CHARACTERIZATION OF NIMA-RELATED KINASE 10 (NEK10): A ROLE IN
CHECKPOINT CONTROL

by

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

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Abstract

Characterization of NimA-related kinase 10 (NEK10): a role in checkpoint control

Larissa S Moniz, Doctor of Philosophy 2010. Department of Medical Biophysics, University of Toronto.

Deregulation of the cell cycle is a hallmark of neoplastic transformation and plays a central role in both the initiation and progression of cancer. Members of the NimA-related kinase (NEK) family of protein kinases are emerging as important players in regulation of the eukaryotic cell cycle during normal cell cycle progression and checkpoint activation in response to genotoxic stresses. The focus of this thesis is NEK10, a previously uncharacterized member of the NEK family. While little is known about the biology of NEK10, recent cancer genomics studies have identified NEK10 as a candidate susceptibility gene at chromosome 3p24 in cancer.

Work herein describes a role for NEK10 in the cellular response to ultraviolet (UV) irradiation. NEK10 was required for the activation of ERK1/2 signaling upon UV irradiation, but not in response to mitogens, such as the epidermal growth factor. NEK10 interacted with Raf and MEK and enhanced MEK activity through a novel mechanism involving MEK autoactivation. Significantly, appropriate maintenance of the G2/M checkpoint following UV irradiation required NEK10 expression and ERK1/2 activation. In support of a conserved role for NEK10 in the cellular response to UV irradiation, nekl-4, the NEK10 C.elegans homologue, affected embryonic sensitivity to UV-irradiation.

In search of regulatory inputs into NEK10, using mass spectrometry, our laboratory identified 19 distinct sites of NEK10 phosphorylation. Characterization of a number of these
sites revealed a role for intermolecular autophosphorylation in achieving full NEK10 catalytic activity through activation loop phosphorylation on S684 and S688. Further, a C-terminal phosphorylation site on NEK10, S933, was found to be a 14-3-3 binding site, and was essential for NEK10 cytoplasmic to nuclear translocation following UV irradiation.

Taken together, my studies have discovered a role for NEK10 in the engagement of the G2/M cell cycle checkpoint and provided a mechanistic insight into the relationship between NEK10 and the Raf/MEK/ERK cascade, and the control of NEK10 subcellular localization. This work will serve as a foundation for future studies aimed at understanding the molecular mechanism of NEK10 action and its function in development and tumourigenesis.
Acknowledgements

There are so many people who have contributed to my work and to my life over the past seven and a half years. Without them this thesis would look very different, and more importantly I would not have had such an enjoyable journey.

First and foremost on this list is my supervisor, Vuk Stambolic. Through your example, you have promoted curiosity, excitement, camaraderie, scientific excellence and most importantly, independent thought. These are qualities that I hope to emulate and share. Thank you for your openness, guidance and patience. I definitely made the right choice.

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Thank you to my committee members: Dan Durocher, Jim Woodgett and Wen-Chen Yeh who have always been supportive, informative and challenging. I have worked hard to earn your respect. This is perhaps especially true for Jim. I have been in awe of you ever since I realized that you have the kinase domain sequence memorized.

Thank you to the other students in MBP who made the long days seem normal and the evenings fun. Thanks especially to Nick and Kellie who are my colleagues and friends. Thank you for sharing wine and laughter, all mixed in with scientific ideas and secret projects.
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<tr>
<td>A</td>
<td>Alanine (Ala)</td>
</tr>
<tr>
<td>aa</td>
<td>Amino acids</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia-telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATR</td>
<td>ATM and Rad3-related</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer 1, early onset</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CAK</td>
<td>CDK activating kinase</td>
</tr>
<tr>
<td>Cdc2</td>
<td>Cell division cycle 2</td>
</tr>
<tr>
<td>Cdc25</td>
<td>Cell division cycle 25</td>
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<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
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<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
</tr>
<tr>
<td>Chk1/2</td>
<td>Checkpoint kinase 1/2</td>
</tr>
<tr>
<td>D</td>
<td>Aspartate (Asp)</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>Double strand break</td>
</tr>
<tr>
<td>E</td>
<td>Glutamate (Glu)</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinases</td>
</tr>
<tr>
<td>esiRNA</td>
<td>Endoribonuclease-prepared small interfering RNA</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>G0</td>
<td>Quiesence</td>
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<td>G1</td>
<td>Gap period 1</td>
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<td>Gap period 2</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
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<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293</td>
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<tr>
<td>HU</td>
<td>Hydroxyurea</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
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<tr>
<td>jck</td>
<td>Junior cystic kidney</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun NH₂ terminal kinases</td>
</tr>
<tr>
<td>kat</td>
<td>Kidney, anemia, testes</td>
</tr>
<tr>
<td>KSR</td>
<td>Kinase suppressor of Ras</td>
</tr>
<tr>
<td>M</td>
<td>Mitosis</td>
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<tr>
<td>Mad1/2</td>
<td>Mitotic arrest deficient 1/2</td>
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<tr>
<td>BubR1</td>
<td>Budding uninhibited by benzimidazoles related 1</td>
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<tr>
<td>MAPK</td>
<td>Microtubule associated/mitogen activated protein kinase</td>
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<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>MMC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organizing centre</td>
</tr>
<tr>
<td>Myt1</td>
<td>Membrane associated tyrosine/threonine 1</td>
</tr>
<tr>
<td>NEK</td>
<td>NimA-related kinase</td>
</tr>
<tr>
<td>nekl</td>
<td>NimA-related kinase (NEK) like</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export sequence</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NimA</td>
<td>Never in mitosis A</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PIKK</td>
<td>Phosphatidylinositol 3-kinase-related kinases</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKD</td>
<td>Polycystic kidney disease</td>
</tr>
<tr>
<td>Plk</td>
<td>Polo-like kinase</td>
</tr>
<tr>
<td>PP-1</td>
<td>Protein phosphatase-1</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>S phase</td>
<td>DNA synthesis phase</td>
</tr>
<tr>
<td>S</td>
<td>Serine (Ser)</td>
</tr>
<tr>
<td>SAC</td>
<td>Spindle assembly checkpoint</td>
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<tr>
<td>SCF-β-TrCP</td>
<td>Skp Cullin F-box complex-β-transducin repeat-containing protein</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single strand DNA</td>
</tr>
<tr>
<td>T</td>
<td>Threonine (Thr)</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine (Tyr)</td>
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CHAPTER 1

GENERAL INTRODUCTION
1.1 The cell cycle

The cell cycle is a series of ordered events, during which time DNA is replicated and segregated between two daughter cells (reviewed in (242)). The accurate completion of cell division is essential for normal organism development and deregulation of this program can result in loss of genomic stability and cell viability. The cell cycle can be divided into four phases: G1, S, G2 and M (Fig 1.1). During G1, the first gap period, the cell integrates information from extracellular signals, the most important of which is the presence of adequate growth factors, to determine if a round of cell division should occur. If nutrients are lacking, the cell may enter a temporary state of quiescence, termed G0, or may undergo differentiation. If conditions are favorable, the cell will prepare to replicate its DNA. This decision occurs at the restriction point (R) in late G1 and once made, irreversibly commits the cell to completing cell division. Following DNA replication in S phase, cells pass through a second gap period, G2, ensuring that replication is completed and preparing for division in mitosis (M). Mitosis is the final stage of the cell cycle and involves the appropriate and equal segregation of genetic material into two new daughter cells.

1.1.1 Cyclin-dependent kinases (Cdks)

Cell cycle progression is controlled by numerous regulatory proteins, which are highly conserved from yeast to mammals. The master regulators of the cell cycle are complexes composed of a regulatory component, a cyclin, and a catalytic component, a serine-threonine cyclin-dependent kinase (Cdk). Yeast have a single Cdk, cell division cycle 28 (cdc28) in *Saccharomyces cerevisiae* and Cdc2 in *Schizosaccharomyces pombe*, whereas
Figure 1.1: The major features of the cell cycle in mammalian cells.

The stages of the cell cycle and the binding of the specified cyclins with the corresponding Cdns at each stage are depicted. G0, G1, S, G2 and M refer to the quiescence, first gap, DNA synthesis, second gap and mitotic phases of the cell cycle, respectively. The restriction point (R) is shown. P refers to phosphorylation. Adapted from: (242).
there are multiple Cdk's in mammalian cells, which are sequentially activated at different stages of the cell cycle (Fig 1.1). In mammalian cells, Cdk4 or Cdk6 complexed with cyclinD, and Cdk2 bound to cyclinE, promote early and late G1 progression, respectively. S phase is controlled largely by cyclinA/Cdk2 complexes while Cdk1 (also known as cdc2 or p34^cdc2) in association with cyclinB and to a lesser extent cyclinA is activated during G2/M and M. The activity of Cdk's is tightly controlled, most notably by protein-protein interactions and phosphorylation (reviewed in (276)).

In order to be activated, Cdk's have to associate with cyclins, which are expressed in a cell cycle dependent manner (31, 94, 275). Interaction with a cyclin controls Cdk activity in two ways, by inducing a permissive conformation of the Cdk catalytic site and by providing substrate specificity (174, 356). In the case of Cdk6, cyclin-binding is necessary and sufficient for activation (357). For other Cdk's, such as Cdk1 and Cdk2, interaction with a cyclin only partially activates the kinase. These kinases also require phosphorylation by the Cdk activating kinase (CAK) within the activation segment (T161 in Cdk1, T160 in Cdk2) (102, 174, 349, 372).

Cdk activity is also subject to negative regulation throughout the cell cycle. The mitotic kinase, Cdk1, is inhibited by phosphorylation at T14 and Y15 within the ATP-binding loop (201). These sites are phosphorylated by the Wee1 and membrane associated tyrosine/threonine 1 (Myt1) kinases, whereas dephosphorylation and Cdk1 activation at the G2/M transition is achieved by the dual-specificity phosphatases, Cdc25 A, B and C (121, 135, 201, 228, 279, 304)(reviewed in (347)).

The G1/S Cdk's are predominantly regulated by their interactions with 2 families of Cdk-inhibitors, the inhibitor of Cdk4 (INK4) and the Cdk interacting protein/kinase
inhibitory protein (Cip/Kip) families (32, 213)(reviewed in (306)). There are four INK4 proteins, p16INK4a, p15INK4b, p18INK4c, and p19INK4d that form stable complexes with the early G1 Cdks, Cdk4 and Cdk6. This interaction inhibits Cdk4/6 activation by preventing their association with cyclinD (154). The Cip/Kip family, composed of p21 (cip1/waf1), p27 (kip1) and p57 (kip2), display lower specificity and can inhibit all cyclin/Cdk complexes to some degree (439)(reviewed in (28)). However, in proliferating cells, Cip/Kip proteins are primarily inhibitory towards cyclinE/Cdk2 complexes. Interestingly, Cip/Kip proteins also promote G1 progression by facilitating the assembly and nuclear translocation of cyclinD/Cdk4/6 complexes (207). Moreover, as cells progress through G1, newly formed cyclinD/Cdk4/6 complexes bind and sequester Cip/Kip proteins allowing downstream activation of cyclinE/Cdk2 (123, 308). In situations of cell cycle arrest, such as when cells undergo senescence or are arrested through contact inhibition or genotoxic stress, upregulation and post-translational modification of Cip/Kip proteins increases their ability to inhibit both cyclinE/Cdk2 and cyclinD/Cdk4/6 complexes (171, 294, 315, 378).

1.1.2 Cyclin/Cdk complexes control cell cycle progression

1.1.2.1 Progression through G1

Cyclin/Cdk complexes control the decision to pass R and to commit to a round of cell division. One of the key mechanistic events regulating this decision is hyperphosphorylation and inactivation of the retinoblastoma protein (pRB), mediated by cyclin/Cdk complexes (reviewed in (124, 143)). Prior to R, unphosphorylated or hypophosphorylated pRB inhibits
transcription of genes required for S phase entry by at least two mechanisms. pRB binds to the transactivation domain of the E2 transcription factor (E2F) preventing transcriptional activation of target genes (103). In addition, in complex with E2F, pRB can bind directly to promoters and actively repress transcription by a mechanism involving chromatin remodeling via histone deacetylase 1 (HDAC1) (458)(reviewed in (243)). Once hyper phosphorylated, pRB loses its ability to interact with E2F, allowing transcription of genes required for DNA replication, such as cyclinE, cyclinA, DNA polymerase subunits, and proliferating cell nuclear antigen (PCNA) (70, 81, 82, 295, 385).

During early G1, low Cdk activity maintains pRB in a hypophosphorylated and active state. Following sustained growth factor stimulation, cyclinD expression is induced, leading to formation of active cyclinD/Cdk4/6 complexes and phosphorylation of pRB on multiple sites (185)(reviewed in (312)). In late G1, E2F-induced cyclinE, complexed with Cdk2, further reinforces pRB phosphorylation, initiating transcription of genes required for S-phase and irreversibly committing the cell to division (144)(reviewed in (143)).

1.1.2.2 Progression through mitosis

The function of mitosis is to faithfully partition the duplicated genome between two daughter cells. Mitosis can be divided into 5 consecutive and morphologically distinct phases: prophase, prometaphase, metaphase, anaphase, and telophase (Fig 1.2) (reviewed in (285)). Early mitotic events include chromosome condensation and nuclear envelope breakdown (prophase). Concurrently, duplicated centrosomes migrate towards opposite ends of the cell and nucleate dynamic microtubules to form the mitotic spindle. Microtubules are
Figure 1.2: The principle events in mitosis.

During prophase, chromatin condensation occurs and previously duplicated centrosomes migrate apart to define the poles of the future mitotic spindle. Concomitantly, nuclear envelope breakdown occurs. During prometaphase microtubules are captured by kinetochores (proteinaceous structures associated with centromere DNA on mitotic chromosomes). After stable bipolar attachment occurs, chromosomes congregate on the metaphase plate during metaphase. Once all chromosomes have been thus attached and positioned, sister-chromatid cohesion is lost at the onset of anaphase and sister chromatids are pulled towards opposite poles. Following chromosome segregation, nuclear envelopes reform and chromatin decondensation begins in telophase. Finally, an actomyosin-based contractile ring is formed and cell division is completed with cytokinesis. Checkpoints regulate cell cycle progression by monitoring genomic integrity. Adapted from: (285).
captured by kinetochores that have formed on mitotic chromosomes (prometaphase), and once sister chromatids have undergone bipolar attachment they align in the centre of the cell at the metaphase plate (metaphase). At the metaphase-anaphase transition, cohesion between sister chromatids is lost and sister chromatids are pulled to opposite ends of the cell (anaphase). Following this, the nuclear envelope begins to reform and chromosomes decondense (telophase). Finally, an actomyosin contractile ring is formed and generates two daughter cells during cytokinesis (reviewed in (285)).

To enter mitosis a cell requires cyclinB/Cdk1 activation. This occurs at the G2/M transition following dephosphorylation of T14 and Y15 (201). At this stage the balance is shifted towards dephosphorylation of T14/Y15 by inactivation of the Wee1 and Myt1 kinases and activation of the Cdc25 phosphatases through phosphorylation by a number of mitotic kinases including Cdk1 and polo-like kinase 1 (Plk1) (17, 32, 247, 284, 403, 425)(reviewed in (225)). Therefore, Cdk1 contributes to a positive feedback loop that enhances its own activity. Activated Cdk1 initiates early mitotic events by phosphorylating a wide variety of targets including, nuclear lamins, condensins, golgi-matrix components, kinesin-related motors and microtubule binding proteins, leading to nuclear envelope breakdown, chromosome condensation, golgi fragmentation, centrosome separation and mitotic spindle assembly, respectively (11, 60, 72, 190, 239).

While Cdk1 activity stimulates mitotic entry, it has to be inhibited for a cell to exit mitosis (281). Cdk1 contributes to it’s own inactivation and to mitotic exit by promoting degradation of cyclinB via the E3 ubiquitin ligase, anaphase promoting complex/cyclosome (APC/C). At the metaphase-anaphase transition, mitotic kinases including Cdk1 and Plk1 phosphorylate and activate APC/C (129, 198, 199, 346). APC/C stimulates mitotic exit by
the timely degradation of mitotic regulators, including cyclinB, Plk1 and Aurora A, as well as inhibitors of chromatid separation, such as securin (157, 365, 368, 463).

The key event in mitosis is the equal segregation of genetic material. To ensure that this occurs, APC/C activity and chromatid separation is inhibited by the spindle assembly checkpoint (SAC), until the point at which all chromatids have undergone bipolar microtubule attachment (335, 336). The SAC is composed of a diffusible signal of kinetochore-interacting proteins, including Mad1/2, Bub1/2, BubRI and Aurora B. These proteins act as molecular sensors and detect unattached kinetochores and changes in microtubule-kinetochore tension that occur following bipolar attachment (reviewed in (282, 309)). The SAC is crucial for genomic stability and in animal cells elimination of this checkpoint results in aneuploidy and cell death (77).

1.1.3 Other cell cycle regulated kinase families: Plk, Aurora, and NEK kinases

While Cdks are considered the master regulators of cell cycle progression, in recent years, a number of other cell cycle regulated kinase families have been characterized. These families, including the Plks, Aurora kinases, and NimA-Related Kinases (NEK) encode serine-threonine kinases that play important roles throughout the cell cycle and particularly during mitosis. Plk and Aurora kinases are discussed below. For information on NEK kinases see Chapter 1.2.

1.1.3.1 Polo-like kinases (Plks)
In vertebrates, there are 4 Plks (Plk1-4). The best characterized mammalian isoform is Plk1, which is most closely related to the single Plk found in yeast and \textit{Drosophila melanogaster} (Polo) and to \textit{Xenopus laevis} Plx1. Plk1 is activated prior to mitotic entry via a molecular mechanism involving transcriptional regulation and activation loop phosphorylation (172)(reviewed in (12)). Phosphorylation is mediated by Aurora A during late G2 and likely by another, currently unidentified, kinase at later points of mitosis (244, 361).

Plk1 contributes to many aspects of mitosis, including regulation of mitotic entry, generation of the microtubule organizing center (MTOC) and cytokinesis (reviewed in (12)). Plk1 promotes mitotic entry by directly phosphorylating Wee1, Myt1 and Cdc25C, leading to their inactivation and activation respectively. This contributes to the positive feedback loop that stimulates Cdk1 activity at G2/M (166, 205, 425). Consistently, depletion of Plx1, in \textit{Xenopus} oocytes, and Plk1, in human cells, causes a delay in early mitotic progression (322, 381). Plks also regulate formation of the mitotic spindle and one of the first phenotypes associated with Polo kinases was the presence of monopolar spindles, seen in both D. \textit{melanogaster polo} mutants, and in cultured human cells with disrupted Plk1 (211, 384). This can be attributed to the function of Plks in centriole duplication, where inhibition or overexpression, of Plk4 in human cells and \textit{polo} in \textit{Drosophila}, results in decreased and increased centriole numbers, respectively. Further, at mitotic entry, Plk1 promotes microtubule recruitment to centrosomes during centrosome maturation (29, 139, 212). In addition, Plks regulate cytokinesis, and in human cells chemical inhibition of Plk1 disrupts cell division. This is mediated by Plk1-dependent activation of RhoA GTPase, which participates in contractile ring formation (310). In addition to these functions, Plk activity is
implicated in maintaining sister chromatid cohesion, recovery from DNA damage induced checkpoints and in asymmetric division during embryonic development (40, 244, 382)(reviewed in (12)).

1.1.3.2 Aurora kinases

Another family with a prominent function in mitosis is the Aurora family of kinases, in mammals consisting of 3 members, Aurora A, B and C (reviewed in (410)). As with Plks, Aurora kinases are expressed in a cell cycle dependent manner. In addition, kinase activation requires phosphorylation and allosteric interaction with specific co-factors. The best-characterized co-factors are targeting protein for Xklp2 (TPX2) for Aurora A, and inner centromere protein (INCENP) for Aurora B and C (95, 223). These interactions induce conformational changes in Aurora kinases required for full activation (21, 364).

