil led to centriole loss and asymmetrical spindle pole formation in human cancer cells lines (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). We also failed to generate a conditional Stil knockout cell line and concluded that we did not generate a clone with the optimal expression level of myc-STIL. It would be of great benefit to generate an antibody which recognises chicken STIL, as we could first determine the optimal expression levels of STIL in DT40. This would allow us to generate clones with the correct expression levels and it would remove the need to myc-tag cStil cDNA prior to expression, which may impact on its conformation or function. The generation of a DT40 cell line conditionally expressing STIL, where expression could be completely turned off, would greatly enhance our current knowledge of STIL. An alternate approach would be to utilise the highly efficient auxin-inducible degron (AID) system. In this system, conditional mutants of essential proteins can be generated with reversible and rapid degradation.
of target proteins, thus, allowing tight control of protein expression (Nishimura et al., 2009). There have been excellent studies in Hela and U2OS cells using STIL siRNA to deplete expression (Tang et al., 2011; Arquint et al., 2012; Vulprecht et al., 2012). However, a conditional knockout in a cell line derived from a chicken lymphoma would be of great interest, as STIL is overexpressed in many cancer types, including lymphomas. Therefore, inhibition of STIL expression could represent a potential gene targeting therapy for the treatment of lymphomas and other cancers in the future (Izraeli et al., 1997; Erez et al., 2004).

It has been shown that in mitosis in the presence of damaged or incompletely replicated DNA, centrosomes can split into fractions containing single centrioles (Hut et al., 2003). Furthermore, DNA damage triggers Chk2-dependent centrosome inactivation in Drosophila embryos leading to mitotic catastrophe (Takada et al., 2003). In the second part of this thesis, we sought to determine the impact DNA damage had on the centrosome. We established that following IR, in G2 phase, single centrioles can become isolated and separated, which we refer to as split centrioles. We found high levels of split centrioles in cancer cells which had mutations in TP53 and BRCA1, and therefore deregulated DNA repair mechanisms, but these mutations alone were not able to drive centrosome amplification. However, following ionising radiation-induced DNA damage, we observed high levels of centrosome amplification in these cell lines. This indicates that centriole splitting potentiates DNA damage-induced centrosome amplification, as split centrioles are licensed for duplication (Tsou and Stearns, 2006b; Saladino et al., 2009). To confirm that this was a general response to DNA damage and not specific to cell lines with abrogated DNA damage responses, we characterised the effect of IR on centrosomes of non-transformed human cells and similarly, we found IR-induced centriole splitting to be a precursor to dose-dependent centrosome amplification.

To validate that centriole splitting is a response to DNA damage and not a G2 phase arrest, we blocked cells at the G2/M phase border, using a CDK1 inhibitor and saw that centrosome amplification occurred but was not preceded by centriole splitting. However, although centrioles do not split and cells do not enter mitosis, centrosomes appear to cycle normally, indicating that centriole disengagement is still occurring prior to reduplication, as has been shown by Prosser et al. (Prosser et al.,
We also found that the DNA replication cycle and the centrosome cycle become uncoupled when cells are arrested with a CDK1 inhibitor. Centrosome separation is not inhibited and centrosomes continue to cycle, while DNA replication is stopped (Hochegger et al., 2007; Smith et al., 2011; Steere et al., 2011).

We went on to show that after IR, cells retain centrosome cohesion components that oppose the separation of centrosomes while they lose positive regulators of separation and reduplication after IR. We determined that split centrioles were structurally normal with associated PCM, but the lack of the maturation marker Cenexin and of Kizuna, the marker of PCM maturation, indicated that they were daughter centrioles with immature PCM (Nakagawa et al., 2001; Oshimori et al., 2006). We found that depletion of C-NAP1 and Rootletin led to high levels of centriole splitting, as has been also shown in U2OS cells (Bahe et al., 2005). Finally, we saw that depletion of C-NAP1 led to the loss of CEP170 and Ninein at the split centriole, while Rootletin-depleted cells had less CEP170 and NEK2-depleted cells less Ninein at split centrioles. As we determined that split centrioles were likely to be daughters and given the fact that CEP170 and Ninein localise to the proximal end of centrioles as well as to the subdistal appendages of mother centrioles, we concluded that depletion of C-NAP1, NEK2 or Rootletin alters the composition of the proximal ends of centrioles, as illustrated in Figure 6.1 (Mogensen et al., 2000; Guarguaglini et al., 2005; Graser et al., 2007a).

