Characterization of NEK10 Tyrosine Kinase Activity in the Cellular Response to DNA Damage

by

Nasir Abbas Haider

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Medical Biophysics
University of Toronto

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Doctor of Philosophy

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Abstract

Protein kinases are signal transduction molecules implicated in most biological processes that, through signal reception, amplification and transduction, direct cellular responses to various stimuli. Deregulation of protein kinases can lead to the formation of many diseases, such as cancer. Kinases are critical for the DNA damage response (DDR) and coordinate a series of processes such as cell cycle arrest, DNA repair, and apoptosis. Defects in the DDR leads to an accumulation of mutations that can promote tumorigenesis.

In response to DNA damage, ATM/ATR, DNA-PKcs, and CHK1/2 kinases orchestrate a signalling program directed towards the tumour suppressor p53, which coordinates the DDR. Emerging studies implicate multiple other kinase cascades in the cellular response to DNA damage including members of the NimA related kinase (NEK) family (NEK1, NEK10 and NEK11).

The work presented in this thesis focuses on NEK10, a largely uncharacterized member of the NEK family. Mutations and lowered expression of NEK10 have been associated with various human malignancies. Previous work implicated NEK10 in the regulation of the G2/M checkpoint in response to UV-C through ERK1/2 pathway
hyperactivation. Work presented herein describes a function for NEK10 in the regulation of p53 transcriptional activity. NEK10 directly phosphorylates p53 on Y327 and mutation of this site (Y327F) results in hypomorphic p53 function and a decrease in the transcription of p53-target genes. Accordingly, NEK10-deficient cells display heightened sensitivity to DNA damaging therapeutics. Furthermore, NEK10 acts as a dual-specificity kinase with prominent tyrosine kinase activity. I have uncovered a key determinant of NEK10 specificity towards tyrosines within its activation loop, allowing for the generation of a tyrosine kinase-specific NEK10 mutant (I693P). In collaboration with Dr. Michael Yaffe, we identified the substrate recognition sequences for the tyrosine kinase activity of NEK10, revealing 77 NEK10 candidate substrates within the human proteome.

Collectively, my studies establish NEK10 as a factor in the cellular response to DNA damage, furthering our understanding of its physiological function. This work also lays a foundation for future studies of NEK10 in the context of tumorigenesis, and establishes a rationale for the exploration of NEK10 inhibitors as sensitizers to genotoxic cancer treatments.
ACKNOWLEDGEMENTS

With the submission of this thesis, I have completed a 7 year journey that has had many ups and downs. Encapsulated within these pages is the culmination of an endeavour I could not have completed without the support and guidance of my loved ones and mentors.

I would first like to thank Dr. Vuk Stambolic, who dislikes the honorific of doctor or professor. I chose to use this title, as it was originally intended, to indicate an individual’s license and ability to teach (*licentia docendi*), as Vuk has truly been my teacher, mentor and friend. Over the years, Vuk has always supported my ideas and provided valued feedback. Whether it be the forwarding of a very technical paper on kinases, or allowing me to come into his office unannounced to ramble and sketch mechanisms on his whiteboard or on a scrap sheet of paper while we discussed my results, Vuk has always been a critical part of my journey towards being a better scientist.

Alongside my supervisor, I would like to thank the members of my supervisory committee: Dr. Jim Woodgett and Dr. Razq Hakem for their guidance and specific suggestions on how to move my project forward.

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lab manager of the Stambolic lab has provided me with a vast deal of technical knowledge and support. And a more general thank you for all your love and support to: Dr. Christian Bassi, Dr. Vanessa Di Palma, Dr. Previn Dutt, and Samaar Mouazz.

Alongside my friends and colleagues within the Stambolic Lab, my friends within the Department of Medical Biophysics have kept me sane throughout the years, through spirited scientific and non-scientific conversations and social outings. Thank you for everything: James, Nick, Peter, Jordan, Jon, and Arshiya.

I would also like to thank my collaborators: Dr. Bert van de Kooij (Yaffe Lab, MIT) for his help with the oriented peptide library screens and Dr. Jiefei Tong for his help with running and interpreting mass spectrometric data.

Most importantly, I would like to thank my family for always being there for me whenever I needed, whether I needed someone to talk to when I was having a difficult time or sharing in my triumph when I had made a new discovery. I love you all: Ami, Abbu, Sadaf, Shahid and Adil.

Finally, I would not have followed the path of a scientist, had it not been for my father: Dr. Masoom Haider. Over the years, he has taught me the value of hard work and perseverance in the face of adversity, imbued me with a love for discovery, and taught me to strive to be the best man I can be.
“Sometimes science is more art than science”

-Dr. Rick Sanchez
TABLE OF CONTENTS

Acknowledgements...........................................................................................................iv

Quotation.............................................................................................................................vi

Table of Contents...............................................................................................................vii

List of Figures and Tables..................................................................................................xiii

List of Abbreviations and Acronyms..................................................................................xvi

CHAPTER 1: Introduction.....................................................................................................1

1.1 Cancer..........................................................................................................................2

1.2 The cell cycle................................................................................................................3

1.3 Regulation of the cell cycle...........................................................................................4

1.3.1 Cyclin dependent kinases and their regulation.......................................................5

1.3.1.1 Progression through G1 and S...........................................................................8

1.3.1.2 Progression through mitosis...........................................................................9

1.4 The DNA damage response (DDR)............................................................................12

1.4.1 CHK1/2 signaling in the checkpoint response......................................................15

1.4.2 p38MAPK/MK2 signaling in the checkpoint response..........................................17

1.5 DNA repair..................................................................................................................19

1.5.1 Single Strand Break Repair (SSBR)..................................................................19
1.5.2 Double strand break repair ................................................................. 21
  1.5.2.1 Non homologous end joining (NHEJ) ............................................ 21
  1.5.2.2 Homologous Recombination (HR) .................................................. 23

1.6 p53 .......................................................................................................... 24
  1.6.1 Discovery of p53 .................................................................................. 24
  1.6.3 The structure of p53 ............................................................................ 25
  1.6.4 p53 in disease and cancer ................................................................. 25
  1.6.5 Transcriptional activity of p53 ............................................................. 26
  1.6.6 Regulation of p53 by protein-protein interaction and posttranslational modifications .......................................................... 29
  1.6.7 Regulation p53 by oligomerization ................................................. 33
  1.6.8 The p53 response to DNA damage ................................................ 34
  1.6.9 p53 and DNA repair ................................................................. 36

1.7 NEK family of protein kinases ................................................................ 37
  1.7.1 NEK kinases in mitotic progression .................................................. 38
  1.7.2 NEK Kinases in the DDR ................................................................. 41

1.8 NEK10 ................................................................................................... 44
  1.8.1 NEK10 in Cancer .................................................................................. 46

1.9 Ph. D. OUTLINE .................................................................................... 49
  1.9.1 Study Rationale .................................................................................. 49
CHAPTER 2: Regulation of p53 dependent transcription by NEK10 mediated phosphorylation

2.1 Introduction

2.2 Results

2.2.1 Loss of NEK10 leads to increased proliferation and DNA replication

2.2.2 NEK10 regulates transcription of p53 target genes

2.2.3 NEK10 phosphorylates p53 on tyrosine 327

2.2.4 NEK10 is required for proper induction of DDR pathways

2.3 Discussion

2.3.1 Nek10 regulates p53 transcriptional activity

2.3.2 NEK10 phosphorylates p53 at Tyrosine 327

2.3.3 NEK10 is required for proper induction of DDR pathways

2.4 Materials and Methods

2.4.1 Antibodies

2.4.2 Cell culture, transfections, viral infections and reagents

2.4.3 Cell lysis, immunoblotting and immunoprecipitations

2.4.4 Protein half-life determination

2.4.5 Immunofluorescence
2.4.6 Cell Proliferation and Clonogenic Assays

2.4.7 RNA isolation, reverse transcription and quantitative real time PCR (qRT-PCR) analysis

2.4.8 Cell cycle analysis by flow cytometry

2.4.9 Annexin V/Prodium Iodide assay for apoptosis

2.4.10 Bacterial protein purification

2.4.11 In Vitro kinase assays

2.4.12 Purification of nuclear proteins

2.4.13 Homologous recombination assay

2.4.14 EdU Pulse Labelling for DNA synthesis determination

2.4.15 Oligomerization Assay

2.4.16 Luciferase Reporter Assays

2.4.17 Electrophoretic Mobility Shift Assay (EMSA)

CHAPTER 3: Characterization of the tyrosine kinase activity of NEK10

3.1 Introduction

3.2 Results

3.2.1 Purification of NEK10 protein

3.2.2 NEK10 is a dual specificity kinase activity
3.2.3 NEK10 requires key residues within the activation loop for tyrosine kinase activity………………………………………………………………………………..98

3.2.4 NEK10 has a specific substrate recognition motif for tyrosine phosphorylation…………………………………………………………………………..……………..101

3.2.5 NEK10 tyrosine kinase activity is required for effective transcription of p53 target genes…………………………………………………………………...105

3.3 Discussion………………………………………………………………………………...110

3.3.1 NEK10 is a dual specificity kinase activity…………………………………..110

3.3.2 NEK10 requires specific residues within the activation loop for tyrosine kinase activity………………………………………………………………….111

3.3.3 NEK10 has a specific substrate recognition motif for tyrosine phosphorylation…………………………………………………………………….112

3.4 Materials and Methods…………………………………………………………………..115

3.4.1 Plasmids………………………………………………………………………………115

3.4.2 Cell culture and transfection……………………………………………………115

3.4.3 Purification of recombinant NEK10 from mammalian cells…………………115

3.4.4 In vitro kinase assays………………………………………………………….116

3.4.5 Western blot…………………………………………………………………….116

3.4.6 Coomassie staining……………………………………………………………117
CHAPTER 4: Future Directions

4.1 Understanding Nek10 activation

4.2 NEK10 in tumorigenesis

4.3 The search for NEK10 substrates

4.4 The significance of Y327 on p53 function

REFERENCES
LIST OF FIGURES AND TABLES

Figure 1.1 The mammalian cell cycle.................................................................6

Figure 1.2 DNA damage dependent responses..............................................14

Figure 1.3 Overview of the DNA damage checkpoint....................................16

Figure 1.4 Basic model of p53 transcriptional activity.....................................28

Figure 1.5 The activation of p53 transcriptional activity after DNA Damage.....31

Figure 1.6 Alignment of 11 mammalian NIMA-related kinases and the NIMA kinase of Aspergillus.................................................................39

Figure 1.7 NEK kinases in the DNA damage responses.................................42

Figure 1.8 NEK10 and its role in the DDR.......................................................45

Figure 1.9 Lowered NEK10 expression is associated with decreased 10 year overall survival........................................................................48

Figure 2.1 NEK10 deletion leads to an increase in cellular proliferation........56

Figure 2.2 NEK10 loss leads to increase in DNA replication and mitotic index.....57

Figure 2.3 Involvement of NEK10 in the expression of p53-responsive genes........59

Figure 2.4 Nek10 regulates p53 transcriptional activity in a kinase dependent manner.........................................................................................61

Figure 2.5 NEK10 phosphorylates p53 on Y327 in vitro..................................62

Figure 2.6 Y327F mutation decreases the expression of p53 target genes.........63
Figure 2.7 Y327 plays a role in the binding of p53 to response elements.................65

Figure 2.8 NEK10 modulates the expression of p53 responsive genes in response to genotoxic agents.................................................................67

Figure 2.9 NEK10 tyrosine phosphorylates p53 in response to ionizing radiation........69

Figure 2.10 NEK10 loss lead to defects in the recruitment of RAD51 and BRCA1 to sites of damage and effects the efficiency of HR.................................70

Figure 2.11 NEK10 loss sensitizes cells to chemotherapeutic agents.........................71

Figure 2.12 Differential cell cycle arrest in response to cisplatin treatment...............72

Figure 3.1. Alignment of human NEK2, NEK7, and NEK10 kinase domains...........92

Figure 3.2 Selection of expression system for NEK10 purification..........................96

Figure 3.3 Purification of NEK10 from HEK293T cells........................................97

Figure 3.4 NEK10 autophosphorylates primarily serine and tyrosine residues.........99

Figure 3.5 NEK10 is phylogenetically distinct from other family members..........100

Figure 3.6 I693 in the activation loop is required for kinase activity.....................102

Figure 3.7 PS-OPLS reveals a distinct substrate recognition motif for tyrosine phosphorylation.................................................................104

Figure 3.8 Identification of potential tyrosine substrates based on Nek10 substrate recognition motifs.........................................................107
Table 3.1 List of candidate Nek10 tyrosine substrates as determined by sequence analysis based on Nek10 substrate recognition motif……………………………………………………………108

Figure 3.9 NEK10 tyrosine kinase activity is required for expression of p53 responsive genes…………………………………………………………………………………………..109
### LIST OF ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>53BP1</td>
<td>p53 binding protein 1</td>
</tr>
<tr>
<td>6–4PP</td>
<td>6–4 pyrimidine–pyrimidine photoproduct</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AKT/PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>APC/C</td>
<td>Anaphase promoting complex/cyclosome</td>
</tr>
<tr>
<td>APC</td>
<td>Anaphase promoting complex subunit 7</td>
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<tr>
<td>APE-1</td>
<td>Apurinic/apyrimidinic endonuclease 1</td>
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<td>APLF</td>
<td>Aprataxin and PNK-like factor</td>
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<tr>
<td>ARC</td>
<td>Activity regulated cytoskeleton associated protein</td>
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<td>ASPP1</td>
<td>Apoptosis stimulating protein of p53 1</td>
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<td>ASPP2</td>
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<td>ATG7</td>
<td>Autophagy-regulated protein 7</td>
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<td>ATM</td>
<td>Ataxia-telangiectasia mutated kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and RAD3-related kinase</td>
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<tr>
<td>ATRIP</td>
<td>ATR interacting protein</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl2-associated X protein</td>
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</table>
BCCIP  BRCA2 and CDKN1A interacting protein
BER     Base excision repair
BLM     Bloom syndrome protein
bp      Base pair
BRCA1   Breast cancer type 1 susceptibility protein
BRCA2   Breast cancer type 2 susceptibility protein
BUB1    Budding uninhibited by benzimidazoles 1
BUB3    Budding uninhibited by benzimidazoles 3
c-Abl   Abelson murine leukemia viral oncogene homologue
CAK     (Cyclin-dependent kinase activating kinase
CAMK    Ca\(^{2+}\)/calmodulin-dependent protein kinase
CARM1   Coactivator-associated arginine methyltransferase 1
CBP/p300 Creb binding protein p300
CD      Common docking motif
Cdc2    Cell division cycle protein 2
Cdc23   Cell division cycle protein 23
Cdc25A/B/C Cell division cycle protein 25
Cdc27   Cell division cycle protein 27
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<td>Cdc28</td>
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<td>CDK5RAP2</td>
<td>CDK5 regulatory subunit-associated protein 2</td>
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<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
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<td>CENP-E</td>
<td>Centromere protein E</td>
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<td>CEP192</td>
<td>Centrosomal protein of 192 kDa</td>
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<td>CEP68</td>
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<td>CETN2</td>
<td>Centrin 2</td>
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<td>CHK1</td>
<td>Checkpoint kinase 1</td>
</tr>
<tr>
<td>CHK2</td>
<td>Checkpoint kinase 2</td>
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<td>CHX</td>
<td>Cyclohexamide</td>
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<td>CIP/KIP</td>
<td>CDK interacting protein/Kinase inhibitory protein</td>
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<td>CK2</td>
<td>Casein kinase 2</td>
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<td>CKI</td>
<td>Cyclin-dependent kinase inhibitor protein</td>
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<td>CLK</td>
<td>Cdc2-like protein kinase</td>
</tr>
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<td>C-Nap1</td>
<td>Centrosomal NEK2-associated protein 1</td>
</tr>
<tr>
<td>CPD</td>
<td>Cyclobutane-pyrimidine dimer</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>CtIP</td>
<td>CtBP-interacting protein</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>dHJ</td>
<td>Double Holiday Junction</td>
</tr>
<tr>
<td>D-loop</td>
<td>Displacement loop</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>DNA protein kinase catalytic subunit</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>DYRK</td>
<td>Dual specificity tyrosine phosphorylation regulated kinase</td>
</tr>
<tr>
<td>E2F1</td>
<td>E2F transcription factor 1</td>
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<tr>
<td>EdU</td>
<td>5-ethynyl-2'-deoxyuridine</td>
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<td>Eg5</td>
<td>Kinesin 5</td>
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<td>ERCC1</td>
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<tr>
<td>ERK1/2</td>
<td>Extracellular signal regulated kinase 1/2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>EXO1</td>
<td>Exonuclease 1</td>
</tr>
<tr>
<td>FHA</td>
<td>Forkhead associated</td>
</tr>
<tr>
<td>G0</td>
<td>Quiescence</td>
</tr>
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<td>G1</td>
<td>Gap period 1</td>
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<tr>
<td>G2</td>
<td>Gap period 2</td>
</tr>
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<td>GADD45α</td>
<td>Growth Arrest and DNA Damage 45 alpha</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GG-NER</td>
<td>Global genome nucleotide excision repair</td>
</tr>
<tr>
<td>γH2A.X</td>
<td>Histone 2A.X phosphorylated on Ser139</td>
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<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase 3 beta</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<td>GWAS</td>
<td>Genome wide association study</td>
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<tr>
<td>H2A.X</td>
<td>Histone 2A.X</td>
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<td>HAT</td>
<td>Histone Acetyltransferase</td>
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<td>HERC2</td>
<td>HECT and RLD domain containing E3 ubiquitin protein ligase 2</td>
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<td>HIPK</td>
<td>Homeodomain interacting protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>hnRNPA0</td>
<td>Heterogeneous nuclear ribonucleoprotein A0</td>
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<td>HR</td>
<td>Homologous repair</td>
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<tr>
<td>HSP90</td>
<td>Heat shock protein 90</td>
</tr>
<tr>
<td>Hzf</td>
<td>Hematopoietic zing finger protein</td>
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<td>iASPP</td>
<td>Inhibitor of apoptosis stimulating protein of p53</td>
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<td>IGFBP3</td>
<td>Insulin-like growth factor-binding protein 3</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>IR</td>
<td>Ionizing radiation</td>
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<tr>
<td>IRIF</td>
<td>Ionizing radiation induced foci</td>
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<td>LFS</td>
<td>Li-Fraumeni syndrome</td>
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<td>LOH</td>
<td>Loss of heterozygosity</td>
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<td>MAPKK</td>
<td>MAP kinase kinase</td>
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<td>MAPKKK</td>
<td>MAP kinase kinase kinase</td>
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<td>MASTL</td>
<td>Microtubule Associated Serine/Threonine Kinase Like</td>
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<td>Mediator of DNA Damage Checkpoint 1</td>
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<td>MK2</td>
<td>MAPK-activated protein kinase 2</td>
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<td>MLC</td>
<td>Myosin light-chain subunit</td>
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<td>MMR</td>
<td>Mismatch repair</td>
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</tr>
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<td>MYC</td>
<td>avian myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>Myt1</td>
<td>Membrane associated tyrosine/threonine 1</td>
</tr>
<tr>
<td>NBS1</td>
<td>Nibrin</td>
</tr>
<tr>
<td>NEDD1</td>
<td>Neural precursor cell expressed, developmentally down-regulated 1</td>
</tr>
<tr>
<td>NEK</td>
<td>NIMA related protein kinase</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export sequence</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NIMA</td>
<td>Never-in mitosis A</td>
</tr>
<tr>
<td>OD</td>
<td>Oligomerization domain</td>
</tr>
<tr>
<td>OGG1</td>
<td>8-oxoguanine glycosylase 1</td>
</tr>
<tr>
<td>PALB2</td>
<td>Partner and localizer of BRCA2</td>
</tr>
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</table>
PBD  Polo box domain
PCM  Pericentriolar material
PCNA  Proliferating nuclear antigen
PCR  Polymerase chain reaction
PDK1  3-phosphoinositide dependent protein kinase-1
pHH3  Phosphorylated Histone H3 on Ser10
PI  Propidium Iodide
PIC  Preinitiation complex
PIKK  Phosphatidylinositol 3-kinase-related kinases
PIRH2  p53-Induced protein with a RING-H2 domain
PKC  Protein Kinase C
PLK1  Polo like kinase 1
PNKP  Polynucleotide phosphatase/kinase
PP-1  Protein Phosphatase 1
PP1γ  Protein phosphatase 1 gamma
pRB  Retinoblastoma protein
PRD  Proline rich domain
PRMT1  Protein arginine N-methyltransferase 1
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-OPLS</td>
<td>Positional scanning oriented peptide library screening</td>
</tr>
<tr>
<td>PTM</td>
<td>Posttranslational modification</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RAF</td>
<td>Rapidly accelerated fibrosarcoma homologue</td>
</tr>
<tr>
<td>Ras</td>
<td>Rat sarcoma homologue</td>
</tr>
<tr>
<td>RBEL1A</td>
<td>Rab-like protein 1A</td>
</tr>
<tr>
<td>RE</td>
<td>Response element</td>
</tr>
<tr>
<td>RET</td>
<td>Rearranged during transfection</td>
</tr>
<tr>
<td>RhoGAP</td>
<td>Rho GTPase activating protein</td>
</tr>
<tr>
<td>RING</td>
<td>Really Interesting New Gene</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication protein A</td>
</tr>
<tr>
<td>RSR</td>
<td>Replication stress response</td>
</tr>
<tr>
<td>SAC</td>
<td>Spindle assembly checkpoint</td>
</tr>
<tr>
<td>SCFβ-TrCP</td>
<td>Skp1/cullin/F-box and β-transducin repeat-containing protein</td>
</tr>
<tr>
<td><strong>Abbreviation</strong></td>
<td><strong>Definition</strong></td>
</tr>
<tr>
<td>------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SILAC</td>
<td>Stable isotope labeling with amino acids in cell culture</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>SRC</td>
<td>Sarcoma homologue</td>
</tr>
<tr>
<td>SSB</td>
<td>Single Strand break</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single stranded DNA</td>
</tr>
<tr>
<td>STAGA</td>
<td>SPT3-TAFII31-GCN5L acetylase</td>
</tr>
<tr>
<td>SV-40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>SWI-SNF</td>
<td>Switch/Sucrose Non-Fermentable</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>TAO</td>
<td>Thousand and one amino acid kinase</td>
</tr>
<tr>
<td>TC-NER</td>
<td>Transcription-coupled nucleotide excision repair</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TESK1</td>
<td>Testis-specific protein kinase 1</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TFIID</td>
<td>Transcription factor II D</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>TFIIH</td>
<td>Transcription factor II H</td>
</tr>
<tr>
<td>TPR</td>
<td>Tetratricopeptide repeats</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>USP7</td>
<td>Ubiquitin-specific-processing protease 7</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td>UV-DDB</td>
<td>UV-damage DNA binding protein</td>
</tr>
<tr>
<td>UVSSA</td>
<td>UV sensitive syndrome A</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
</tr>
<tr>
<td>WRN</td>
<td>Werner syndrome ATP-dependent helicase</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>XLF</td>
<td>XRCC4-like factor</td>
</tr>
<tr>
<td>XPA</td>
<td>Xeroderma pigmentosum complementation group A</td>
</tr>
<tr>
<td>XPB</td>
<td>Xeroderma pigmentosum complementation group B</td>
</tr>
<tr>
<td>XPC</td>
<td>Xeroderma pigmentosum complementation group C</td>
</tr>
<tr>
<td>XPD</td>
<td>Xeroderma pigmentosum complementation group D</td>
</tr>
<tr>
<td>XPG</td>
<td>Xeroderma pigmentosum complementation group G</td>
</tr>
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XRCC4  X-ray repair cross-complementing protein 4
γ-TuRC  γ-tubulin ring complex
CHAPTER 1:
Introduction
1.1 Cancer

Cancer is a term that defines a broad spectrum of diseases in which abnormal cells acquire a limitless capacity to divide, grow and invade surrounding tissues [1-3]. Cancer development leads to the formation of solid masses (tumours) that are capable of locally effecting organ function and distally, through the spread of cancer cells via the blood and lymph systems, lead to formation of metastases. Roughly half of all Canadians will develop cancer at some point in their lifetime and 1 in 4 Canadians are expected to die from the disease [2]. Cancer is the leading cause of death in Canada with a mortality rate higher than that of cardiovascular diseases (30.2% vs 19.2%). In the past two decades, our understanding of cancer, both of its causes and the ways in which we diagnose and treat the disease, has greatly improved, leading to decreases in both cancer incidence and cancer related mortality.