Aurora A is required for mitotic entry and for bipolar spindle formation. At the G2/M transition, Aurora A directly phosphorylates and activates Plk1 and Cdc25B (90, 244, 361). Together, these events promote cyclinB/Cdk1 activation and mitotic entry. Aurora A also regulates the mitotic spindle by promoting centrosome maturation and separation, and in Drosophila, C.elegans, and Xenopus model systems, inhibition of Aurora A kinase activity results in monopolar spindle formation (27, 128, 142, 230).

Of the three mammalian Aurora kinases, Aurora B is most closely related to the single Aurora homologue found in lower eukaryotes. Primary functions of Aurora B feature during chromatid separation and cytokinesis. Aurora B regulates appropriate chromatid segregation by multiple mechanisms (reviewed in (410)). For example, Aurora B activity
stabilizes bi-polar kinetochore-microtubule attachments and is required to maintain the SAC prior to the metaphase-anaphase transition (76, 149, 210). Aurora B also plays an essential role in cytokinesis, and depletion in C.elegans embryos and Drosophila S2 cells leads to impaired cytokinesis and polyploidy (126, 358). Aurora B may have an additional function during chromatin condensation either by phosphorylating histone H3 (S10) and/or by chromosomal targeting of the Condensin I complex, which drives condensation (125, 226). The mechanism of Aurora B signaling for this event appears to differ between organisms and has not been fully defined in mammalian cells (reviewed in (410)).

Little is known about Aurora C, the third Aurora kinase, whose expression is low in most adult tissues, with prominent expression only seen in the testis (189). In HeLa cells, ectopic expression of Aurora C can rescue multinucleation defects caused by Aurora B depletion. Taken together with the fact that it interacts with INCENP, Aurora C may have partially redundant functions with Aurora B in cells (223).

1.1.4 Checkpoint signaling

All eukaryote cells have multiple molecular mechanisms to identify and repair damaged DNA and preserve genomic integrity (reviewed in (353)). An important aspect of this process is activation of a checkpoint, or cell cycle arrest, to allow the cell time to repair damage. Cell cycle arrest can be triggered at G1/S, intra-S and G2/M by damage caused by endogenous sources, such as stalled replication forks, or by exogenous agents including ultraviolet (UV) radiation, ionizing radiation (IR), reactive oxygen species (ROS), and certain chemotherapeutic agents. Upon successful repair, the cell will re-enter the cell cycle.
However, if the damage is too severe, apoptosis may be initiated. Loss of checkpoint or repair functions promotes propagation of damaged DNA and can have deleterious consequences on cell and organismal viability.

1.1.4.1 ATM and ATR signaling in the checkpoint response

The DNA damage checkpoint is controlled by phosphorylation cascades initiated by two phosphatidylinositol 3-kinase-related kinases (PIKK), ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) (Fig 1.3). ATM was originally identified as the causal mutation in the neurodegenerative disease ataxia telangiectasia (A-T) (355)(reviewed in (215)). Cells established from patients with A-T display hypersensitivity to ionizing radiation and defects in G1/S and G2/M checkpoints (161, 184, 300). ATR is the human homologue of the S.pombe gene rad3, mutants of which display checkpoint defects following UV, IR, or inhibition of DNA replication by hydroxyurea (7, 25, 55). Mutations in ATR were identified in patients with Seckel syndrome, which is characterized by dwarfism and mental retardation (134). Fibroblasts derived from a patient with Seckel syndrome exhibit impaired DNA damage response and hypersensitivity to UV irradiation and mitomycin C (MMC) (290).

ATM is primarily activated by agents that cause double strand breaks, such as IR, whereas ATR responds to accumulation of single strand DNA caused by stalled replication forks, UV-induced damage or DNA crosslinks, and to a lesser extent by IR-induced damage (44, 290, 424). While the exact molecular mechanisms differ, both kinases are activated following recruitment to sites of DNA damage by multi-protein complexes, and...
Figure 1.3: The main features of the DNA damage checkpoint.

Activation of the cell cycle checkpoint results in rapid Cdk inhibition. Cell cycle arrest is triggered by activation of the PIKKs ATM and ATR at double strand breaks and single strand DNA, respectively. ATM/ATR phosphorylate and activate a number of downstream effectors including p53, and the p38/MK2 and Chk1/2 kinases. Activated p53 acts as a transcription factor for a number of proteins required for G1/S arrest including p21, GADD45, and 14-3-3σ. p38/MK2 are activated by both ATM/ATR-dependent and -independent mechanisms and act parallel to Chk1/2. The three effector kinases, Chk1, Chk2 and MK2, directly phosphorylate and inhibit members of the Cdc25 family of Cdk-activating phosphatases. Adapted from (235, 331).
phosphorylate numerous substrates with overlapping specificity (254, 371)(reviewed in (54)).
The best-characterized downstream targets of ATM/ATR are the effector kinases, checkpoint kinase 1 and 2 (Chk1 and Chk2), and the tumour suppressor protein p53 (44, 137, 173, 256, 398).

In mammalian cells, the p53 transcription factor mediates a cell’s decision to undergo cell cycle arrest or to commit apoptosis following cellular stress (reviewed in (417)). In the absence of stress, p53 is downregulated by an interaction with the E3 ubiquitin ligase, murine double minute 2 (Mdm2). In addition to promoting the ubiquitination and degradation of p53, Mdm2-binding inhibits interaction of p53 with transcriptional co-activators and blocks transactivation (204, 273). Following DNA damage, ATM/ATR stabilizes p53 through direct and indirect mechanisms. ATM/ATR directly phosphorylate p53 at S15, inhibiting its interaction with Mdm2, and Mdm2 at S395, promoting its autodegradation (44, 380, 398). ATM/ATR activation of other kinases such as Chk1/2 and Ablison murine leukemia viral oncogene homolog 1 (Abl1) may also promote p53 stability through their phosphorylation of p53 and Mdm2 (reviewed in (265)). One of the key targets of p53 is p21, which is rapidly induced following DNA damage and inhibits cyclinD/Cdk4/6 and cyclinE/Cdk2 complexes (86, 227). In addition to p21, p53 regulates the expression of a number of anti-proliferative genes and while p53 can contribute to all checkpoints, the G1/S cell cycle arrest is dependent on functional p53 (184). Many human tumours and immortalized cell lines have compromised p53 activity and G1/S arrest following damage. In these cases, the G2/M checkpoint takes on increasing importance for maintaining genomic stability.

1.1.4.2 Chk1/2 signaling in the checkpoint response
Chk1 and Chk2 are non-homologous serine-threonine kinases that act downstream of ATM/ATR (231, 255). Phosphorylation of two sites within the non-catalytic C-terminus by ATM/ATR, increase Chk1 activity, likely by relieving autoinhibition (120, 186, 231)(reviewed in (51)). By comparison, Chk2 is phosphorylated within the non-catalytic N-terminus by ATM/ATR following DNA damage, promoting Chk2 dimerization, and facilitating autophosphorylation, both within the autoinhibitory loop and the activation loop (216, 256, 437, 441)(reviewed in (5)). While Chk1 and 2 phosphorylate many of the same downstream targets, they also have distinct functions, highlighted by the phenotypes of mice deficient for their genes. Chk1-null mice die early during embryogenesis, while Chk1-deficient embryonic stem (ES) cells and embryos display a defective G2/M arrest following UV and IR (231, 387). In contrast, mice deficient for Chk2 are viable, whereas mouse embryonic fibroblasts (MEFs) derived from them are radioresistant and display mild checkpoint defects in response to IR (155, 169, 386). These and other studies suggest that Chk1 is the major checkpoint kinase, while Chk2 has a specific function in IR-induced apoptosis and plays a supporting role in other circumstances (reviewed in (19)).

One of the key targets of Chk1/2 are the Cdk-activating phosphatases, Cdc25A, B and C, which are inhibited through phosphorylation by Chk1/2. Cdc25A promotes cyclin/Cdk activation at all stages of the cell cycle, whereas Cdc25B/C act predominantly during G2/M to dephosphorylate and activate Cdk1 (177, 209, 268, 272)(reviewed in (80)). Following genotoxic stress, Cdc25A is polyubiquitinated and degraded via the ubiquitin ligase Skp Cullin F-box complex-β-transducin repeat-containing protein (SCF-β-TrCP). Interaction between Cdc25A and SCF-β-TrCP requires phosphorylation of a DSG motif within Cdc25A
which occurs in a Chk1/2-dependent manner (42, 79). In mammalian cells, Chk1/2 phosphorylates Cdc25A, priming it for further DSG phosphorylation by NEK11 (79, 176, 260). In contrast to Cdc25A, Cdc25B/C are functionally inactivated by Chk1/2 phosphorylation, which creates 14-3-3 binding sites and leads to their cytoplasmic sequestration, away from their nuclear target Cdk1 (62, 64, 307)(reviewed in (35)). In addition, using a *Xenopus* egg/embryo system, Chk1 but not Chk2, was shown to inhibit Cdc25A through phosphorylation at a C-terminal site (T504 in *Xenopus* Cdc25A), which promoted 14-3-3 binding and inhibited Cdc25 interaction with cyclin/Cdk complexes (409). Sites equivalent to T504 exist in mammalian Cdc25A, B and C indicating that phosphorylation of this residue may contribute to phosphatase-inactivation in all three proteins.

### 1.1.4.3 p38 signaling in the checkpoint response

The serine-threonine kinase, p38, has been implicated in checkpoint control parallel to Chk1/2 (reviewed in (331)). p38 kinases are members of the family of mitogen activated protein kinases (MAPKs) and are part of the general stress response. They are activated by a variety of stimuli including osmotic stress, heat shock, UV, IR and reactive oxygen species (ROS) and, depending on the stimuli and cell context, can exert pro-survival, pro-apoptotic or pro-differentiation effects (reviewed in (418)).

Following genotoxic stress, p38 plays a critical role in checkpoint initiation by promoting inactivation of Cdc25B/C (41, 423)(reviewed in (331)). The p38-associated and activated kinase, MAPK activated protein kinase 2 (MK2, also known as Mapkap kinase 2),
phosphorylates Cdc25B and Cdc25C and, in a similar manner as Chk1/2 phosphorylation, this leads to 14-3-3 binding and cell cycle arrest (41, 248). Consistent with an integral role in checkpoint function, MK2-depleted U2OS cells (human osteosarcoma cells) are defective in both G1/S and G2/M checkpoints after UV irradiation (248). Several lines of evidence indicate that checkpoint control may be a conserved function of p38. In fission yeast, the p38/MK2 homologues, Sty1/Srk1, are required for G2/M arrest following osmotic stress, via a mechanism that involves Srk1 phosphorylation of Cdc25, 14-3-3 binding and cytoplasmic sequestration (2).

The mechanism of p38 activation is not completely understood, but appears to differ depending on the specific genotoxic stress involved. Raman et al. demonstrated that in HeLa cells, thousand and one amino acid (Tao) kinases, which are directly phosphorylated by ATM, were required for p38 activation in response to IR (327). Interestingly, this likely reflects a stimulus-specific response, as in ATM-deficient and ATR-defective fibroblasts, p38/MK2 activation was inhibited in response to doxorubicin, but not UV irradiation (330).

1.2. NimA-related kinases (NEKs)

1.2.1 Never in mitosis A (NimA)

The NEK family of serine-threonine kinases, which in mammals consists of 11 members, are the least characterized of the mitotic kinases. Never in mitosis A (NimA) is the founding member of the NEK family and was identified in the filamentous fungus, Aspergillus nidulans, in a screen for temperature sensitive mutations that blocked entry into
mitosis (278). At the non-permissive temperature, mutants of nimA arrest in late G2 with duplicated spindle pole bodies, which are the fungal equivalent of centrosomes (293). Conversely, overexpression of NimA caused premature entry into mitosis marked by chromosome condensation and aberrant mitotic spindle formation (278, 297).

NimA expression and activity is controlled in a cell cycle-dependent manner and by transcriptional, post-translational modification and proteolytic mechanisms, is restricted to a brief window during the G2-M transition and into mitosis (reviewed in (298)). In all eukaryotes studied to date, activation of p34^{cd2} is sufficient to initiate mitosis. The sole exception, is A.nidulans, where there is also a requirement for NimA (296). Unlike in higher eukaryotes, mitosis in A.nidulans is closed, meaning that nuclear envelope breakdown does not occur. In cells mutant for nimA, active p34^{cd2} remains in the cytoplasm suggesting that NimA is required to transport active cyclinB/p34^{cd2} complexes into the nucleus prior to mitosis (436). Interestingly, NimA itself is activated downstream of p34^{cd2}, likely via direct phosphorylation (451). Additional mitotic roles for NimA include induction of chromosome condensation through phosphorylation of histone H3 and a potential function in regulating nuclear membrane fission during mitotic exit (67, 69).

The function of NimA in promoting cell cycle progression in A.nidulans raised the possibility that homologues of NimA existed in higher eukaryotes. Consistent with this, overexpression of NimA in S.pombe and in human HeLa cells induced chromosome condensation in the absence of other mitotic events such as microtubule spindle assembly or p34^{cd2} activation (240, 289). Furthermore, overexpression of a catalytically-inactive NimA delayed entry into mitosis, suggesting that it may be interfering with the activity of an endogenous kinase or sequestering a conserved substrate (240). Indeed, NimA-related
kinases (NEKs) have been identified in higher eukaryotes where the family expanded through evolution. While a single NimA homologue exists in yeast, 2, 4 and 11 homologues were identified in *D.melanogaster, C.elegans* and mammals, respectively (Fig 1.4). While they display a high degree of sequence similarity within their kinase domains, NEK family members share little homology outside this region, indicative of a divergence in function. In support of this notion, the only gene that can functionally complement the *nimA* mutation is *nim-1* from the related fungus *Neospora crassa* (320). Neither of the yeast homologues of *nimA* (*fin1* in *S.pombe; KIN3 in *S.cerevesiae*) or NEK2, the closest mammalian homologue is able to rescue the cell cycle defect incurred by defects in *nimA* (202, 288).

### 1.2.2 Organization of NEK proteins

NimA has an N-terminal catalytic domain and, in terms of substrate specificity, exhibits specificity towards N-terminal hydrophobic residues and a phenylalanine at position –3, relative to the target residue (FR/KR/KS/T) (241). NimA contains coiled-coiled domains, which are important for oligomerization, and PEST sequences, which mediate ubiquitin-dependent proteolysis, a process that may be required for *A.nidulans* to exit mitosis (Fig 1.5) (319). Despite low overall sequence homology, the organizational features of NimA are broadly conserved among mammalian NEK kinases. For instance, all NEK kinases, except NEK10, contain N-terminal catalytic domains, whereas NEK4, 6 and 7 are the only family members that do not contain coiled-coiled motifs. Moreover, 6 of 11 mammalian NEK kinases contain putative PEST sequences.
Figure 1.4: Phylogenetic tree of NimA-related kinases.

Alignment of NimA-related kinase catalytic domains was generated using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).
Figure 1.5: Key structural features of NimA-related kinases.

Alignment of the key structural features of NimA-related kinases. Adapted from (288).
Outside regions of homology, certain NEK kinases contain unique protein domains that point to the acquisition of novel functions compared to the ancestral NimA. NEK8 and NEK9 feature regulator of chromosome condensation (RCC1) repeats. These repeats are also found in RCC1, a guanine nucleotide exchange factor (GEF) for the small GTPase, Ras-related nuclear protein (Ran). While the function of RCC1 repeats has not been defined in NEK8, in NEK9 this domain acts as a negative regulator of NEK9 catalytic activity and can interact with Ran. However, the consequence of this interaction for either NEK9 or Ran function is not clear (340). Additional unique domains in NEK family members include a predicted DEAD-box helicase-like domain in NEK5 and a cluster of armadillo repeats in NEK10 (288).

### 1.2.3 Mammalian NEK kinases

#### 1.2.3.1 NEK kinases in cell cycle progression

Based on sequence homology within the kinase domain, NEK2 is the closest mammalian NimA homologue. Unlike NimA however, NEK2 is not essential for mitotic entry, but regulates centrosome separation during mitosis. NEK2 localizes to centrosomes during interphase and early mitosis and interacts and phosphorylates several centrosomal proteins including cNap-1, Rootletin and β-catenin (14, 15, 110, 111). It has been proposed that the balance between phosphorylation and dephosphorylation of target substrates by NEK2 and its’ interacting partner, protein phosphatase-1 (PP-1), controls centrosome separation (150, 263). In support of a role for NEK2 in centrosome regulation,
overexpression of NEK2 in U2OS cells results in premature centrosome splitting during interphase, whereas expression of catalytically inactive NEK2 inhibits centrosome segregation and increases the rate of spindle defects and multinucleated cells (98, 111). Consistently, knockdown of NEK2 substrates cNap-1, Rootletin and β-catenin in human cells also causes defects in centrosome separation and spindle formation (14, 15).

In addition to centrosomes, there is preliminary evidence that NEK2 localizes to condensed chromatin during mitosis, and the midbody and kinetochores of dividing NIH3T3 cells (138). NEK2 interacts with certain centromeric proteins, including Hec1 and Mad1 and, in HeLa cells, NEK2 phosphorylation of Hec1 is required for proper chromosome alignment at metaphase (85, 238). Further, knockdown of NEK2 in HeLa cells leads to displacement of the centromeric protein Mad2 from kinetochores and impaired chromosome segregation (238). Taken together, these studies indicate that NEK2 plays multiple roles in coordinating cell division.

NEK6, 7 and 9 also participate in proper mitotic progression. NEK6 and NEK7 are highly related and are composed almost entirely of catalytic domains, which share 87% identity (181). They were originally identified based on their ability to phosphorylate p70 S6 kinase in vitro (23). However, this kinase is likely not a NEK6/7 target in vivo (234). Instead, there is increasing evidence that NEK6 and NEK7 are required for mitotic progression acting downstream of NEK9 (24, 292). All three kinases display elevated catalytic activity during mitosis, at which time NEK9 interacts with and activates both NEK6 and NEK7 (238). During interphase, NEK6 and NEK7 are repressed by a unique autoinhibitory mechanism, which is relieved by NEK9 in mitosis (334). Activation of NEK6 and NEK7 results from
interaction with NEK9’s non-catalytic C-terminal tail, which relieves autoinhibition, and direct NEK9-mediated phosphorylation within NEK6/7 activation loops (334, 340).

In HeLa cells, knockdown of either NEK6 or NEK7 leads to apoptosis following mitotic arrest (292). NEK6 or NEK7 deficiency in HeLa cells results in fragile spindle formation during mitosis and a prolonged activation of the SAC (292). If the SAC is inhibited, NEK6- or NEK7-depleted cells will continue to progress through mitosis but will then arrest during cytokinesis. Therefore, NEK6 and NEK7 function at multiple points during mitosis to regulate microtubule organization at the spindle poles, mitotic spindle and central spindle (292). Consistently, inhibition of NEK9, resulting from microinjection of α-NEK9 antibodies, impairs spindle assembly and chromosome alignment during metaphase (340). Interestingly, despite their strong homology, NEK6 and NEK7 are not redundant and cannot compensate for the loss of the other kinase (292). This may be explained by their differential tissue distribution and subcellular localization (101, 292).

In addition to a mitotic role, NEK9 may also function during interphase, when it interacts with the facilitates chromatin transcription complex (FACT), which modulates chromatin structure (52). Finally, HeLa cells depleted for NEK9 exhibit slower progression through G1 and S, implicating NEK9 in control of transcription and/or DNA replication (52).

1.2.3.2 NEK kinases in checkpoint control

In addition to roles in normal cell cycle progression, many members of the NEK family have been implicated in checkpoint control and the DNA damage response. Thus far, the molecular mechanism by which NEK11 contributes to checkpoint function has been best
characterized. Meliexetian et al. demonstrated that in response to IR, NEK11 was activated through Chk1-mediated phosphorylation (260). It has been previously established that Chk1 also phosphorylates Cdc25A on S76 (131). This event primes Cdc25A for further phosphorylation within the DSG motif, which is required for SCF β-TrCP-mediated ubiquitination and degradation of Cdc25A (176). Significantly, NEK11 has been identified as the Cdc25A DSG-motif kinase (260). Consistently, HeLa cells depleted for NEK11 display elevated levels of Cdc25A protein and fail to undergo IR-induced G2/M arrest (260).