Centriole splitting and amplification following IR may be mechanism to rid an organism of cells with damaged DNA, by causing multipolar mitoses. However, we know that many cancer cells can overcome these multipolar mitoses (Ring et al., 1982; Guidotti et al., 2003; Ganem et al., 2009; Silkworth et al., 2009). It would be interesting to determine the kinetics of centriole splitting and amplification after IR by following irradiated, non-transformed cells using time-lapse microscopy. This would allow us to determine the frequency with which split centrioles reduplicate and would also allow us to investigate the cellular impact of entering mitosis with split centrioles. It would also allow us to assess whether non-transformed cells can cluster their split or amplified centrioles as efficiently as transformed cells. Experiments to investigate the role of Plk1 and Separase in IR-induced centriole splitting would expand our understanding of this relatively undefined phenomenon.
The observation that depletion of C-NAP1, Rootletin and NEK2 appear to alter the proximal end of split centrioles is very interesting but needs to be investigated further. Further microscopy analysis is needed using specific proximal and distal centriole markers to confirm what structural components of the centriole are lost following C-NAP1, Rootletin or NEK2 depletion and also whether the proximal ends of engaged centrioles are altered too. Immuno-EM against CEP170 and Ninein following C-NAP1, NEK2 and Rootletin depletion would be an exciting experiment to carry out, as it would allow us to confirm the precise centriolar localisation of these centriolar components following loss of centrosomal cohesion. Immuno-EM would also confirm if C-NAP1 and Rootletin are present at the proximal ends of engaged centrioles, which would also be important in determining what role they play in DNA damage-induced centriole splitting.

In the final part of this study we sought to determine if IR had an impact on the primary cilium. IR does not impede primary cilium formation directly, as we found comparable levels of ciliated cells in irradiated and unirradiated populations. We also found that IR does not affect the structure of a primary cilium, although the cell cycle arrest following IR can allow the maturation of younger mother centrioles and the formation of additional primary cilia (Anderson and Stearns, 2009; Mahjoub and Stearns, 2012). This led us to study the DNA damage response in irradiated, ciliated cells and we found that HR may be limited in ciliated cells compared to non-ciliated cells in the same population. Cell cycle analysis using FACS is important to confirm that ciliated and non-ciliated cells have comparable cell cycle distributions as any slight differences could alter the DNA repair mechanisms utilised by the cell. Immunoblot and IF analysis using antibodies against NHEJ and HR components on hTERT-RPE1 and other ciliated cells would allow us to study this phenotype further.

Following this, we investigated the impact loss of centrosomal cohesion had on ciliogenesis. We found that cells depleted of C-NAP1 and Rootletin had impaired primary cilium formation, with the cilia in C-NAP1 depleted cells being shorter than those in control cells (Figure 6.1). NEK2 depletion led to lower levels of ciliation after IR. As other proximal end centriolar components have also been implicated in ciliogenesis, again we suspect that an alteration to the structure of the proximal end of the mature centriole may affect ciliogenesis (Graser et al., 2007a; Li et al., 2007; Pearson et al., 2009; Knorz et al., 2010). We found that centrioles which become
isolated after IR are rarely ciliated but we concluded that isolated mother centrioles are not likely to be impaired in primary cilium formation. Serial section EM would be a useful tool in determining if multiple cilia which formed after IR, emerged from a single ciliary pocket, as such a phenomenon has been described when Plk4 overexpression drives centrosome amplification (Mahjoub and Stearns, 2012). Identifying novel protein-protein interactions between ciliary proteins and C-NAP1, Rootletin or NEK2 using co-immunoprecipitation and mass spectrometry would be an exciting insight into how centrosome cohesion proteins and ciliary proteins interact and how alterations at the proximal end of centrioles may affect ciliogenesis (Ishikawa et al., 2005; Graser et al., 2007a).

Figure 6.1. The impact of IR and C-NAP1 depletion on centriole structure and ciliogenesis. Ninein and CEP170 localise to both the subdistal appendages and proximal end of centrioles. C-NAP1 and Rootletin localise to the proximal end/fibrous linker between centrioles. Following IR, there is no alteration of Ninein, CEP170, C-NAP1 or Rootletin localisation at engaged or split daughter centrioles. Additionally, IR does not impact on primary cilium formation. Following C-NAP1 depletion (+/- IR), Ninein and CEP170 localise to the engaged centrioles but not the daughter centrioles. This indicates that proximal end of split daughter centrioles (and probably engaged centrioles) are altered following C-NAP1 depletion, while the subdistal appendages are unaffected. This alteration in turn, may affect the capacity of a mother centriole for ciliogenesis. A similar, but less dramatic phenotype is seen following Rootletin depletion, where Ninein is unaffected and NEK2 depletion, where CEP170 is unaffected. Other proximal end components involved in ciliogenesis, such as POC1 or ALMS1 could also be displaced following C-NAP1, Rootletin and/or NEK2 depletion, thus, impairing ciliogenesis.
References


References


References


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Yang, J., M. Adamian, and T. Li. 2006. Rootletin interacts with C-Nap1 and may function as a physical linker between the pair of centrioles/basal bodies in cells. Mol Biol Cell. 17:1033-1040.