Cancer was first defined by Hippocrates who used the word cancer (Karkinos) to describe a tumour whose malformed vasculature, to his eye, resembled a crab with its legs spread outwards [4]. Many years of research and medical practice have found cancer to be a spectrum of complex diseases that share a series of cellular traits or hallmarks [1-3]. Tumour cells invariably exhibit broad genomic alterations, ranging from single nucleotide changes to large chromosomal and genomic rearrangements [5]. The road to tumorigenesis from a healthy cell is thought to involve a multistep route to transformation, during which a cell acquires competitive traits allowing for it to outcompete the other cells in a given niche [1, 3, 6, 7]. Simplistically, carcinogenic mutations can be broken into two classes: those that produce oncogenes with dominant gain of function and those that lead to recessive loss of function of tumor suppressor genes [8]. These
genetic aberrations invariably lead to perturbations in cellular signalling networks responsible for the control of multiple aspects of cellular physiology such as growth, differentiation, motility, apoptosis, senescence, angiogenesis, DNA homeostasis, and metabolism [1, 3].

Many cancer-associated mutations map to genes encoding protein kinases whose physiological function is to carry out specific phosphorylation of amino acids on protein substrates. Phosphorylation can affect protein activity, localization, protein-protein interactions, and stability [9-11]. Kinases act as molecular switches, capable of quickly relaying signaling information between the outside of the cell and the intercellular space, as well as between various intracellular compartments. Many kinase substrates are involved in the processes that are significant in cancer such as growth, proliferation, differentiation, DNA homeostasis and apoptosis. Gain or loss of function mutations in kinases can greatly modulate signalling pathway dynamics, leading to broad effects on cellular physiology. These properties have made kinases promising targets for therapeutic intervention and is driving large efforts towards development of small molecules that specifically modulate kinase enzymatic activity and function [10, 12]. The prototypical example of a therapeutic kinase inhibitor is Imatinib (Gleevec), a BCR-ABL oncogenic kinase inhibitor used to treat chronic myelogenous leukemias (CML) and acute lymphoblastic leukemias featuring the BCR-ABL gene fusion [13-15]. Many more kinase inhibitors are in preclinical and clinical development as anti-cancer therapeutics.

1.2 The cell cycle

The cell cycle is a highly ordered series of events during which a cell duplicates its genetic information and segregates into two daughter cells [16]. Deregulation of this
process can lead to uncontrolled proliferation and unscheduled entry into the cell cycle, a hallmark of neoplastic transformation and tumorogenesis [3, 17]. Progression through the cell cycle requires timely activation of multiple transcriptional and translational programs [18]. The cell cycle can be broken down into 4 distinct phases: G1, S, G2, and the M phase (Figure 1.1). There are two major phases of activity: the S phase (interphase), where the genome is duplicated, and the M phase (mitosis), where the duplicated genome is segregated into two daughter cells. In between these phases there are the two gap periods: G1 (prior to S phase), and G2 (prior to mitosis). During the G1 phase, the cell determines whether the conditions are appropriate for DNA replication by integrating multiple extracellular and intracellular signals, such as the presence of adequate growth factors/nutrients and the potential presence of DNA damage. During this phase, if conditions are not conducive to growth, the cell will either enter a state of quiescence, known as the G0, or halt cell cycle progression prior to the S phase (G1/S arrest). If conditions are suitable for DNA replication, the cell will enter the S phase. At the second gap period, the G2, the cell ensures that DNA replication is complete and prepares for cellular division during the M phase. If the genome is improperly duplicated or damaged, the cell cycle can also be arrested at the G2/M checkpoint. The cell cycle is finalized upon induction of mitosis, and the formation of two daughter cells.

1.3 Regulation of the cell cycle

Control of the cell cycle is a conserved process from yeast to higher multicellular eukaryotes. The major regulators of the cell cycle are the cyclin dependent kinases (CDKs) and their regulatory subunits, cyclins. More recent data reveal that multiple other kinases are crucial to the proper cell cycle progression and cell cycle checkpoint integrity.
### 1.3.1 Cyclin dependent kinases and their regulation

In yeast, cell cycle progression is regulated by a single CDK, cdc28 in *Saccharomyces cerevisiae* and Cdc2 in *Schizosaccharomyces pombe*, which bind to the specific cyclins at different phases of the cell cycle [19]. During evolution, the number of CDKs has increased and humans have twenty distinct CDKs (CDK1-20) [20]. Of those, only CDKs 1, 2, 4, and 6 are clearly associated with cell cycle progression. These CDKs can be subdivided into the interphase CDKs (CDK 2, 4, 6) and a mitotic CDK (CDK1).

CDKs require association with their regulatory partners, cyclins, for activation. Cyclins are transcriptionally and posttranslationally regulated in a cell cycle-dependent manner. They connect with their cognate CDK’s kinase domain through multiple hydrophobic interactions, which lead to a structural change in the CDK active site, allowing access to specific substrates and their subsequent phosphorylation [21-24]. For CDK 1 and 2, cyclin A and cyclin B1, respectively, binding makes a threonine within the CDK’s activation segment accessible for phosphorylation by CAK (Cyclin-dependent kinase activating kinase) [21, 25-28]. CAK phosphorylation is not required for all CDKs, as the sole requirement for CDK6 activation is cyclin D binding [29]. The mechanism of activation of CDK4 by cyclin D is less clear, as structural studies have shown that their interaction does not impose an active kinase conformation [30, 31].

CDKs are also subject to negative regulation in a cell cycle dependent manner via interactions with CKI (Cyclin-dependent kinase inhibitor) proteins or via inhibitory phosphorylation. One mode of CDK downregulation involves interactions with either INK4 or Cip/Kip family proteins during the G1 and S phases [32, 33]. INK4 proteins (p16\[^{\text{INK4a}}\], p15\[^{\text{INK4b}}\], p18\[^{\text{INK4c}}\], and p19\[^{\text{INK4d}}\]) associate with the monomeric forms of CDK4/6 and the
Figure 1.1 The mammalian cell cycle.

The phases of the cell cycle are indicated with their appropriate CDK-cyclin pairs. The major steps required for the transition from G1 to S phases and G2 to M phase are illustrated as well.
distort the CDK kinase domain, preventing the D-type cyclin interaction or activation by CAK [34, 35]. Cip/Kip proteins (p21\textsuperscript{Cip1/Waf1}, p27\textsuperscript{Kip1}, p57\textsuperscript{Kip2}) interact and inhibit CDK2-cyclin E and CDK2-cyclin A complexes leading to G1/S arrest [33, 36]. Cip/Kip family members may also participate in the assembly and nuclear translocation of catalytically active CDK4/6-cyclin D complexes [37-39]. This leads to an intriguing scenario where Cip/Kip proteins are sequestered into CDK4/6-cyclin D complexes allowing for the downstream activation of CDK2-cyclin E complexes [40-43].

The Cip/Kip family member p21\textsuperscript{Cip1/Waf1}, which mainly functions as an inhibitor of interphase CDKs (CDK2/4/6) and an initiator of G1/S arrest, has additional functions, specifically in the G2/M transition and mitotic progression. In response to genotoxic stress, p21\textsuperscript{Cip1/Waf1} interacts with CDK1-cyclin A, blocking its activation and leading to a G2/M arrest [44]. Furthermore, p21\textsuperscript{Cip1/Waf1} plays a function in mediating the degradation of cyclins A2 and B1 in response to DNA damage, via the downregulation of the early mitotic inhibitor 1 (Emi1) and activation of the anaphase promoting complex (APC/C) [45, 46]. p21\textsuperscript{Cip1/Waf1} may also be important for proper mitosis, as deletion or knockdown of p21\textsuperscript{Cip1/Waf1} in HCT116 cells induced prolonged mitosis, aberrant centrosome number, cytokinesis failure and polyploidy [47-49].

Inhibitory phosphorylation is another mode of CDK downregulation. The glycine rich loop within the kinase domain of CDK1/2 (Thr14 and Tyr15) is subject to inactivating phosphorylation by WEE1 and the membrane associated tyrosine/threonine 1 (MYT1) kinases prior to the G2/M transition or in response to DNA damage [23, 50-55]. This phosphorylation does not cause major structural changes in CDK1/2, but does reduce the affinity of CDK for its substrates [56]. In order for the G2/M transition to proceed and for
cell to enter mitosis, these inhibitory phosphates must be removed by the dual specificity phosphatases CDC25 A, B, and C [57, 58]. While CDC25B has been reported to initiate mitosis by activation of CDK1-cyclin B1, which leads to subsequent CDC25C activation at the G2/M boundary [59-61], CDC25A appears essential for the activation of CDK/cyclin throughout the cell cycle [61-63].

1.3.1.1 Progression through G1 and S

One of the key regulators of entry into the cell cycle is the retinoblastoma protein (pRB). During early G1, unphosphorylated or hypophosphorylated pRB represses the genes required for cell cycle progression [18, 64, 65]. pRB achieves the downregulation of multiple cell cycle regulators by its interaction with a range of proteins, such as the SWI/SNF complex, histone deacetylases (HDACs), polycomb proteins and methylases that cause epigenetic changes at promoters, as well as inactivation of the E2F1 transcription through sequestration [18, 66, 67].

pRB-mediated cell cycle repression is relieved in response to mitogenic signals by the expression of D-type cyclins (D1, D2, D3), which complex with and activate CDK4/6 [18, 68], which in turn hyperphosphorylate pRB on multiple sites [69, 70]. Hyperphosphorylated pRB disassociates from E2F1 and its other binding partners, allowing for the transcription of genes cyclin E, cyclin A, DNA polymerase subunits, and proliferating cell nuclear antigen (PCNA) genes, which are required for progression into S phase and DNA replication [68, 70-75]. The increase in cyclin E expression induced by derepression of E2F1 allows for the activation of CDK2, which further reinforces the hyperphosphorylated state of pRB, driving irreversible commitment to DNA replication [65, 76-78].
1.3.1.2 Progression through mitosis

Mitosis is the last stage of the cell cycle (M phase) and it can be divided into 5 distinct phases: prophase, prometaphase, metaphase, anaphase, and telophase (reviewed in [79] and summarised below). During prophase, chromatin condenses into chromosomes (chromatin condensation), the nuclear envelope collapses, and duplicated centrosomes migrate towards opposite ends of the cell and begin to form the mitotic spindle via recruitment of γ-tubulin. During prometaphase, microtubules emerging from the centrosome poles attach to protein structures called kinetochores that have formed on the mitotic chromosomes. When the sister chromatids have undergone bipolar attachment to the mitotic spindle via kinetochore-bound microtubules, the cell progresses to metaphase. During metaphase, the centromeres of the attached chromosomes align at the center of the cell, along a plane that is equidistant from both centrosome poles, called the metaphase plate. Aligned sister chromatids are split and pulled towards opposite poles of the cell. The final stage of mitosis, telophase, begins when the daughter chromatids reach the centrosomal poles at distal ends of the cell. Two nuclear envelopes begin to form within each daughter cell around each set of chromatids. Following nuclear envelope formation, the mitotic chromosomes decondense into expanded chromosomes. To end this phase, an actomyosin contractile ring forms and breaks the mitotic structure into two daughter cells via a process known as cytokinesis.

Critical to the initiation and progression of mitosis is CDK1, which during the G2/M transition and mitosis, phosphorylates a range of proteins involved in chromosomal condensation, mitotic spindle formation, nuclear envelope breakdown and Golgi disassembly [80, 81]. To date, CDK1 has been reported to have over 300 potential protein
substrates, including the kinesin-related motor protein Eg5, lamin, and condensin [79, 82-87]. CDK1 is subject to multiple layers of regulatory control, to prevent premature mitotic entry, and to ensure proper mitotic exit. The kinase activity of CDK1-cyclin B1 emerges in late G2 and peaks at metaphase, before being inactivated prior to anaphase [88]. Although CDK1 is expressed during all phases of the cell cycle, the increase in transcription of cyclin B1 during late G2 and early mitosis leads to the formation of an active CDK1-cyclin B1 complex [80, 89]. Activation of CDK1 requires phosphorylation of Thr161 in the activation loop by CAK [28]. However, CDK1-cyclin B1 complexes are kept inactive by the phosphorylation of CDK1 at Thr14 and Tyr16 by MYT1 and WEE1, respectively, and require dephosphorylation by the CDC25 family phosphatases (CDC25A/B/C) for mitotic entry [23, 50-55, 57]. CDK1-cyclin B1 further activates all CDC25 phosphatases in a positive feedback manner [90-92].

CDK1 is also controlled by PLK1, which, via phosphorylation of CDC25B/C, promoting their nuclear retention [93-95]. Furthermore, CDK1 phosphorylates MYT1 and WEE1, priming them for PLK1 binding through its PBD domain. Upon binding, PLK1 phosphorylates MYT1 and WEE1, leading to their inhibition and degradation, respectively [55, 96-101]. In the case of WEE1, phosphorylation at Ser123 primes it for phosphorylation at Ser121 by CK2 (casein kinase 2) and its degradation via ubiquitin ligase SCFβ-TrCP (Skp1/cullin/F-box and β-transducin repeat-containing protein) [100, 102, 103]. PLK1 also appears to control localization of cyclin B1, which, during the G2/M transition localizes to the centrosome where it is phosphorylated by PLK1 on Ser147, leading to cyclin B1 nuclear accumulation [104].
Other kinases are also important for the regulation of CDK1-cyclin B1 complex activity. For example, centrosomal Aurora A phosphorylates CDC25B on Ser353, promoting its phosphatase activity [105, 106]. In contrast, CHK1 and NEK11 act via CDC25A/B, as CHK1 and NEK11 mediated phosphorylation of CDC25A leads to its degradation and inactivation of CDC25B [107-109].

The fidelity of chromosomal segregation subsequent to mitotic initiation and progression is subject to further control by a multilayered surveillance mechanism known as the spindle assembly checkpoint (SAC). The SAC acts at the metaphase-anaphase boundary by delaying anaphase until all chromatids have undergone bipolar microtubule attachment [110, 111]. Moreover, the SAC is essential to genome stability, as its deregulation leads to aneuploidy and cell death [112]. Unattached kinetochores interact with a host of SAC sensor proteins, such as BUB1, BUBR1, BUB3, CENP-E (centromere protein E), MAD1 (mitotic arrest deficient 1), MAD2 and MPS1, leading to formation of the diffusible mitotic checkpoint complexes (MCCs) consisting of BUB3, BUBR1, and MAD2. MCCs inhibit the E3 ubiquitin ligase APC/C\textsuperscript{CDC20} (Anaphase promoting complex) through binding to and sequestration of CDC20 [111]. After all the kinetochores are properly attached, the spindle-assembly checkpoint is terminated to allow APC/C\textsuperscript{CDC20} activation and progression into anaphase [111, 113]. APC/C\textsuperscript{CDC20} is further stimulated through phosphorylation by CDK1-cyclin B1 and PLK1 [114-117]. This implicates CDK1-cyclin B1 in a negative feedback loop that results in its own degradation. APC/C\textsuperscript{CDC20} mediates the degradation of multiple proteins such as securin, PLK1, Aurora A, and cyclin B1 to ensure timely exit from mitosis [117-122].
Tapering of CDK1 activity during anaphase is critical for cytokinesis, as during early mitosis CDK1-cyclin B1 inactivates the motor protein responsible for formation of the contractile ring, Myosin II. CDK1-cyclin B1 phosphorylates MLC (myosin light-chain subunit), the regulatory subunit of Myosin II. This inhibitory phosphorylation is relieved during anaphase by APC/C\(^{Cdc20}\)-mediated inactivation of CDK1-cyclin B1 [123].

1.4 The DNA damage response (DDR)

The protection and proper maintenance of our genomes through continuous cell divisions and in response to exogenous environmental insults is vital to ensure the normal transmission of genetic material through generations and the prevention of a plethora of diseases, including cancer. DNA damage can be a product of the endogenous processes arising from cellular metabolism, or from exposure to exogenous stressors. During normal DNA replication, potentially mutagenic DNA mismatches can be caused by the introduction of improper dNTPs [124]. Another genome threat due to normal cellular functioning arise from the formation of reactive oxygen species (ROS) by the metabolic processes. ROS can attack and oxidize DNA bases leading to both potential single strand breaks and double strand breaks (SSBs and DSBs, respectively). Collectively, the endogenous genomic insults have been estimated to result in \(10^5\) genomic lesions per cell per day [125, 126].

Exposure to high energy electromagnetic radiation, such as cosmic rays, ionizing radiation (IR) from medical treatments or imaging, and UV rays from sunlight are thought to be major sources of exogenous DNA damage. They can result in the formation of DSBs and SSBs either by oxidation of DNA bases, or in the case of UV, by the formation of pyrimidine dimers and 6-4 photoproducts. Exposure to cigarette smoke has been
estimated to cause over 1000 lesions per cell over the course of 40 years [127, 128]. Commonly used chemotherapeutic drugs also act as genotoxic agents and induce various forms of DNA damage, such as interstrand crosslinks (cisplatin), base alkylation (temozolomide), and inhibition of topoisomerase I/II (etoposide).

To address this multitude of threats and attacks to genomic integrity, cells possess a complex network of surveillance, recognition and repair pathways that are collectively referred to as the DNA damage response (DDR). This response leads to cell cycle arrest, allowing for specialized machinery to detect and repair the damaged DNA, or if the damage is deemed too extensive, to induce cell death. The DDR can be divided into distinct layers whereby: 1) damaged DNA is detected by a series of sensor proteins, 2) various DNA repair factors are recruited to the site of damage and effector signalling proteins are activated, and 3) the lesion is repaired. The following section will focus on the initiation of the DDR, with an emphasis on checkpoint activation and a brief overview of the pathways involved in DNA repair (Figures 1.2, 1.3).

Central to DDR signalling cascades are the phosphatidylinositol 3-kinase-related kinases (PIKKs): ataxia-telangiectasia mutated (ATM), ATM and RAD3-related (ATR), and DNA-PKcs. Upon detection of a DSB, ATM and ATR undergo autoactivation and proceed to phosphorylate and activate CHK1, CHK2 and p53 [129]. ATM/ATR/DNA-PKcs are also crucial in the initiation of DNA repair and the selection of repair choice [129]. In addition to initiating a cascade of phosphorylation-based signalling events that ultimately lead to cell cycle arrest, ATM/ATR also trigger a signalling cascade that recruits multiple factors to the sites of damage. Critical to the initiation of repair of double stranded breaks
**Figure 1.2 DNA damage dependent responses**

DNA is constantly damaged by endogenous stress processes arising from DNA and cellular metabolism or from exposure to exogenous agents such as ionizing radiation, UV irradiation, and chemotherapeutic agents. These stressors cause various types of damaging chemical changes to DNA. Each type of DNA damage elicits a distinct signalling pathway tailored towards the repair of the lesion.
is the phosphorylation of H2A.X on Ser139 (γH2AX) by ATM. Phosphorylation of this histone protein can extend 1-2 megabases from the site of damage, and acts as a platform for the recruitment of additional proteins required for repair and amplification of the DSB signal [130-132]. Two proteins that are central to the cellular response to DSBs are BRCA1 and 53BP1, which govern the DSB repair pathway choice between HR and NHEJ (see below) [133-135].