In addition to NEK11, NEK1 and NEK2 also participate in IR-induced checkpoints. Following this insult, NEK1−/− cells are defective in the G1/S and G2/M checkpoints and in their ability to repair DNA, leading to accumulation of double strand breaks (313). Moreover, in HK2 and HeLa cells, NEK1 expression and catalytic activity are elevated in response to IR (50). Conversely, IR inhibits NEK2 activity in a PP-1-dependent manner, which is essential for the radiation-induced inhibition of centrosome splitting (266). In addition to IR, the catalytic activity of NEK1, NEK2, NEK6 and NEK11 is sensitive to genotoxic stresses such as UV and etoposide (104, 218, 286, 313). Thus, various NEK kinases participate in the cellular response to genotoxic stress and can act as positive and negative regulators of damage-induced checkpoints.

### 1.2.3.3 NEK kinases in ciliagenesis

Cilia are organelles that protrude from the cell surface and are structurally and functionally similar to flagella (reviewed in (93)). A cilium is classified as a motile or non-motile/primary cilium and is composed of a cylindrically organized, microtubule-based...
cytoskeleton, called an axoneme. Motile cilia function to move fluid and debris, and are found on various cell types including the efferent ductules of the testies, the ependymal lining of the brain and epithelial cells of the respiratory tract and oviduct (93). Until recently considered vestigial structures, primary cilia are present in most cells. They are generated during interphase from the mother centriole, which serves as the basal body (reviewed in (68))(375). The cilia dissemble prior to mitosis, allowing the centriole to nucleate the mitotic spindle (406)(reviewed in (324)). Both during development and in adulthood, the primary cilium performs key roles in coordinating extracellular conditions with cellular responses. Indeed, mutations in ciliary proteins are the basis of a number of human genetic disorders termed ciliopathies, including retinal degeneration, polycystic kidney, liver and pancreatic diseases and abnormalities in neural tube closure (reviewed in (26)).

A link between NEK kinases and ciliagenesis originates from identification of loss-of-function mutations in NEK1 and NEK8 as the causal events in independent mouse models of polycystic kidney disease (PKD) (232, 408). Two mouse models of PKD feature loss-of-function mutations in NEK1. The kidney, anemia, testes (kat) allele results from an internal deletion (bp 791-2105), whereas the kat2J allele contains a base pair insertion at position 966, which generates a premature stop codon (408). The junior cystic kidney (jck) mouse contains a glycine 488 to valine (G488V) mutation in NEK8, which alters the subcellular localization of NEK8. Immunohistochemical staining of mouse kidneys revealed that NEK8 is localized to the apical cytoplasm of duct collecting cells in wildtype mice but exhibits diffuse cytoplasmic staining in jck mice (232). Further supporting a causal role for NEK8 in PKD, zebrafish embryos depleted for NEK8 with antisense morpholinos develop pronephric cysts (232).
While the molecular mechanism by which NEK1 and NEK8 cause PKD is not fully understood, both proteins have been shown to regulate ciliagenesis. In primary kidney epithelial cells derived from wildtype mice, NEK8 localizes to primary cilia (370). This localization is lost in cells derived from jck mice and correlates with increased cilia length. Conversely, IMCD3 cells, derived from the inner medullary collecting duct of the murine kidney, overexpressing wildtype NEK1 do not contain primary cilia (430). Supporting the importance of NEK1 kinase activity in ciliagenesis, a catalytically inactive mutant of NEK1 localizes to primary cilia, but fails to affect cilia formation.

Appropriate regulation of cilia is tightly linked to cell cycle progression. The primary cilium is nucleated by the centriole, which needs to be liberated for proper spindle formation to occur during mitosis (406)(reviewed in (324)). Indeed, cell cycle proteins contribute to cilia assembly. For instance, mutations in Fa2, a NEK protein in Chlamydomonas, cause defects in both flagellar detachment and progression through the G2/M transition (246). A further example is Aurora A, which regulates centrosome maturation and mitotic entry during the cell cycle (27, 90). This kinase is also activated during cilium disassembly and is necessary for cilia-resorption in the human retinal epithelial pigment cell line (hTERT-RPE1) (321).

There may be a correlation between an expansion of NEK kinases in the genomes of higher eukaryotes, and the need to coordinate primary cilia with cell cycle progression (323). For example, Aspergillus nidulans and yeast are non-ciliated and contain a single NimA/NEK protein. Drosophila and C.elegans have 2 and 4 NEK kinases, respectively. While these organisms contain ciliated cells, these are terminally differentiated and do not coordinate cilia function with the cell cycle. In contrast, organisms such mammals,
Chlamydomonas and Tetrahymena, contain proliferating ciliated cells and feature an expansion in the number of NEK kinases they contain, with 11, 10 and 35 members, respectively. In these organisms, NEKs have been implicated in regulating cilia and/or centrioles/centrosomes (reviewed in (323)). For example, in mammals, in addition to NEK1 and NEK8, NEK2, 6, 7 and 9 associate with centrioles and/or the mitotic spindle. Future studies aimed at this largely unexplored aspect of the biology of NEK kinases will shed further light on their relationship with basal bodies, cilia and centrioles (reviewed in (323)).

1.3 NEK10

1.3.1 NEK10

NEK10 is a previously uncharacterized member of the NEK family and is evolutionarily its most divergent member. NEK10 is located on human chromosome 3p24 and encodes a 1125 amino acid protein. Unlike other NEK and NimA-related kinases, which feature N-terminal catalytic domains, NEK10’s kinase domain is centrally located. In addition, NEK10 contains coiled-coiled domains flanking the catalytic domain, a putative PEST sequence in the C-terminus and 4 N-terminal armadillo repeats (Fig 1.5).

Armadillo repeats are related to the HEAT (Huntington, Elongation Factor 3, PR65/A, TOR) domain and together they are found in less than 10 human kinases (249). Each armadillo repeat is composed of approximately 40 amino acids, which form three α-helices (162). It has been shown that multiple contiguous armadillo repeats are required to create a functional domain (156). Armadillo repeats have been implicated in coordinating
protein-protein interactions. For example, β-catenin’s armadillo repeats mediate interactions with several partner proteins including axin, cadherin and adenomatous polyposis coli (APC) (reviewed in (440)). Another well-characterized interaction is between the armadillo repeats of importin-α, which is part of the nuclear import machinery and the nuclear localization sequences of target proteins (151). The significance of armadillo repeats in NEK10 is currently unknown.

Judged by sequence homology within the catalytic domain, NEK10 is most similar to the mitotic kinases NEK6 and NEK7. Its closest homologue, however, is the uncharacterized C.elegans protein nekl-4, which also contains armadillo repeats and a centrally located catalytic domain. NEK10 and nekl-4 share 72% homology within the kinase domain and 34% overall homology. By comparison, NEK10 shares only 54% homology within the kinase domain with NEK6/7. nekl-4 expression as detected by in situ hybridization is ubiquitous during C.elegans embryogenesis, but is restricted during larval and adult stages to a limited number of cells, likely either muscle or nerve cells near the pharynx (http://nematode.lab.nig.ac.jp/). In large-scale, loss of function screens, knockdown of nekl-4 has not been associated with any of the common phenotypes examined, such as sterility, lethality or defects in movement or egg laying, and its physiological function is currently unknown (http://www.wormbase.org/).

1.3.2 NEK10 and carcinogenesis

Growing evidence indicates that NEK10 may participate in oncogenesis. Whole genome sequencing of primary tumours and immortalized human cancer cell lines uncovered
more than a 1000 somatic mutations within the coding sequences of the 518 predicted human protein kinases (66, 136). Mutations in NEK10 were found in primary tumours (ovarian mucinous carcinoma (A66K), large cell carcinoma (R878M)) and cultured cell lines (metastatic melanoma (E379K), and lung neuroendocrine carcinoma (P1115L)) (136). None of the identified mutations map to the catalytic domain of NEK10, and their effect on protein function is currently unknown. Somatic mutations fall into two classes, driver mutations, which confer a growth advantage and are positively selected, and passenger mutations, which occur by chance and are not subject to selection. Based on the frequency of mutations, NEK10 was defined as one of 120 kinases predicted to contain a driver mutation (136). Of note, NEK10 mutations were found with the same frequency (4/33) as the mutations of B-Raf and liver kinase B1 (LKB1), kinases previously implicated in tumourigenesis (66).

A further suggestion of potential NEK10 involvement in cancer came from a genome wide association study (GWAS) involving over 37,000 breast cancer samples and 40,000 controls, which identified a strong breast cancer susceptibility locus within 3p24 (p value = 4.1 x 10^{-23}). Importantly, the sub-region of 3p24 identified by this GWAS contains only two genes, NEK10 and solute carrier family 4, sodium bicarbonate co-transporter, member 7 (SLC4A7).

Prompted by the reports of NEK10 mutations in human cancers, our lab explored NEK10 expression profiles in a series of breast cancers. Mining the NKI295 breast cancer dataset, consisting of 295 breast cancer patients, we found that low NEK10 expression was significantly associated with >20% reduction in 5-year and overall disease-free survival (p = 0.0014) (Fig 1.6C) (411). When cross-referenced with key pathology parameters, low NEK10 expression was associated with a number of characteristics indicative of poor
Figure 1.6: Low NEK10 expression is associated with poor prognosis in breast cancer.

NEK10 expression was analysed in the NKI295 breast cancer dataset. Low NEK10 expression was significantly associated with:
A) triple negative status (P<0.0001).
B) poorly differentiated tumours (P<0.001).
C) a greater than 20% reduction in 5-year and overall disease-free survival (P=0.0014).
prognosis including triple negative status and poorly differentiated tumours (Fig 1.6A, 1.6B). Consistent with this data, low NEK10 expression was associated with higher tumour grade in an independent cohort of 88 breast cancers isolated and analyzed at Princess Margaret Hospital/Ontario Cancer Institute (in collaboration with Dr. Wey-Liang Leong, Surgeon-Scientist, PMH). Finally, compared to nonmalignant control tissue, reduced NEK10 expression was found in high-grade serous ovarian carcinoma and in a series of nasopharyngeal carcinomas (362, 401). Taken together, these studies strongly implicate NEK10 expression as a possible risk factor for disease severity in several human cancers.

1.4 ERK1/2 signaling

Work described in Chapter 2 links NEK10 to regulation of the ERK1/2 pathway. This section will provide a brief overview of ERK1/2 signaling, with emphasis on its function in mammalian systems.

1.4.1 Organization of MAPK signaling modules

Mitogen activated protein kinase (MAPK) pathways are highly conserved kinase cascades. They are activated by a wide variety of extracellular stimuli and are critical for numerous cellular processes such as proliferation, differentiation, migration, and apoptosis (reviewed in (48)). The three best-characterized MAPK subfamilies are extracellular regulated kinases (ERK1/2) (also known as MAPK1/2), Jun NH2 terminal kinases

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(JNK1/2/3, also known as stress activated protein kinases (SAPK1/2/3)) and p38α/β/γ/δ kinases.

MAPK cascades are three-tiered signaling modules consisting of a MAPK, a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK) (reviewed in (203)). MAPKKK are serine-threonine kinases that are activated in response to extracellular stimuli by phosphorylation and/or interactions with small GTP binding proteins such as Ras or Rho. MAPKKK phosphorylate and activate the dual specificity kinases, MAPKK, which in turn activate the MAPK by phosphorylation of a conserved TXY motif within the activation loop. MAPKs are proline-directed serine-threonine kinases and phosphorylate substrates on S/T residues that are directly followed by a proline residue.

ERK1/2 were the first identified MAPK subfamily and are strongly activated by growth factors, serum and phorbol esters (57, 342). In addition, ERK1/2 show lower levels of activation to a wide variety of stimuli including cytokines, G-protein coupled receptor activation and genotoxic stress (99, 229, 233). JNK1/2 and p38 MAPK families are most strongly activated by cellular stresses including UV irradiation, genotoxic stress and in the case of p38, osmotic stress (141, 152)(reviewed in (182)).

1.4.2 ERK1/2 signaling

The canonical ERK1/2 signaling module is composed of Raf (MAPKKK), MEK (MAPKK) and ERK (MAPK) kinases which are activated downstream of the small GTPase Ras (Fig 1.7) (reviewed in (259)). The ERK cascade is one of the most extensively studied signaling pathways and plays a central role in organismal development and homeostasis and
Treatment of cells with mitogenic stimuli such as growth factors, peptide hormones, neurotransmitters and serum activates canonical ERK signaling. Ligand binding activates cell surface receptors, such as receptor tyrosine kinases (RTKs), and through adaptors, such as growth factor receptor-bound protein 2 (Grb2) and exchange factors, such as son of sevenless (SOS), stimulates GTP binding to Ras. The active, GTP-bound form of Ras binds to effectors, including Raf kinase, and recruits it to the plasma membrane where it is activated. Following its activation, Raf kinase phosphorylates and activates MEK. Activated MEK then activates ERK by phosphorylation on tyrosine and threonine residues. Active ERK phosphorylates numerous substrates in the cytoplasm and the nucleus. Depending on the cell-type and stimulus involved, ERK activation can lead to proliferation and differentiation and when deregulated oncogenesis and growth arrest. Scaffold proteins of ERK cascade components can increase the efficiency, specificity and cellular compartmentalization of ERK signaling. Adapted from: (259, 429).
when deregulated, in carcinogenesis. Activating mutations in Ras and Raf are frequently found in tumours and ERK signaling is best characterized in the context of growth factor stimulation and control of cell proliferation.

1.4.2.1 MAPKKK: Raf kinase

Raf is the primary MAPKKK involved in ERK signaling and constitutes the first step in the kinase cascade. There are three Raf isoforms in mammals, A-Raf, B-Raf and C-Raf (also known as Raf-1) (165). B-Raf is most closely related to the single Raf isoform present in lower eukaryotes such as *D. melanogaster* (DRaf) and *C. elegans* (lin-45) (457). The three kinases have unique and overlapping functions. A-Raf knockout mice are viable, but exhibit neurological and intestinal defects after birth (318). Deletion of either B-Raf or Raf-1 in mice results in embryonic lethality around midgestation (164, 267, 434). Interestingly, in response to growth factors, both A-Raf- and Raf-1-deficient fibroblasts exhibit normal ERK activation whereas ERK activation is reduced, but not abolished in cells derived from B-Raf-deficient embryos (164, 267, 318, 434).

Raf-1 was originally identified as the cellular homologue of the viral oncogene v-raf, and is the best characterized of the three isoforms (328). More recently, interest in B-Raf has been sparked by the discovery that activating mutations in B-Raf frequently occur in human tumours (65). In contrast, little is known about the physiological role of A-Raf, although it is predicted that it’s mechanism of activation is similar to Raf-1 (252)(reviewed in(429)). Raf-1 activation following growth factor stimulation is complex and involves protein-protein interactions, changes in subcellular localization and multiple phosphorylation events. In
unstimulated cells, Raf-1 is cytoplasmic and is held in an autoinhibited state by phosphorylation and 14-3-3 binding at S259 and S621 (220, 407). Following growth factor stimulation, Raf-1 is recruited to the plasma membrane by an interaction with the effector domain of the activated GTPase Ras (220, 274). This promotes dephosphorylation of inhibitory phospho-sites and phosphorylation of positive regulatory sites including S338, Y341 and T491 and S494 within the activation loop (71, 220)(reviewed in (74)). Candidate upstream kinases that contribute to phosphorylation of the activating sites include protein kinase B (PKB, also known as Akt) for S259, p21-activated kinase (Pak) for S338 and Src and Janus kinase (JAK) kinases for Y341 (96, 191, 251, 462).

In comparison to Raf-1, activation of B-Raf is simpler in response to extracellular stimuli, requiring only Ras binding and activation loop phosphorylation (252). Raf-1 contains five activating phosphorylation sites, while in B-Raf only three appear to play similar functions. This includes S728 (equivalent to S621 in Raf-1), which mediates 14-3-3 binding, and activation loop sites T598 and S601 (equivalent to T491 and S494 in Raf-1) (reviewed in (429)). In Raf-1 phosphorylation of Y341 and S338 are also required for activation. In contrast, B-Raf exists in a “primed” state and in B-Raf these residues are replaced by an aspartic acid (D448) and are constitutively phosphorylated (S445), respectively (253). The fact that B-Raf exists in a primed state may contribute to the fact that it, but not Raf-1 or A-Raf, is found mutated in human tumours. Raf-1 and B-Raf are also subject to feedback phosphorylation by ERK (16, 83, 337). In the case of Raf-1, the significance of this event is not fully understood whereas for B-Raf, phosphorylation acts to negatively regulate its activity.
Defining the specific roles of B-Raf and Raf-1 in ERK activation is further complicated by the ability of B-Raf and Raf-1 to homo- and heterodimerize. The importance of Raf dimerization for ERK activation was highlighted by the discovery of oncogenic mutants of B-Raf, which have reduced catalytic activity but still activate ERK (420). This seemingly contradictory property can be explained by the fact that these B-Raf mutants bind and stimulate Raf-1 catalytic activity (117, 420). Systematic evaluation of Raf dimers revealed that B-Raf/Raf-1 heterodimers display higher catalytic activity compared to homodimers/monomers of B-Raf, which themselves have higher activity than the homodimers/monomers of Raf-1 (117, 348). Interestingly, heterodimers composed of one active and one inactive Raf kinase exhibit similar activity to heterodimers composed of two active kinases (348). This suggests that enhanced catalytic activity is a result of the physical interaction and is not caused by intermolecular phosphorylation. Initially, the fact that growth factor-stimulated ERK activation is compromised in B-Raf- but not Raf-1-null fibroblasts was interpreted to indicate that B-Raf was the predominant kinase for MEK (164, 267, 434). Nevertheless, subsequent studies demonstrated that loss of B-Raf also impaired Raf-1 activity, suggesting that the effect on ERK phosphorylation likely arises from a combined decrease in B-Raf and Raf-1 activity within the cell (117).

MEK1/2 are the only well-defined Raf substrates. However there is accumulating evidence that Raf-1 has kinase-independent functions in apoptosis. Raf-1 deficient embryos display increased apoptosis, while Raf1−/− cells are hypersensitive to pro-apoptotic stimuli such as etoposide and α-Fas antibody (164, 267). Hypersensitivity to apoptosis in the absence of Raf-1 is independent of MEK, as it can be rescued by expression of a catalytically impaired mutant of Raf-1 (Raf-1 Y340F Y341F), but appears dependent on the ability of

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Raf-1 to interact with, and inhibit, other pro-apoptotic kinases, including apoptosis signal-regulating kinase 1 (ASK1) and MST2 (164, 291, 443) (reviewed in (116)).

In addition to Raf kinases, other MAPKKKs that activate MEK in a cell-type and stimulus-specific manner exist. For instance, the serine-threonine kinase, mouse sarcoma (Mos), acts as the MAPKKK in germ cells where it is necessary for MEK/ERK activation during oocyte maturation (316, 414) (reviewed in (350)). Oocyte maturation occurs during ovulation and is the process by which oocytes arrested at the first meiotic prophase reenter meiosis in response to hormones such as progesterone (reviewed in (350)). These cells subsequently arrest at the second meiotic metaphase (metaphase II) as mature unfertilized oocytes. Mos and MAPK are essential components of the cytostatic factor (CSF), which is responsible for metaphase II arrest (140, 414) (reviewed in (350)). Another stimulus-specific MAPKKK is tumour progression locus 2 (Tpl-2, also known as MAP3K8 and Cot), implicated in innate immunity. Tpl-2 links toll-like receptor (TLR) activation to tumour necrosis factor (TNF) production by regulating ERK1/2. This is best exemplified by the lack of ERK1/2 activation and TNF-α production in LPS-treated macrophages isolated from Tpl-2 knockout mice (87). Interestingly, in MEFs, Tpl-2 is also implicated in activating ERK1/2 and JNK1/2 in response to TNF-α (63).