Appendix I. List of Primers

Table I gives the list of primers obtained from Sigma Aldrich and used for PCR-based cloning and DNA sequencing of \textit{cStil} cDNA.

Table I. Primer sequences used for PCR cloning and sequencing

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’-3’)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIL1-F-N1</td>
<td>CTCGAGATGTCCTTTCAGTTTCCC</td>
<td>cloning of \textit{cStil} cDNA</td>
</tr>
<tr>
<td>SIL1-F-C1</td>
<td>CTCAAGCCATAGCTCCCCTTCAGTTTTC</td>
<td>cloning of \textit{cStil} cDNA</td>
</tr>
<tr>
<td>SIL1-Rev</td>
<td>CCCGGGAAACAAGTCTCGCGAGCTG</td>
<td>cloning of \textit{cStil} cDNA</td>
</tr>
<tr>
<td>SIL F2</td>
<td>AACCACACACACAGGTCCCATAGCAG</td>
<td>sequencing of internal \textit{cStil} cDNA sequence</td>
</tr>
<tr>
<td>SIL R2</td>
<td>CAGCTCCCATATTTCCATGATAC</td>
<td>sequencing of internal \textit{cStil} cDNA sequence</td>
</tr>
<tr>
<td>5’ arm forward</td>
<td>GTCGACGGGTAGAGCAAGTCTGC</td>
<td>cloning of 5’ arm (\textit{Stil} K/O)</td>
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<tr>
<td>5’ arm reverse</td>
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<td>cloning of 3’ arm (\textit{Stil} K/O)</td>
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<td>3’ arm forward</td>
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<td>cloning of 3’ arm (\textit{Stil} K/O)</td>
</tr>
<tr>
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<td>GCCGCCCGGATCCTGTAACGGTTATCAG</td>
<td>cloning of 3’ arm (\textit{Stil} K/O)</td>
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<tr>
<td>5’ probe forward</td>
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<td>cloning of 3’ probe (\textit{Stil} K/O)</td>
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</table>
Appendix II. Full length cStil cDNA

Full length chicken Stil cDNA sequence with the ATG and STOP codon underlined and indicated in red. Underlined and indicated in green are the base pair positions that differ to that of the predicted cDNA sequence.

```
1 atgctccctctcagttcctcccgtctcttaagttcagtctctctcggtctgagcttcggacagtcttcctccttcccttctttgtttgcaagtgtcattcaaggactaaattgaaagggattgtaaaagcaggatttgctggttacaggaagagctctgtttgcatctgttttcttcagagtgtgctcatttaagttgacagtctctgtttgcttcatcacttgtcatcactgtcatcactctgtacattcaagttgcctctctgttgagcatggtggtggccaaaattgctatagcatgccaatgtaataagcatgtgtgaccttgcataaatgcagactggtttacccatgatgggaactcgcttcttccttgccagctcaaccccctacaatcctcttcacattgtgctgctctggttagccagaaggccattgtgacgtaatctctggccacagcagaaatccaaatagcgctttgacggtggaggaaatggtgctacctttacagcagctcacattgtgctgctctggttagccagaaggccattgtgacgtaatctctggccacagcagaaatccaaatagc
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Appendix

3541 aaaaagactg atgaagaaac tcccccttct atgaaagtct ccacgagaga gttgactgct
3601 aatgctcttc taccagaatt gaacagccct atattaagaa acattacaaa tgaatatttttt
3661 tttccccgggact caacaaagc aatgaaacg tccgggctaa tttaaagga tttgaagtca
3721 aacacaaatat tgttacctcg gaaggttaga ttaccggag acacactggcag gaaagtttgga
3781 gaagatattc aagtattcag tggaaatctcg cagcctccag gccccctgaa attaagctcag
3841 tcaaaacagtctg tgaattctctg tggcaccatt cttgatgctg aacaactcag gcagcgccg
3901 aagttgttctga

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Appendix III. Publications and presentations

Publications

- C-NAP1 and rootletin restrain DNA damage-induced centriole splitting and facilitate ciliogenesis
  
  **Conroy PC**, Saladino C, Dantas TJ, Lalor P, Dockerty P, Morrison CG.
  Cell Cycle, 11:20, 3769 - 3778; October 15 2012

- Centriole separation in DNA damage-induced centrosome amplification
  Saladino C, Bourke E, **Conroy PC**, Morrison CG.

Poster presentations

- DNA damage-induced loss of centrosome cohesion and primary ciliogenesis
  **Conroy PC**, Saladino C, Morrison CG. Poster presented at the “Centrosomes and Spindle pole bodies” conference, Barcelona, 2nd-6th October 2011

- Causes and consequences of centrosome amplification
  **Conroy PC**, Saladino C, Morrison CG. Poster presented at the Irish Association for Cancer Research, Annual Meeting, Galway, 3rd-5th March 2010

- Causes and consequences of centrosome amplification