1.4.1 CHK1/2 signaling in the checkpoint response

Downstream of ATM/ATR are the two non-homologous serine-threonine kinases, CHK1 and CHK2, which act as effector kinases for checkpoint integrity [136-138]. In the absence of DNA damage, CHK2 exists as an inactive monomer and in response to IR or other genotoxic stressors is phosphorylated by ATM/ATR on Thr68 [139-142], an event that enhances CHK2 dimerization and activation by facilitating the interaction of one CHK2 monomer with the Forkhead-associated (FHA) domain of another. Structural studies of CHK2 have revealed that FHA-kinase and FHA-FHA interactions are important for dimerization and to effectively orient the kinase domains of two CHK2 monomers for trans autophosphorylation on Thr383/T387, which leads to its full activation [139, 140, 143]. CHK1, on the other hand, becomes an ATR substrate under the conditions of DNA damage or replicative stress. ATR-mediated phosphorylation of CHK1 at Ser317/Ser345 within its non-catalytic C-terminal region requires the mediator protein Claspin [144-146] and causes CHK1 activation through a relief of autoinhibition [136, 147-151].

The primary CHK1/2 substrates related to checkpoint induction are the CDC25 family of phosphatases, primarily CDC25A, which governs the G2/M and Intra-S phase checkpoints [152-154]. In response to damage, CHK1 phosphorylates CDC25A on Ser76,
Figure 1.3 Overview of the DNA damage checkpoint

In response to DNA damage, the cell initiates a signalling cascade leading to the ultimate inhibition of CDK activity causing cell cycle arrest. ATM/ATR are activated in response to damage and phosphorylate CHK1/2 and in combination with TAO, p38MAPK/MK2. This leads to downstream activation of p53, WEE1, hnRNPA0, and inhibitory phosphorylation of CDC25A/B/C.
and promotes additional phosphorylation events resulting in its degradation by the E3 ubiquitin ligase, SCF$^{β-TRCP}$. Mechanistically, CHK1 promotes the degradation of CDC25A via the phosphorylation and activation of NEK11, which then goes on to phosphorylate the pool of CDC25A that has been already phosphorylated by CHK1 on Ser76. This dual phosphorylated form of CDC25A is then recognized and degraded by SCF$^{β-TRCP}$ [109, 153, 155]. Unlike CDC25A, CDC25B/C is functionally inactivated via cytoplasmic sequestration [156-158]. In response to genotoxic stress, CDC25B/C are phosphorylated on Ser309/323 and Ser216, respectively, leading to 14-3-3 binding and nuclear exclusion [159-163]. Interestingly, CHK1 has also been reported to phosphorylate and increase WEE1 activity in multiple organisms, suggesting another route for CHK1 in mediating the G2/M checkpoint [164-166].

### 1.4.2 p38MAPK/MK2 signaling in the checkpoint response

p38MAPK and its substrate MK2 (p38MAPK/MK2) also respond to general stresses (heat shock or osmotic stress), or genotoxic stressors (IR, UV, cisplatin, TMZ, etoposide) [138, 167-171]. Context-dependent activation of the p38MAPK/MK2 signalling complex can also lead to multiple other outcomes including cellular differentiation, checkpoint induction, changes in cytoskeletal structure, and/or apoptosis. Activation of p38MAPK/MK2 occurs via a Mitogen activated protein kinase (MAPK) cascade. Upon stimulation, a MAP kinase kinase kinase (MAPKKK) will activate a MAP kinase kinase (MAPKK/MKK), which subsequently phosphorylates p38MAPK on its activation loop and results in p38MAPK/MK2 complex activation. The MAPKKK and MAPKKs responsible for p38MAPK/MK2 activation appear to be context-dependent. In response to UV, IR, and hydroxyurea, the MAPKKK, thousand and one amino acid kinase (TAO), activates
p38MAPK downstream of ATM activation [172]. MKK3, MKK4 and MKK6 are the MAPKKs responsible for activation of p38MAPK in response to UV irradiation [173, 174]. Interestingly, p38MAPK/MK2 may also operate downstream of ATM/ATR in response to cisplatin, camptothecin and doxorubicin, but not UV irradiation [169]. p38MAPK and MK2 phosphorylate a host of targets including transcription factors, p53, and multiple proteins involved in post-transcriptional regulation of mRNA and mRNA translation [167, 175-180].

Following a genotoxic insult, p38MAPK/MK2 is required for the induction of the G2/M checkpoint [138, 168, 170, 171, 181, 182]. As with CHK1/2, MK2 phosphorylates CDC25B/C on Ser323 and Ser216, respectively, leading to functional inactivation by 14-3-3 [138, 168, 170]. Mass spectrometry, oriented peptide library, and in vitro studies have identified MK2 as one of the kinases responsible for phosphorylation of CDC25 phosphatases in response to genotoxic stress [168, 169, 183].

The p38MAPK/MK2 complex becomes particularly vital for checkpoint induction and cell survival in the context of p53 mutation or loss. MK2 depletion in p53-deficient MEFs led to an increase in both cisplatin and doxorubicin sensitivity [169]. Furthermore, loss of MK2 in p53-deficient cells causes a defective G1/S and G2/M checkpoint in response to genotoxic treatment [169]. This reliance on p38MAPK/MK2 activation in p53-deficient cells may be due to their ability to activate the RNA binding protein hnRNPA0 [184]. Upon cisplatin treatment, hnRNPA0 transcriptionally stabilizes p27^Kip1 and GADD45α mRNAs, leading to checkpoint induction in a p53-independent manner [184].
1.5 DNA repair

Given the multitude of chemical changes caused by various types of DNA damage, multiple and partially overlapping DNA repair pathways exist to address any genotoxic insult (Figure 1.2). These pathways can be separated into two broad categories: single strand break repair (SSBR) which encompasses nucleotide excision repair (NER), base excision repair (BER) and DNA mismatch repair (MMR), and double strand break repair (DSBR), which includes homologous recombination (HR) and non-homologous end joining (NHEJ).

1.5.1 Single Strand Break Repair (SSBR)

NER can eliminate the widest range of DNA lesions, specifically those caused by UV-induced DNA damage, such as the cyclobutane–pyrimidine dimers (CPDs) and the 6–4 pyrimidine–pyrimidine photoproducts (6–4PPs). These lesions are detected by one of two NER sub-pathways, through global genome NER (GG-NER) or through transcription-coupled NER (TC-NER) (reviewed in [185] and summarised below).

The GG-NER pathway is responsible for detection of altered base pairing throughout the genome. The helix distorting lesion is detected by XPC, in complex with RAD23B and CETN2, through interaction with unpaired ssDNA. In the case of UV-induced CPDs, the UV-DDB complex detects and binds to the lesion to stimulate XPC recruitment. XPC binding to the lesion further recruits the TFIIH complex (containing XPB and XPD helicases), which verifies the lesion and extends the open DNA confirmation caused by the lesion. XPF-ERCC1 and XPG endonucleases then excise the lesion and a short region flanking region, by creating incisions on the 5' and 3' ends of the lesion,
resulting in the excision of a 22-30bp region of ssDNA. These incisions are coordinated by the action of XPA, XPG and RPA to ensure only the damage strand is excised. The resultant gap is then filled by the action of DNA polymerase and ligated by DNA ligase 1 or XRCC1-DNA ligase 3.

The TC-NER pathway acts to detect and repair transcription blocking lesions and is initiated by stalled RNA polymerase II. Stalled RNA polymerase II recruits CSA and CSB, which induce the recruitment of multiple factors required for TC-NER such as UVSSA, USP7 and XPA-binding protein 2. RNA polymerase II is then backtracked away from the lesion on the transcribed DNA strand to give access to the NER incision machinery. The resulting gap region is then filled and ligated by the same machinery as in GG-NER.

BER is responsible for the recognition and repair of small non-helix distorting damaged bases, such as the uracil insertions, abasic sites, 8-oxoguanines, and deaminated bases. The damaged base is first cleaved by a DNA glycosylase, such as OGG1 (8-oxoguanine glycosylase 1), forming an apurinic site, and then an endonuclease, such as the APE1 (Apurinic/apyrimidinic endonuclease 1), recognises this abasic region and cleaves the DNA backbone 5' to the abasic site, leading to a single strand break. The resultant gap is processed and either a single nucleotide is replaced or a patch of 2-10 new nucleotides is synthesized to fill the gap [186, 187]

MMR is a highly conserved pathway in both prokaryotes and eukaryotes that corrects mismatches that occurring as a result of DNA replication errors (reviewed in [188], [189], [190], and, [191], and summarised below). The mismatched bases are recognized and bound by MutSα (MSH2-MSH6) or MutSβ (MSH2-MSH3) heterodimers.
Upon recognition of the lesion by MutSα/β, MutLα nicks the 3’ or 5’ sides of the mismatched bases on the newly synthesized DNA strand. The nicked DNA is then excised by the EXO1 nuclease, in cooperation with RPA to protect the unprotected ssDNA segment. The resultant gap is filled by DNA polymerase ε/δ in complex with PCNA, and ligated by DNA ligase 1.

1.5.2 Double strand break repair

DSBs are highly cytotoxic DNA lesions that are mainly caused by radiotherapy or exposure to radiomimetic drugs, but are also formed under periods of replication stress, when a stalled replication fork encounters a SSB [129]. DSBs are also transiently formed during normal cellular processes such as, meiosis, telomere maintenance, and in V(D)J recombination, class-switch recombination and somatic hypermutation that occur in developing B and T cell lymphocytes for the generation of immunoglobulin and T-cell receptor (TCR) diversity [129, 192]. DSBs are highly deleterious to cellular function due to the inherent nature of this lesion, as both strands of DNA are broken, effectively resulting in discontinuous chromosomes. If left unrepaired, DSBs can cause mutations and chromosomal translocations leading to cell death or neoplastic transformation [193]. To cope with DSBs, mammalian cells use two major overlapping pathways whose activity is constrained to specific cell cycle phases: NHEJ throughout the cell cycle and HR in S and G2 phases [133].

1.5.2.1 Non homologous end joining (NHEJ)

While widely considered to be a highly error-prone repair mechanism, NHEJ repairs approximately 80% of irradiation-induced DSBs through a series of defined steps, from initial recognition of the break, to the final ligation of the DSB [194-196]. In NHEJ, a
DSB is recognized by the Ku70/Ku80 heterodimer within seconds [197, 198]. Once bound to DNA, the Ku heterodimer serves as a protein scaffold for the recruitment of other NHEJ proteins to the site of damage, including DNA-PKcs, XRCC4, DNA Ligase IV, XLF, and APLF [199]. Interaction of DNA-PKcs with Ku70/Ku80 at broken DNA ends can only occur in the presence of DNA and leads to the formation of an active DNA-PK complex that protects and stabilizes the ends of the break from non-specific processing by nucleases [200-203].

Depending on the nature of the DSB, different processing enzymes are required to prepare the break for ligation to finalize the repair process, including, but not limited to Artemis, PNKP, APLF, DNA polymerases μ and λ, WRN, and Aprataxin [199]. For example, Artemis, WRN and APLF act as nucleases with differing preferences and directionality to process single stranded DNA overhangs near the break, to leave a blunt-ended DSB that can be ligated [204-207]. PNKP, which possesses both phosphatase and kinase activity, can add phosphates to 5’ OH groups or remove phosphates from 3’ OH groups to create a ligatable configuration at the site of damage [208].

The final step of NHEJ is the ligation of the break by DNA ligase IV. Autophosphorylated DNA-PKcs disassociates from the break allowing for final ligation of the DSB by DNA ligase IV, which is recruited to the DSB when complexed with XRCC4 and XLF4 [198, 209, 210]. Despite the prevalence of NHEJ as a DSB repair mechanism, its lack of use of a repair template strand and the nature of the end-processing steps can lead to the formation of multiple genomic scars through the addition or loss of genetic information.
1.5.2.2 Homologous Recombination (HR)

In contrast to NHEJ, HR is considered a largely error-free and non-mutagenic form of DNA repair. While NHEJ ligates two free ends to repair the DSB, HR utilizes a sister chromatid as a template for repair, through a highly orchestrated series of events. Given this requirement, HR only operates in DSB repair during the late S and G2 phases of the cell cycle [133, 211].

HR can be broken down into discrete steps, from initial DSB recognition to its repair. In response to DNA damage, the heterotrimeric MRN complex (Mre11-Rad50-Nbs1) detects and binds to the DSB [212]. This complex then governs the recruitment and activation of ATM at the site of damage, which in turn nucleates multiple factors required for HR-mediated resolution of the DNA lesion [213-216]. The ends of the DNA at either side of the DSB are resected by the endonuclease activity of Mre11 (3’-5’) in a CtIP and BRCA1-dependent manner, leading to the exposure of two regions of ssDNA on either side of the break [135, 217-219]. Further 5’-3’ resection is catalysed by the EXO1 and BLM nucleases [220]. After resection, RPA coats the resultant ssDNA and stabilizes and protects these regions from degradation and secondary structure formation. Rad51, a DNA recombinase, replaces RPA on ssDNA, in a process mediated by BRCA2 in a PALB2-dependent manner [221-224]. Rad51-bound DNA forms a nucleoprotein filament which performs a DNA sequence homology search [225, 226]. After identifying a homologous region, Rad51 mediates the invasion of a homologous DNA duplex on a sister chromatid by the damaged DNA strand, forming a displacement loop (D-loop) [225, 227, 228]. DNA polymerase η then uses the homologous region on the sister chromatid as a template to synthesize new DNA from the 3’ end of the invading strand [229]. This
process is resolved through synthesis dependent strand annealing (SDSA), where the invading strand is disassociated from the D-loop, and anneals to the complementary end of the resected damaged DNA duplex, and DNA synthesis completes the process [228]. An alternative outcome of HR is a crossover event, where both resected ends of the DSB (second-end capture) are captured by the D-loop to form a double-Holliday junction (dHJ) [230]. In certain cases, the D-loop may be misarranged into a replication fork in the absence of a second DNA end, in a process termed break induced replication (BIR) [231]. Both the formation of dHJ crossovers and the BIR can be deleterious to cell function, and lead to a loss of heterozygosity in the areas distal to the DSB [228].

1.6 p53

Loss of function of the TP53 gene is found in over 50% of all human cancers. The TP53 gene and its product the p53 protein have been extensively studied, with over 90,000 published works to date (PubMed). This section provides a brief overview of p53 biology, with a focus on its function in transcriptional activation and the DDR.

1.6.1 Discovery of p53

In 1979, multiple studies of the SV-40 virus-transformed cells discovered a 55kDa protein that interacted with the SV-40 Large T-antigen [232-235]. The identified protein, named p53, was originally classified as an oncogene, as its expression was increased in SV-40 transformed cells [236]. Further studies corroborated the oncogenic potential of p53, as cloned human and murine p53 were found to cooperate with Ras and other oncogenes to transform cells [237-239]. Nevertheless, accumulating evidence began pointing towards p53 function as a tumour suppressor. TP53 was found to be inactivated and/or deleted in human and murine cancers [240-242]. The "oncogene or tumour
suppressor” question was answered in 1989 when it was discovered that the human and murine p53 cDNA clones from transformed cells, until that time, were TP53 mutant variants [243-246]. Studies using the first wildtype p53 cDNA showed that it had anti-proliferative properties, that it was mutated and underwent loss of heterozygosity (LOH) in cancers [243, 247-251]. Taken together, these studies led to the reclassification of p53 as a tumour suppressor.

1.6.3 The structure of p53

The human p53 protein is a 393 amino acid DNA sequence-specific transcription factor with defined functional domains, an N-terminal transactivation domain (TAD) (1-42 a.a.), a proline rich domain (PRD) (61-92 a.a.), a DNA binding domain (DBD) (101-300 a.a.), an oligomerization domain (OD) (325-356 a.a.), and a C-terminal basic domain (CTD) (364-393 a.a.) [252, 253]. Together, these functional domains coordinate to regulate transcription of multiple genes in a context-dependent manner. p53 is regulated through various PTMs and differential protein-protein interactions (expanded on in 1.6.6).

1.6.4 p53 in disease and cancer

The TP53 gene is located on chromosome 17p13.1. Somatic TP53 mutations occur at variable rates in cancers, from less than 5% in cervical cancer and 10% in leukemia, to 80% in small-cell lung cancer and 90% in ovarian cancer [254, 255]. The significance of p53 in tumorigenesis is further supported by research in multiple transgenic animal models. Mice harbouring loss or mutation of p53 display spontaneous and DNA damage- and oncogene-induced tumours [256-271]. Restoration of wild type p53 function in these models leads to tumour regression and extended survival [256-271].
Most cancer-associated mutations in the \textit{TP53} gene are missense mutations (>76.7%), which are mainly found in the DBD (93.1%), followed by the OD (1.6%) (http://p53.iarc.fr/). \textit{TP53} has several mutational hotspots within the DBD, where the most frequent mutations are R175H, Y220C, G245S, R248Q/W, R249S, R273C/H, and R282W. These mutations disrupt the DBD of p53 either by destabilizing its structure or removing residues crucial to DNA binding, effectively decreasing its transcriptional activity [272, 273].

Germline mutations of \textit{TP53} are the cause of Li-Fraumeni (LFS) and Li-Fraumeni-like (LFL) syndromes. This rare hereditary disorder leads to a predisposition to early onset cancers such as breast cancer, osteosarcoma, soft tissue sarcoma, brain cancer, and adrenocortical carcinoma [274]. Mutations associated with these diseases are mainly missense mutations (75.2%) found in the DBD (72.7%) and in the OD (19.6%) (http://p53.iarc.fr/).

\textbf{1.6.5 Transcriptional activity of p53}

The high degree of cancer-associated mutations within the p53 DBD indicate that the key to p53 tumour suppressive function lies in its transcriptional activity. As a transcription factor (TF), p53 is involved in a host of cellular processes, including, but not limited to, cell cycle control, apoptosis, senescence, metabolism, immune response and migration [275]. p53 transactivates genes such as p21, MDM2, BAX, PUMA, and GADD45α, and acts as a transcriptional repressor of genes such as MYC, SURVIVIN and VEGFA (reviewed in [275]). The activation of a p53 target gene occurs in two steps, first p53 recognizes and binds to a p53 response element (RE) in its target genes via the DBD, followed by recruitment of multiple transcriptional modifiers (Figure 1.4) [276, 277].
Crucial to the transactivation activity of p53 is its binding to and recognition of cis p53 response elements (RE) [278, 279]. The consensus RE for p53 binding is made up of two 10-base sequences: RRRCWWGYYY...n...RRRCWWGYYY (R=purine, Y=pyrimidine, W=A or T and n=spacer of 0–13 nucleotides) [275, 278, 280]. p53-responsive genes have differing numbers of p53 REs, for example the genes for $p21$, $MDM2$, and insulin-like growth factor-binding protein 3 ($IGFBP3$) contain more than two p53 REs [281]. These REs display high or low affinities for p53 depending on their sequence, leading to a threshold of p53 mediated transactivation for the expression of individual genes. For example, proapoptotic genes such as $BAX$ and $PUMA$ have low affinity p53 REs and a high threshold for p53 transactivation and can only be transcribed in situations where the degree of DNA damage is irreparable and high levels of active p53 are present in the cell. As a general guiding principle, having more than one p53 binding site in the 5’ promoter region of a gene would increase the responsiveness and sensitivity of a given gene to p53 [275, 282, 283]. Of the identified p53 response elements, more than 50% are within the 5’ promoter enhancer region, and ~25% reside within the first intron [284].

Following RE binding, p53 stimulates transcription through the recruitment of transcriptional regulatory components, histone acetyltransferases (HATs), and chromatin remodelling complexes (CRCs) [276, 277, 285-287]. RE-bound p53 associates with HATs, such as p300 or STAGA, leading to the acetylation of nucleosomal histones promoting the recruitment and activity of nucleosomal remodelling complexes such as SWI/SNF [282, 288-295]. Recruitment of SWI/SNF complexes causes the displacement of nucleosomes from transcriptional initiation sites [296-302].
Figure 1.4 Basic model of p53 transcriptional activity
1) Tetrameric p53 binds to a p53 RE in the promoter of a p53 responsive gene. 2) p53 recruits transcriptional coactivators such as histone acetyltransferases HATs (p300/CBP), which acetylates p53 and chromatin. 3) Acetylated chromatin recruits chromatin remodelling complexes (CRCs) which promotes histone displacement. 4) The RNA pol II preinitiation complex (PIC) binds to the open promoter and leads to transcriptional elongation and expression of the p53 responsive gene.
Alongside HATs, the arginine methyltransferases PRMT1 and CARM1 are involved in p53 transcriptional activation through the modification of local chromatin structure [288]. PRTM1 and CARM1 interact with p53 and cooperatively function to methylate histone modification, and maintain histone acetylation by p300 through the of histone deacetylases (HDACs) [288, 303-306].

The transcription of eukaryotic genes requires a multi-subunit complex known as the preinitiation complex (PIC) [307]. The PIC is made of RNA polymerase II, Mediator complex and TFII-A, -B, -D, -E, -F, and –H [307-311]. To initiate PIC formation and transcription, TFIID nucleates the complex by first binding to a TATA box in the promoter of the gene to be transcribed [312]. p53 interacts with TFIID and TFIIH components to recruit them to the promoters, nucleating PIC formation and transcriptional initiation [313-321]. p53, alongside its function in TFIID and TFIIH recruitment, directs transcription through interaction of the p53 TAD and CTD with the Mediator complex [308, 322-324]. The Mediator complex is a multi-subunit scaffold within the PIC that transmits signals from transcriptional activators to RNA polymerase II to enhance transcriptional initiation [325]. Interaction with p53 induces a structural change in Mediator and promotes mRNA elongation by RNA polymerase II [323].