1.4.2.2 MAPKK: MEK1/2

In the ERK1/2 cascade, the dual specificity kinases, MEK1/2 are the MAPKK which lie immediately downstream of the MAPKKK. MEK1 and MEK2 are highly homologous, displaying 80% identity, and perform both overlapping and non-redundant roles within the
cell. MEK2-null mice are viable and fertile and cells derived from them exhibit normal ERK activation (22). By contrast, mice null for MEK1 are embryonic lethal. Interestingly, MEFs derived from these mice display normal ERK2 activation, indicating that this function of MEK1 may be compensated for by MEK2 (127). Consistent with distinct physiological roles, MEK1 may be regulated independently from MEK2. MEK1, but not MEK2, is subject to positive (S298 by Pak1) and negative (T292 by ERK) regulatory phosphorylation and in certain circumstances may be preferentially activated. For instance, in serum-stimulated NIH3T3 cells MEK1, but not MEK2, forms a complex with Ras and Raf-1 (175).

Generally, MEK1/2 are activated by phosphorylation of S218 and S222 (in MEK1) within the activation loop by the upstream MAPKKK, most commonly Raf kinases (459). An additional level of regulation can be provided by other phosphorylation events, in a context-dependent manner. For instance, in adherent cells, MEK1 activation is promoted by phosphorylation on S298 by Pak1, which can be inhibited through feedback phosphorylation on MEK1 T292 by ERK1/2 (109). Phosphorylation of S298 has also been shown to promote MEK1 autophosphorylation in the context of cell adhesion (303). ERK1/2 are the only established MEK1/2 substrates, a testament to the high degree of substrate specificity of MEK1/2.

1.4.2.3 MAPK: ERK1/2

ERK1/2 (also known as p42/p44MAPK and MAPK1/2) are serine-threonine kinases originally identified as growth-factor stimulated kinases, which phosphorylate microtubule-associated protein-2 (MAP-2) and myelin basic protein (MBP) (159, 341). Since their
discovery, many lines of investigation have revealed their essential functions in numerous cellular processes. Despite the high degree of homology between ERK1 and ERK2 (85% amino acid identity), knockout mice display distinct phenotypes. ERK1-deficient mice are viable but display defective thymocyte maturation (299). In particular, ERK1 is critically required for positive selection of thymocytes, as well as for thymocyte proliferation (299). In contrast, ERK2-null mice are embryonic lethal at gastrulation (E6.5), due to a defect in mesoderm differentiation (450). Immunohistochemical analysis of ERK2−/− embryos does not reveal any activation specific-ERK staining, indicating that ERK2 but not ERK1 is active during early embryogenesis (450). Interestingly, ERK2−/− embryos (E6.5 and E7.5) and ES cells display normal proliferation, suggesting that at this early stage in development, ERK signaling is dispensable for control of proliferation.

ERK1/2 are activated by phosphorylation of T202/Y204 (in ERK1) within the TEY motif by the dual specificity MAPKKs, MEK1/2 (43, 460). Phosphorylation of these sites is necessary and sufficient for ERK activation and dephosphorylation of either site by serine-threonine, tyrosine or dual specificity phosphatases is a powerful means of downregulation. The dual specificity MAPK phosphatases (MKPs) are the largest family of phosphatases that act primarily on MAPKs. There are 10 MKPs, which display varying degrees of specificity towards the ERK, p38 and JNK MAPKs (reviewed in (187)). Many of the MKPs are present at low levels in unstimulated cells and are transcriptionally induced upon appropriate extracellular stimuli including serum, growth factors and cytokines (36, 92). Significantly, expression and protein stability of several MKPs is MAPK dependent (reviewed in (187, 305)). For example, MKP-1 (also known as DUSP-1) protein is undetectable in quiescent CCL39 cells, a Chinese hamster fibroblast cell line, but is induced within 30 minutes of
serum stimulation in an ERK-dependent manner (36, 37). The increase in MKP1 protein levels is mediated by increased gene expression, and direct phosphorylation by ERK. This indicates that ERK signaling can stimulate feedback loops and negatively regulate its own activation.

Unlike Raf and MEK, ERK1/2 have numerous downstream targets, both in the cytoplasm and in the nucleus. These include MAPK activated protein kinases (MAPKAPs) such as ribosomal S6 kinases (RSKs), mitogen and stress activated protein kinases (MSKs) and MAPK integrating kinases (MNKs), cytoskeletal proteins, tau and paxillin, and nuclear substrates, including the transcription factors, E-26 like protein 1 (Elk-1) and myelocytomatosis viral oncogene homolog (Myc), and FBJ murine osteosarcoma viral oncogene homolog (fos) and jun, which comprise the activator protein 1 (AP-1) transcription factor (reviewed in (454)). ERK1/2 are proline-directed kinases and phosphorylate serine/threonine residues that are followed by a proline residue (133). Based on the looseness of this consensus sequence, ERK1/2 can theoretically phosphorylate 1000’s of proteins. However, additional specificity is provided by the presence of docking motifs on both ERK1/2 and putative targets.

ERK kinases recognize at least two motifs: the docking (D) motif and the docking site for ERK and FXFP (DEF, also known as FXF) motif (170, 449). The D motif is recognized by most MAPKs with varying specificity. In contrast, the DEF motif is specifically targeted by ERK1/2, but not by related MAPKs, p38 or JNK (170, 448). The D and DEF motifs, either alone or in combination, are found in many proteins that interact with ERK (170). For example, the transcription factor elk-1 contains a D motif and a DEF motif. The former mediates interactions with ERK and JNK1/2, whereas the latter binds ERK alone (170). In
comparison kinase suppressor of ras (KSR), which interacts with ERK but not JNK or p38 contains a DEF motif alone (170). The presence of two ERK interaction motifs in elk-1 is consistent with the stronger affinity that ERK has for elk-1 compared to KSR which contains one interaction motif (170).

In addition to docking motifs present on substrates, ERK1/2 itself contains protein sequences that facilitate protein-protein interactions. The C-terminal common docking (CD) site, also present on other MAPKs, mediates interaction with D-domains, whereas the ERK docking site (ED), only found on ERK1/2, recognizes the DEF motif (75, 219, 391, 392). The modular system of recognition motifs allows MAPKs to achieve substrate specificity and explains how different MAPK families can have partially overlapping substrate specificities.

1.4.3 Molecular mechanisms of ERK signaling regulation

ERKs are activated by a wide array of extracellular stimuli, which promote various biological outcomes in a cell-type-, tissue- and stimulus-specific manner. For instance, sustained ERK activation stimulates proliferation in fibroblasts, whereas it is associated with neurite outgrowth and differentiation in PC12 cells (rat pheochromocytoma cells) (280, 404). Even within the same cell type, ERK activation can produce different phenotypes depending on the strength and duration of the signal. In PC12 cells, nerve growth factor (NGF) and epidermal growth factor (EGF) stimulation leads to sustained and transient ERK activation, respectively. While transient ERK activation drives proliferation, sustained is responsible for differentiation (404, 405). The wide spectrum of ERK targets necessitates their tight regulation.
1.4.3.1 Regulation by subcellular localization

In most unstimulated cells, ERK is cytoplasmic. As much as half of ERK activity is associated with microtubules where it influences cytoskeleton dynamics (160, 333). ERK is also retained in the cytoplasm by association with a number of cytoplasmic proteins, including MEK1/2 (113). Following stimulation by certain extracellular stimuli, such as growth factors and phorbol esters, active ERK can translocate to the nucleus via both passive diffusion and active transport (3, 49, 221). The importance of ERK nuclear translocation is highlighted by the fact that it is required for a number of ERK functions such as stimulation of NIH3T3 cell proliferation and PC12 cell differentiation (39, 339).

Nuclear translocation facilitates access of ERK to its nuclear substrates. For example, in primary human foreskin fibroblasts (HFFs), ERK nuclear translocation is induced by phorbol 12-myristate 13-acetate (PMA) but not EGF (431). RSK, a cytoplasmic target of ERK1/2, is phosphorylated to equal levels in PMA- and EGF-treated cells. However in PMA-, but not EGF-treated cells, this is accompanied by the accumulation of c-fos, a nuclear ERK1/2 target, and cyclinD1, which is a transcriptional target of c-fos. Interestingly, the difference in nuclear localization is independent of the amplitude and duration of ERK1/2 activation, which is similar in response to either stimuli (431). Therefore, differences in subcellular localization provide flexibility to generate distinct phenotypic responses to different extracellular stimuli.
1.4.3.2 Regulation by scaffolds

Scaffold proteins act as docking platforms that serve to colocalize the components of a particular signaling cascade. The first identified MAPK scaffold was Ste5 in budding yeast (53, 200). Since then, numerous proteins that fulfill similar functions have been characterized. MAPK scaffolds interact with multiple components of the MAPK cascades and modulate the strength, duration and location of activation (reviewed in (73)). Furthermore, they can provide signal specificity by promoting activation in response to particular stimuli and access to specific substrates (45).

KSR is the best characterized ERK1/2 scaffold and was concurrently identified in \textit{D.melanogaster} and \textit{C.elegans} in screens for modifiers of Ras signaling (196, 383, 396). In cultured cells, KSR constitutively binds MEK and ERK, and following mitogenic stimulation, Raf (397, 438, 455). KSR promotes ERK activation by bringing MEK/ERK into close proximity to Raf, thereby facilitating cascade activation (344). In addition, KSR binding may directly stimulate Raf catalytic activity (326). In cells derived from KSR1-null mice, the amplitude and duration of ERK activation following growth factor stimulation is decreased (197). Furthermore, while these cells proliferate normally, their tumourigenic potential in response to oncogenic Ras expression is lowered (197).

Other scaffolds with capacity to provide stimulus and substrate specificity to the ERK cascade exist. Ectopic expression of one of these, MAPK organizer-1 (MORG-1), in NIH3T3 cells, promotes ERK1 activation in response to PMA and lysophosphatidic acid, but not EGF, whereas in PC12 cells, another scaffold, regulator of G-protein signaling-12 (RGS-12),
is required for NGF-, but not fibroblast growth factor (FGF)-induced neurite outgrowth (416, 433). Finally, through direct interaction with ERK cascade components, scaffold proteins can direct ERK activation to specific subcellular compartments, such as MEK partner 1 (MP-1) to endosomes, similar expression to fgf genes (Sef) to the Golgi, and Paxillin to focal adhesions (167, 395, 402).

1.4.4 Physiological roles of ERK1/2 signaling

ERK1/2 are activated by numerous extracellular signals, which produce discrete outcomes in a cell- and stimulus-specific manner. Traditionally, ERK1/2 signaling has been associated with promoting proliferation and cell transformation. Nevertheless, ERK1/2 activation also contributes to cell differentiation, migration, embryonic development and the cellular response to genotoxic and inflammatory stresses.

1.4.4.1 ERK1/2 signaling in oncogenesis

Deregulated ERK1/2 signaling can contribute to cell transformation and tumourigenesis. Activation of ERK1/2 signaling is found in many tumours due to the activating mutations in its upstream regulators (receptor tyrosine kinases (RTKs), Ras, and Raf) or downregulation of its negative regulators (Sprouty, neurofibromatosis type 1 (NF1)) (reviewed in (183)). ERK activation likely contributes to tumourigenesis through multiple mechanisms, promoting proliferation, survival, motility and angiogenesis. Curiously, mutations in MEK and ERK have thus far not been identified in human cancers.
Activating Ras mutations are found in approximately 30% of human tumours with the highest incidence in pancreatic, colon and lung cancers (reviewed in (33, 183)). Ras acts via several downstream effectors that can contribute to transformation. While ERK activation is not necessary for Ras-mediated NIH3T3 transformation, constitutive activation of Raf or MEK is sufficient to promote cell transformation and tumourigenesis in mouse models (250, 328, 377).

Even though Raf-1 was initially identified as a viral oncogene, mutations in Raf-1 or A-Raf have not been found in human tumours (328). Nevertheless, activating mutations in B-Raf are found in approximately 7% of human tumours, most commonly in melanoma, colorectal, ovarian and papillary thyroid carcinomas (65). The V600E mutation accounts for 80% of B-Raf mutations found in cancer and encodes a constitutively active, Ras-independent protein kinase (65). Other mutations in B-Raf have been identified at much lower frequencies and include at least three mutants that display decreased catalytic activity, yet retain the ability to activate the ERK pathway through a Raf-1-dependent mechanism (65, 420).

1.4.4.2 ERK1/2 signaling in cell cycle regulation

Early work on ERK1/2 linked them to activation by a variety of growth signals. A close correlation was also found between sustained ERK1/2 activity and DNA synthesis (179, 262). Further work has established that entry into S-phase requires sustained ERK signaling through till late G1 (427, 444). During G1, one of the key events downstream of ERK1/2 is cyclinD1 induction (427). The cyclinD1 promoter contains an AP-1 binding site...
and sustained ERK likely induces cyclinD1 expression by promoting stabilization of fos and jun family members, which form the AP-1 transcription factor (18). This is consistent with the ability of c-fos to act as a molecular sensor for ERK signal duration (280). c-fos is unstable in conditions of transient ERK activation (280). However, following prolonged activation, ERK phosphorylates the C-terminus of c-fos. This stabilizes c-fos and exposes a DEF domain allowing further ERK-mediated phosphorylation. The hyperphosphorylated form of c-fos is active and can promote expression from AP-1 target genes and cellular transformation (280).

In addition to fos and jun, ERK targets the transcription factor c-Myc to promote G1 progression. ERK stabilizes c-Myc by direct phosphorylation at S62, leading to an upregulation of c-Myc targets, including cyclinD2, Cdk4, and Cdc25A (359) (reviewed in (261)). Finally, continuous ERK activation is required through G1 to repress expression of up to 100 antiproliferative genes (444). The importance of this inhibition is demonstrated in NIH3T3 cells where ectopic expression of a number of these genes, including JunD and Gadd45α, inhibits serum-stimulated proliferation (444).

Mammalian cells are only sensitive to growth factor stimulation prior to passing R. However, there is accumulating evidence that ERK is also important at later stages of the cell cycle. After the G1/S transition, inhibition of ERK activity can delay entry into mitosis and increase its duration, indicating that ERK signaling participates in both the G2/M transition and mitotic progression (338) (reviewed in (47)). ERK signaling may impact these processes by promoting activation and nuclear localization of the cyclinB1/Cdk1 complex. CyclinB1/Cdk1 is primarily cytoplasmic in interphase and accumulates in the nucleus prior to mitosis (84). In Xenopus oocytes, ERK directly phosphorylates the cytoplasmic retention
sequence (CRS) of cyclin B1, which in conjunction with phosphorylation by Plx, increases its nuclear localization at the G2/M transition (419). ERK activity also regulates proteins that control Cdk1 (p34\textsuperscript{cdc2}) activation. During Xenopus oocyte maturation, RSK, phosphorylates and inhibits Myt1, a p34\textsuperscript{cdc2} inhibitory kinase (301). Additionally, in both Xenopus oocytes and human cells, ERK activates the Cdk-phosphatase, Cdc25C, by direct phosphorylation on T48 (422). Finally, localization of active ERK on the spindle and midbody during mitosis suggests that it may also influence chromosome dynamics during mitosis (432)(reviewed in (47)).

Somewhat paradoxically, while ERK1/2 activation promotes proliferation and transformation in many cultured cell lines, its hyperactivation can lead to differentiation or cell cycle arrest in both primary and immortalized cells (352, 363)(reviewed in (47)). ERK-mediated cell cycle arrest is accompanied by elevated levels of the Cdk inhibitors p21 and p16\textsuperscript{INK4a} and can be overcome by co-expression of the adenoviral oncogene E1A or ablation of a tumour suppressor such as p53 or p16\textsuperscript{INK4A} (363, 461). This suggests that there is selective pressure to mutate p53 or p16\textsuperscript{INK4a} during tumourigenesis to avoid cell cycle arrest. Indeed, Ras mutations are often found in combination with p53 or p16\textsuperscript{INK4a} mutations in human tumours (363). ERK hyperactivation may also promote cell cycle arrest through a Cdc25A dependent mechanism. In Xenopus oocytes, Cdc25A degradation is induced by expression of constitutively active MEK, by a mechanism involving both ERK and p90RSK activity (168). Taken together, these studies suggest that in the presence of deregulated mitogenic stimulation, cells may induce senescence as a tumour suppression mechanism to prevent inappropriate cell growth and proliferation.
1.4.4.3 ERK1/2 signaling in development and differentiation

Early characterization of ERK signaling in *Drosophila* and *C. elegans* revealed their roles in eye and vulval development, respectively (30, 208). In addition to defining the hierarchal order of the ERK cascade, these studies highlighted a function for ERK signaling in organism development and cellular differentiation. This has been substantiated by studies examining the activation pattern of ERK during embryogenesis in zebrafish, *Xenopus*, *Drosophila*, and mice, where it is largely FGF-dependent (59, 61, 114, 115). Despite the high level of proliferation that is occurring in the embryo, active ERK displays restricted spatio-temporal localization, suggesting that it is predominantly involved in tissue patterning during embryogenesis (59). Whereas ERK1^-/-^ mice are viable, ERK2^-/-^ mice are early embryonic lethal (E6.5) due to a defect in mesoderm differentiation. Interestingly ERK2-null embryos display normal proliferation, indicating that at this stage ERK signaling is required for differentiation but not proliferation. Other transgenic mouse models have demonstrated critical functions of ERK signaling in placental angiogenesis, and bone and brain development during embryogenesis, and in adipogenesis, learning, and memory in adult organisms (34, 148, 257, 258) (reviewed in (351)).

Developmental functions of ERK signaling are further highlighted by a class of developmental syndromes termed RASopathies caused by germline mutations in several constituents of the ERK pathway, such as Raf-1 germline mutations associated with Noonan and Leopard syndromes (302). RASopathies display partially overlapping phenotypes including facial and cardiac abnormalities, neurocognitive delays and malignancy (reviewed
in (399)). Finally, a genetic link has been discovered between ERK1 and autism. Approximately 1% of all cases of autism are associated with a duplication or deletion in chromosome 16p11.2 a locus that encodes ERK1 (206, 428).

1.4.4.4 ERK1/2 in the response to genotoxic stress

The JNK and p38 kinases are strongly activated by cellular stress and are considered the predominant MAPKs involved in the stress response. However, ERK activation in response to these stimuli can also be detected, albeit to a lesser extent than in response to mitogens. In particular, ERK1/2 activation has been demonstrated following UV, IR, etoposide, adriamycin and MMC exposure, as well as following breast cancer 1, early onset (BRCA1) overexpression, implicating ERK1/2 in the cellular response to genotoxic stress (329, 390, 446).

ERK1/2 activation following genotoxic insults has been linked with appropriate cell cycle arrest. Yan et al. demonstrated that BRCA1 overexpression in MCF7 cells, which led to a G2/M cell cycle arrest, was marked by Cdc25C degradation, and Wee1 and ERK1/2 activation (446). Notably, in this system, checkpoint activation and Cdc25C and Wee1 regulation, required ERK1/2 activation (446). ERK1/2 activation was also required for appropriate hydroxyurea-induced, S-phase arrest, in MCF-7, NIH3T3 and HCT116 cells, where inhibition of ERK1/2 was associated with decreased induction of γH2AX foci and ATR nuclear localization (435). In addition, MCF-7 cells treated with a MEK1/2 specific inhibitor, U0126, had defects in the G2/M cell cycle arrest after IR (445). Interestingly, this may be a MEK2 specific response, as in IR-treated HeLa cells, expression of dominant
negative MEK2, but not MEK1, inhibited initiation and recovery of cells from G2/M arrest (1).

1.5 Ph.D. Outline

1.5.1 Study Rationale

Deregulation of the cell cycle is a hallmark of neoplastic transformation and participates in both the initiation and progression of cancer. Several kinase families play an indispensable role in governing the mammalian cell cycle including Cdk, Aurora, Polo and NEK kinases. The focus of this thesis is the biology of NEK10, an unexplored member of the NEK family.

In Chapter 2, I discuss the importance of NEK10 for G2/M arrest and MEK autoactivation in response to UV irradiation. In Chapter 3, I examine the mechanism of NEK10 regulation by phosphorylation and subcellular localization. Given that loss of NEK10 has been correlated with poor prognostic characteristics in breast cancer, these studies will provide insight into the molecular function of NEK10 and its role in tumourigenesis.

1.5.2 Thesis Aims

1. Investigate the cellular role of NEK10 in response to UV irradiation

2. Investigate the role of phosphorylation in regulating NEK10
CHAPTER 2

NEK10 ENHANCES MEK AUTOPHOSPHORYLATION FOLLOWING UV IRRADIATION

A version of this chapter is in revision with MCB
2.1 Abstract

Appropriate cell cycle checkpoint control is essential for the maintenance of cell and organismal homeostasis. Members of the NimA-related kinase (NEK) family of serine/threonine protein kinases have been implicated in regulation of various aspects of the cell cycle. We report the cloning and characterization of NEK10, a novel member of the NEK family and demonstrate a role for NEK10 in the cellular UV response. NEK10 was required for the activation of ERK1/2 signaling upon UV irradiation, but not in response to mitogens, such as epidermal growth factor. NEK10 physically associated with Raf-1 and MEK1 in a Raf-1-dependent manner, and the formation of this complex was necessary for NEK10-mediated MEK1 activation. NEK10 did not affect the kinase activity of Raf-1 but instead promoted autophosphorylation-dependent activation of MEK1. The appropriate maintenance of the G2/M checkpoint following UV irradiation required NEK10 expression and ERK1/2 activation. Consistently, the NEK10 C.elegans homologue, nekl-4, modulated UV-irradiation sensitivity during embryogenesis. Taken together, our results uncover a conserved role for NEK10 in the cellular response to UV irradiation.
2.2 Introduction

The NEK kinases are a family of cell cycle regulated, serine-threonine kinases. The founding member of the family, the *Aspergillus nidulans' Never in mitosis A* (NimA) is essential for mitotic entry (293). Based on the amino acid homology within their respective catalytic domains, 11 mammalian NEK kinases have been identified (249) and several have been shown to play diverse roles both during mitosis and at the other phases of the cell cycle. Further, NEK family members have been implicated in checkpoint control and the DNA damage response.

Within the NEK family, the molecular mechanism of NEK11 impact on checkpoint control is the best characterized. The Cdk1-activating phosphatase, Cdc25A, undergoes ubiquitin-mediated degradation following genotoxic stress, and this event promotes cell cycle arrest (42). NEK11 directly phosphorylates Cdc25A, enhancing its interaction with the E3 ubiquitin ligase, SCF β-TrCP (260). Significantly, HeLa cells depleted for NEK11 display elevated levels of Cdc25A protein and fail to undergo IR-induced G2/M arrest (260).

In addition to NEK11, other NEK kinases are regulated by genotoxic stresses. In HeLa cells, NEK2 is inactivated following ionizing radiation, which appears to be essential for radiation-induced inhibition of centrosome splitting (266). Conversely, NEK1 expression and catalytic activity are elevated in HK2 and HeLa cells treated with IR, whereas *kat2J/NEK1* cells are deficient in their ability to repair DNA following this genotoxic stress (50, 313). Finally, the catalytic activity of NEK1, NEK2, NEK6 and NEK11 appears sensitive to genotoxic stresses such as ultraviolet (UV) radiation, IR, and etoposide (104,
Thus, various NEK kinases participate in the cellular response to genotoxic stress and can act as positive and negative regulators of various damage-induced checkpoints.

Many cellular stresses, including UV irradiation, lead to activation of MAPK families, JNK/SAPK, p38 and ERK1/2. While UV-induced JNK activation mediates activation of the AP-1 transcription factor and the cell’s survival response, p38 is required for engagement of the G2/M checkpoint (41, 423, 456). The physiological relevance and the mechanism of ERK1/2 activation in response to UV irradiation are less well characterized. Nevertheless, activation of ERK1/2 is emerging as an important aspect of G2/M checkpoint control in a cell type- and stimulus-specific manner. For instance, ERK1/2 activation by IR and etoposide in MCF7 and NIH3T3 cells is required for G2/M arrest (390, 445).

Here, we describe the cloning of human NEK10, a novel member of the NEK family and a recently identified candidate susceptibility gene in cancer (4, 66, 136). Our results demonstrate a role for NEK10 in the maintenance of the G2/M checkpoint following UV irradiation. Mechanistically, NEK10 was found to act as a positive regulator of ERK1/2 signaling in response to UV irradiation, but not mitogenic stimuli, by forming a complex with Raf-1 and MEK1 and enhancing MEK autoactivation. Importantly, our data indicate that checkpoint regulation may be a conserved function of NEK10 between C.elegans and mammalian cells.

2.3 Materials and Methods

All materials were obtained from Sigma unless otherwise indicated. UV-C irradiation (254nm) was performed using a UV stratalinker 2400 (Stratagene, La Jolla, CA, USA).
**Plasmids**

NEK10 cDNA was isolated by PCR from a skeletal muscle cDNA library (Clontech HL5505u) based on the longest predicted NEK10 transcript and was confirmed by sequencing (249). The resulting cDNA was subcloned into the EcoRI and Kpn1 sites of 3xFLAG-CMV-7.1. Deletion mutants of NEK10 were generated by standard recombinant DNA procedures (details available upon request). Catalytically inactive NEK10 (KD) was generated by site-directed mutagenesis of lysine 548 to arginine. pEBG-Raf-1 was provided by J.Woodgett. Catalytically inactive Raf-1 (KD) was generated by site-directed mutagenesis of lysine 375 to tryptophan. pMCL HA-MEK1, pMCL HA-MEK1 K97M (KD) by M.Cobb, pcDNA HA-MEK1, MEK1Δ270-307, Pak1 K299R (KD) by A.Catling.

**Cell Culture and Transfection**

All cells were cultured in DMEM/10% FBS unless otherwise indicated. HEK293 cells were transfected using the calcium phosphate method. For knockdown using esiRNA, cells were transfected with Dharmafect #1 (Dharmacon) according to manufacturer’s instructions. Briefly, 1ug of esiRNA was transfected per 35mm plate on Day 1 and Day 2. Media was changed 24 h following transfection. For FACS analysis, cells were UV irradiated on Day 3 and gathered 24 h later. For Semi-Quantitative (Semi-Q) RT PCR RNA was extracted on Day 3. For preparation of whole cell lysates, cells were lysed on Day 4.
**Cell Lysis and Immunoprecipitation**

Cells were lysed in CHAPS lysis buffer (40 mM Hepes, pH 7.5, 0.3% CHAPS, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na₂VO₃, 20 mM β-glycerophosphate, 1mM DTT and protease inhibitors). Immunoprecipitations were performed with Anti-Flag M2-Agarose or Glutathione Sepharose 4 Fast Flow (GE Healthcare) for 2 h at 4°C, washed 4 times with CHAPS lysis buffer containing 220mM NaCl, and eluted by boiling in sample buffer.

**Western Blot Analysis and Antibodies**

Whole cell lysates or immunoprecipitates were resolved by SDS–PAGE and blotted to PVDF membranes. Proteins were probed using appropriate primary antibodies from the following sources: α-ERK1/2 (#9102), α-pERK1/2 (T202/Y204) (#9101), α-pMEK1/2 (S217/221) (#9121) α-pS298 MEK1 (#9128), α-pJNK1/2 (T183/T185) (#9251), α-pRaf-1 (S338) (#9427) from Cell Signaling, α-Raf-1 (C20) (#sc-227), Vinculin from Abcam, α-Flag M2 from Sigma, α-GST from GE Healthcare, and α-HA (12CA5) was harvested from supernatants of the corresponding hybridoma.

**NEK10 in vitro Kinase Assay**

Flag-NEK10 immunoprecipitates were washed 2 times with CHAPS Lysis buffer + 0.4M LiCl and 2 times with NEK10 kinase assay buffer (KAB) (50mM MOPS pH7.4, 10mM MgCl₂, 10MnCl₂, 2mM EGTA, 20mM β-glycerophosphate, 1mM DTT). Kinase assays were performed in a 40ul reaction containing 30ul KAB supplemented with 5uM ATP and 5uCi γ-32P ATP and either recombinant Histone H3, or GST-Raf-1 or HA-MEK immunoprecipitated and eluted from HEK293 cells. Reactions were carried out for 30min at
30°C and stopped with SDS-loading buffer. Reactions were separated by SDS-PAGE and detected by autoradiography.

**esiRNA Preparation**

esiRNA was produced as described in Kittler *et al.* 2005 (192). Briefly, using Flag-NEK10 cDNA as a template, 1kb fragments of NEK10 were PCR amplified with primers containing T7 promoter sequences. The PCR primer sequences for esiNEK10#2 were: sense: 5’-cgttaatacgactcactatatagggacttgaagctcctctgg-3’, antisense: 5’-cgttaatacgactcactatagggatgataaagctgct-3’. For esiNEK10#3 were: sense: 5’-cgttaatacgactcactatatgggtatatggtatatgtg-3’, antisense: 5’-cgttaatacgactcactatatgggtatatggtatatgtg-3’. As a control esiRNA was generated against dsRed.

PCR product was used as a template for *in vitro* transcription reaction using MEGAscript kit (Ambion). Resulting RNA was denatured, annealed and digested with GST-RNaseIII (provided by L.Pelletier) at 26°C for 5 h, followed by 37°C for 1 h. Digested RNA was purified using Q-Sepharose.

**Semi-Quantitative PCR**

For RNA preparation, cells were harvested, and total RNA extracted using RNeasy Mini kit (Qiagen). The first-strand cDNA was prepared with 0.5ug of RNA using qScript cDNA SuperMix (Quanta Biosciences). In preliminary experiments, the relative amounts of cDNA and the range of PCR cycles that permit the linear amplification of NEK10 and β-Actin were determined. The PCR primer sequences for NEK10 were: sense: 5’-
ATGAGGGATCCATGTTATCAGGAAATAC-3’, antisense: 5’-TGGGGCTCTGCACAAAGTA-3’, and those for β-Actin were: sense 5’-GCCAACCGCGAGAAGATGACC-3’, antisense: 5’-CTCCTTAATGTCACGCACGATTTC-3’

The PCR conditions for NEK10 were 94°C for 2 min, followed by 30 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1 min. PCR conditions for β-Actin were the same except the amount of cDNA used as template was 10 times lower than for NEK10. Using ImageJ, the relative expression of the NEK10 mRNA was evaluated by calculating the band intensity ratio of NEK10/β-Actin.

**FACS analysis**

Cell cycle distributions were determined by the flow cytometric analysis of propidium iodide–labeled cells. Cells were collected, fixed in 70% ethanol, and stored at –20°C overnight. They were then washed with PBS and incubated with RNaseA and propidium iodide. For quantification of mitotic cells, cells were collected, fixed in 70% ethanol, and stored at –20°C overnight. They were washed with PBS and incubated with α-pS10 H3 antibody (1:1000) (provided by P.Cheung) for 1 hr at room temperature. Cells were washed three times and incubated with α-mouse Alexa Fluor 488 (1:200) (Molecular Probes) for 30 min at room temperature. Cells were washed one time and incubated with RNaseA and propidium iodide. Cell cycle analysis was done by FACScan flow cytometry (BD Biosciences, San Jose, CA).

**Cloning of nekl-4 RNAi feeding vector**
nekl-4 sequences corresponding to nucleotides #1-1000 (nekl4 RNAi #1) and #1019-2050 (nekl4 RNAi#2) were amplified from *C.elegans* cDNA, cloned into an RNAi vector L4440 (provided by W.B. Derry) and sequenced.

**RNAi by Feeding in *C.elegans***

Gene expression was inhibited using the feeding method (400). Bacteria expressing double-stranded RNA (dsRNA) to *nekl-4* were grown on nematode growth media (NGM) plates supplemented with 25ug/ml Carbenicillin and 2.5 mM isopropyl--d-thiogalactopyranoside to induce dsRNA production. Bacteria expressing an empty RNAi vector (L4440) were used as controls.
2.4 Results

2.4.1 Cloning and characterization of NEK10, a novel NimA kinase

A cDNA for NEK10, a previously uncharacterized member of the NEK family, encoding an 1125aa protein was isolated by PCR from a human skeletal muscle cDNA library. Amino acid sequence alignment within the kinase domain revealed that NEK10 is the most divergent member of the NEK family sharing 54% homology within this region with its closest mammalian relatives NEK6 and 7. Unlike other NEK kinases that feature N-terminal kinase domains, NEK10 contains a centrally located catalytic domain (Fig 2.1A). In addition, NEK10 contains 4 N-terminal armadillo repeats, coiled-coiled repeats flanking the catalytic domain, and a putative PEST sequence near the C-terminus. Judged by RT-PCR, NEK10 appears to be ubiquitously expressed at low levels in most mouse tissues with prominent expression in the mammary gland, lung, spleen and kidney (Fig 2.1B).

2.4.2 NEK10 is a stimulus-specific modulator of ERK1/2

In attempting to define a cellular role for NEK10 we observed that ectopic expression of NEK10 in HEK293 cells resulted in an increase in activation-specific phosphorylation of ERK1/2 (Fig 2.2A). Curiously, under conditions of lower NEK10 expression, which did not significantly enhance ERK1/2 activation in non-stimulated cells, NEK10 expression did not affect ERK1/2 phosphorylation elicited by mitogenic stimuli, such as the epidermal growth factor (EGF) (Fig 2.2B) but did enhance ERK1/2 phosphorylation in response to UV
Figure 2.1: Cloning and characterization of NEK10, a novel NimA-related kinase.

A) Predicted domain structure of full-length NEK10.
B) Expression analysis of NEK10 in adult mouse tissues by RT-PCR (top). Expression analysis of β-Actin was used as control (bottom).
Figure 2.2: NEK10 is a stimulus-specific modulator of ERK1/2.

A) NEK10 stimulates ERK1/2 activation-specific phosphorylation. HEK293 cells were transfected with indicated constructs. Lysates were immunoblotted as indicated.

B) NEK10 enhances ERK1/2 activation in response to specific stimuli. HEK293 cells were transfected with Flag-NEK10 and treated with UV (250 J/m²) or EGF (1ng/ml) for 30 min before lysis. U0126-treated cells were incubated with 20µM U0126 for 1 h prior to UV irradiation. Lysates were immunoblotted as indicated.

C) NEK10 enhances UV-induced MEK1 activation. HEK293 cells were transfected with the indicated constructs and treated with UV (250 J/m²) for 30 min before lysis. Lysates were immunoblotted as indicated.

D) NEK10 does not effect UV-induced Raf-1 activation. HEK293 cells were transfected with the indicated constructs and treated with UV (250 J/m²) for 30 min before lysis. Lysates were immunoblotted as indicated.
irradiation (Fig 2.2B). The effect of NEK10 expression on ERK1/2 activation post-UV was dependent on MEK1/2 activity, as it was sensitive to the pretreatment of cells with the MEK1/2 inhibitor U0126 (Fig 2.2B). Two other MAPK sub-families, the JNK and p38 kinases, are known to be robustly activated by stress stimuli including UV irradiation (reviewed in (343)). Nevertheless, NEK10 expression did not enhance activation-specific phosphorylation of JNK or p38 following UV irradiation (Fig 2.2B).

Mitogenic stimulation leads to ERK1/2 activation by phosphorylation within its activation loop (T202/Y204 in ERK1) by the dual specificity kinases MEK1/2, which are themselves activated by phosphorylation (S217/S221 in MEK1) by the serine-threonine kinase Raf-1 (reviewed in (343)). We investigated if NEK10 affected activation of Raf-1 and MEK1/2. Judged by activation-specific MEK1 phosphorylation, cells ectopically expressing NEK10 displayed enhanced activation of MEK1 following UV irradiation (Fig 2.2C). Significantly, although Raf-1 was activated following UV irradiation, as measured by S338 phosphorylation, NEK10 did not enhance Raf-1 activation (Fig 2.2D).

To examine the function of endogenous NEK10 in human cells, esiRNA against two distinct regions of the human NEK10 cDNA were generated (Fig 2.3A, top panel). HEK293 cells were transfected with NEK10 esiRNA or dsRed esiRNA as control, and the efficiency of knockdown determined by RT-PCR. While both of the NEK10 esiRNA pools successfully decreased endogenous NEK10 transcript, esiNEK10#2 consistently produced greater knockdown, resulting in up to 70% decrease compared to controls (Fig 2.3B). Both NEK10 esiRNAs efficiently reduced the expression of transfected Flag-NEK10 protein, further demonstrating their specificity towards NEK10 mRNA and protein (Fig 2.3C).
Figure 2.3: EsiRNA knockdown of NEK10.

A) NEK10 esiRNA. Two independent pools of esiRNA were generated against NEK10. 
B) Knockdown of NEK10 transcript in HEK293 cells. HEK293 cells were transfected with pools of esiRNA against NEK10 or dsRed (Control) and knockdown confirmed by semi-quantitative RT-PCR. 
C) Knockdown of NEK10 protein in HEK293 cells. HEK293 cells were co-transfected with pools of esiRNA and Flag-NEK10. Lysates were immunoblotted as indicated (lower panel).
The requirement for NEK10 in ERK1/2 activation was examined in HEK293 cells depleted for endogenous NEK10. While NEK10 knockdown impaired ERK1/2 activation following both high (250J/m²) and low (20J/m²) doses of UV irradiation (Fig 2.4A, 2.4C), it had no effect on ERK1/2 activation in response to EGF stimulation (Fig 2.4B, 2.4C) or on the activation of JNK or p38 following UV irradiation (Fig 2.4B). Consistent with the effect of ectopic NEK10 expression on MEK1/2 and Raf-1 phosphorylation, UV-irradiated NEK10-depleted cells displayed impaired MEK1/2 but not Raf-1 phosphorylation (Fig 2.4B). Thus, NEK10 appears to specifically mediate ERK1/2 activation in response to UV irradiation.

2.4.3 NEK10, Raf-1 and MEK1 form a ternary complex

To further probe the mechanism of ERK1/2 activation by NEK10, its ability to directly interact with components of the ERK1/2 signaling cascade was examined. While an interaction between co-transfected Flag-NEK10 and HA-H-Ras could not be detected, Flag-NEK10 co-precipitated both with GST-Raf-1 from transfected HEK293 cells (Fig 2.5A) and endogenous Raf-1 (Fig 2.5B). Possible association of NEK10 with MEK1 was also tested. A weak interaction between the two proteins was significantly enhanced by co-expression with GST-Raf-1 suggesting that Raf-1 may bridge the NEK10-MEK1 association (Fig 2.6A). To explore this further, we used a previously characterized mutant MEK1Δ270-307, which lacks its proline-rich repeat and is unable to interact with Raf-1 (Fig 2.6C left) (46). Upon co-expression, NEK10 associated with wildtype, but not Δ270-307 MEK1, in a Raf-1-dependent manner, further demonstrating that the interaction between NEK10 and MEK1 requires Raf-1
**Figure 2.4: NEK10 is required for UV-induced MEK1/2 and ERK1/2 activation.**

**A)** Knockdown of NEK10 impairs ERK1/2 activation following UV irradiation. HEK293 cells transfected with esiRNA were UV irradiated (250 J/m²) and harvested 30 min later. Lysates were immunoblotted as indicated.

**B)** Knockdown of NEK10 impairs MEK1/2 activation following UV irradiation. HEK293 cells transfected with esiRNA were UV irradiated (250 J/m²) or stimulated with EGF (0.1ng/ml) and harvested 30 min later. Lysates were immunoblotted as indicated.

**C)** Knockdown of NEK10 does not affect mitogenic ERK1/2 activation. HEK293 cells transfected with esiRNA were UV irradiated (20 J/m²) or stimulated with EGF (1ng/ml) and harvested 30 min later. Lysates were immunoblotted as indicated.
Figure 2.5: NEK10 interacts with Raf-1.

A) NEK10 co-immunoprecipitates with Raf-1. HEK293 cells were transfected with GST-Raf-1 and Flag-NEK10 or vector control. NEK10 was immunoprecipitated with M2 Flag agarose. Co-precipitated GST-Raf-1 was detected by immunoblotting of immunoprecipitates. Whole cell lysates were run as input controls.

B) NEK10 co-immunoprecipitates with endogenous Raf-1. HEK293 cells were transfected with either vector control or Flag-NEK10. NEK10 was immunoprecipitated with M2 Flag agarose. Co-precipitated Raf-1 was detected by immunoblotting of immunoprecipitates with α-Raf-1 antibody. Whole cell lysates were run as input controls.
Figure 2.6: NEK10 interacts with the Raf-1-MEK1 complex.