### 1.6.6 Regulation of p53 by protein-protein interactions and posttranslational modifications

The transcriptional activity of p53 is regulated and fine-tuned via a series of posttranslational modifications (PTMs), including ubiquitination, phosphorylation, acetylation, and methylation, which modulate p53's interactions with the specific regulatory proteins and the DNA [265, 326-335]. The most well-defined regulator of p53
is the RING-finger type E3 ubiquitin ligase, MDM2. Together with its partner MDMX, MDM2 targets p53 for degradation (Figure 1.5) [264, 336-356]. The N-terminus of MDM2 serves as a docking site for the TAD of p53, and upon association induces a conformational shift that allows for additional contact between MDM2 and the p53 DBD [357-360]. MDM2/MDMX mediates p53 polyubiquitination on multiple lysine residues (Lys370, Lys372, Lys373, Lys381, Lys382 and Lys386) within the CTD and targets it for ubiquitin-mediated proteolysis [343-346, 361-363]. Binding of MDM2/MDMX to the p53 TAD also impacts p53 transcriptional activity through competing for its interactions with the coactivators, such as p300/CBP, perturbing the conformation of the p53 DBD, and recruitment of HDACs to effect nucleosome occupancy at promoters [364-369]. Interestingly, p53 and MDM2 form a negative feedback loop in which p53 stimulates transcription of MDM2, thereby regulating its own turnover and the p53 response [370].

DNA damage promotes ATM/ATR-mediated phosphorylation of p53 Ser15 and increasing its transcriptional activity [371]. In parallel, MDM2 undergoes multi-site phosphorylation by ATM/ATR, primarily on Ser395, which suppresses MDM2/p53 DBD interaction [372-380]. p53 Ser15 phosphorylation leads to further p53 phosphorylation on Thr18 and Ser20 by CK1 and CHK2, respectively, which uncouples p53 from MDM2-mediated degradation and nuclear export [326, 334, 381-403]. Mechanistically, phosphorylation of these residues inhibits p53-MDM2 interaction via electrostatic repulsion between the phosphorylated p53 N-terminus and MDM2 [404]. Moreover, Ser15/Thr18 phosphorylation stimulates interaction of p53 with p300/CBP, a histone acetyltransferase (HAT) that binds to the p53 TAD, acetylates its C-terminal domain, and blocks p53 polyubiquitination [265, 291, 292, 294, 326, 327, 361, 385-387, 405-424].
Figure 1.5 The activation of p53 transcriptional activity after DNA Damage.

Basally, p53 is downregulated as a homotetramer by MDM2 mediated ubiquitylation and degradation. In response to DNA damage or other stressors, p53 is phosphorylated on Ser15 in by ATM/ATR/DNA-PKcs, leading to the disruption of the p53-MDM2 interaction. This leads to accumulation of p53 and further activating modification of p53 by phosphorylation and acetylation. Activated p53 homotetramers bind to p53 RE containing promoters and transactivate the transcription of p53 responsive genes leading to cell cycle arrest, DNA repair and/or apoptosis.
p300/CBP acetylates Lys370, Lys372, Lys373, Lys381, Lys382, and Lys386, the same lysines that are targeted by MDM2, effectively competing with MDM2-mediated ubiquitination and downregulation of p53 [328, 419, 420]. Further, p53 CTD acetylation enhances the recruitment of transcriptional co-activators and histone modifiers promoting p53-dependent transactivation of genes [282, 328, 419, 425, 426]. Phosphorylation of residues within the p53 CTD impacts its DNA binding ability. Specifically, phosphorylation of Ser315 (CDKs), Ser376, and (CAK) Ser378 (PKC) promote p53 binding to DNA and confer limited DNA binding site preference [427-430].

Outside of the ubiquitin-phosphorylation-acetylation cascade regulating the p53 response, other p53 PTMs and protein-protein interactions effect p53 activity, directing its impact towards choices of cell cycle arrest or apoptosis. High levels of DNA damage can induce the phosphorylation of Ser46 on p53 via HIPK2, DYRK2 and PKCδ favoring specific transactivation of proapoptotic p53 response genes, p53DINP1 and p53AIP1 [431-435]. Cofactors such as Hzf, ASPP1, ASPP2 and iASSP bind to the DBD of p53 and act as determinants of p53 promoter selectivity. In response to genotoxic stress, ASPP1 and ASPP2 steer p53 towards induction of an apoptotic program, whereas iASPP binding to the same site was found to promote cell cycle arrest [436, 437]. Interestingly, phosphorylation of p53 at Ser46 promotes binding of Pin1 and subsequent displacement of iASPP from the DBD of p53, thereby inducing apoptosis [438].

Acetylation and methylation of p53 also factor into the cellular decision between growth arrest and death. Acetylation of Lys120 in the p53 DBD by Tip60/hMOF, in response to DNA damage, promotes transactivation of apoptotic genes [419, 439-441]. Conversely, acetylation of Lys320 by PCAF leads to preferential transactivation of cell
cycle arrest genes [442-444]. p53 methylation also features in the choice of response to DNA damage, where PRMT5 methylation of the tetramerization domain of p53 (R333, R335, R337) promotes apoptosis over cell cycle arrest [445].

1.6.7 p53 oligomerization

Structural studies have revealed oligomerization as a determinant of p53 transcriptional activity. p53 binds to REs as a homotetramer consisting of two p53 head to tail dimers [252, 253, 446, 447]. The p53 tetramer displays 100-fold greater affinity for p53 REs compared to the monomeric p53, as each p53 DBD recognizes a single pentanucleotide repeat within the RE [448, 449]. In addition to increasing the affinity of p53 for DNA, p53 oligomers mask its NES sequence and lead to p53 nuclear accumulation [450]. Under basal conditions, there are varying levels of p53 monomers, dimers, and tetramers within the cell, and in response to DNA damage the equilibrium swings towards the tetrameric form [451]. Tetramerization also affects p53 posttranslational modifications. Damage-induced p53 Ser20 phosphorylation by CHK2, which inhibits its interaction with MDM2, requires tetramerization [452]. Further, acetylation of Lys382 by p300 requires interaction with the p53 tetramer [417, 453, 454]. Interestingly, the E3 ubiquitin ligase Pirh2 preferentially targets the tetrameric form of p53 [455].

Posttranslational modifications and protein-protein interactions also impact p53 oligomerization. Phosphorylation of Ser392 stabilizes the p53 tetrameric form in response to various stressors [456-458]. Furthermore, nitration of Y327 in response to nitric oxide has been proposed as a regulator of p53 oligomerization and activation, independent of Ser15 phosphorylation [459]. Alongside these modifications, multiple proteins
preferentially interact with p53 tetramers to increase their stability (i.e. c-Abl, Atg7, MYBBP1A, BCCIP, RhoGAPs, APE-1) or inhibit p53 oligomerization via interaction with the OD (i.e. RBEL1A, S100, ARC, HERC2) [460-472].

1.6.8 The p53 response to DNA damage

Early work on p53 revealed its possible function in the cellular response to DNA damage, as nuclear p53 increased in response to UV irradiation, and was implicated in cell cycle control after irradiation [473, 474]. Our knowledge on the impact of p53 in the cellular response to DNA damage has vastly expanded since these initial studies [473, 475-478]. Upon stabilization and activation in response to DNA damage, p53 carries out an extensive and coordinated transcriptional program to control cell cycle arrest, DNA repair and apoptosis (Figure 1.5) [275, 284, 479, 480]. The CDK inhibitory protein p21, is considered to be the primary target for p53, as its transcription is rapidly induced following DNA damage [481, 482]. Inhibitory binding of p21 to CDK4/6-cyclin D1 and CDK2-cyclin E leads to G1/S arrest [33, 36]. p21 also binds to PCNA, inhibiting DNA replication during S phase [483]. Alongside its function in G1/S control, p53 also acts in the G2/M checkpoint regulation through transactivation of GADD45α, which inhibits CDK1 activity, and 14-3-3σ which interacts with and sequesters CDC25C from the nucleus [158, 484]. The cell cycle arrest elicited by p53 allows time for DNA damage repair, but if the extent of DNA damage is deemed irreparable, p53 induction can also lead to apoptosis [485, 486], via the control of expression of genes involved in the mitochondrial death pathway, such as BAX, NOXA, PUMA and APAF1 [284, 479, 480, 487-490].
The critical impact of p53 on the choice to initiate transcriptional programs that can lead to cell cycle arrest or apoptosis, respectively, suggests that the p53 pathway may have the ability to sense the fidelity of DNA repair. If the damage is repaired, the p53 response is attenuated and the cell can re-enter the cell cycle, whereas the presence of unrepaired or too numerous DNA lesions directs a cellular death outcome. Cell type, genetic background, and the duration and intensity of the stress are important factors in determining the outcome of p53 pathway activation [491-494].

One potential mechanistic model for the p53-dependent decision between growth arrest and cell death is based on the differential p53 affinity for different classes of promoters [275, 278, 280, 282, 283, 486, 495-500]. Promoters of cell cycle arrest genes, such as p21 and GADD45α, possess high affinity p53 REs, whereas promoters of apoptotic genes, such as PUMA and BAX, feature low affinity p53 REs [281]. Therefore, levels of p53 activation may be decisive in the cell fate choice, with low p53 induction causing growth arrest, whereas high levels would lead to apoptosis. Supporting this, p53 is basally preloaded onto promoters of growth arrest genes (p21, PCNA, GADD45α, and 14-3-3σ), but not onto proapoptotic genes, in the absence of DNA damage [501]. Promoter-bound p53 mediates the recruitment and assembly of PICs onto transcriptional start sites, priming growth arrest genes for rapid expression in case of cellular stress caused by DNA damage, whereas the expression of proapoptotic genes occurs with slower kinetics and at later time points [501]. This hypothesis is consistent with the concept of prolonged p53 activation caused by unrepaired or persistent damage leading to apoptosis [502]. Another possibility is that posttranslational modifications are responsible for fine tuning the p53 response by integrating input from multiple pathways.
Individual PTMs and combinations of PTMs would direct p53 through the formation of a "barcode" that would provide a p53 response tailored to the particular form of encountered damage [333, 334, 480, 493, 494, 503].

**1.6.9 p53 and DNA Repair**

Outside of its function in the control of checkpoint induction and apoptosis, p53 also directly promotes or suppresses multiple DNA repair processes in a context- and damage-dependent manner (NER, BER, MMR, NHEJ, and HR) [504, 505]. The effects mediated by p53 on these DNA repair pathways occur through both transactivation-dependent and -independent mechanisms.

Homozygous mutant p53 fibroblasts from Li-Fraumeni patients and human fibroblasts lacking p53 display heightened UVC sensitivity, and are defective in the removal of CPDs from genomic DNA, indicative of defective NER [505-511]. After UVC irradiation, p53 transactivates the expression of DDB2 and XPC, which are both involved in the recognition of 6-4 photoproducts and CPDs [512-517]. Moreover, p53 directly binds to several components of the NER pathway, including XPB, XPD and CSB, and stimulates their detection of DNA lesions [518-520].

The stimulatory effect of p53 in BER is also well-characterized, and includes transactivation-dependent and -independent mechanisms [521, 522]. p53 promotes expression of OGG1, and further goes on to interact with, and stimulate OGG1 protein activity, resulting in the removal of 8-oxoG DNA lesions [523, 524]. p53 also stabilizes the interaction of DNA polymerase β, the BER DNA polymerase essential for replacing the excised base(s), with an abasic site [525-527]. Interestingly, p53 controls BER in a dose- and cell cycle-dependent manner, as low doses of genotoxic exposure prompt p53-
dependent BER, whereas the high doses inhibit BER and lead to p53-dependent apoptosis [528]. During the G0-G1 stages of the cell cycle p53 enhances BER, whereas in the G2-M phases it reduces it [529]. Similar to NER and BER, p53 also supports MMR function [530-533], likely through the p53 dependent transactivation of the MMR component, hMSH2 [534, 535].

Compared to the other DNA repair mechanisms, the significance of p53 in the repair of DSBs remains largely uncharacterized. The literature supports a primarily inhibitory effect of p53 on HR through transcription-dependent and -independent mechanisms [536, 537]. p53 is thought to suppress RAD51 transcription, but also directly interacts with and inhibits the function of RAD51, RAD54 and RPA in HR [538-546].

1.7 NEK family of protein kinases

Alongside the CDK, Aurora and PLK kinase families, the NEK (NimA related kinase) family of protein kinases have emerged as key players in the regulation of both cell cycle progression and the DDR. The founding member of the NEK family is the never-in-mitosis A (NIMA) protein of the filamentous fungus, *Aspergillus nidulans*. NIMA was first identified in a genetic screen for *A. nidulans* genes involved in the cell division cycle, where a heat-sensitive mutation of NimA led to a reduction in the mitotic index and a defect in mitotic spindle formation [547]. Loss of function NimA mutations lead to G2 arrest, whereas overexpression of NimA induces premature mitotic entry [548, 549]. NimA also affects mitotic progression via phosphorylation of Histone H3, inducing chromosomal condensation, and its function in transporting Cdc2 into the nucleus for mitotic initiation [548, 550]. In *A. nidulans*, NimA is thought to be as important as the mammalian Cdc2-
cyclin B1 complex as a master regulator of mitotic progression, as degradation of NimA is required for mitotic exit [551].

Homologues of NimA have been identified in multiple eukaryotes from *S. cerevisiae* to humans, with an expansion of the family of NimA related kinases (NEK) in higher multicellular eukaryotes. Humans possess 11 genes that encode NEK1 to NEK11, each with distinct functions and features (Figure 1.6). None of the human NEKs fully recapitulate NimA biology in *A. nidulans*, but have diversified into control of a host of processes spanning mitotic initiation, mitotic spindle formation, cytokinesis, cell cycle checkpoint induction and DNA repair [552, 553].

NEK family kinases, with the exception of NEK10, possess an N-terminal serine/threonine kinase domain. Interestingly, NEK kinase domains do not share high degree of amino acid sequence similarity with either NimA or each other (only ~40-50% homology), with the exception of NEK6 and NEK7 that display 85% sequence identity within their kinase domains and carry out similar functions in spindle assembly [552, 553].

### 1.7.1 NEK kinases in mitotic progression

NEK2, NEK9, NEK6, and NEK7 constitute a signalling block that participates in proper mitotic initiation and mitotic spindle formation. Although not essential for mitotic entry, the closest human NIMA homologue, NEK2 is required for proper centrosomal disjunction and separation during mitotic onset [554, 555]. NEK2 phosphorylates various target proteins that impact the disassembly of the proteinaceous linker between the two centrioles that form a centrosome [552]. Disassembly of this filamentous tether allows the centrioles to migrate to the opposite poles of the mitotic cell and nucleate a mitotic spindle. During interphase, the expression of NEK2 is variable with the highest expression at S
Figure 1.6 Alignment of 11 mammalian NIMA-related kinases and the NIMA kinase of *Aspergillus*.

The relative positions of significant motifs and regions are indicated [305].
and G2 phases [555-557]. NEK2 localizes mainly to centrosomes and is a part of the microtubule organizing center (MTOC), where it phosphorylates C-Nap1 and Rootletin, which form the intercentriolar linker, leading to centriole dissolution and mitotic onset [554, 558-561]. More recent data have identified other NEK2 substrates, such as CEP68, centlein, Dishevelled, and β-catenin, which also participate in the centrosome cycle [562-565]. Expression of a catalytically inactive form of NEK2 or knockdown of NEK2 substrates induces defects in centrosome separation upon mitotic onset and spindle assembly leading to the formation of multinucleated cells [554, 558, 562, 564]. During interphase, NEK2 is maintained in a catalytically inactive, dephosphorylated state, via the interaction with MST-2 and the protein phosphatase PP1γ [566]. Upon mitotic onset, MST-2 is phosphorylated by PLK1, leading to dissolution of this heterotrimeric complex, allowing for NEK2 activation by autophosphorylation [567]. Interestingly, PLK1 may have a further effect on the mitotic activity of NEK2, by regulating NEK2-mediated stabilization of β-catenin at mitotic centrosomes [568].

NEK6, NEK7, and NEK9 also act as regulators of the mitotic spindle [569-572]. RNAi mediated knockdown or expression of catalytically inactive mutants of NEK6, NEK7 or NEK9 induces defects in centrosome separation, weak spindle formation during mitosis, and prolonged spindle checkpoint activation resulting in apoptosis [569, 573, 574]. Mice deficient for NEK7 exhibit severe growth retardation and very few NEK7−/− mice live past one month of age [575]. On a cellular level, NEK7−/− MEFs display increased polyploidy and genome instability, increased centrosome numbers in binucleated cells, and cytokinesis failure. Interestingly, despite the strong homology between NEK6 and NEK7, NEK6 was unable to fully compensate NEK7 deletion [569].
NEK9 acts as an upstream activator of both NEK6 and NEK7 during mitosis. During prophase, NEK9 is concentrated at centrosomes and undergoes activation loop autophosphorylation (Thr210), and is subsequently phosphorylated by CDK1-cyclin B1 and PLK1, resulting in its full activation [570, 571, 573, 576, 577]. In mitosis, NEK9 interacts with and phosphorylates NEK6 and NEK7 on their activation loops (NEK6: Ser206 and NEK7: Ser195) [570, 578, 579]. Structural studies have indicated that direct binding of a non-catalytic region of NEK9 allosterically activates both NEK6 and NEK7 through the disruption of their autoinhibitory conformation, the so-called “Tyr-Down” conformation, whereby a tyrosine residue points into the active site of the kinase domain, interacts with the activation loop, and blocks the αC helix [580, 581]. Activated NEK6/7 is then able to phosphorylate the kinesin-related motor protein Eg5 inducing separation of centrosomes [85, 571, 573, 579, 582]. Interestingly, NEK5, a relatively uncharacterized NEK family member may also influence interphase centrosome assembly in a manner similar to NEK7 [583]. Cells depleted of NEK5 display reduced levels of C-NAP1, NEK2, γ-tubulin, CEP192, CDK5RAP2, and pericentrin, and a defect in centrosomal separation [583].

1.7.2 NEK Kinases in the DDR

Alongside their well-characterized roles in cell cycle progression and mitosis, emerging evidence is pointing toward functions for NEK kinases in both the early and late stages of the cellular response to DNA damage (Figure 1.7). The two best characterized NEK kinases in the DDR are NEK1 and NEK11.
Figure 1.7 NEK kinases in the DNA damage responses

NEK kinases play a diverse number of roles in the cellular response to DNA damage as indicated. NEK1 interacts with ATR/ATRIP and is crucial to the activation of ATR in response to DNA damage. Further downstream, NEK11 has been shown to be involved in the regulation of CDC25A. In response to IR, NEK11 is activated via CHK1 and goes on phosphorylate Cdc25A leading to beta-TrCP mediated polyubiquitylation and degradation of CDC25A. In parallel, Nek10 has been shown to form a ternary complex with RAF and MEK1/2 and activate ERK1/2 in an UV dependent manner leading to G2/M arrest. To halt mitotic progression, in response to IR, NEK2 is inhibited in a ATM and PP-1 dependent manner. NEK8 plays roles both in response to DNA damaging agents as well as in response to replicative stress. NEK8 was found to play a key role in the recruitment of RAD51 to DSBs. Alongside this NEK8 was found to interact with ATR-ATRIP and CHK1 and inhibit CDK-cyclin A in response to aphidocin
NEK1\(^{-/-}\) primary MEFs, as well as HK2 human cancer cells treated with NEK1 siRNA, are more sensitive to IR, and fail to arrest at G1/S and G2/M checkpoints [584, 585]. NEK1 expression and activity increase in response to IR, followed by the formation of NEK1 foci that co-localized with \(\gamma\)H2A.X and MDC1 at sites of DNA damage [584, 585]. NEK1 participates in the initiation and priming of the ATR response, as it is regulates the expression of ATRIP (ATR partner protein), the basal activity of ATR, and the formation of the ATR-ATRIP complexes [586]. Accordingly, cells lacking NEK1, or those that express NEK1 non-functional variants, are unable to properly repair their DNA [585].

NEK11 is part of a regulatory loop involved in CHK1-mediated checkpoint control after IR and irinotecan [109, 587]. Depletion of NEK11 in HeLa and HCT116 cells resulted in defective G2/M arrest in response to IR [109, 587]. Following irradiation, NEK11 is activated via CHK1, and goes on to phosphorylate CDC25A on Ser273, which is required for SCF\(^{\beta-TRCP}\)-mediated polyubiquitination and degradation of CDC25A, a critical step in inducing G2/M arrest [109, 588].

RNAi library screens have pointed to NEK8 and NEK9 as effectors within the ATR-CHK1-mediated replication stress response (RSR). An siRNA screen to identify genes involved in the formation of DSBs in response to replication stress found NEK8 knockdown to cause aphidocolin hypersensitivity. Although NEK8 did not affect checkpoint fidelity, it was found to decrease cyclin A-associated CDK activity, and directly interact with ATR-ATRIP and CHK1 [589]. NEK8 also impacts replication fork stability through regulation of RAD51 localization to DSBs. NEK8\(^{-/-}\) MEFs were sensitive to hydroxyurea, a well characterized replication inhibitor [590]. Similar to NEK8, NEK9 loss
was found to cause hypersensitivity to replication stress, decreased CHK1 activation after stress and spontaneous DSBs [591].

Certain members of the NEK kinase family, such as NEK2 and NEK6, are downregulated in response to DNA damage. NEK2 kinase activity is reduced in response to IR in an ATM- and PP-1 dependent manner, resulting in a decrease in centrosomal splitting following IR [592, 593]. Similarly, NEK6 kinase activity is abolished in response to IR and UV irradiation [594]. Interestingly, NEK6 may control p53- and DNA damage-induced senescence, as loss of NEK6 or the expression of kinase-dead NEK6 causes premature senescence upon treatment with doxorubicin and camptothecin [595, 596]. Taken together, multiple lines of evidence implicate NEK family kinases as both positive and negative effectors and regulators of the cellular response to DNA damage and replication stress.

1.8 NEK10

NEK10 is the most divergent NEK family member, with unique protein architecture. At the amino acid sequence level, the kinase domain of NEK10 is closely related to that of NEK6 and NEK7. However, unlike other NEKs, which feature N-terminal kinase domains, the kinase domain of NEK10 is centrally located and flanked by two coiled coil domains (Figure 1.8). The N-terminal half of NEK10 harbours 4 armadillo repeats of unknown function [597-600].