A) Co-immunoprecipitation of NEK10 with Raf-1 and MEK1. HEK293 cells were transfected with the indicated constructs. NEK10 was immunoprecipitated with M2 Flag agarose. Co-precipitated proteins were detected by immunoblotting of immunoprecipitates. Whole cell lysates were run as input controls.

B) NEK10 interaction with Raf1 and MEK1 is not modulated by UV irradiation. HEK293 cells were transfected with the indicated constructs. Cells were UV irradiated (250 J/m²) 30 minutes prior to lysis. Protein precipitation and immunodetection was performed as in A).

C) Raf-1 mediates the assembly of a complex between NEK10, MEK1 and Raf-1. HEK293 cells were transfected with the indicated constructs. Raf-1 was precipitated with Glutathione Sepharose (left) and NEK10 with M2 Flag agarose (right). Co-precipitating proteins were detected by immunoblotting with the indicated antibodies. Whole cell lysates were run as input controls. * denotes a non-specific band.
binding to MEK1 (Fig 2.6C right). NEK10 does not appear to be required for association between Raf-1 and MEK1, as complex formation was unaffected by co-expression of NEK10. Interestingly, the ternary complex containing NEK10, Raf-1 and MEK1 formed in the absence of specific cell stimulation and was not modulated upon UV irradiation, suggesting that the complex is present under cycling, non-stimulated conditions (Fig 2.6B).

To map the interaction between Raf-1 and NEK10, we designed a series of NEK10 deletion mutants and tested their ability to interact with Raf-1 and MEK1 (Fig 2.7A). All of the generated mutants retained the ability to interact with Raf-1 and MEK1, except the NEK10 protein composed of amino acids 1-478 (NEK10 Nt) encompassing the N-terminal third of the protein, which includes the armadillo repeats (Fig 2.7A). A predicted coiled-coiled domain (aa 784-817) was common to all of the remaining NEK10 proteins, raising the possibility that it mediates the Raf-1/MEK1/NEK10 interaction. Nevertheless, NEK10 protein lacking this region (NEK10 ΔCtΔcc) retained the ability to interact with Raf-1 and MEK1 (Fig 2.7B), implying that NEK10 and Raf-1 interact through multiple sites.

2.4.4 UV irradiation promotes MEK1 autophosphorylation

The ability of NEK10 to enhance MEK but not Raf-1 activation following UV irradiation (Fig 2.4B), combined with the lack of any apparent effect of NEK10 on Raf-1/MEK1 association (Fig 2.6C), prompted us to further explore the mechanism(s) by which NEK10 impacts MEK activity. We first probed the possibility that Raf-1 or MEK were targets of direct phosphorylation by NEK10, by performing NEK10 in vitro kinase assays using affinity-purified GST-Raf-1 or HA-MEK1 as substrates. While Flag-NEK10
**Figure 2.7: NEK10 and Raf-1 exhibit a multi-site interaction.**

**A)** NEK10 and Raf-1 exhibit a multi-site interaction. Schematic representation of NEK10 constructs depicting full-length NEK10 and truncated NEK10 fragments (left). HEK293 cells were transfected with the indicated constructs. NEK10 was immunoprecipitated with M2 Flag agarose. Co-precipitated GST-Raf-1 and HA-MEK1 were detected by immunoblotting of immunoprecipitates (right). Whole cell lysates were run as input controls.

**B)** Mapping the multi-site interaction between NEK10 and Raf-1. Schematic representation of NEK10 constructs depicting full-length NEK10 and truncated NEK10 fragments (left). HEK293 cells were transfected with the indicated constructs. Protein precipitation and immunodetection was performed as in **A)** (right).
immunoprecipitated from HEK293 cells readily autophosphorylated and phosphorylated a
generic serine/threonine kinase substrate, Histone H3, it failed to phosphorylate both Raf-1
and MEK1 in vitro (Fig 2.8).

We reasoned that NEK10 might enhance MEK activation by an unidentified kinase or
phosphatase. An alternative explanation was that NEK10 promotes MEK
autophosphorylation following UV irradiation. To explore this latter possibility, we
examined the role of autophosphorylation in MEK activation following UV irradiation using
two MEK inhibitors with different modes of action. U0126 is a direct, non-ATP-competitive
inhibitor of MEK catalytic activity, whereas PD98059 prevents phosphorylation of MEK by
upstream activators, such as Raf-1, by binding and sequestering inactive forms of MEK (9,
100). In response to EGF stimulation, MEK phosphorylation was inhibited by pretreatment
with PD98059, but was unaffected by incubation with U0126 (Fig 2.9A). Interestingly, both
MEK inhibitors impaired MEK phosphorylation following UV irradiation (Fig 2.9A). This
result indicates that MEK activation proceeds by distinct mechanisms following UV
irradiation and EGF stimulation. In particular, UV-induced MEK activation requires MEK
catalytic activity.

Consistent with this, both wildtype MEK1 and MEK1 KD were comparably
phosphorylated at S217/S222 upon EGF stimulation, whereas phosphorylation of MEK1 KD
at these sites following UV irradiation was reduced (Fig 2.9B). We next assessed the effect
of NEK10 on MEK1 phosphorylation. While NEK10 expression did not affect EGF-
stimulated MEK1 phosphorylation, it enhanced UV-induced phosphorylation of wildtype but
not catalytically inactive MEK1 (MEK1 KD) (Fig 2.9B). Significantly, NEK10-induced
MEK1 phosphorylation was also sensitive to U0126 (Fig 2.9C). Taken together, these data
Figure 2.8: NEK10 does not phosphorylate Raf-1 or MEK1 in vitro.

HEK293 cells were transfected with wildtype (WT) or kinase dead (KD) Flag-NEK10, GST-Raf-1 KD or HA-MEK1 KD. Flag-NEK10 immunoprecipitates were used in in vitro kinase assays with Histone H3 (0.8ug), or immunopurified and eluted GST-Raf-1 or HA-MEK1 as substrate. Kinase assays were separated by SDS-PAGE and phosphorylation detected by autoradiography.
**Figure 2.9: NEK10 promotes MEK1 autophosphorylation following UV irradiation.**

**A)** UV irradiation stimulates MEK1 autocatalytic activity. HEK293 cells were transfected with the indicated constructs and treated with U0126 (10uM) or PD98059 (500uM) for 1 hour prior to treatment with UV (250 J/m²) or EGF (1ng/ml). Cells were lysed 30 minutes later. Lysates were immunoblotted as indicated.

**B)** MEK1 requires catalytic activity for activation loop phosphorylation following UV irradiation. HEK293 cells were transfected with the indicated constructs and treated with UV (250 J/m²) or EGF (1ng/ml). Cells were lysed 30 minutes later. Lysates were immunoblotted as indicated.

**C)** NEK10 promotes U0126-sensitive MEK1 activation following UV irradiation. HEK293 cells were transfected with the indicated constructs and treated with U0126 (20uM) for 1 hour prior to UV (250 J/m²). Cells were lysed 30 minutes later. Lysates were immunoblotted as indicated.
reveal a distinct mechanism of MEK activation following UV irradiation that requires MEK autocatalytic activity. NEK10 specifically enhances MEK autoactivation in response to UV, but does not promote MEK activation in response to mitogens.

2.4.5 NEK10 promotes MEK1 autophosphorylation by a mechanism involving Raf-1

MEK1 autoactivation has been implicated as a non-canonical means of stimulating ERK1/2 signaling, specifically in the context of cell attachment (303). In rat embryo fibroblasts, Pak1 phosphorylation of MEK1 at S298 led to MEK1 autophosphorylation at S217/S222 (303). We examined the requirement for S298 phosphorylation in UV-induced MEK autophosphorylation using a catalytically inactive, dominant-negative mutant of Pak1 (Pak1 KD). While MEK1 phosphorylation at S298 was abolished by expression of Pak1 KD, there was no inhibition of S217/S222 phosphorylation by Pak1 KD following UV irradiation, indicating that MEK autoactivation in this context does not require S298 phosphorylation (Fig 2.10A).

Autoactivation of MEK upon UV irradiation raised the possibility that Raf-1 may be dispensable for MEK activation in response to this stimulus. However, MEK1Δ270-307, which does not bind to Raf-1 (Fig 2.6C), was not phosphorylated upon UV irradiation or the concomitant NEK10 overexpression (Fig 2.10B), indicating that Raf-1 is required for MEK activation following UV irradiation. Interestingly, Raf-1 catalytic activity was not necessary for this effect, as ectopic expression of catalytically inactive Raf-1 (Raf-1 KD) enhanced UV-induced MEK1 phosphorylation (Fig 2.10C). Significantly, and consistent with a distinct mechanism of MEK activation in response to mitogenic stimuli, Raf-1 KD appeared to act in
Figure 2.10: UV-induced MEK1 autophosphorylation requires Raf-1.

A) S298 phosphorylation is not required for UV induced MEK1 autoactivation. HEK293 cells were transfected with the indicated constructs and treated with UV (250 J/m²) or EGF (1ng/ml). Cells were lysed 30 minutes later. Lysates were immunoblotted as indicated.

B) NEK10 requires Raf-1 interaction with MEK1 to enhance MEK1 activation. HEK293 cells were transfected with the indicated constructs and treated with UV (250 J/m²). Cells were lysed 30 minutes later. Lysates were immunoblotted as indicated.

C) Raf-1 enhances UV-induced MEK1 phosphorylation independent of Raf-1 catalytic-activity. HEK293 cells were transfected with the indicated constructs and treated with UV (250 J/m²) or EGF (1ng/ml). Cells were lysed 30 minutes later. Lysates were immunoblotted as indicated.
a dominant-negative manner and reduced MEK1 activation in response to EGF (Fig 2.10C). Taken together, these data suggest that Raf-1 plays a non-catalytic role in MEK autoactivation following UV irradiation.

2.4.6 NEK10 participates in the maintenance of the G2/M checkpoint following UV irradiation

In response to genotoxic stress, such as ionizing radiation and the intercalating agent etoposide, ERK1/2 activity is required for appropriate checkpoint function, and inhibition of ERK1/2 by pretreatment with MEK1/2 inhibitors or expression of dominant negative MEK1 led to a reduction in G2/M arrest (390, 445). While ERK1/2 activation by UV irradiation has been well documented, a role for ERK1/2 signaling in regulating the G2/M checkpoint following UV irradiation has not been established (264, 317, 325). We explored the cell cycle distribution of HEK293 cells following UV irradiation by propidium iodide (PI) staining and flow cytometry. Twenty-four hours after UV-irradiation, a 25% increase in the G2/M population could be detected, indicative of a G2/M checkpoint arrest (Fig 2.11). Pretreatment of cells with the MEK1/2 inhibitor U0126 markedly attenuated the G2/M arrest in HEK293 cells, indicating that ERK1/2 is required for the response to UV irradiation (Fig 2.11).

NEK10’s ability to promote UV-stimulated ERK1/2 activation prompted us to further investigate its function in the UV response. Cell cycle distribution of HEK293 cells depleted of NEK10 by esiRNA was profiled under basal conditions or following UV-irradiation. NEK10 depletion did not affect the cell cycle profile of unsynchronized cycling HEK293
Figure 2.11: ERK1/2 activation is required for maintenance of the G2/M checkpoint following UV irradiation.

HEK293 cells were treated with U0126 (10uM) starting 1hr prior to UV irradiation (20J/m²) and harvested 24h following irradiation. DNA content was measured by propidium iodide staining. Error bars represent s.e.m. Asterisks denote statistical significance (* P < 0.04, **P < 0.006).
cells, indicating that NEK10 is not essential for normal cell cycle progression in this system (Fig 2.12A, 2.12B). Nevertheless, following UV irradiation, cells depleted for NEK10 by two independent esiRNA pools, consistently displayed a decreased G2/M population compared to control cells (Fig 2.12A, 2.12B). While this is indicative of an impaired G2/M checkpoint, a similar result would be also observed if NEK10-depleted cells were arresting earlier in the cell cycle at the G1/S transition. To distinguish between these possibilities, we measured the proportion of mitotic, cycling UV-irradiated cells by staining for phospho-Serine 10 Histone H3. In control cells, UV irradiation led to a greater than 3-fold reduction in the proportion of mitotic cells, whereas in NEK10-depleted cells only a modest decrease in the mitotic cell population could be detected (Fig 2.12C). Based on the fact that cells transfected with esiNEK10#2, which displays greater potency in NEK10 knockdown (Fig 2.3B), consistently displayed a more severe G2/M arrest defect compared to cells transfected with esiNEK10#3 (Fig 2.12A, 2.12C), NEK10 may exert a dose-dependent effect on the UV-induced G2/M arrest.

2.4.7 NEK10 has a conserved role in the UV response

To further explore the physiological function of NEK10 in the UV response we exploited *C. elegans* as a model organism. There are 4 NEK-like kinases in *C. elegans*, nekl-1, -2, -3 and -4, which correspond to mammalian NEK9, 8, 7 and 10 respectively. NEK10’s closest homologue is the *C. elegans* protein nekl-4 (previously designated D1044.8), which also contains armadillo repeats and a centrally located kinase domain. Remarkably, NEK10 and nekl-4 share 72% homology within the kinase domain and 34% overall homology (Fig
Figure 2.12: NEK10 activation is required for maintenance of the G2/M checkpoint following UV irradiation.

A) Knockdown of NEK10 decreases the G2/M population in UV irradiated cells. HEK293 cells were transfected with esiRNA. Cells were treated with UV (20J/m²) and harvested 24 h later and their DNA content measured by propidium iodide staining. The proportion of cells in G2/M was quantified. Representative images are shown.

B) Quantitation of G2/M phase cells in NEK10 knockdown cells from three independent experiments. Error bars represent s.d. Asterisks denote statistical significance (** P < 0.0002).

C) NEK10 maintains the G2/M arrest following UV irradiation. HEK293 cells were transfected with esiRNA and treated with UV (20J/m²). 24h following UV irradiation, the percentage of mitotic cells was determined by dual staining with propidium iodide (DNA) and anti-pS10-H3.
2.13A). By comparison, NEK10 shares only 54% homology within the kinase domain with its closest mammalian homologues NEK6 and NEK7.

The role of nekl-4 in the UV response was explored by measuring the radiation sensitivity of nekl-4-depleted embryos. N2 strain worms (F0) were fed on bacteria containing two independent RNAi against nekl-4 or empty vector as control, and the F1 adults were used in subsequent experiments. Consistent with several large-scale RNAi screens (132, 180, 245, 374), no obvious defects in worm viability, development or progeny survival were detected upon nekl-4 knockdown using our RNAi constructs.

*C.elegans* embryos display differential sensitivity to UV irradiation at various stages of development (145). We therefore irradiated embryos at three stages: pachytene nuclei stage, fertilization/early embryogenesis and gastrulation/early morphogenesis and determined the survival rate of control or nekl-4-depleted embryos by measuring the rate of egg hatching. Twenty-four hours after laying, non-irradiated embryos, both control and nekl-4-depleted, displayed close to a 100% survival as measured by hatching rate (Fig 2.13B). Following UV irradiation of embryos in either pachytene nuclei stage or fertilization/early embryogenesis, no significant differences in the hatching rates could be detected between control and nekl-4-depleted eggs (Fig 2.13C). However, when embryos were UV irradiated during gastrulation/early morphogenesis, an approximately 30% decrease in survival of nekl-4-depleted embryos compared to controls was observed (Fig 2.13D). Thus, nekl-4 appears to mediate radiation sensitivity in *C.elegans* embryos, further supporting the notion that NEK10 has a conserved role in the UV response.
Figure 2.13: NEK10 homologue, nekl-4, mediates the UV response in *C. elegans* embryos.

A) Alignment of amino acid sequences of catalytic domains of human NEK10 with human NEK6 or *C. elegans* nekl-4 using BlastP. Amino acid sequences are shown in bold. Identical residues are indicated, and similar residues indicated by +.

B) nekl-4 is not-essential for *C. elegans* embryonic survival. Young adult hermaphrodites (F0) were placed on RNAi producing plates. Young F1 adults were transferred to fresh RNAi-producing plates and after 5 hours F2 eggs were counted. 24 hours after laying unhatched eggs were scored. Survival was calculated as the percentage of eggs that hatched compared to total number of eggs laid. Shown are results of two independent experiments. Error bars represent s.e.m..

C) nekl-4 knockdown does not affect sensitivity of *C. elegans* embryos to UV irradiation prior to gastrulation. Young adult hermaphrodites (F0) were placed on RNAi-producing plates overnight. Worms (F0) were transferred to fresh RNAi-producing plates and allowed to lay eggs (F1) for 5 hours. Young F1 adults were transferred to unseeded plates and were UV irradiated (20J/m²). Irradiated F1 worms were then placed on fresh RNAi-producing plates and allowed to lay eggs (F2) for 8 h (early embryogenesis). After 8 hours F1 worms were transferred to fresh RNAi plates and allowed to continue to lay eggs for an additional 16h (pachytene nuclei stage). Survival was calculated as in B). Shown are results from two independent experiments performed in duplicate or triplicate. Error bars represent s.e.m.

D) nekl-4 knockdown increases sensitivity of late stage *C. elegans* embryos to UV. Young adult hermaphrodites (F0) were placed on RNAi-producing plates overnight. Worms (F0) were transferred to fresh RNAi-producing plates and allowed to lay eggs (F1) for 5 hours. Young F1 adults were transferred to fresh RNAi-producing plates for 5 hours. F2 eggs were counted and irradiated with UV (20 J/m²). Survival was calculated as in B). Shown are results from 4 independent experiments performed in duplicate or triplicate. Error bars represent s.e.m. Asterisks represent statistical significance (* P < 0.02).
2.5 Discussion

In this study we demonstrate a function for a previously uncharacterized member of the NEK family, NEK10, in promoting MEK/ERK activation and G2/M arrest in response to UV irradiation. Our results indicate that NEK10 is a stimulus-specific modulator of ERK1/2 signaling, as its expression enhanced MEK and ERK activation in response to UV irradiation, but not to mitogenic stimuli such as EGF (Fig 2.2B). Consistent with this, NEK10 depletion led to impaired MEK/ERK activation in response to UV irradiation but not to EGF stimulation (Fig 2.4B, 2.4C). Our results indicate that the specificity of the ERK signaling response to UV can be attributed to the ability of NEK10 to promote a non-canonical mechanism of MEK activation following UV irradiation. Interestingly, this mechanism requires MEK catalytic activity (Fig 2.9A, 2.9B) and Raf-1 binding (Fig 2.10B), but not Raf-1 kinase activity (Fig 2.10C).

2.5.1 NEK10 promotes MEK autoactivation

ERK1/2 has traditionally been associated with mitogenic stimulation and regulation of cell proliferation, but is also activated by a diverse range of other stimuli, including cytokines and various stresses, such as UV and IR, as well as hypoxia (269, 325, 390). One of the means of achieving control in ERK1/2 signaling, is the differential interaction of ERK1/2 cascade components with various scaffolding proteins (reviewed in (195)). These interactions have been found to modulate the stimulus specificity, amplitude and duration of
pathway activation, as well as to impact subcellular localization, access to substrates and cellular outcome (45).

NEK10 promoted MEK autoactivation in response to UV, but not following EGF stimulation (Fig 2.9C), suggesting a distinct mechanism of MEK activation from the canonical Ras/Raf/MEK cascade. This is reminiscent of the ability of p38α to undergo MAPKK-independent autophosphorylation in response to specific stimuli. In HEK293 cells, p38α autoactivation occurred following stimulation with TNF or peroxynitrite, but not anisomycin or sorbitol (122). Significantly, autophosphorylation was stimulated by binding to TAB1 (transforming growth factor-β-activated protein kinase 1 (TAK1)-binding protein 1) (122).

MEK autoactivation has been previously described in the context of cell adhesion, where it was induced by Pak1-mediated S298 phosphorylation (303). Significantly, UV-induced MEK autophosphorylation is independent of S298 phosphorylation, indicating that it proceeds by a distinct mechanism (Fig 2.10A). Our data are consistent with the formation of a MEK autoactivation-competent complex consisting of NEK10, Raf-1 and MEK. Interestingly, while NEK10 association with Raf-1 and MEK1 was needed for enhanced MEK1 activation in response to UV (Fig 2.10B), the formation of the ternary complex was not sensitive to UV (Fig 2.6B). Further, Raf-1 protein, but not its catalytic activity, was required for MEK1 activation following UV (Fig 2.10C). The ability of Raf-1 to bridge the association between NEK10 and MEK (Fig 2.6A) points towards a scaffolding, non-catalytic role for Raf-1 in the formation of the MEK autoactivation complex.

The fact that signaling by the NEK10/Raf/MEK module depends on MEK autocatalysis, points towards the existence of distinct spatial and temporal controls for MEK
activation in response to specific stimuli. In the case of UV, NEK10 participation in this complex may promote association with additional regulators, facilitate a permissive change in MEK conformation leading to its autoactivation and/or govern access to specific substrates. Uncoupling of MEK activation from RTKs, Ras, and their other effectors allows for discrete control of MEK/ERK signaling targets leading to specific outcomes, such as engagement of the G2/M checkpoint in response to UV (Fig 2.11, 2.12). Whether MEK activation is dependent on autocatalysis and NEK10 following other non-mitogenic stimuli, such as genotoxic agents, cellular stressors or cytokines, remains to be determined.

2.5.2 NEK10’s role in checkpoint control

ERK signaling has previously been implicated in regulation of cell cycle checkpoints in response to ionizing radiation and etoposide in MCF-7 and NIH3T3 cells, respectively (390, 445, 446). Our results revealed that ERK1/2 activation was required for engagement of the G2/M checkpoint following UV-irradiation of HEK293 cells (Fig 2.11). A similar phenotype was also observed in UV-irradiated cells depleted for NEK10 by esiRNA (Fig 2.12B). Interestingly, we did not observe any impact of NEK10 knockdown on normal cell cycle progression, indicating that unlike it’s closest homologues NEK6/7, NEK10 is not required for mitotic progression (Fig 2.12B) (188, 452, 453). Taken together, our results suggest that NEK10-mediated ERK1/2 activation participates in the UV-induced G2/M arrest. To further define NEK10’s role in the response to genotoxic stress, future studies may explore the ability of NEK10 to impact checkpoints at earlier stages of the cell cycle, both in response to genotoxic agents, or endogenous replication stress. Furthermore, the possibility
that NEK10 is required for appropriate DNA repair in addition to checkpoint control, akin to other kinases including ATR and NEK1 should be pursued (50)(reviewed in (106)).

2.5.3 Characterization of *C. elegans* nekl-4 during the UV response

Examination of the *C. elegans* NEK10 homolog, nekl-4, further supports the notion that NEK10 kinases have a conserved function in the UV response. Namely, *C. elegans* embryos depleted for nekl-4 displayed increased lethality in response to UV irradiation, as measured by the rate of egg hatching (Fig 2.13D). This phenotype is reminiscent of the increased radiation sensitivity observed in worms depleted for genes involved in checkpoint functions and DNA repair, including the xeroderma pigmentosum complementation group A (XPA, formerly known as rad-3), suppressor of mek-null (smk-1, formerly known as rad-2) and *C. elegans* Fanconi anemia complementation group D2 (FANCD2) (217) (145). Interestingly, the hatching defect was only apparent when nekl-4 depleted embryos were irradiated during gastrulation/early morphogenesis (0-5h post laying) when the embryo is undergoing the majority of its cell divisions, but not during earlier stages of development (Fig 2.13C, 2.13D) (10). This is similar to the phenotypes of mutants of *rad-2* and *xpa*, which are involved in nucleotide excision repair, who displayed increased radiation sensitivity as they passed through embryogenesis (13, 145).

The original *C. elegans* radiation sensitive (*rad*) mutants were identified using embryonic lethality as a measure of UV sensitivity, and some of them were later shown to play roles in checkpoint signaling and DNA repair (146, 217). However, such experiments do not directly test checkpoint function, as *C. elegans* embryos do not undergo cell cycle arrest.
Therefore, as an extension of our work, the role of nekl-4 in cells in the adult germline, which undergo cell death or cell cycle arrest following DNA damage, can be investigated (119). These responses are spatially separated within the gonad and produce morphological changes, which can be monitored microscopically (118). In the mitotic region of the gonad, UV-irradiated germ cells undergo cell cycle arrest, marked by a decrease in mitotic figures and reduced density of enlarged nuclei. In contrast, cells within the pachytene region of the gonad undergo apoptosis following irradiation. This can be measured by scoring the number of germ cell corpses in the germline of live animals by Nomarski microscopy. Compared to wildtype worms, checkpoint mutants exhibit decreased cell death, whereas worms defective in DNA repair have higher levels of cell death (118).

Our work in mammalian cells demonstrates an interaction between NEK10 and Raf/MEK/ERK, which may contribute to checkpoint activation. Suggestive of a conserved role for MEK/ERK in checkpoint function in lower eukaryotic systems, Drosophila embryos lacking MEK exhibit faster cell division and fail to arrest following ionizing radiation, indicating an involvement in both intrinsic and extrinsic checkpoint control (271). Interestingly this function was independent of Raf (271). In C.elegans, mutations in lin-45/mek-2/mpk-1 (Raf/MEK/ERK) are not associated with increased sensitivity to genotoxic stress, however strong phenotypes of null mutations, including sterility and lethality, may preclude identification of a milder phenotype. Therefore, the radiosensitivity of mutants containing alleles that weakly inactivate ERK1/2 signaling should be studied either alone or in combination with nekl-4 knockdown.
CHAPTER 3

CHARACTERIZATION OF NEK10 REGULATION BY PHOSPHORYLATION
3.1 Abstract

Phosphorylation is a primary means of transmitting information from extracellular stimuli to defined cellular outputs. The activity of protein kinases is tightly controlled by multiple mechanisms, including post-translational modification and allosteric regulation. In search of regulatory inputs into NEK10, using mass spectrometry, our laboratory identified nineteen distinct sites of NEK10 phosphorylation. While the majority of the detected phosphorylation events depended on NEK10 catalytic activity, likely due to prominent autophosphorylation, several phospho-sites were also found on the catalytically inactive NEK10, indicative of phosphorylation by other kinases. In addition to the NEK10 activation loop phosphorylation sites, which were obligate for catalytic activity, a C-terminal NEK10 phosphorylation site, predicted to be a target of an upstream kinase(s) was characterized. This phospho-site was essential for NEK10 cytoplasmic to nuclear translocation following UV irradiation. Moreover, phosphorylation of this site facilitated interaction of NEK10 with 14-3-3, revealing a possible role in control of NEK10 subcellular localization. Taken together, this work defines a role for phosphorylation in NEK10 regulation.
3.2 Introduction

Protein kinases constitute one of the most common protein domains in the eukaryotic proteome, representing 2% of all encoded proteins, and participate in control of most aspects of cell physiology and function (345). Consistent with their potent biological activity, kinase catalytic activity is strictly controlled, temporally and spatially.

The kinase domain is a well-conserved structure composed of two lobes, a smaller N-terminal lobe and a larger C-terminal lobe (reviewed in (163)). The N lobe is composed of a five-stranded β-sheet and one α-helix (αC) whereas the C lobe is predominantly α-helical. The C lobe bears the catalytic loop, which contains residues that are directly involved in catalysis of phosphate transfer from ATP to a serine, threonine or tyrosine residue on the substrate protein. ATP binds in the cleft between the N and C lobes and is coordinated by interactions with the glycine-rich phosphate-binding (P) loop. The kinase substrate is correctly positioned by the centrally located activation loop, which is phosphorylated in most active kinases. These motifs (αC helix and catalytic, P and activation loops) have to be appropriately spatially coordinated for a kinase to achieve full activity. The transition from an inactive to an active kinase conformation is controlled by several mechanisms, including phosphorylation and allosteric regulation.

Activation loop phosphorylation is the most common means of regulating kinase activity (reviewed in (287)). In kinases that require activation loop phosphorylation for activation, the unphosphorylated activation loop collapses into the catalytic core, blocking the interaction of the kinase with both ATP and substrate (reviewed in (163)). Following phosphorylation, the activation loop undergoes a large conformational change, moving away
from the catalytic core, allowing the kinase domain to bind and catalyze phosphate transfer to substrates. Certain kinases require additional phosphorylation events to achieve full activity. For example, while PKB is partially activated by activation loop phosphorylation (T308 in PKB1), phosphorylation within the hydrophobic motif (S473 in PKB1), located outside of the kinase domain is required to stabilize the active conformation (8, 108, 447).

Allosteric regulation of kinases can influence catalytic activity either alone or in combination with phosphorylation events. A well-studied example of this is the relationship between cyclins and Cdks, in which cyclin-binding induces an active conformation within the $\alpha_C$ helix of a Cdk (174). The conformation of the $\alpha_C$ helix is directly related to the ability of a kinase to bind ATP. This is mediated by the formation of an ion pair between a conserved glutamate (E51 in Cdk2) within the $\alpha_C$ helix and the ATP-coordinating lysine (K33 in Cdk2). In the absence of cyclin-binding, the E51-K33 interaction is disrupted in Cdks, preventing nucleotide binding and catalysis (174). Certain Cdks, such as Cdk2, also require activation loop phosphorylation for full activity, whereas others, including Cdk5 and Cdk6, can be activated by cyclin binding alone (349, 357, 393). Kinase activity can also be modulated by autoinhibitory mechanisms. For example, the presence of autoinhibitory or pseudosubstrate sequences in the non-catalytic regions of proteins, such as in Src and calcium/calmodulin-dependent protein kinase (CaMK) kinases respectively, limits kinase activity until activating stimuli cause a change in conformation that relieves the inhibition (130, 270, 366).

Protein phosphorylation represents a major mediator of signal transduction. Phosphorylation can influence protein conformation and enzymatic activity, as well as create binding sites for domains that bind phospho-residues. For example, SH2 domains, FHA
domains and 14-3-3 proteins target motifs that contain phospho-tyrosine, phospho-threonine and phospho-serine/phospho-threonine residues, respectively (89, 194, 283). Thus, phosphorylation can induce protein-protein interaction and in the case of modular proteins, nucleate multiprotein complexes. Coupling a dynamic event such as phosphorylation/dephosphorylation with protein interaction provides flexibility and increases the efficiency with which information can be transmitted through the cell.

Our previous work described a role for NEK10 in mediating the cellular UV response. NEK10 was found to participate in activation of MEK/ERK and the establishment of the G2/M checkpoint following UV irradiation. The mechanism of NEK10 regulation following irradiation remains unknown. To address this, the role of phosphorylation in regulation of NEK10 activity, protein interactions and subcellular localization was explored.

3.3 Materials and Methods

For additional materials and methods see Chapter 2.3.

Plasmids

myc-14-3-3η was provided by R.Rottapel. For list of additional plasmids see Chapter 2.3.

Cell Culture and Transfection

HEK293 cells were cultured and transfected as in Chapter 2.3. HeLa cells were transfected with Effectene (Qiagen) according to manufacturer’s instructions.
Cell Lysis, Immunoprecipitation and Kinase Assays

Cell lysis, immunoprecipitation and kinase assays were performed as in Chapter 2.3.

Western Blot Analysis and Antibodies

Whole cell lysates or immunoprecipitates were resolved by SDS–PAGE and blotted to PVDF membranes. Proteins were probed using appropriate primary antibodies from the following sources: α-GFP (sc-8334) and α-pTyr (PY99) (sc-7020) from Santa Cruz, α-Flag M2-FITC from Sigma and α-HA (12CA5) and α-Myc (9E10) were harvested from supernatants of the corresponding hybridoma. Phosphospecific antibodies against NEK10 (α-pNEK10), phosphorylated on S684 and S688 were raised by immunizing rabbits with the peptide CEQENpSKLTpSVVG linked to keyhole limpet hemocyanin (KLH) (Antibodies Inc.). Phosphospecific antibodies were purified from rabbit serum by two-step affinity chromatography on a sulfolink coupling gel column on sulfolink coupling gel column (Pierce) with covalently coupled dephosphopeptide (first step) and phosphopeptide (second step) immunogen. For a list of additional antibodies see Chapter 2.3.

[^32P]Orthophosphate labeling in cells

Transfected HEK293 cells were incubated overnight with 1mCi of[^32P]orthophosphate in phosphate-free DMEM + 10% dialyzed/heat inactivated FBS. After treatments, cells were washed twice with ice cold PBS(–/-) and lysed with CHAPS lysis buffer. After lysates were clarified, Flag-NEK10 was immunoprecipitated with Anti-Flag M2-Agarose. Immunoprecipitates were washed four times with lysis buffer. Immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membrane and exposed by autoradiography.
Phosphoamino acid Analysis

Immunoprecipitates of radiolabelled proteins (from orthophosphate labeling in cells or in vitro kinase assays) were separated by SDS-PAGE and transferred to PVDF membrane. The protein band of interest was identified and cut out of the PVDF membrane. The piece of membrane was soaked in 0.5% polyvinyl pyrollidone (PVP-360) in 100mM acetic acid, at 37C for 30 min, washed twice in water and then incubated with 10ug of TPCK-treated trypsin in fresh 50mM ammonium bicarbonate pH8.0, at 37C overnight. The next morning reactions were spiked with 10ug of trypsin and incubated, at 37C for 3 additional hours. At the end of the reaction the supernatant was removed and reserved. The membrane was washed one time with water and the wash liquid pooled with reserved supernatant. Pooled liquid was lyophilized in a centrifugal vacuum concentrator (Speedvac). Once dried, peptides were hydrolyzed in 5.7M HCl, at 110C for 1h, and subsequently lyophilized. Samples were resuspended in pH 1.9 buffer (2.5% formic acid (88%), 7.8% glacial acetic acid), which contained 15 parts buffer to 1 part cold phosphoamino acid standards (1.0mg/ml of each phosphoserine (P-S), phosphothreonine (P-T) and phosphotyrosine (P-Y) in water). Phosphoamino acids were separated on cellulose-TLC plates in the first dimension from cathode (-) to anode (+) at 1.5kV for 20 min in buffer pH 1.9. The plate was rotated 90 degrees counterclockwise and electrophoresed in the second dimensionn from cathode (-) to anode (+) at 1.3kV for 16 min in buffer pH 3.5 (5% glacial acetic acid, 0.5% pyridine). The location of the phosphoamino acids was mapped by ninhydrin staining, and the presence of $^{32}$P-labelled amino acids detected by autoradiography.
**Immunofluorescence**

For immunofluorescence experiments cells were grown on glass coverslips. Cells were fixed for 10 min in 2% PFA in PBS and permeabilised for 2 min in 0.2% Triton X-100 in water, at room temperature. Staining was performed at 37°C. Samples were blocked for 30 min in blocking buffer (1.5% BSA/1.5% goat serum in PBS), and incubated for 1h with fitc-conjugated antibodies in blocking buffer and washed. Samples were mounted with Mowiol/DAPI and analysed by inverted fluorescence microscopy (Zeiss Axiovert 200M).
3.4 Results

3.4.1 Biochemical characterization of NEK10 catalytic activity

To gain insight into NEK10 catalytic properties we developed a NEK10 *in vitro* kinase assay. The assay conditions were based on those previously established for NEK10’s closest mammalian relatives NEK6 and NEK7 (23). Flag-NEK10 immunoprecipitated from transiently transfected HEK293 cells was used as the kinase source. To generate a catalytically-inactive kinase (NEK10 KD), the conserved ATP-binding lysine (K548) was mutated to arginine (R). NEK10 immunopurified from exponentially growing cells was catalytically active, as measured by autophosphorylation, as well as phosphorylation of various histones, including 1, 2 and 3. NEK10 however failed to phosphorylate β-casein, another generic kinase substrate (Fig 3.1A).

Phosphoamino acid analysis of NEK10, autophosphorylated *in vitro*, revealed phosphorylated serine, threonine and tyrosine residues, with the majority of phosphate incorporated into serine (Fig 3.1B). Based on sequence homology, NEK10 is predicted to be a serine-threonine kinase. Surprisingly, its ability to autophosphorylate on tyrosine suggested that it might possess dual-specificity activity, at least towards self. Consistent with this, immunoprecipitates of wildtype NEK10 contained a single band, which corresponded to NEK10, which was reactive with the phospho-tyrosine specific antibody (PY99: α-pTyr). However, catalytically inactive NEK10 was not detected with this antibody indicating that catalytic activity is required for NEK10 tyrosine phosphorylation *in vivo* (Fig 3.1C). Furthermore, the phospho-tyrosine signal was increased when wildtype NEK10
Figure 3.1: Characterization of NEK10 catalytic activity.

A) Substrate preference of NEK10. HEK293 cells were transfected with wildtype (WT) or kinase dead (KD) Flag-NEK10. NEK10 was immunoprecipitated with M2 Flag agarose and immunoprecipitates used in \textit{in vitro} kinase assays with 0.4\mu g of \(\beta\)-Casein, Histone mix or Histone H3. Kinase assays were separated by SDS-PAGE and phosphorylation detected by autoradiography.

B) NEK10 autophosphorylates on serine, threonine and tyrosine \textit{in vitro}. HEK293 cells were transfected with Flag-NEK10. NEK10 was immunoprecipitated with M2 Flag agarose and immunoprecipitates subjected to \textit{in vitro} kinase assays. Kinase assays were separated by SDS-PAGE, transferred to PVDF membrane and exposed by autoradiography. The band corresponding to NEK10 was cut out and subjected to phospha-amino acid analysis. pS, phosphoserine, pT, phosphothreonine, pY, phosphotyrosine.

C) Wildtype but not KD NEK10 is tyrosine phosphorylated \textit{in vivo} and \textit{in vitro}. HEK293 cells were transfected with wildtype (WT) or kinase dead (KD) Flag-NEK10. NEK10 was immunoprecipitated with M2 Flag agarose and immunoprecipitates subjected to \textit{in vitro} kinase assays plus or minus ATP. Kinase assays were separated by SDS-PAGE, transferred to PVDF membrane and blotted with indicated antibodies.
immunoprecipitates were subjected to an in vitro kinase assay (Fig 3.1C), further supporting the notion that NEK10 possesses dual serine/threonine and tyrosine kinase activity.

3.4.2 NEK10 is a phosphoprotein

To examine if phosphorylation was required for NEK10 activity, kinase assays were performed on NEK10 immunoprecipitates that had been pretreated with calf intestinal phosphatase (CIP) predicted to robustly dephosphorylate NEK10. CIP treatment abolished NEK10 catalytic activity illustrating the importance of phosphorylation for NEK10 kinase activity (Fig 3.2A).

To explore NEK10 phosphorylation status in vivo, Flag-NEK10 was immunoprecipitated from transiently transfected HEK293 cells metabolically labeled with [32P]orthophosphate. While both wildtype and NEK10 KD were phosphorylated in vivo, wildtype NEK10 displayed significantly higher levels of phosphate incorporation. This implies that the majority of NEK10 phosphorylation may depend on its autocatalytic activity (Fig 3.2B upper). Interestingly, phosphoamino acid analysis of orthophosphate labeled NEK10 showed that NEK10 was phosphorylated on serine and tyrosine but not threonine in vivo (Fig 3.2B lower).

To comprehensively identify sites of NEK10 phosphorylation, liquid chromatography tandem mass spectrometry (LC/MS/MS) was performed on wild-type and catalytically inactive Flag-NEK10 immunopurified from HEK293 cells (Protana). Mass spectrometric data were analyzed with the bioinformatics database system Mascot (Matrix Science, London) and every phosphorylation site identified was confirmed manually. Wildtype
Figure 3.2: Characterization of NEK10 phosphorylation.

A) NEK10 requires phosphorylation for catalytic activity. HEK293 cells were transfected with indicated constructs. NEK10 was immunoprecipitated with M2 Flag agarose and immunoprecipitates treated with calf intestinal phosphatase (CIP), for 30min at 30°C, prior to performing in vitro kinase assays. Kinase assays were separated by SDS-PAGE and phosphorylation detected by autoradiography.

B) NEK10 is phosphorylated in vivo. HEK293 cells were transfected with indicated constructs. NEK10 was immunoprecipitated with M2 Flag agarose from cells labeled with [32P]orthophosphate. Immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membrane and exposed by autoradiography (top). The band corresponding to NEK10 was cut out and subjected to phosphoamino acid analysis (bottom). pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.