Previous work from our laboratory has shown NEK10 involvement in the cellular response to UVC irradiation (Figure 1.8b) [601]. NEK10 was found to induce ERK1/2 hyperactivation in response to UVC in human cancer cell lines (HEK293T, MCF-7, and
Figure 1.8 NEK10 and its role in the DDR

A) The structure of NEK10, with domains of significance indicated. B) Reported role for NEK10 in G2/M induction and fidelity by the formation of a ternary complex with RAF and MEK. This leads to MEK autoactivation and ERK dependent G2/M arrest.
MCF10A). NEK10 formed a ternary complex with RAF-1 and MEK1/2, in a RAF-1 dependent manner, and this complex was necessary for UVC-induced MEK1/2 autoactivation and subsequent ERK1/2 hyperactivation. Upon UVC treatment, the NEK10-ERK1/2 axis was found to be crucial for G2/M checkpoint maintenance [601].

1.8.1 NEK10 in Cancer

Large scale cancer genomic studies have implicated NEK10 in carcinogenesis. A comprehensive genome wide association study (GWAS) identified a strong breast cancer susceptibility locus within a sub-region of human chromosome 3p24 containing the NEK10 gene (p value =4.1 x 10^{-23}) [602]. A follow-up study found this NEK10 SNP to associate with breast cancer susceptibility in BRCA2 carriers [603]. Cancer genome sequencing projects have also reported NEK10 mutations in several human cancers. For instance, NEK10 was one of the only 21 protein kinases in the whole kinome (518 genes) that displayed multiple nonsynonymous somatic mutations within a group of 26 primary lung neoplasms and seven lung cancer cell lines [604, 605]. To date, 240 unique coding NEK10 mutations have been reported in COSMIC, mapping to various regions. The most prevalent affected loci are P611L (5 mutations) within the kinase domain and E882K (6 mutations) in the C-terminal region [204]. In renal clear cell carcinoma, NEK10 is homozygously deleted in 13% of the cases (56/488) (TCGA Research Network: [http://cancergenome.nih.gov/](http://cancergenome.nih.gov/)).

Outside of the multiple mutations found in NEK10 in various cancer types, lowered NEK10 expression associates with poor breast cancer prognosis [601]. Low NEK10 expression correlates with a >20% reduction in 5-year and overall disease-free survival,
as well as higher tumour grade (p = 0.0014) [601]. Moreover, lowered NEK10 expression separates with a decrease in 10 year overall survival in lung (p = 3.7 x 10^{-7}), breast (p = 1.9 x 10^{-4}) ovarian (p = 4.8 x 10^{-4}), and gastric cancers (p = 0.017) in a combined analysis of the NCBI Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA) and The Cancer Genome Atlas (TCGA) datasets using KM plotter (Figure 1.9) [606]. Taken together, these studies indicate lowered NEK10 expression as a potential risk factor for cancer progression and severity, and highlight the need for further studies into the function of NEK10 in carcinogenesis.
Figure 1.9 Lowered NEK10 expression is associated with decreased 10 year overall survival.

Lowered NEK10 expression separates with a decrease in 10 year overall survival in lung (p=3.7 x 10^{-3}), breast (p=1.9 x 10^{-4}) ovarian (p=4.8 x 10^{-3}), and gastric cancers (p=0.017) in a combined analysis of the NCBI Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA) and The Cancer Genome Atlas (TCGA) datasets using KM plotter online tool [606].
1.9 Ph. D. OUTLINE

1.9.1 Study Rationale

The DNA Damage Response (DDR) is a cellular process responsible for the maintenance of genomic stability in response to a host of endogenous and environmental insults. Recent work has uncovered key functions for the members of the NIMA Related Kinase (NEK) family, such as NEK1, NEK8, NEK9, NEK10 and NEK11 in the DDR. NEK10 is a largely uncharacterized member of the NEK kinase family that has been implicated in the response to UV irradiation. However, NEK10’s physiological function is yet to be determined.

In chapter 2, I used CRISPR-mediated genetic deletions of NEK10 in A549 cells to uncover a role for NEK10 in the control of p53 mediated transcription. Mechanistically, I found that NEK10 phosphorylates Y327 on p53, leading to increased transcriptional activity of p53.

In chapter 3, I sought to characterize the dual specificity kinase activity of NEK10. In collaboration with Dr. Michael Yaffe’s group at MIT, I determined the substrate recognition sequence for the tyrosine kinase activity of NEK10 and used this consensus sequence to compile a list of candidate NEK10 substrates.
1.9.2 Thesis Objectives

-Determination of the physiological relevance of NEK10 by using \textit{NEK10}-deficient cell lines

-Characterisation of the function of NEK10-mediated tyrosine phosphorylation of p53 on Y327

-Characterisation of the tyrosine specific kinase activity of NEK10
CHAPTER 2:
Regulation of p53 dependent transcription by NEK10 mediated phosphorylation
2.1 INTRODUCTION

The eukaryotic cell responds to various forms of DNA damage through distinct DNA damage response (DDR) pathways. These molecular circuits first function to recognize the damaged DNA and suppress cell cycle progression, to provide time for repair of genetic material or, if the damage is deemed irreparable, carry out an apoptotic programme. Deregulation of DDR networks leads to genetic abnormalities, chromosomal instability and accumulation of mutations that can initiate neoplastic transformation and, ultimately tumorigenesis. DDR networks are subject to complex regulation which integrates multiple input signals to titer the cellular response to DNA damage. One of the master regulators of the cellular response to DNA damage is the tumour suppressor p53.

p53 is a transcription factor that coordinates an extensive gene expression program, both basally and in response to various forms of cellular stress. p53 transcriptional targets govern a series of cellular processes, including cell cycle control, apoptosis, senescence, DNA repair, metabolism, immune response and migration [275, 278, 279, 478, 607-609]. The transcriptional activity of p53 is dependent on its ability to bind to the p53 response elements (RE) within gene promoters, leading to upregulation of genes involved in the control of cell cycle control, apoptosis and DNA repair, and repression of pro-growth genes [275, 278, 279, 610, 611]. Loss of p53 can lead to genomic instability and the acquisition of mutations that effect proliferation, transformation, therapeutic resistance and metastasis [478, 612]. TP53 germline mutations are the cause of the hereditary disorders: Li-Fraumeni syndrome (LFS) and Li-Fraumeni-like syndromes (LFL), rare diseases that lead to a predisposition to early onset
cancers [274]. Somatic mutations of TP53 are some of the most frequent in human cancers with an incidence greater than 50% (http://p53.iarc.fr/).

In response to even low levels of DNA damage or stress, p53 protein is modulated through multiple posttranslational modifications, including phosphorylation, ubiquitination, methylation, acetylation, and SUMOylation, that act to direct and titrate the cellular p53 response [284, 332, 607, 613]. For example, in healthy cycling cells, p53 protein is kept at low levels by its negative regulator, the E3 ubiquitin ligase, MDM2 and its co-regulator MDMX [339, 343]. Following DNA damage, ATM/ATR phosphorylates p53 on S15, disrupting its interaction with MDM2, leading to an accumulation of p53 protein and increased transcription of multiple genes associated with the cellular response to DNA damage [379, 380, 403, 614-616].

Alongside p53, multiple other inputs have been implicated in control of the cellular response to DNA damage. Recently, members of the NEK kinase family have been linked to cell cycle checkpoint control and DNA repair. In response to ionizing radiation (IR), NEK1 and NEK11 have been shown to positively regulate checkpoint integrity and DNA repair, whereas inactivation of NEK2 or NEK6 is required for proper checkpoint engagement [109, 585, 587, 588, 592-594, 617-619]. Moreover, both NEK8 and NEK9 have been shown to participate in DNA repair and the response to replicative stress [589-591], whereas NEK10 has been found to control G2/M checkpoint integrity in response to ultraviolet (UV) irradiation [601].

Cancer genomic characterization efforts have correlated NEK10 status with cancer incidence and outcome. Cancer genome sequencing projects have reported NEK10
alterations and mutations in ~1% of human cancers [604, 605, 620-622]. Moreover, a comprehensive genome wide association study (GWAS) identified a strong breast cancer susceptibility locus within a sub-region of human chromosome 3p24 containing \textit{NEK10} [602]. Further, lowered \textit{NEK10} expression has been associated with poor breast cancer prognosis and higher tumour grade [601].

In this study, we describe a function for \textit{NEK10} in the regulation of p53 transcriptional activity. Work in knockout cell lines lacking \textit{NEK10} demonstrates its involvement in the control of cellular growth, DNA replication and sensitivity to genotoxic agents. Mechanistically, \textit{NEK10} regulates p53 through direct phosphorylation of Y327 within the p53 oligomerization domain
2.2 RESULTS

2.2.1 Loss of NEK10 leads to increased proliferation and DNA replication

To directly probe the biological function of NEK10, we generated A549 lung adenocarcinoma cell lines with a loss of Nek10 function by CRISPR mediated deletion of exon 24 of the NEK10 gene, which contains the crucial “DFG” motif required for kinase activity (A549 NEK10 Δ/Δ cells). Deletion of exon 24 led to a loss of NEK10 expression at the mRNA and protein level. Cells that had been subject to the NEK10 deletion strategy but did not undergo exon 24 deletion were used as matched controls for the experiments in this chapter (A549 NEK10 +/+ cells).

Phenotypic characterization of NEK10 Δ/Δ cells, revealed an increase in their proliferation and colony forming ability (Figure 2.1 a-b). Despite the differences in cellular proliferation, cell cycle distributions of NEK10 +/+ and NEK10 Δ/Δ cells were indistinguishable, pointing to the intact basal checkpoint fidelity in the absence of NEK10 (Figure 2.2 a). Considering that propidium iodide staining of asynchronous cells only provides a “snapshot” of the DNA content of a population of cells, we pulse labeled cells with 5-ethynyl-2’-deoxyuridine (EdU), a nucleoside analog of thymidine that is incorporated into DNA during active DNA synthesis. Measurement of the proportion of cells with active DNA synthesis (EdU+ cells), showed increased DNA synthesis in NEK10 Δ/Δ cells (Figure 2.2 b). Consistently, staining of cells with phosphorylated Histone H3 (Ser10), a mitotic marker, indicated a higher proportion of NEK10 Δ/Δ cells were undergoing mitosis, further supporting a role for NEK10 in the control of cellular proliferation (Figure 2.2 c).
Figure 2.1 NEK10 deletion leads to an increase in cellular proliferation.

A) Proliferation curve of A549 Nek10 +/- and A549 Nek10 Δ/Δ cells measured using SRB assay over a 72 hour time period. Relative growth was calculated relative to absorbance at t=0 for each cell line (p<0.002, t test, n=3, bars represent SEM). B) Colony forming assay of Nek10 +/- and Nek10 Δ/Δ A549 cells. 100 cells were seeded and plating efficiency is indicated (p<0.05, t test, n=3, bars represent SEM)
Figure 2.2 NEK10 loss leads to increase in DNA replication and mitotic index.

A) Cell cycle distribution was determined using propidium iodide staining for total DNA content. G1, S and G2 fractions were determined using FlowJo. B) Nek10 +/- and Nek10 Δ/Δ A549 cells were pulse labelled with EdU for 2 hours. Flow cytometry was performed to determine the proportion of cells with active DNA replication. C) Asynchronous cells were stained with phospho-H3 (Ser10) antibody to determine the amount of mitotic cells. (p<0.01, t test, n=3, bars represent SEM).
2.2.2 NEK10 regulates transcription of p53 target genes

To explore the mechanism of NEK10-mediated control of cellular proliferation and DNA synthesis, we assessed the cellular levels of p53 and the p53-responsive genes p21 and MDM2, which are crucial for the regulation of these processes [33, 36, 481, 482, 623, 624]. When compared to parental A549 and NEK10 +/+ cells, NEK10 Δ/Δ cells displayed lowered expression of p21 and MDM2 (Figure 2.3 a). This was not due to alterations in p21 or p53 stability, as their half-lives were indistinguishable between wildtype and NEK10 knockout cells (Figure 2.3 b). We next explored the mRNA levels of multiple p53 target genes (p21, p53, MDM2, GADD45α, BAX, PUMA, and CDC25A). Quantitative reverse transcription PCR (RT-qPCR) analysis showed decreases in all of the assayed p53 responsive genes, with the greatest reduction in the expression of p21, p53, and MDM2 (Figure 2.3 c). Consistent with this observation, re-expression of NEK10 in NEK10 Δ/Δ cells lead to an increase in the expression of both p21 and MDM2, while leaving p53 levels unaffected (Fig 2.3 d).

As p21 acts as an inhibitor of the activity of multiple cyclin dependent kinases (CDKs), as well as the other proteins that are involved in cell cycle progression and DNA replication, we sought to determine if the decrease in p21 expression affected its interaction with CDK2 [33, 36]. The reduction in p21-bound CDK2 was proportional to the overall decrease in p21 expression in NEK10 Δ/Δ A549 cells, indicating that the observed changes in proliferation may be due to a decrease in p21 expression (Figure 2.3 e).
Figure 2.3 Involvement of NEK10 in the expression of p53-responsive genes.

A) Immunoblot of A549 Nek10 +/+ and Nek10 ΔΔ cells for the expression of indicated proteins.
B) Cycloheximide pulse chase experiment to determine half-life of indicated proteins.
C) qRT-PCR of p53 responsive genes in Nek10 +/+ and Nek10 ΔΔ A549 cells to determine relative expression levels in the context of Nek10 loss (p<0.01, t test, n=3, bars represent SEM).
D) Immunoblot of Nek10 ΔΔ A549 cells reconstituted with indicated lentiviral constructs.
2.2.3 NEK10 phosphorylates p53 on tyrosine 327

To determine the mechanism of p53 regulation by NEK10, we assessed the impact of NEK10 on p53-dependent transcription. While transfection of NEK10 led to an increase in the p53-dependent transcription of a luciferase reporter coupled to a 5' p21 response element, this effect was fully dependent on NEK10 kinase activity, as expression of a kinase dead NEK10 (A546F) led to no increase in p53 transcriptional activity (Figure 2.4).

We sought to determine if NEK10 directly phosphorylates p53. Active FLAG-NEK10 from transfected 293 cells readily phosphorylated GST-p53 in vitro (Figure 2.5a). Further, overexpression of NEK10 in 293T cells led to an increase in tyrosine phosphorylated p53, indicating the potential site of phosphorylation is a tyrosine residue (Figure 2.5b). We next queried the Phosphosite™ database for reported sites of p53 tyrosine phosphorylation found in phospho-mass spectroscopic studies and found 3 candidate sites: Y126, Y220 and Y327 [625]. As both Y126 and Y220 had been identified previously as being sites for Src phosphorylation, we focused on Y327 which was identified in 15 instances in independent mass spectrometric studies (Figure 2.5c) [625-628]. We next generated a p53 protein with a Y327F substitution, which largely abolished its phosphorylation by NEK10 in vitro (Figure 2.5d).

Prompted by these results, we investigated the possible function of Y327 in p53 transcriptional activity. Judging by expression of a panel of p53-target genes, p53 Y327F acted as a hypomorph to WT p53 upon expression in HCT116 p53 -/- cells, showing partial impairment in transcriptional activity, albeit to a lesser extent than the one caused by the p53 L344P mutant that does not oligomerise and as such lacks transcriptional activity (Figure 2.6 a-e) [629].
Figure 2.4 NEK10 regulates p53 transcriptional activity in a kinase dependent manner.

A luciferase reporter assay was performed to monitor for the effect of Nek10 on p53 transcriptional activity. H1299 cells were transfected with HA-p53, pGL3-p21 5'RE, Renilla and the indicated FLAG-NEK10 constructs. Relative p21 promoter activity was measured in relative to cells overexpressing FLAG (p<0.05, t test, n=3, bars represent SEM).
Figure 2.5 NEK10 phosphorylates p53 on Y327 in vitro.

A) Radioactive in vitro kinase assay performed using purified FLAG-NEK10 WT and kinase dead (A546F) incubated with GST alone or GST-p53. B) 293T cells transfected with the indicated constructs were lysed under denaturing conditions and phosphotyrosine proteins were immunoprecipitated. Immunoblots for the amount of pY-p53 tyrosine were performed subsequently.

C) Schematic representation of the structure of p53 highlighting the location of Y327 with relevant domains indicated.

D) Radioactive in vitro kinase assay performed using purified FLAG-NEK10 WT and the indicated GST-p53 constructs. Relative phosphate transfer was quantified and is shown (p<0.05, t test, n=3, bars represent SEM).
Figure 2.6 Y327F mutation decreases the expression of p53 target genes

A) qRT-PCR of p53 responsive genes in HCT116 p53 −/− cells reconstituted with indicated constructs to determine relative expression levels compared to empty vector (EV). B) Heatmap of qRT-PCR of p53 responsive genes indicating expression of indicated gene relative to empty vector (EV). C) qRT-PCR of p53 responsive genes in HCT116 p53 −/− cells reconstituted with indicated constructs to determine relative expression levels compared to WT-p53 (*= p<0.05, **=p<0.005, t test, n=3, bars represent SEM). D) Heatmap of qRT-PCR of p53 responsive genes indicating expression of indicated gene relative to WT-p53. E) Immunoblot of HCT116 p53 −/− cells reconstituted with indicated His-p53 constructs. Quantifications of expression levels of p21 relative to cells overexpressing WT-p53 are indicated. F) Immunoblot of H1299 cells reconstituted with indicated variants of FLAG-Nek10 and His-p53. Quantifications of expression levels of p21 relative to cells overexpressing FLAG and WT-p53 are indicated.
The relationship between p53 Y327 and NEK10 kinase activity was further assessed by co-expression of WT or kinase dead (A546F) NEK10 with WT p53 or p53 Y327F, respectively, in H1299 cells (p53-null) (Figure 2.6f). The expression of p21 was increased by the co-expression of WT p53 and WT NEK10, pointing to NEK10 as an inducer of p53 transcriptional activity. This effect was highly dependent on the Y327 site, as the increase in p21 levels diminished in cells co-expressing WT NEK10 and p53 Y327F. Accordingly, the kinase-dead mutant of NEK10 (A546F) failed to effect p21 levels in combination with WT p53 (Figure 2.6f).

Given the observed defects in transcriptional activity upon Y327F mutation, we sought to uncover the specific role of Y327 on p53 function. Despite its location in the oligomerization domain of p53, and previously reported data indicating that nitration of this amino acid influenced oligomerization and transcriptional activity, Y327F mutation failed to influence p53 oligomerization (Figure 2.7a) [459, 630]. Nevertheless, judging by electrophoretic mobility shifts assays (EMSA) p53-Y327F was partially defective in binding to a canonical p53 RE, indicating a defect in DNA binding (Figure 2.7b).

2.2.4 NEK10 is required for proper induction of DDR pathways

In light of the possible role for NEK10 in the regulation of p53 and previous work pointing towards a role for NEK10 in the DNA damage response, led us to investigate the effect of NEK10 loss in the context of DNA damage [601]. In response to cisplatin treatment, NEK10 loss impaired induction of certain p53 responsive genes, such as p21, GADD45α, and PUMA (Figure 2.8 a). Further, IR treatment led to a rapid and sustained increase in both p21 and p53 protein levels in the NEK10 +/+ cells, with maximum levels reached 30 minutes after exposure, in contrast to the NEK10 Δ/Δ cells which took up to
Figure 2.7 Y327 plays a role in the binding of p53 to response elements

A) HCT116 p53 +/- expressing indicated constructs were lysed and lysates were treated with 0.025% glutaraldehyde to cross link proteins and immunoblotted to assess the degree of p53 oligomerization. The expression of total p53 in the lysate prior to cross linking is indicated as input.

B) Electrophoretic mobility shift assay (EMSA) was performed using nuclear lysates from H1299 cells expressing indicated constructs that had been incubated with a fluorescent p53 RE' probe. The reactions were run on a non-denaturing polyacrylamide gel and imaged to determine the DNA binding capacity of WT p53 compared to p53 Y327F. The expression of total p53 in the nuclear lysate used in the EMSA is indicated as input.
4 hours to reach maximum levels of p21 expression (Figure 2.8 b, c). Moreover, this effect did not depend on differential phosphorylation of p53 by ATM, as evidenced by equivalent levels of p53 Ser15 phosphorylation in both cell lines (Figure 2.8 a, b). Moreover, IR exposure prompted tyrosine phosphorylation of WT p53, which was abolished by p53 Y327F substitution (Figure 2.9a). Consistently, in response to IR, the p53 Y327F mutant failed to fully support expression of p21 and MDM2 (Figure 2.9b).

We next assayed the recognition of DNA damage and the efficiency of DNA repair in NEK10 Δ/Δ cells. Comparable formation of γH2A.X and 53BP1 foci, key indicators of double stranded breaks, in NEK10-deficient and wildtype cells at 4 and 24 hours after irradiation indicated that there is no defect in the recognition or clearing of DSB breaks in the absence of NEK10 (Figure 2.10 a,b). Downstream of DSB recognition, multiple repair pathways are activated, resulting in the recruitment and assembly of multi-protein complexes[631]. Both BRCA1 and RAD51 are key proteins involved in homologous recombination. Compared to WT cells, NEK10 Δ/Δ cells displayed reduced levels of BRCA1 and RAD51 recruitment to sites of DNA damage after irradiation (Figure 2.10 c, d). Despite this decrease in recruitment of BRCA1 and RAD51, 24 hours after irradiation, both WT and NEK10-null cells resolved these foci equally. Nevertheless, NEK10 Δ/Δ cells were deficient in the repair of a DR-GFP plasmid-based reporter system, a quantitative measure of homologous recombination mediated DNA repair (Figure 2.10 e) [632, 633].

Considering the NEK10-dependent impairment in the p53 function and DNA repair, we probed the effect of NEK10 loss on cellular sensitivity to escalating doses of ionizing radiation, cisplatin, or olaparib using clonogenic assays (Figure 2.11 a-c). This apparent treatment-specific effect on clonogenic survival was paralleled by the impact of NEK10
Figure 2.8 Nek10 modulates the expression of p53 responsive genes in response to genotoxic agents

A) Nek10 +/- and Nek10 Δ/Δ A549 cells were exposed to 5 uM of cisplatin and were lysed 20 hours after treatment.

B) Nek10 +/- and Nek10 Δ/Δ A549 cells were exposed to 5 Gy of IR and were lysed at the indicated time points after treatment. C) Quantifications of expression levels of p21 relative to p21 levels of Nek10 +/- cells at T0 are indicated (n=3, bars represent SEM, *=p<0.005)
loss on DNA damage-induced cell cycle arrest. In response to cisplatin treatment and in contrast to NEK10 +/- cells, NEK10 Δ/Δ cells were impaired in induction of G2/M arrest (Figure 2.12 a, b), whereas such differences were not apparent in the same cells treated with IR (Figure 2.12 c, d). Compared to NEK10 +/- cells, the cisplatin-specific G2/M arrest defect in NEK10 Δ/Δ cells was accompanied by heightened cell death, evidenced by the increased proportion of both the sub-G1 and Annexin V+/PI+ cells, (Figure 2.12 e, f).
Figure 2.9 NEK10 tyrosine phosphorylates p53 in response to ionizing radiation

A) H1299 cells transfected with the indicated constructs were treated with 5 Gy of ionizing radiation and lysed 30 minutes after treatment under denaturing conditions and phosphorylation proteins were immunoprecipitated. Immunoblots for the amount of pY-p53 tyrosine were performed subsequently. B) H1299 cells transfected with the indicated constructs were lysed under denaturing conditions 30 minutes after IR treatment and immunoblots were performed to monitor the expression of the indicated proteins. Quantifications of expression levels of p21 relative to untreated cells are indicated.
Figure 2.10 NEK10 loss leads to defects in recruitment of RAD51 and BRCA1 to sites of damage and affects the efficiency of HR.

A) D) A549 Nek10 +/- and A549 Nek10 Δ/Δ were exposed to 5 Gy of irradiation for the indicated times and immunostained for γH2AX, 53BP1, BRCA1, and RAD51 as indicated and DAPI was used as a DNA/nuclear stain. Cells containing >5 foci were scored as positive. (p<0.01, t test, n=3, bars represent SEM). E) Nek10 +/- and Nek10 Δ/Δ A549 cells were transfected with DsRed and I-Scel cut pDR-GFP plasmids. The relative efficiency of repair was calculated as the ratio of GFP+ cells to DsRed+ cells (p<0.001, t test, n=3, bars represent SEM).
Figure 2.11 Nek10 loss sensitizes cells to chemotherapeutic agents.

A)  Nek10 +/- and Nek10 ΔΔ A549 cells were treated with increasing doses of Olaparib, cisplatin or IR and assessed for clonogenic survival 10-12 days after treatment. Graph represents percent of cells that survive after treatment compared to untreated control (p<0.005, t test, n=3, bars represent SEM).

B)  Nek10 +/- and Nek10 ΔΔ A549 cells were treated with increasing doses of Olaparib, cisplatin or IR and assessed for clonogenic survival 10-12 days after treatment. Graph represents percent of cells that survive after treatment compared to untreated control (p<0.005, t test, n=3, bars represent SEM).

C)  Nek10 ΔΔ A549 cells were treated with increasing doses of Ionizing Radiation and assessed for clonogenic survival 10-12 days after treatment. Graph represents percent of cells that survive after treatment compared to untreated control (p<0.005, t test, n=3, bars represent SEM).
Figure 2.12 Differential cell cycle arrest in response to cisplatin treatment.

A) Cells were treated with 5 uM of cisplatin overnight and allowed to recover for 24 H prior to being analysed for DNA content with propidium iodide. B) Quantification of G2 populations for cisplatin treated cells as an indicator of G2/M arrest (p<0.01, t test, n=4, bars represent SEM). C) Cells were treated with 5 Gy of IR and allowed to recover for the indicated times prior to being analysed for DNA content with propidium iodide. D) Quantification of G2 populations for IR treated cells as an indicator of G2/M arrest (p<0.01, t test, n=3, bars represent SEM) E) Cells were treated with 5uM cisplatin for 20 hours and cells were assessed for their proportion of sub-G1 cells, an indicator of cell death, at the indicated time points after treatment (p<0.05, t test, n=3, bars represent SEM). F) Cells were treated with indicated doses of cisplatin and assessed for apoptosis with Annexin V/propidium iodide staining 24 hour after overnight cisplatin treatment.


2.3 DISCUSSION

The cellular response to DNA damage is a complex network of many intersecting pathways that are modulated or activated in a context dependent manner whether it be the phase of the cell cycle in which the damage is encountered, the type/degree of damage, the extracellular milieu, or the mutational status of the cell itself. However central to many of these response pathways is the tumour suppressor p53 [332, 478, 480, 634, 635]. To date, p53 has been shown to have multiple regulatory inputs through protein-protein interactions and posttranslational modifications that titrate its activation and function. The work described above highlights the potential for the protein kinase NEK10 to play a key role in the regulation of p53 transcriptional activity, both under basal conditions and in response to IR and cisplatin.

2.3.1 Nek10 regulates p53 transcriptional activity

When using CRISPR-mediated deletion of NEK10 we discovered an increase in cellular proliferation and DNA replication (Figure 2.1, 2.2). This was unexpected as previous work with siRNA mediated knockdowns of NEK10 did not affect proliferation [601]. Consistently, deletion or knockdown of other NEK family kinases, including the close NEK10 homologues, NEK6/7 has only been reported to either lead to a decrease in cellular proliferation or cell death [553, 575, 636]. In order to delve deeper into the mechanism behind the observed defects, we chose to assess the levels of the tumour suppressor p53 and its key target p21 as they are both key regulators of cell cycle progression [623, 624]. We noticed a decrease in the expression of p53 target genes, primarily p21 that was independent of p53 protein stability, this pointed to a potential role
for NEK10 in the regulation of p53 activation (Figure 2.3a, c). We also observed an increase in p53 expression, which may be due to the p53 RE that has been recently discovered in the human promoter for p53 and may contribute to the overall phenotypes observed [637].

2.3.2 NEK10 phosphorylates p53 at Tyrosine 327

Prompted by the potential role for NEK10 in the stimulation of p53 target gene expression, we went on to look at the effect of NEK10 kinase activity on the expression of p21 (Figure 2.4). We observed a marked increase in transcriptional activity in a kinase activity manner, suggesting a potential role for NEK10 kinase activity in the regulation. Further, NEK10 appeared to phosphorylate Tyrosine 327 and mutation of this residue to a phenylalanine (Y327F) reduced the expression of p53 target genes in a stimulus dependent manner and make act as a hypomorphic p53 mutant (Figure 2.5, 2.6).

When attempting to assess the effect of Y327F mutation on p53 function we found that it did not have a stark effect on the oligomerization ability of p53, despite its location in the oligomerization domain of p53 (Figure 2.7 a). This is in concordance with studies in yeast indicating that Y327 mutation has no effect on oligomerization [629, 638]. In contrast, computational studies and studies assessing the effect of nitration of Y327 in response to nitric oxide treatment pointed a role for Y327 in the stabilization of p53 dimers and tetramers [459, 630]. To further assess the effect of Y327F on p53 function, we found a decrease in the DNA binding ability of Y327F mutant p53, indicating the potential that this mutant, although capable of forming tetramer, leads to a decrease in DNA-binding capacity (Figure 2.7b). This defect may possibly occur through an action at a distance effect, whereby this mutation leads to improper positioning of the
DBD in relation to the p53 RE or tyrosine phosphorylation may act to increase DBD capacity of p53.

2.3.3 NEK10 is required for proper induction of DDR pathways

The requirement for NEK10 in p53-dependent transcription was further highlighted upon treatment with ionizing radiation or cisplatin, both inducers of p53 activation and transcriptional activity (Figure 2.8 a, b) [639, 640]. Each stimulus elicited a slightly different response as in response to IR, p53 and p21 protein levels increased rapidly in NEK10 +/+ cells compared to NEK10 Δ/Δ cells (Figure 2.8 b). In the case of cells treated with cisplatin, there was no obvious modulation of either p53 protein levels or p53 Ser15 phosphorylation, however NEK10 Δ/Δ cells failed to induce the expression of multiple p53 target genes (Figure 2.8 a).

Furthermore, despite not having been characterized previously, tyrosine phosphorylation of p53 increases in an IR-dependent manner (Figure. 2.9 a). This may point to a potential mechanism whereby Y327 phosphorylation acts to stimulate the transcriptional activity of p53 in response to genotoxic treatment as the Y327F mutation decreases the induction of p21 in response to IR (Figure 2.9b). The crosstalk between Y327 and S15 phosphorylation is unclear, as NEK10 may act to increase total p53 levels and the relevance of Ser15 in this process is nebulous. There may be a Ser15 independent function for Y327 phosphorylation as suggested upon nitration of p53 on Y327, or an additive effect which leads to rapid and sustained activation of p53 in response to specific genotoxic stresses [459]. It is likely the latter as Ser15 phosphorylation is induced in response to IR and has been shown to be required for p53 transcriptional activity [641].
The attenuated p53 response in **NEK10 Δ/Δ** cells likely contributed to the differential clonogenic survival when compared to the **NEK10 +/+** cells (Figure 2.11) [501, 642-647]. NEK10-deficient cells are markedly more sensitive to higher doses of cisplatin but not IR, which is surprising given that p53 activity has been previously associated with cellular apoptosis in response to cisplatin treatment (Figure 2.11b) [640]. Moreover, **NEK10 Δ/Δ** cells failed to engage an effective G2/M arrest in response to cisplatin, possibly due to the lack of induction of GADD45α, a key mediator of the G2/M checkpoint, but were capable of eliciting apoptosis (Figures 2.8a, 2.12 a, b, e, f) [484, 648, 649]. This selective p53 response is similar to that elicited by PRMT5 methylation of p53 upon UV treatment, where a cell cycle arrest program rather than execution of the p53 apoptotic program is induced [445].

Multiple other NEK family members such as NEK1, NEK8, NEK9, and NEK11 have been shown to play key roles in DNA repair [109, 584-591, 650]. To probe the function of NEK10 in this process we assessed the recruitment and clearance of various factors that recognize DSBs (γH2A.X and 53BP1) and factors involved in HR/NHEJ (BRCA1, RAD51 and 53BP1) in the context of NEK10 loss (Figure 2.10). Upon treatment with radiation NEK10 deficient cells displayed defective recruitment of RAD51 and BRCA1 at 4 and 8 hours after irradiation. However, it appeared that both **NEK10 +/+** and **NEK10 Δ/Δ** were able to clear and potentially fully repair the DSBs caused by IR. To determine if the decrease in the recruitment of RAD51 and BRCA1 observed in **NEK10 Δ/Δ** cells was indicative of a defect in HR, we used a DR-GFP reporter based system to assess HR fidelity and observed a reduction is the amount of Sce-I cleaved DR-GFP plasmids repaired by HR (Figure 2.10 e).
This suggests that the failure to timely recruit HR factors may result in ineffective HR, in a NEK10 dependent manner. However, radiation induced DSBs appear to be fully repaired in both $NEK10^{+/+}$ and $NEK10^{Δ/Δ}$ cells as measured by the clearance of IRIFs. This observation may imply that $NEK10^{Δ/Δ}$ cells rely on alternative modes of DSB repair such as NHEJ or that $NEK10^{Δ/Δ}$ cells bypass improperly repaired DSBs and DSB induced arrest. As such DNA-PKcs and Ku70/80 foci, both markers of NHEJ and DR-GFP reporter systems that measure NHEJ should be used to probe this hypothesis [632, 651]. In parallel the proportion of cells with CtIP foci should also be measured, as it has been shown to be crucial for DNA repair pathway choice [652, 653]. Furthermore, given the cell cycle dependence of DNA repair, the repair pathway choice during various cell cycle phases should also be assessed [654].

The dependence of this DNA repair effect on p53 transcriptional activity remains an open question as p53 has largely been shown to suppress HR, in order to suppress unscheduled recombination events [504]. This largely occurs through the suppression of transcription of factors associated with HR such as RAD51 and BRCA1 [538, 655, 656]. The observed defect in HR may be explained by the decrease in the expression of specific p53 target genes in NEK10-null cells. A recent study uncovered p21 as a promoter of HR in response to Mitomycin C [657]. The deletion or loss p21 was further shown to lead to an increase in NHEJ activation in place of HR. Another study identified the p53 isoform Δ133p53, as a regulatory target of p53 that is induced upon IR treatment. It was shown to upregulate the transcription of RAD51, LIG4, and RAD52 through a novel p53-RE effecting HR activation [658].
The work in this chapter, lays out a potential for mechanism by which NEK10 activates and supports p53 transcriptional activity under conditions of genotoxic treatment through the phosphorylation of Y327. Further, it identifies the phosphorylation of Y327 as a potential mechanism whereby the cell may elicit a specific p53-dependent transcriptional program that in response to DNA damage leads to G2/M arrest. Taken together, these results may provide a better understanding of the role of NEK10 mutation or loss in tumorigenesis and clarify the role of p53 in the sensitivity or resistance to various radiotherapeutic and chemotherapeutic regimens.
2.4 MATERIALS AND METHODS

Unless otherwise stated all products were purchased from Sigma-Aldrich

2.4.1 Antibodies

The following antibodies were purchased from Santa Cruz Biotechnology:
GAPDH FL-335 (sc-25778), RAD51 H-92 (sc-8349), p53 FL-393 (sc6243), p-Tyr PY-99 (sc-7020), CDK2 (sc-163), GADD45α C-4 (sc-6580), BRCA1 D-9 (sc-6954). FLAG M2 (F3165) and FLAG M2 Agarose were from Sigma-Aldrich. Phospho-Histone H2A.X (Ser139) (JBW301) (05-636), and p-Tyr 4G10 (05-321) were from Millipore. The antibody against 53BP1 (A300-272A) was from Bethyl Laboratories. Anti-p21 (556431) was from BD Pharmingen. MDM2 Ab-1 (OP46) was from Calbiochem. Anti-PUMA (NB500-261). Anti-p53 S15 (9284) was Cell Signalling Technology.

2.4.2 Cell culture, transfections, viral infections and reagents

HEK293T, HEK293T NEK10 Δ/Δ, A549, A549 NEK10 Δ/Δ, HCT116 p53+/+, HCT116 p53−/−, and H1299 cells and their derivatives were maintained in DMEM (Corning), supplemented with 10% FBS (Wisent) and Pen/Strep (100 mg/ml, Hyclone).

A549 NEK10 Δ/Δ were generated through a CRISPR-Cas9 nickase strategy targeting exon 24 of the NEK10 genetic locus. A549 cells were transfected with pSpCas9n(BB)-2A-Puro (Addgene) and single guide RNAs (sgRNAs) (5’ TAAGGTATCATGCCCTCAT 3’ and 5’ CTACACTTCATAACCAA 3’). 48 hours post transfection, Cas9 nickase expressing cells were selected for using 1.5 μg/mL puromycin (Sigma-Aldrich) for a further 48 hours. Single cell clones were picked and grown in 24 well plates and assessed for NEK10 deletion and expression by genomic PCR and qRT-PCR, respectively.
Transient transfections of HEK293T cells were performed by using calcium phosphate transfections. Transfection of other cell lines was achieved using PolyJet™ according to manufacturer's instructions.

All plasmids used were designed and cloned in the Stambolic lab with the exception of plasmids encoding pDR-GFP (Addgene), and pLVX-3xFLAG-NEK10 series of constructs (Gift of Dr. Michael Yaffe).

For expression in mammalian cells, NEK10 was cloned into 3xFLAG-CMV-7.1, and p53 was cloned into pcDNA3-His. For expression of recombinant proteins in bacterial cells, p53 was cloned into pGEX2TK. All point mutants were generated by using the QuickChange site-directed mutagenesis kit (Stratagene).

Cells were subjected to the following genotoxic treatments: Ionizing radiation was administered by the Gammacell 40 Exactor Cs137 Irradiator (Best Theratronics) at a dose rate of 0.95 Gy/min. Cisplatin (Hospira) was reconstituted in a 0.9% NaCl solution, and diluted in DMEM (Corning), supplemented with 10% FBS (Wisent) and Pen/Strep (100 mg/ml, Hyclone) prior to treatment. Olaparib (Astra-Zeneca) was reconstituted in DMSO, and diluted in DMEM (Corning), supplemented with 10% FBS (Wisent) and Pen/Strep (100 mg/ml, Hyclone) prior to treatment.

2.4.3 Cell lysis, immunoblotting and immunoprecipitations

Unless indicated otherwise, cells were lysed in Laemmli sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol), normalized for total protein content, resolved by SDS-PAGE, and transferred to PVDF membranes (Millipore). Membranes were blocked in 5% BSA and probed with the indicated antibodies.
For immunoprecipitations, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, supplemented with fresh 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate and a protease inhibitor cocktail (Sigma)) and sonicated followed by an incubation on ice for 20 minutes. Insoluble material was removed by centrifugation at 15,000 × g for 15 minutes at 4°C. Samples were then equalized using the Bradford protein assay, and incubated with the indicated antibody. Immunoprecipitations were performed by gentle end over end rotation for 3 hours, followed by incubation with Protein A/G-Sepharose beads for 45 minutes, and then immune complexes were washed four times in cold RIPA buffer and resuspended in Laemmli loading buffer.

For denaturing immunoprecipitations, cells were lysed in Laemmli buffer (50 mM Tris-HCl pH 6.8, 2% Triton X-100, 10% glycerol) and sonicated followed by an incubation on ice for 10 minutes. Insoluble material was removed by centrifugation at 15,000 × g for 10 minutes at 4°C. Samples were then equalized using the Bradford protein assay, diluted 15-fold with TBS +1% Tx-100 (50mM Tris pH 7.6, 125mM NaCl, 5% Glycerol, and 1% Triton X-100, supplemented with fresh 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate and a protease inhibitor cocktail (Sigma)) and incubated with the indicated antibody. Immunoprecipitations were performed by gentle end over end rotation overnight followed by incubation with Protein A/G-sepharose beads for 45 minutes, and then immune complexes were washed four times in cold Wash buffer (50mM Tris pH 7.6, 250mM NaCl, 5% Glycerol, and 1% Triton X-100, supplemented with fresh 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate and a protease inhibitor cocktail (Sigma)) and resuspended in Laemmli loading buffer.
2.4.4 Protein half-life determination

Cells were incubated with 200 μg/mL of cyclohexamide (Sigma-Aldrich) for the indicated times and lysed in Laemmli buffer loading buffer. Protein levels were detected via western blot.

2.4.5 Immunofluorescence

Cells were seeded onto glass coverslips in 24-well dishes, treated as indicated, and washed twice with PBS. Cells were then fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature and then simultaneously blocked, permeabilised and incubated with primary antibody in a PBS solution containing: 0.25% Saponin, 10% Normal Goat Serum, 0.1% Triton X-100, and 0.5% BSA for 3 hours at 37°C. The following primary antibody dilutions were used: RAD51 1:500, 53BP1 1:500, γH2A.X 1:1000, BRCA1 1:100. After three washes with TBS-T, 5 minutes each, at room temperature, coverslips were incubated for 45 minutes with 1:400 dilution of goat anti-rabbit IgG conjugated to Alexa 546 or goat anti-mouse IgG conjugated to Alexa 488 (Invitrogen Molecular Probes). After three washes with TBS-T, for 5 minutes each, coverslips were stained with DAPI. Coverslips were then washed with 3x TBS-T, 1x ddH2O, and 1x 70% ethanol. After air drying, coverslips were mounted in MOWIOL, and set overnight at 4°C. Images were taken using the AxioImager Microscope (Zeiss) and acquired using Metamorph software. For foci analysis, 100-200 cells were counted per analysis and image analysis was performed using ImageJ.

2.4.6 Cell Proliferation and Clonogenic Assays

Cell number was assessed indirectly by using the Sulforhodamine B (SRB) assay as described in [659].
Clonogenic assays were performed via seeding 100-1,000 cells/well in a 6 well plate and treating as indicated. Cells were allowed to recover and grow for 14-16 days. Cells were then washed twice with PBS and fixed and dyed with a solution containing: 0.5% Crystal Violet (w/v), 0.5% formal saline (w/v), 50% ethanol (v/v), and 145 mM NaCl for 20 minutes at room temperature. Cells were then washed three times in ddH_2O and air dried overnight. Plates were scanned and colonies were manually counted in ImageJ with the CellCounter plugin.

2.4.7 RNA isolation, reverse transcription and quantitative real time PCR (qRT-PCR) analysis

Total RNA was isolated from cells after the indicated treatments using the EZ-10 DNAaway RNA Miniprep kit (Bio Basic). Reverse transcription to generate cDNAs was performed using the qScript cDNA Supermix (Quantabio). Subsequent quantitative real-time PCR analysis was performed using PerfeCTa SYBR Supermix with 20 ng of cDNA per reaction and 200 nM of the following specific primers:

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AGCCTAAGATCATCAGCAATG</td>
<td>ATGGACTGTGAGTCATGAGTCTTT</td>
</tr>
<tr>
<td>p21</td>
<td>AGGCACCGAGGCACCTCAGAG</td>
<td>AGTTGTAAGAATCTGTTCATGCTG</td>
</tr>
<tr>
<td>p53</td>
<td>ATGGAGGAGCCTGCTAGAATGC</td>
<td>GCAGCGCCCTACAACCTCGTC</td>
</tr>
<tr>
<td>MDM2</td>
<td>AAGAGACCTGGTACGAAAGAC</td>
<td>TTTCTCTGTTCCTCAATTCGCTCT</td>
</tr>
<tr>
<td>GADD45α</td>
<td>TGCTCAGCAAGGCTTGTGAGT</td>
<td>GCTTGCCCGCTTGATACA</td>
</tr>
<tr>
<td>BAX</td>
<td>TGGAGCTGGCAGAGATGATTG</td>
<td>GAAGTGGCAGTCAAGAACATG</td>
</tr>
<tr>
<td>PUMA</td>
<td>GGGCCAGACTGTGATTCCT</td>
<td>ACTTGCTTCTCTAAACCTATG</td>
</tr>
</tbody>
</table>
Quantitative real-time PCR was performed on the Roche LightCycler 480, and relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method using GAPDH as a reference gene [660].

2.4.8 Cell cycle analysis by flow cytometry

For both forms of cell cycle analysis used, cells were seeded into 6cm plates, treated as indicated, trypsinized, and fixed with ice cold 70% ethanol (-20°C) with gentle vortexing. Cells were then fixed on ice for one hour.

For total DNA content analysis for cell cycle distribution, samples were washed twice with PBS and stained with 50 μg/mL propidium iodide and 20 μg/mL RNAse A in PBS + 0.1% Triton X-100. Stained cells were analysed on a FACSCanto flow cytometer (BD Biosciences) and CellQuest software (BD Immunocytometry Systems). Ten thousand events were analyzed for each sample on FlowJo.

For the quantification of mitotic cells, indirect immunofluorescence was used on ethanol fixed cells using an anti-phospho–H3 (Ser10) antibody (Millipore). Briefly, cells were washed twice with PBS, and permeabilised with 0.25% Triton X-100 and incubated on ice for 15 minutes. Cells were washed twice with PBS+ 1% BSA and incubated with 0.25 μg anti-phospho–H3 (Ser10) antibody with 1% BSA for 1 hour at room temperature. Cells were then incubated in goat anti-mouse IgG conjugated to Alexa488 (1:300 dilution) with 1% BSA at room temperature for 30 minutes under light protection. Cells were counterstained with a DNA content stain consisting of 50 μg/mL propidium iodide and 20 μg/mL RNAse A in PBS. Stained cells were analysed on a FACSCanto flow cytometer (BD Biosciences) and CellQuest software (BD Immunocytometry Systems). Twenty thousand events were analyzed for each sample on FlowJo.
2.4.9 Annexin V/Propidium Iodide assay for apoptosis

For Annexin V/PI assays, cells were evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (BD Pharmingen, San Diego, CA, USA). Briefly, cells were trypsinized, washed with PBS and 2x10^5 cells were stained with 5 μL of Annexin V-FITC and 10 μL of 50 ng/mL of Propidium iodide in 200 μL binding buffer (10mM HEPES, pH 7.4, 140mM NaCl, 2.5mM CaCl₂) for 15 minutes at room temperature in the dark. The proportion of apoptotic cells was determined using a FACSCanto flow cytometer (BD Biosciences) and CellQuest software (BD Immunocytometry Systems). Ten thousand events were analyzed for each sample on FlowJo.

2.4.10 Bacterial protein purification

GST-p53 and GST-p53 Y327F were produced in BL21 E.coli cells. Single colonies of transformed bacteria were inoculated as starter cultures and grown overnight at 37°C with gentle agitation. Starter cultures were diluted into large scale production cultures to an OD₆₀₀ of 0.05-0.1. Production cultures were allowed to grow until an OD₆₀₀ of 0.5 had been reached, subsequent to which cultures were incubated with 0.5 mM of IPTG for 4 hours. Cells were pelleted at 6000xg for 15 minutes, followed by snap freezing in liquid nitrogen. Cells were then thawed in a 37°C water bath and lysed with PBS +1% Triton X-100 + lysozyme. Cells were sonicated in 15 second pulses, followed by a 15 second recovery on ice, for a total of three cycles, and then incubated on ice for 30 minutes. Insoluble material was removed via centrifugation at 15,000xg for 15 minutes. The supernatant was then incubated with 20 μL/mL of Glutathione Sepharose 4B resin (GE) for 2 hours. Beads were washed three times with lysis buffer, and incubated with elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl pH 7.6, 150 mM NaCl), three times
for 10 minutes. Eluted fractions were pooled and protein amount and purity was
determined via SDS-PAGE Coomassie staining.

2.4.11 In Vitro Kinase Assays

Kinase assays were performed by the incubation of 100-200 ng of purified NEK10
recombinant protein (purification description in 3.4.3) at 30°C for 30 minutes in kinase
assay buffer (50mM Tris pH 7.6, 10mM MgCl₂, 10mM MnCl₂, 2mM EGTA, 20mM β-
glycerophosphate, 1 mM Na₃VO₄, and 1mM DTT), supplemented with 5μCi [γ-³²P] ATP,
20µM ATP and 1µg of GST, GST-p53 or GST-p53 Y327F. Reactions were terminated
using electrophoresis buffer followed by boiling for 5 minutes. Samples were resolved
with SDS-PAGE, and imaged on a Typhoon Imager (GE Healthcare Lifesciences).
Relative activity was determined using densitometry on ImageJ.

2.4.12 Purification of Nuclear proteins

Subsequent to treatment or transfections, nuclear proteins were purified as
described in Luo et al. prior to western blot or EMSA [661].

2.4.13 Homologous recombination Assay

To assess HR efficiency cells were transfected with 0.5 µg DsRed and 4.5 µg of I-
SceI endonuclease cut pDR-GFP plasmids. Two days post transfection, cells were
trypsinized and 20,000 cells were counted and analysed using a FACSCanto flow
cytometer (BD Biosciences) and CellQuest software (BD Immunocytometry Systems).
The percent of GFP+ cells corresponding to the efficiency of DNA DSB repair and the
percent of DsRed+ cells indicating the efficiency of transfection were quantified. The
relative efficiency of repair was calculated as the ratio of GFP+ cells to DsRed+ cells.
2.4.14 EdU Pulse Labelling for DNA synthesis determination

To determine percentage of cells with active DNA synthesis, a modified EdU pulse labeling protocol that utilized Click chemistry was used. Briefly, cells were grown and pulse labelled with 10 μM EdU (Setareh Biotech) for 2 hours at 37°C with 5% CO₂. Cells were then trypsinized and fixed with 4% paraformaldehyde, permeabilised with 0.1% Triton X-100, and washed with PBS +1% BSA. Permeabilised cells were dyed with a Click labelling dye (8 μM FAM-Azide 488 (Lumiprobe), 2 mM CuSO₄, 20 mg/mL ascorbic acid) for 30 minutes in the dark at room temperature. 10,000 cells were counted and analysed using a FACSCanto flow cytometer (BD Biosciences) and CellQuest software (BD Immunocytometry Systems).

2.4.15 Oligomerization Assay

Cells were treated as indicated and lysed with a buffer containing: 25mM HEPES pH 7.5, 150mM NaCl, and 1% NP-40, supplemented with fresh 1 mM dithiothreitol (DTT), 0.1 mM sodium orthovanadate and a protease inhibitor cocktail (Sigma). Lysates were incubated on ice for 20 minutes, and insoluble material was removed by centrifugation at 15,000 × g for 15 minutes at 4°C. Samples were then equalized using the Bradford protein assay, and treated with 0.025% Glutaraldehyde (v/v) for 30 minutes on ice. To stop the cross linking, reactions were quenched with the addition of 100 mM Glycine pH 2.5.

2.4.16 Luciferase Reporter Assays

50,000 H1299 cells were plated in a 24 well plate and transfected the next day with combinations of 331ng pGL3-p21, 3.7ng of Renilla luciferase, 20ng HA-p53 and 20ng of the indicated 3xFLAG-CMV-7.1 NEK10 construct. 24 hours post transfection, luciferase activity was measured as per manufacturer’s instructions for the Dual-Luciferase®
Reporter Assay system (Promega). Luminescence was measured on a Polarstar Omega plate reader (BMG Labtech).

**2.4.17 Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear proteins were purified as described above and equalized to a concentration of 5 μg/μL. Binding reactions were performed by the incubation of 5μg of nuclear protein with IRDye 700 p53 consensus oligonucleotide (LI-COR) in binding buffer (10mM Tris-HCl pH 7.6, 50mM KCl, 10mM MgCl₂, 0.25 Tween-20, 200ng sonicated fish sperm DNA, and 3.5mM DTT) for 20-30 minutes at RT. Binding reactions were then evaluated on a 4% TBE polyacrylamide gel. Shifts were imaged using an Odyssey Classic scanner (LI-COR).
CHAPTER 3:
Characterization of the tyrosine kinase activity of NEK10
3.1 INTRODUCTION

Protein phosphorylation is a posttranslational modification by which cells regulate the majority of their signalling networks. Phosphorylation is carried out by protein kinases, and they comprise one of the largest protein families, with approximately 518 members in humans, encoded by >2% of the human genome [597]. Within the kinase family there are two broad phylogenetic groups, which can be classified as kinases that phosphorylate serine/threonine residues and a smaller group of tyrosine kinases. Protein kinases regulate a host of processes including growth, differentiation, motility, metabolism, apoptosis, and the cellular response to DNA damage. Deregulation of protein kinase function, through mutation or a loss of regulatory control has been associated with multiple cancers, and inherited developmental and metabolic diseases [11].

Protein kinases are defined by a conserved catalytic core of approximately 250-300 amino acids and can be flanked by non-conserved regulatory domains that are unique to individual kinases. The catalytic core of all kinases features a series of conserved sequence motifs that are spread amongst 12 subdomains (Figure 3.1) [662]. These subdomains are the constituents of the two structurally and functionally distinct lobes (N and C lobe, respectively). Together, these two lobes form a dynamic structure that acts as a molecular switch that responds to various regulatory stimuli (reviewed in [663] and summarized below).

The N-lobe of the catalytic core is essential for ATP binding and is comprised of a five stranded β-sheet with a helical subdomain known as the αC-helix. More specifically, there are two unique and highly conserved sequence motifs within the N-lobe: the glycine
rich loop (GxGxxG), and the AxK motif. The glycine-rich loop lies between the β1 and β2 strands and is crucial for positioning of the ATP γ-phosphate for catalysis. The AxK motif is located within the β3 strand and mediates the interaction with the αC-helix. The lysine in the AxK motif forms a salt bridge with a glutamate residue in the αC-helix that is highly conserved across the kinome and is required for kinase activation. The proper positioning of the αC-helix is a critical step in kinase activation, as it interacts with many key regions within the kinase domain to stabilize the active conformation. In the inactive kinase conformation, the αC-helix is disordered or rotated into a position that is not optimal for catalysis.

In contrast to the N-lobe, the C-lobe is primarily helical, with a single β-sheet. This lobe acts as a substrate binding domain, and undergoes fewer conformational changes during catalysis. C-lobe possesses a highly dynamic region that acts as an activation segment for the kinase that is crucial for catalysis. The activation segment is canonically defined as a region flanked by a HRD and an APE motif, and contains residues essential for phosphotransfer from ATP to a phosphoacceptor substrate residue. The catalytic loop contains the HRD motif which is needed for catalysis, whereas the activation loop region contains the DFG motif which coordinates Mg$^{2+}$ ions that participate in the catalysis. The spatial conformation of the activation segment is crucial for kinase activation, as it is involved in positioning of both kinase lobes, as well as the coordination of the substrate within the kinase active site. In inactive kinases, the activation segment can adopt multiple conformations that can mask the active site of a kinase or destabilize positioning of key regions of the kinase required for full activation.
Figure 3.1. Alignment of human NEK2, NEK7, and NEK10 kinase domains.

The secondary elements shown in the alignment were derived from the structure of NEK7 (PDB ID 2WQM). Alignments were performed in CLUSTAL Omega and figure was generated using ESPript 3.0.
Specific phosphorylation or movements of the activation segment serve to expose the active site of the kinase and coordinate substrate interaction and proper positioning of the substrate via interactions with the “P+1” loop [664]. Activating kinase phosphorylation can occur either via cis or trans autophosphorylation or by another kinase. Subsequent to activation segment phosphorylation, both lobes coordinate to form a cleft where the $\gamma$-phosphate of ATP becomes available for phosphotransfer to the hydroxyl group of a phosphorylatable serine, threonine or tyrosine ($P_0$ residue).

The catalytic activities of certain kinases are also subject to allosteric regulation. For example, alongside activation loop phosphorylation, CDK family kinases require the binding of a cyclin for full activation (reviewed in [666]). Moreover, kinases such as PKC, SRC, and CaMK kinases possess pseudosubstrate or autoinhibitory regions within their regulatory domains that mask the active site or hold the kinase domain in an inactive conformation [667-670].

The biological function of a kinase is defined by its spectrum of substrates. However, unlike non-enzymatic protein-protein interactions, the majority of kinase-substrate interactions are weak and highly transient. Substrate targeting to a kinase is a product of weak hydrophobic and electrostatic interactions between the substrate and the kinase active site. Amino acid sequence and structural analyses have shown that protein kinases recognize amino acid sequences surrounding the phosphoacceptor residue, and that these residues confer substrate specificity [671-673]. Targeting of a substrate to a kinase can also be driven by interaction with other regions of the kinase domain or other regulatory domains. For example, the MAPKs such as ERK1/2, p38, and JNK have a
common docking motif (CD) within their catalytic domains that interact with D-motifs on their specific substrates such as MK2, and MNK1 [674].

Given that little is known about the physiological function of NEK10, work in this chapter describes efforts aimed to determine the substrate specificity of NEK10 and ultimately to identify its substrates. This work also led to a detailed understanding of the determinants of NEK10’s dual specificity kinase activity.
3.2 RESULTS

3.2.1 Purification of NEK10 protein

To study the kinase activity of NEK10 and to gain insight into its biological function, a source of pure and active NEK10 was needed. Both the full length NEK10 and the NEK10 kinase domain alone were expressed in bacterial, insect, and mammalian overexpression systems. Although large amounts of NEK10 were recovered from the bacterial and the insect expression systems, the purified kinase displayed little to no catalytic activity (0-0.06 nmol/ng/min) when tested against Histone H3 as a generic kinase substrate (summarized in figure 3.2). I next expressed and immunopurified FLAG-NEK10 from transiently transfected 293T cells, yielding on average 0.8 μg of purified protein/mg of total cell lysate, with specific activity of 0.75-1.1 nmol/min/mg measured against Histone H3 (Figure 3.3 a, b). To further increase the specific activity of recombinant NEK10, purified NEK10 was preincubated with 100 μM ATP, as previous work from our laboratory has shown that autophosphorylation leads to full NEK10 activation [625]. This led to an approximate 5-fold increase in NEK10 specific activity (3-5 nmol/min/mg) against the histone H3 (Figure 3.3 c).

3.2.2 NEK10 is a dual specificity kinase activity

Previous preliminary work from our laboratory has suggested that, despite belonging to the NEK family of Ser/Thr kinases, NEK10 may autophosphorylate on serines, threonines and tyrosines, indicating possible dual specificity [625]. In agreement with these observations, active preparations of NEK10 from 293T cells are tyrosine phosphorylated and undergo further tyrosine autophosphorylation in in vitro kinase assays (Figure 3.4a).
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**Figure 3.2 Selection of expression system for NEK10 purification.**

Small scale purifications and reactions were performed using the indicated constructs and expression systems. The relative levels of expression and kinase activity against a generic H3 substrate were determined via coomassie staining and radioactive *in vitro* kinase assays.
Figure 3.3 Purification of NEK10 from HEK293T cells

A) Coomassie stained gel of protein purification process. WC=Whole Cell Lysate, PCL=Precleared Lysate, FT=Flowthrough, W1=Wash 1, W4=Wash 4, E=Eluted Protein. B) Purified recombinant NEK10 was incubated with 5uCi [γ-32P] ATP, 20uM ATP and, 1ug Histone H3. Kinase assays were separated by SDS-Page and phosphorylation detected by autoradiography. C) Purified recombinant NEK10 was preincubated with indicated concentrations of ATP prior to kinase assay. The specific activity of NEK10 against a generic Histone H3 substrate is indicated.
To identify unique sites of NEK10 tyrosine autophosphorylation and verify its dual specificity, *in vitro* autophosphorylation assays were performed with purified WT NEK10 and its kinase-dead mutant NEK10 A546F, both in the presence or absence of ATP. The products of these reactions were subjected to liquid chromatography tandem mass spectrometry, for the purpose of identifying phosphorylated peptides. All NEK10 proteins were detected with ~80% coverage. Wild-type FLAG-NEK10 immunopurified from transfected 293T cells displayed 31 unique sites of phosphorylation in the absence of exogenous ATP, with a further 6 additional phosphosites revealed upon addition of ATP (Figure 3.4b). In the absence of ATP, WT NEK10 was only phosphorylated on serine and tyrosine moieties and underwent further autophosphorylation on serine, tyrosine and threonine residues upon ATP addition. Of the 37 phosphorylation sites found in WT NEK10, only 5 serine sites were also phosphorylated in immunopurified FLAG-NEK10 A546F. Of note, FLAG-NEK10 A546F displayed no sites of tyrosine phosphorylation. This indicates that the majority of NEK10 serine and tyrosine phosphorylation requires NEK10 kinase activity (Figure 3.4b).

### 3.2.3 NEK10 requires key residues within the activation loop for tyrosine kinase activity

The tyrosine activity of NEK10 appears to be distinct from the other members of the NEK kinase family, with NEK1 being the only other NEK kinase with measureable *in vitro* tyrosine activity [675]. The kinase domain of NEK10 is also the most phylogenetically divergent within the NEK family, as it only shares 54% sequence identity with its closest homologues NEK6/7 (Figure 3.5). To explore the molecular determinants of NEK10
Figure 3.4 NEK10 autophosphorylates primarily serine and tyrosine residues. A) In vitro kinase assay of purified NEK10 in the presence of ATP shows an increase in tyrosine autophosphorylation. B) In vitro kinase reactions of both WT NEK10 and kinase dead NEK10 (A546F) were submitted for liquid chromatography tandem mass spectrometry.
Figure 3.5 NEK10 is phylogenetically distinct from other family members.
The kinase domains of NEK kinase family members were aligned using CLUSTAL Omega, and phylogenetic analysis was performed using an average distance method based on percentage sequence identity. The distances are indicated on each leaf of the phylogenetic tree.
kinase specificity, the activation segments of all members of the NEK kinase family were aligned using CLUSTAL-Omega (Figure 3.6 a, b). The alignment region was defined by two boundaries, the “HRD” motif of the catalytic loop, and the “APE” motif of the activation loop plus 4 C-terminal amino acid residues. Four amino acids were found to be unique to the activation segment of NEK10: T657, I693, S696, and C697. These four residues are conserved throughout evolution, as per CLUSTAL-Omega alignments of NEK10 homologs from nematodes to humans (Figure 3.6b).

To probe the importance of the conserved residues for NEK10’s tyrosine activity, T657, I693, S696, and C697 were replaced with residues conforming to those found in other NEK kinases: T657>K, I693>P, S696>M, and C697>S (Figure 3.6c). While both T657K and I693P NEK10 mutations abolished tyrosine autophosphorylation, T657K considerably decreased the overall catalytic activity of NEK10, whereas I693P appeared to specifically reduce the tyrosine catalytic activity of NEK10. I693 lies within the “P+1” loop of the NEK10 activation loop and may be crucial to the interaction with tyrosine containing substrates. Supporting this, a consensus sequence for the activation segment of tyrosine kinases, generated using CLUSTAL-Omega, contains an isoleucine residue at an equivalent position. This points to the possibility of I693 acting as a key determinant of NEK10 tyrosine kinase activity.

3.2.4 NEK10 recognizes a specific substrate motif for tyrosine phosphorylation

Given that NEK10 substrates, other than itself, have not been identified, we sought to determine the substrate specificity of NEK10. A positional scanning oriented peptide library screen (PS-OPLS) was performed in collaboration with Dr. Michael Yaffe (MIT), a method routinely used to definitively determine kinase substrate sequence
Figure 3.6. I693 in the activation loop is required for kinase activity. A) The catalytic and activation loops of NEK kinase family members were aligned with CLUSTAL Omega, residues unique to NEK10 are indicated with arrows. B) The catalytic and activation loops of NEK10 homologues from the indicated species were aligned using CLUSTAL Omega to determine the degree of conservation of unique amino acids. C) Purified NEK10 and activation loop mutants were analysed for their ability to autophosphorylate on tyrosines via radioactive kinase assay for the total kinase activity and via western blot for tyrosine activity.
Specificity [673, 676-680] (Figure 3.7a). This method takes advantage of the fact that protein kinases phosphorylate their substrates based on the primary amino acid sequence of the region surrounding the acceptor phosphosite [671-673].

PS-OPLS utilizes a library of 198 distinct peptide mixtures, within each mixture are peptides (16 amino acids in length) tagged with a C-terminal biotin tag. Within each peptide, a central Ser/Thr/Tyr that acts as the phosphoacceptor, whereas a second fixed amino acid is located at any of the respective residues N- or C-terminal to the phosphoacceptor site, from five before to four residues after the phosphoacceptor site (Figure 3.7a) [676, 679]. At all other positions, a degenerate mixture containing all 20 naturally occurring amino acids except Cys, Ser, Thr or Tyr is present. In each peptide mixture, target phosphorylation is determined by the position and identity of the second fixed residue. Each mixture is incubated with the kinase of interest and γ^{32}P-ATP to quantify kinase specificity and substrate recognition sequence.

To delineate the specific substrate recognition sequences for NEK10's serine/threonine and tyrosine activities, a modified PS-OPLS approach was taken. WT NEK10 and NEK10 I693P (serine/threonine-specific, tyrosine-deficient mutant) were independently incubated with PS-OPLS peptide libraries, to determine the substrate recognition sequences for the combined NEK10 serine/threonine/tyrosine-specific activity and the NEK10 serine/threonine-specific activity, respectively. To define the substrate recognition sequence for the tyrosine activity of NEK10, the results of the NEK10 I693P PS-OPLS were then subtracted from those of the WT NEK10 PS-OPLS. The tyrosine activity of NEK10 has a strong preference for hydrophobic residues (F/H/L) at the +1 position relative to the phosphoacceptor site (Figure 3.7b). In contrast, NEK10 displayed
Figure 3.7 PS-OPLS reveals a distinct substrate recognition motif for tyrosine phosphorylation. A) Representative schematic of PS-OPLS strategy for determining protein kinase substrate recognition motif. B) Substrate phosphorylation motif for tyrosine phosphoacceptor residues and for serine/threonine residues made using Seq2Logo.
no apparent substrate recognition sequence for serine/threonine phosphorylation (Figure 3.7b).

To search for candidate substrates for NEK10, the Phosphosite™ database was queried for proteins containing pY-[F/H/L] (Figure 3.8a, b). Based on the work presented in Chapter 2, only proteins involved in cellular processes related to cell cycle control, DNA repair or DNA damage processes were queried, yielding 538 candidate tyrosine phosphoacceptor sites. The search was further narrowed to the phosphosites that have been detected 5 or more times in tandem mass spectrometry based experiments, and had not been previously reported as targets of other kinases, reducing the numbers of candidates to 77 target sites of NEK10 tyrosine phosphorylation within 62 unique proteins (Table 3.1). Among the candidate NEK10 target sites is p53 Y327, which, as reported in Chapter 2 was discovered as a target of NEK10 phosphorylation using a functional approach (Section 2.2.3), thus validating the modified PS-OPLS screen for the identification of NEK10 candidate substrates.

### 3.2.5 NEK10 tyrosine kinase activity is required for effective transcription of p53 target genes

Given that in Chapter 2, loss of NEK10 was reported to lead to an increase in A549 cell proliferation and a defect in transactivation of p53 target genes, we sought to determine the significance of NEK10’s tyrosine activity on these phenotypes. To that end, *NEK10 Δ/Δ* A549 cells were stably reconstituted by expression of NEK10, NEK10 D665N (kinase dead), or NEK10 I693P (Ser-specific, Tyr-deficient), respectively (Figure 3.9). Re-expression of WT NEK10 led to a decrease in cellular proliferation, whereas NEK10
D665N or NEK10 I693P were found not to affect this process, consistent with the importance of tyrosine kinase activity for the NEK10’s biological effects in A549 cells (Figure 3.9a). Moreover, re-expression of WT NEK10, but not NEK10 D665N or NEK10 I693P, led to an increase in p21 protein and p53-target mRNA levels (Figure 3.9 b-c).
**Figure 3.8 Identification of potential tyrosine substrates based on Nek10 substrate recognition motif.**

**A)** Workflow for determination of candidate Nek10 tyrosine substrates. Phosphotyrosine sequences were taken from Phosphosite™ database, and filtered using the shown parameters.

**B)** Cellular processes used as a filter for candidate substrate proteins.
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Table. 3.1 List of candidate Nek10 tyrosine substrates as determined by sequence analysis based on Nek10 substrate recognition motif.
Figure 3.9 NEK10 tyrosine kinase activity is required for expression of p53 responsive genes.

A) Proliferation curve of Nek10 Δ/Δ cells reconstituted with the indicated constructs (p<0.01, t test, n=3, bars represent SEM). B) Immunoblot of Nek10 Δ/Δ cells reconstituted with the indicated constructs. C) qRT-PCR of p53-responsive genes in Nek10 Δ/Δ cells reconstituted with the indicated constructs. (p<0.05, t test, n=3, bars represent SEM)
3.3 DISCUSSION

3.3.1 NEK10 is a dual specificity kinase activity

The results presented in this chapter reveal that NEK10 is a dual specificity kinase with both serine- and tyrosine-directed activity, and limited threonine-directed activity (Figure 3.4). The only other member of the NEK family with tyrosine kinase activity is NEK1, which displays weak tyrosine kinase activity in vitro [675]. The majority of kinases are thought to have exclusive Ser/Thr- or Tyr-targeted activity, however there are a handful of kinases that display dual specificity. One of the most well-known dual specificity kinases is MEK1/2, which phosphorylates both phosphoacceptor sites within the TxY motif in the activation loop of ERK1/2, which are required for ERK1/2 activation [681].

The majority of the other reported dual specificity kinases, such as GSK-3α, GSK-3β, and DYRKs, appear to only undergo tyrosine autophosphorylation. GSK-3β and DYRKs both undergo intramolecular tyrosine phosphorylation in a transitional intermediate state as they mature off the ribosome. This cis tyrosine autophosphorylation of the activation loop leads to their transformation into obligate serine/threonine kinases [682-684]. Proposed mechanisms for the significance of the cis tyrosine autophosphorylation include increased interaction with Hsp90 chaperones in the case of GSK-3β, or with an intramolecular scaffolding region for DYRK2 [682, 685]. Interestingly, a recent report has pointed to the possibility that the tyrosine autophosphorylation of DYRK2 stabilizes the kinase domain into a predominantly serine/threonine kinase, as the activation site mutants lead to enhanced tyrosine activity [684, 686, 687].
Unlike GSK3-β and DYRKs, NEK10 appears to tyrosine autophosphorylate in \textit{trans} and is capable of maintaining its dual specific activity, as suggested by \textit{in vitro} kinase assays (Figure 3.4). This however does not negate the possibility that NEK10 also autophosphorylates in \textit{cis}. Further understanding of the molecular tyrosine autophosphorylation will require full structure/function analysis to determine if NEK10 possesses regions within or outside the kinase domain that regulate kinase activity. Autophosphorylation of these regions could affect specificity of NEK10 kinase activity, as these regions may be acting as scaffolds for proper folding of the kinase domain, similar to class 2 DYRKs [685]. Another potential mechanisms by which autophosphorylation of regions outside the kinase domain may contribute to the full activation of NEK10 is through a relief of autoinhibition, as observed in PLK4 [688].

3.3.2 NEK10 requires specific residues within the activation loop for tyrosine kinase activity

The kinase activation segment has a major function in driving substrate specificity, and upon full kinase activation is positioned in such a manner that allows for phosphorylation of the intended substrate. Recent work has pointed towards key residues within the activation loop and kinase domain that act as “determinants of specificity” that are crucial for substrate and phosphoacceptor specificity [689, 690]. Moreover, cancer associated mutations in several kinases at these residues altered kinase activity and specificity [691].

NEK10 I693 appears to be a key residue that acts as a unique determinant of phosphoacceptor preference (Figure 3.6). Upon mutation to a proline, that is present at the equivalent residue in all other NEK kinases, NEK10 loses its ability to
autophosphorylate on tyrosines. Structural comparisons of serine/threonine and tyrosine kinases have pointed towards residues within the “P+1” loop as being responsible for the discrimination between serine/threonine and tyrosine phosphor-acceptors by controlling the distance between the substrate acceptor site and the kinase active site [692, 693]. I693 may act in such a manner within the NEK10 “P+1” loop, to allow for a tyrosine phosphoacceptor residue to be properly positioned within the kinase active site for effective phosphotransfer. It is possible that the replacement of an isoleucine with a bulkier proline residue may lead to a restructuring of the kinase activation segment and active site that allows for effective phosphotransfer to serine substrates but excludes tyrosine substrates. Full characterization of the determinants of NEK10 tyrosine kinase activity will require a structural model of active NEK10 in complex with a substrate, to understand the extent to which I693 is involved in substrate interaction and positioning.

3.3.3 NEK10 has a specific substrate recognition motif for tyrosine phosphorylation

Using PS-OPLS we found a unique substrate recognition sequence for NEK10 tyrosine substrates: pY-[F/H/L], and used this motif to mine the Phosphosite™ database to identify 77 candidate phosphorylation sites within 62 proteins (Figure 3.7, Figure 3.8, Table 3.1). Interestingly, the list generated by this supervised search highlighted multiple proteins involved in mitotic progression (Aurora A, APC7, CDC23, CDC27), and the DDR (ATM, p53, 53BP1, Claspin, Top2A).

Of the candidate substrates identified, APC7, CDC23 and CDC27 are scaffolding subunits of the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase critical to mitotic progression [694]. All three of these subunits form homodimers and are composed of 14 tetratricopeptide repeats (TPR) that are essential for APC/C assembly
and function. The candidate phosphorylation sites Y360, Y353 and Y740 lie within the TPR superhelices of APC7, CDC23 and CDC27, respectively, and their phosphorylation could effect APC/C holoenzyme conformation, activity and stability and thereby effect mitotic progression and exit.

Another interesting potential substrate is 53BP1, as the candidate phosphosite (Tyr1552) lies within its second Tudor domain. The tandem Tudor domains of 53BP1 are crucial for localization to DSBs and function in NHEJ, where they recognize mono and dimethylated histone H4 at lysine 20 (H4K20me1 and H4K20me2) [695-697]. NMR studies have shown that 53BP1 Tyr1552 interacts with H4 Lys16, and phosphorylation of this residue may effect the interaction between 53BP1 and H4K20me1-2, modulating the choice of DNA repair between NHEJ and HR [698].

Although the PS-OPLS based approach used for defining Nek10 specificity is a powerful tool for substrate identification, the resulting consensus substrate recognition sequence that was identified is short and lacks complexity (pY-[F/H/L]), yielding a multitude of candidate targets (77 sites). A number of the identified target sites thus may be false positives and be temporally or spatially exclusive from NEK10 in vivo, or may be targeted by kinases distinct from NEK10. Further, although the Phosphosite™ database contains a large number of phosphosites from published studies (~10,000 studies) in addition to the mass spectrometry-based studies performed by the Cell Signalling Technologies (CST), the available data may not be fully representative of the conditions under which NEK10 is active. This applied approach also fails to take into account 3-dimensional motifs or potential docking and scaffolding interactions that may be essential to NEK10 substrate phosphorylation [699].
On the other hand, despite the identification of multiple candidate NEK10 target sites for tyrosine phosphorylation, the generated substrate list likely does not encapsulate the full spectrum of NEK10 substrates, as we were unable to identify a consensus substrate recognition sequence for the serine kinase activity of NEK10. Intriguingly, it may be possible that the NEK family of kinases as a whole have a loosely defined substrate recognition motif, as a PS-OPLS for NEK2 identified: [F/L/M]-[+/Y/A/N/Q; no P]-[no-P]-[pS/pT]-[F; no P]-[R/H/x] [700]. Their specificity may arise from spatio-temporal control of kinase-substrate interaction, as in the case of NEK2 its substrate specificity is linked to its centrosomal localization [554, 558-565, 700].

Taken together, these studies have expanded our understanding of the dual specificity kinase activity of NEK10. Biochemical characterization allowed us to dissect its dual specific activity, and identify a list of candidate tyrosine substrates using a PS-OPLS based approach. The list of candidate substrate were involved in processes related to the DDR and cell cycle control and more specifically those involved in DDR initiation and control (ATM, 53BP1, p53, claspin, Top2A), and mitotic progression/exit (APC/C subunits). The list of candidate substrates contains many interesting targets for future study and characterization which would aid in the determination of NEK10’s physiological significance in vivo. Phosphorylation of bona fide NEK10 substrates would also indicate potentially novel modes for the regulation of these substrates. Moreover, study of NEK10 substrates would lead to an understanding of the role NEK10 alteration plays in tumorigenesis.
3.4 MATERIALS AND METHODS

Unless otherwise stated all reagents were purchased from Sigma-Aldrich

3.4.1 Plasmids

The 3xFLAG-CMV-7.1 NEK10 construct was used for all the experiments. Mutants of NEK10 were generated by site-directed mutagenesis.

3.4.2 Cell culture and transfection

HEK293T cells were cultured in DMEM/10% FBS. Cells were seeded at a density of 3x10^6 per 15 cm dish 24 hours prior to transfection with 20 μg of NEK10 plasmid using the calcium phosphate method. Cells were reseeded 24 hours after transfection at a ratio of 2:5, and allowed to grow for 48 hours.

A549 NEK10 Δ/Δ cells were engineered to express pLVX-FLAG, FLAG-NEK10 WT, FLAG-NEK10 D665N and FLAG-NEK10 I693P via lentiviral transduction. In short, transduction was performed with lentiviral particles in the presence of protamine sulfate (5 μg/mL) for 20 hours. Subsequent to transduction, cells were reseeded into 200 μg/mL Hygromycin (Sigma) and selected until negative control cells died (3-5 days).

3.4.3 Purification of recombinant NEK10 from mammalian cells

Cells were rinsed with PBS, flash-frozen in liquid nitrogen, and stored at −70°C. Cell pellets were lysed with CHAPS lysis buffer (40mM HEPES pH 7.5, 0.3% CHAPS, 120 mM NaCl, 1 mM EDTA, 20 mM β-glycerophosphate, 50mM NaF, 1 mM DTT, 1 mM Na3VO4, and protease inhibitor cocktail). Prior to immunoprecipitation, cell lysates were precleared using Protein-A sepharose beads (Sigma). Immunoprecipitation was carried out with M2-Flag Agarose beads (Sigma) and washed sequentially three times each with
lysis buffer containing 0.4 M LiCl, kinase assay buffer (50 mM Tris pH 7.6, 10 mM MgCl₂, 10 mM MnCl₂, 2 mM EGTA, 20 mM β-glycerophosphate, 1 mM Na₃VO₄, and 1 mM DTT), and storage buffer (50 mM Tris pH 7.6, 125 mM NaCl, 5% glycerol, 1 mM DTT, protease inhibitor cocktail). Recombinant NEK10 was eluted with 200-300 ng/mL 3xFlag peptide (Sigma) in storage buffer. Peptide was removed and the purified protein concentrated using Amicon Ultra centrifugal filters (50kDA cut off). Prior to storage of the purified protein at -20°C, the concentration of glycerol was increased to 20%.

3.4.4 \textit{In vitro} kinase assays

Protein was assayed for activity by the incubation of 100-200 ng of purified NEK10 at 30°C for 10 minutes in kinase assay buffer (50 mM Tris pH 7.6, 10 mM MgCl₂, 10 mM MnCl₂, 2 mM EGTA, 20 mM β-glycerophosphate, 1 mM Na₃VO₄, and 1 mM DTT), supplemented with 5 μCi [γ-³²P]ATP, and 20 μM ATP. Reactions were terminated using electrophoresis buffer followed by boiling for 5 minutes. To determine specific activity, the sample was resolved by SDS-PAGE and sections of the gel with Histone H3 were excised and scintillation counting was performed to determine the ³²P incorporation.

3.4.5 \textit{Western blot}

Whole cell lysates, immunoprecipitates, or purified protein was resolved by SDS–PAGE and blotted to PVDF membranes (Millipore). Proteins were probed using appropriate primary antibodies from the following sources: α-pTyr (PY99) (sc-7020) from Santa Cruz Biotechnology, and α-Flag M2-FITC from Sigma.
3.4.6 Coomassie staining

Protein was separated by SDS-PAGE, and gels were first washed in ddH20, before being incubated for 1 hour with Coomassie Brilliant Blue R-250 (CBB R-250). Subsequent to staining, gels were destained with destain buffer (20% MeOH/10% Acetic Acid), and incubated with gel storage buffer (30% MeOH/5% glycerol) prior to mounting. Protein amounts were determined with densitometry using ImageJ™.
CHAPTER 4:
FUTURE DIRECTIONS
The studies described in this thesis demonstrate a function for NEK10 in the regulation of p53 transcriptional activity, and reveal an unexpected preference of NEK10 kinase activity for tyrosine residues. My studies uncovered a single residue within the NEK10 activation loop, I693, that is responsible for NEK10 preference for tyrosine as substrate. These findings lay a foundation for future work on the understanding of NEK10 function in cancer development, and the biological significance of lowered NEK10 expression in human cancers.

4.1 Understanding NEK10 activation:

Posttranslational modifications and protein-protein interactions

While this thesis provides a partial understanding of the potential downstream effectors and biological outcomes of NEK10 activity, little is known about the modes of NEK10 regulation. NEK10 appears to be constitutively active in cycling cells and its activity or localization do not appear to be modulated following exposure to IR (Haider et al, unpublished). NEK10 may be regulated by posttranslational modifications or by differential protein-protein interactions. In chapter 3, mass spectrometric analysis identified 4 sites of NEK10 phosphorylation independent of NEK10 kinase/autophosphorylation activity (Figure 3.4). One of these phosphosites, S933, appears to be a candidate target for phosphorylation by AGC or CHK1/2 kinases. The use of shRNA or chemical inhibitors of these kinases, and active monitoring of NEK10 S933 phosphorylation may uncover the identity of the kinase(s) responsible for S933 phosphorylation and provide context for the conditions of its modulation.
Another potential posttranslational modification effecting NEK10 activity may be poly-ADP-ribosylation (pADPr/PAR), or NEK10's interaction with PARylated proteins. A recent study has identified a pADPr-binding motif within the N-terminus of NEK10 (98-105 a.a.) [701]. PARylation is a posttranslational modification elicited by the PARP family of enzymes, and has been found to modulate cell cycle progression, DNA repair, and apoptosis [702, 703]. Monitoring the PARylation status and subcellular localization of NEK10 in response to IR, H₂O₂ and UVC, in the presence or absence of PARP1/2 shRNA or inhibitors (olaparib), would determine the extent of NEK10 PARylation and provide context to the conditions under which PARylation is a relevant NEK10 modification. PARylation status and localization of NEK10 mutant proteins lacking the putative pADPr binding motif should be assessed using a similar approach. Moreover, this thesis revealed that NEK10 Δ/Δ cells were more sensitive to treatment with olaparib (PARP1/2 inhibitor) (Figure 2.11a). Given the growing prospects for the use of olaparib and other PARP1/2 inhibitors in the clinical management of BRCA1/2-mutant cancers, it would be important to determine what effect NEK10 status has on tumour response to PARP inhibitor treatment [704].

The NEK10 protein-protein interactions also remain largely unexplored. Mass spectrometric-based proteomics analysis, through the use of complementary affinity purification coupled to mass spectrometry (AP-MS) and BioID proximity biotinylation approaches, would serve to identify the interactome of NEK10 [705]. In short, stable cell lines harboring a single copy of an inducible BirA-FLAG-NEK10 cassette would be generated to express NEK10 as a bait protein for AP-MS and BioID. The cellular expression of NEK10 would be induced, both under basal conditions and in response to
IR, for parallel AP-MS and BioID analysis. Cell stimulation could be used to distinguish stable and transient NEK10 interactions. Further work on the generated NEK10 interactome would aid in the understanding of NEK10 function in the greater DDR network, and unveil the involvement of NEK10 in additional signalling pathways and cellular processes.

4.2 NEK10 in tumorigenesis

Genome wide association studies and sequencing efforts have identified NEK10 mutations and lowered expression associating with worse prognosis in multiple cancer types (Chapter 1.8.1). The use of mouse models of NEK10 loss would be instrumental in understanding its role in tumour development and potential sensitivity to genotoxic agents. Our group is currently developing conditional NEK10 knockout mouse models, which will be powerful tools in determining the physiological function of NEK10 (Dutt et al., unpublished). Work presented in chapter 2 suggests a potential tumour suppressive function for NEK10 through modulation of p53 transcriptional activity, particularly in response to genotoxic stressors (IR, cisplatin, olaparib). To begin to elucidate the tumour suppressive properties of NEK10, it would be pertinent to challenge \( \text{NEK10}^{-/-} \) mice with genotoxic stressors, such as low doses of gamma-radiation. The effect of irradiation on tumorigenesis and survival in \( \text{NEK10}^{-/-} \) mice should be compared to the increased radiation-induced tumorigenesis and lethality observed in \( \text{p53}^{-/-} \) mice [706, 707].

These models would also provide some much needed tools for the study of the cellular functions of NEK10. Mouse embryonic fibroblasts (MEFs) and thymocytes generated from the NEK10-knockout mice could be assessed for maintenance of genome integrity, apoptosis, and growth arrest in response to various genotoxic treatments. The
study of knockout MEFs immortalized with RAS and/or E1A would be invaluable in the
determination of NEK10's function in cellular transformation, senescence, and tumour
formation [708, 709]. Tumours and mammary epithelial cell lines developed from NEK10
 mice would provide an insight into the significance of NEK10 in tumour development
and could be used as a model to assess NEK10 loss as a predictor of response to
chemotherapeutic treatments. As with the study of the NEK10 interactome, the
identification of NEK10 substrates would facilitate exploration of further NEK10
function(s).

4.3 The search for NEK10 substrates

In chapter 3, the tyrosine kinase activity of NEK10 was defined and the molecular
mechanism driving dual specificity was identified within the NEK10 activation loop (I693).
This mutation allowed me to build a list of candidate NEK10 substrates, including multiple
regulators of the DDR (ATM, p53, 53BP1, APC/C subunits). To validate these candidates
as bona fide substrates, SILAC (Stable isotope labeling with amino acids in cell culture)
mass spectrometric analysis of phosphorylated proteins should be performed in NEK10
Δ/Δ cell lines expressing WT NEK10, NEK10 A546F, and NEK10 I693P [710-712].
Promising candidates should be verified as direct NEK10 substrates via in vitro kinase
assays. Subsequently, verified substrates should be assessed for involvement in DDR
and tumorigenesis through the use of NEK10 phosphorylation site specific mutations in
NEK10 +/- and NEK10 Δ/Δ cell lines.

4.4 The significance of Y327 on p53 function

In chapter 2, I uncovered p53 Y327 as a site of NEK10 phosphorylation that
modulates p53 transcriptional activity. To delve deeper into the significance of p53 Y327
on transcriptional activity and determining the cellular outcome in response to DNA damage, an HCT116 cell line, or another wild type p53 cell line, should be developed harbouring a p53 Y327F mutation at the endogenous p53 locus. The transcriptional profile of these cells should be compared to isogenic HCT116 p53+/+ and HCT116 p53−/− cells via RNA-seq and a p53 program-specific microarray, under basal conditions, and after IR and cisplatin treatment. Transcriptional profiling should be coupled with monitoring for cell cycle checkpoint induction, apoptosis, and senescence. The proposed study could be extended towards the development of a library of isogenic HCT116 cell lines expressing mutants of p53 PTM sites, such as S46A, K120R, K320R, S392A, that are involved in the selection of cellular outcome between growth arrest and cell death [431-435, 438, 442-444].
REFERENCES


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