C) Characterization of NEK10 phosphorylation by mass spectrometry. Flag-NEK10 and Flag-NEK10 KD were immunoprecipitated from transiently transfected HEK293 cells. Immunoprecipitates were separated by SDS-PAGE and stained with Coomassie. The stained band corresponding to NEK10 was excised and subjected to phospho-mass spectrometry.
NEK10 displayed eighteen distinct sites of phosphorylation (Fig 3.2C). Of these, four were also found on NEK10 KD suggesting that the majority of NEK10 phospho-sites arise either as a result of autophosphorylation or are dependent on NEK10 catalytic activity. Interestingly, one additional site of phosphorylation, S846, appeared to be unique to NEK10 KD (Fig 3.2C).

Consistent with previous results (Fig 3.2B), wildtype NEK10 was phosphorylated on serine and tyrosine but not threonine, whereas NEK10 KD was exclusively phosphorylated on serine (Fig 3.2C). Using Scansite (http://scansite.mit.edu/), which searches for motifs that are likely to be phosphorylated by specific kinases or bind to domains such as SH2, 14-3-3 or PDZ domains, we analysed NEK10 protein sequence to determine if any of the identified phosphorylation sites were putative targets of known protein kinases or part of phospho-binding motifs. pS933 was predicted to be within a 14-3-3 binding sequence, as well as a target of CaMK2G and PKB kinases, while pS352 and pS797 displayed limited homology to sites targeted by casein kinase 2 (CK2) and ATM, respectively. None of the other sites displayed significant homology to the established kinase consensus sequences.

3.4.3 NEK10 requires activation loop phosphorylation for full catalytic activity

Two phosphorylation sites identified exclusively on wildtype but not KD NEK10, S684 and S688, are located within the activation loop of the catalytic domain. Phosphorylation at equivalent residues in other kinases, including NEK6, is important for their activation. NEK6 phosphorylation at S206 (equivalent to NEK10 S688) by Nek9 and to
a lesser degree S202 (equivalent to NEK10 S684) is required for NEK6 to achieve full catalytic activity (24).

To explore the importance of activation loop phosphorylation for NEK10 catalytic activity, S684 and S688 were mutated to alanine (A), a non-phosphorylatable residue, individually, or in combination. Mutation of either S684 or S688 strongly impaired NEK10 catalytic activity, although the impact was more pronounced following mutation of S688 (Fig 3.3A). Replacement of S684, but not S688, with the phospho-mimetic residue, aspartic acid (D), restored full NEK10 catalytic activity (Fig 3.3A). Interestingly, the double mutant S684D/S688D was also impaired for catalytic activity. These results indicate that both S684 and S688 are required for full catalytic activity. However, S688 may play a primary role in NEK10 activation, as was the case for the equivalent residue in NEK6 (24).

The involvement of S684/S688 phosphorylation in control of NEK10 catalytic activity prompted us to develop a polyclonal antibody against a phosphorylated peptide encompassing S684 and S688 (α-pNEK10). Wildtype NEK10, immunoprecipitated from cycling HEK293 cells, was detected by α-pNEK10, but the signal was markedly reduced when either S684 or S688 were mutated to an alanine, and completely abolished in the S684A/S688A double mutant (Fig 3.3B). This indicates that α-pNEK10 specifically recognizes NEK10 phosphorylated on S684 and S688.

Mass spectrometry analysis did not identify S684 and S688 as phospho-sites on NEK10 KD. Consistently, α-pNEK10 did not react with Flag-NEK10 KD immunoprecipitated from HEK293 cells (Fig 3.3B). The lack of NEK10 KD activation loop phosphorylation suggested that this might occur as a result of autophosphorylation. Activation loop autophosphorylation can occur by an intramolecular (in cis) mechanism, as
Figure 3.3: NEK10 requires activation loop phosphorylation for full catalytic activity.

A) Examination of catalytic activity of NEK10 activation loop mutants. HEK293 cells were transfected with indicated constructs. NEK10 was immunoprecipitated with M2 Flag agarose and immunoprecipitates used in in vitro kinase assays with 0.4mg of Histone H3. Kinase assays were separated by SDS-PAGE and Histone H3 phosphorylation detected by autoradiography. Whole cell lysates were run as input controls.

B) Characterization of α-pNEK10 antibody. HEK293 cells were transfected with indicated constructs. NEK10 was immunoprecipitated with M2 Flag agarose and immunoprecipitates separated by SDS-PAGE and immunoblotted with indicated antibodies.

C) Wildtype NEK10 stimulates NEK10 KD activation loop phosphorylation in vivo. HEK293 cells were transfected with indicated constructs. NEK10 was immunoprecipitated and immunoblotted as in B). Whole cell lysates were run as input controls. * indicates Flag-NEK10 KD. Δ indicates GFP-NEK10

D) NEK10 dimerizes with wildtype and KD NEK10. HEK293 cells were transfected with indicated constructs. Flag immunoprecipitates were separated by SDS-PAGE and co-precipitating proteins detected by immunoblotting with indicated antibodies. Whole cell lysates were run as input controls.
in glycogen synthase kinase 3 α/β (GSK3α/β) and dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK) or by an intermolecular (in trans) mechanism, as in Chk2, NEK2 and EGF receptor (EGFR) (6, 56, 158, 237, 332). In support of NEK10 autophosphorylation occurring in trans, expression of increasing levels of wildtype, but not KD GFP-NEK10, led to phosphorylation of the activation loop of co-expressed Flag-NEK10 KD in vivo (Fig 3.3C). Dimerization is a common feature of kinases that undergo intermolecular autophosphorylation (311). Indeed, HA-NEK10 co-precipitated with both wildtype and KD Flag-NEK10, consistent with NEK10 dimerization (Fig 3.3D). Taken together, our results are consistent with a mechanism of activation loop phosphorylation that involves NEK10 intermolecular autophosphorylation.

### 3.4.4 NEK10 localization, but not catalytic activity, is modulated by UV irradiation

Considering that my previous work has implicated NEK10 in mediating the UV response, I was interested in examining how NEK10 may be regulated by UV irradiation. Significantly, certain NEK family members exhibit sensitivity to a variety of genotoxic stresses. In response to stimuli such as IR, UV and etoposide, NEK1, NEK2, NEK6 and NEK11 catalytic activity is modulated (218, 266, 286, 313). For instance, IR activates NEK1 and NEK11, whereas NEK2 and NEK6 are inhibited under this condition (218, 260, 266, 313). In addition, following IR, NEK1 relocalizes from the cytoplasm to nuclear foci within 10 minutes of IR treatment, and this persists for up to 8 hours (313).

We first examined if UV irradiation affected NEK10 catalytic activity. Flag-NEK10 was immunoprecipitated from UV-irradiated HEK293 cells and its kinase activity assayed
using Histone H3 as substrate. At both early (30 minutes) and late (3 hours) time points following UV irradiation, NEK10 activity was not modulated (Fig 3.4A). Consistently, activation loop phosphorylation of NEK10 at S684/S688 was unaffected by UV (Fig 3.4B).

We next explored NEK10 subcellular localization following UV irradiation in HeLa cells, using indirect immunofluorescence. In cycling cells, ectopically expressed NEK10 displayed predominantly cytoplasmic localization (Fig 3.4C). However, 3h post-UV irradiation, prominent NEK10 relocalization to the nucleus was observed (Fig 3.4C).

3.4.5 NEK10 nuclear localization requires S933 phosphorylation

During the characterization of NEK10 localization, the subcellular distribution of a phosphorylation mutant of NEK10, NEK10 S933A, was examined. S933 was identified by mass spectrometry as being phosphorylated on both wildtype and kinase dead NEK10. Significantly, NEK10 S933A, displayed impaired nuclear localization following UV irradiation when compared to wildtype NEK10 (Fig 3.5). Even in the absence of UV, NEK10 S933A exhibited reduced nuclear localization compared to wildtype protein, suggesting that in cycling cells NEK10 may constantly shuttle between the cytoplasm and the nucleus.

3.4.6 S933 is a 14-3-3 binding site

NEK10 S933 is located within a consensus binding site for 14-3-3 proteins, which are known to regulate localization, stability and activity of their target proteins (reviewed in (413)). Consistent with this, Flag-NEK10 and myc-14-3-3η co-precipitated from transfected
Figure 3.4: NEK10 undergoes nuclear translocation following UV irradiation.

A) UV irradiation does not affect NEK10 catalytic activity. Flag-NEK10 was immunoprecipitated from transiently transfected HEK293 cells treated with UV irradiation (100 J/m²) for indicated times. Immunoprecipitates were used in *in vitro* kinase assays with histone H3 as a substrate. Kinase assays were separated by SDS-PAGE and phosphorylation detected by autoradiography. Whole cell lysates were run as input controls. Results show relative quantity of phosphate incorporated onto histone H3 and are from three independent experiments performed in duplicate or triplicate. Histone H3 phosphate incorporation was normalized against whole cell levels of Flag-NEK10.

B) UV irradiation does not affect NEK10 activation loop phosphorylation. Flag-NEK10 wildtype and KD, were immunoprecipitated from transiently transfected HEK293 cells treated with UV irradiation for indicated times and doses. Immunoprecipitates were separated by SDS-PAGE and probed with indicated antibodies.

C) Subcellular localization of NEK10. HeLa cells were transfected with Flag-NEK10 and treated with UV irradiation (100 J/m²) for 3h. Expressed proteins were visualized by indirect immunofluorescence staining. Nuclei were visualized by DAPI.
Figure 3.5: S933 phosphorylation is required for NEK10 nuclear localization.

HeLa cells were transfected with indicated constructs and treated with UV (100 J/m²) for 3 hours prior to fixation. Indirect immunofluorescence staining was performed. Greater than 90 cells were counted per sample. Presented are the results of three independent experiments. Error bars represent s.e.m. Asterisks represent statistical significance (* P < 0.05).
HEK293 cells (Fig 3.6A). Intriguingly, this interaction was not modulated by UV irradiation. Moreover, NEK10 S933A retained the ability to interact with 14-3-3 (Fig 3.6A).

In addition to S933, NEK10 contains other predicted 14-3-3 binding sites and may engage in multi-site interactions with 14-3-3. To probe for this, a truncated NEK10 protein, composed of the C-terminal 339aa of NEK10 (Flag NEK10 Ct), and including S933, was utilized. Similar to the full-length protein, Flag-NEK10 Ct co-precipitated with 14-3-3η in a UV-independent manner. However, S933 mutation in Flag-NEK10 Ct abolished its interaction with 14-3-3η (Fig 3.6B), demonstrating that NEK10 S933 is a bona fide 14-3-3 binding site.

In addition to impacting protein subcellular localization, 14-3-3 binding has also been shown to modulate protein-protein interactions and kinase catalytic activity. Judging by the comparable activity of NEK10 S933A to wildtype NEK10, 14-3-3 binding to this site does not appear to affect the catalytic function of NEK10 (Fig 3.6C). My previous work demonstrated that NEK10 interacts with Raf-1 (Fig 2.5) and that the NEK10 C-terminus is sufficient to mediate this interaction (Fig 2.7A). Raf-1 is a well-characterized 14-3-3 binding protein and it’s interactions with a number of proteins including A20 and Protein Kinase Cζ are bridged by a 14-3-3 dimer (412, 415). Nevertheless, 14-3-3 binding to S933 was dispensable for the NEK10/Raf-1 interaction, as endogenous Raf-1 co-precipitated to equal degrees with Flag NEK10 Ct and Flag NEK10 Ct S933A (Fig 3.6D).
Figure 3.6: NEK10 binds 14-3-3.

A) Co-immunoprecipitation of NEK10 with 14-3-3. HEK293 cells were transfected with the indicated constructs. NEK10 was immunoprecipitated with M2 Flag agarose. Co-precipitating proteins were detected by immunoblotting with the indicated antibodies. Whole cell lysates were run as input controls.

B) NEK10 S933 is a 14-3-3-binding site. HEK293 cells were transfected with the indicated constructs. NEK10 was immunoprecipitated with M2 Flag agarose. Co-precipitating proteins were detected by immunoblotting with the indicated antibodies. Whole cell lysates were run as input controls.

C) NEK10 S933 phosphorylation is not required for catalytic activity. HEK293 cells were transfected with indicated constructs and treated with UV irradiation (100 J/m²) for indicated times. Flag immunoprecipitates were used in *in vitro* kinase assays with Histone H3 as a substrate. Kinase assays were separated by SDS-PAGE and Histone H3 phosphorylation detected by autoradiography. Whole cell lysates were run as input controls.

D) NEK10-Raf-1 interaction does not require NEK10 S933 phosphorylation. HEK293 cells were transfected with Flag-NEK10 or Flag-NEK10 S933A. NEK10 was immunoprecipitated with M2 Flag agarose. Co-precipitating proteins were detected by immunoblotting with the indicated antibodies. Whole cell lysates were run as input controls.
3.5 Discussion

Work described in this chapter examined the role of phosphorylation in regulation of NEK10 catalytic activity and subcellular localization. This data indicates that NEK10 is fully active in cycling cells. Furthermore, that NEK10 catalytic activity requires activation loop phosphorylation, which likely occurs as a result of autophosphorylation (Fig 3.3.A, 3.3B). While no effect of UV irradiation on NEK10 catalytic activity was observed (Fig 3.4A), NEK10 underwent nuclear translocation following irradiation (Fig 3.4C). Significantly, this was dependent on phosphorylation of S933, a residue within one of the predicted NEK10 14-3-3 binding sites (Fig 3.5, 3.6B).

3.5.1 Characterization of NEK10 catalytic activity

NEK10 displayed robust autophosphorylation and had a preference for histones compared to β-casein as a generic substrate (Fig 3.1A). This may indicate NEK10’s preference for basic over acidic substrates. NEK kinases can be grouped according to their general substrate specificity, with NimA, NEK1, 2 and 3 exhibiting robust phosphorylation of β-casein, and NEK6, 9 and 10 with no activity towards this substrate (112, 147, 222, 340, 389). The substrate specificity of NEK10 is consistent with its greater homology to NEK6 compared to NEK1/2/3. Analysis of NEK6 substrate specificity demonstrated that it had a preference for an aromatic residue at the –4 and +1 position, relative to the phospho-target site, and did not tolerate a proline at +1 (F/YXXXpS/TF/Y) (234). It would be informative
to determine if NEK10 shared similar substrate specificity to NEK6, using a method such as oriented peptide library screening (373).

### 3.5.2 NEK10 undergoes activation loop autophosphorylation

Eighteen sites of serine and tyrosine phosphorylation were detected within wildtype NEK10 (Fig 3.2C). Five serine phosphorylation sites were identified within NEK10 KD, indicating that the majority of NEK10 phosphorylation is dependent on NEK10 catalytic activity. While some of these sites are undoubtedly sites of autophosphorylation, others might be targeted by unidentified upstream kinases. In such a scenario, limited NEK10 autophosphorylation may serve to prime NEK10 for additional phosphorylation events or may alter NEK10 protein conformation allowing it to interact with upstream kinases.

Two likely sites of NEK10 autophosphorylation are S684 and S688 within its activation loop. Similar to other kinases, phosphorylation of these sites was required for activity and mutation of these residues rendered NEK10 inactive (Fig 3.3A). Several pieces of evidence implicate these residues as targets of intermolecular autophosphorylation. First, S684 and S688 were identified as phosphorylation sites on wildtype, but not NEK10 KD, by both mass spectrometry and immunoblotting with a phosphospecific antibody (Fig 3.2C, 3.3B). Second, in vivo, NEK10 expression induces activation loop phosphorylation of NEK10 KD, indicating that phosphorylation may occur in trans (Fig 3.3.C). Finally, NEK10 was found to dimerize with both wildtype and catalytically inactive NEK10 (Fig 3.3.D), a feature associated with kinases that autophosphorylate in trans. For example, NEK2 and NEK9 are activated by autophosphorylation and are able to dimerize by a mechanism that
requires their coiled-coil motifs (reviewed (323)). In contrast, NEK6 and NEK7 do not dimerize and are activated by NEK9-mediated activation loop phosphorylation (24). This suggests that for NEK kinases, the ability to dimerize may be predictive of an activation mechanism by autophosphorylation.

3.5.3 NEK10 exhibits tyrosine kinase activity

An interesting feature of NEK10 phosphorylation is the apparent absence of tyrosine phosphorylation on catalytically inactive NEK10 and the ability of wildtype NEK10 to autophosphorylate on tyrosine \textit{in vitro} (Fig 3.1B,C, 3.2C). Despite sequence homology to serine-threonine kinases, tyrosine autophosphorylation has been noted for certain kinases, including GSK3, DYRK and CK2 (56, 78, 236, 237). Autophosphorylation of serine-threonine kinases on tyrosine may be kinetically- and sterically-favored compared to tyrosine phosphorylation of exogenous substrates, especially when the reaction occurs in \textit{cis}, as is the case for GSK3 and DYRK. Thus, to further explore NEK10 as a bona fide dual specificity enzyme, it should be systematically tested for its ability to phosphorylate exogenous substrate(s) on S/T and Y residues (224).

Of note, a member of the NEK family, NEK1, was originally identified as a dual-specificity kinase (222). NEK1 expression in bacteria was accompanied by tyrosine phosphorylation of bacterial proteins, and \textit{in vitro} NEK1 displayed weak activity towards a generic acidic tyrosine kinase substrate, poly(Glu:Tyr). However, the physiological relevance of NEK1’s tyrosine kinase activity is unclear.
3.5.4 NEK10 subcellular localization is regulated by UV irradiation

Following up on our studies with NEK10 and the UV response, we were interested in determining if NEK10 was regulated by UV irradiation. While we did not observe any change in catalytic activity (Fig 3.4A) or binding to Raf-1 (Fig 2.6B), we detected a cytoplasmic to nuclear translocation of NEK10 following UV irradiation (Fig 3.5). The physiological relevance of such behaviour is currently unknown, however, UV-dependent relocalization may serve as a means to control access of NEK10 to distinct pools of substrates, or interacting regulatory proteins in a stimulus-dependent manner. Future work should explore if localization impacts NEK10 in checkpoint control. For example, NEK10-null MEFs, which are being generated in our laboratory, could be reconstituted with wildtype or S993A NEK10 and the fidelity of the G2/M arrest measured following UV irradiation.

Parallels can be drawn between the control of NEK10 subcellular localization and that of NEK1. The latter exhibits predominantly cytoplasmic localization in cycling cells, but translocates to the nucleus following IR, where it is found at discrete foci in the nucleus and colocalizes with \( \gamma \text{H2AX} \) (313). NEK1 shuttling between the cytoplasm and nucleus likely depends on the presence of functional nuclear localization sequences (NLS) and nuclear export sequences (NES) (153). Based on these studies, it would be of interest to explore NEK10 subcellular localization following other genotoxic stresses and to further examine subnuclear localization of NEK10. Using publicly available prediction tools (http://www.cbs.dtu.dk/services/NetNES/; http://cubic.bioc.columbia.edu/predictNLS/), 3 putative NES, but no NLS, were identified within NEK10. Examining the localization of NEK10 NES point mutants or NEK10 truncation mutants, possibly in the context of
pharmacological modifiers of nuclear transport, such as leptomycin B, may identify regions of NEK10 that regulate its subcellular localization.

3.5.5 14-3-3 proteins regulate NEK10 subcellular localization

My data reveal that phosphorylation of NEK10 S933, a residue within a site of 14-3-3 binding, participates in control of NEK10 subcellular localization (Fig 3.6B). The 14-3-3 family are small, acidic, dimeric proteins that bind to phosphoserine/phosphothreonine residues within the consensus sequences: RSX(pS/sT)XP or RXFX(pS/pT)XP (442). 14-3-3 binding can have multiple consequences for the target protein. In the case of Raf-1, 14-3-3 binding influences catalytic activity and protein-complex formation. In non-stimulated cells, 14-3-3 binding to phosphorylated S259 inhibits Raf-1 by promoting an inactive conformation. In contrast, following mitogenic stimulation, 14-3-3 binding to S621 promotes Raf-1 activation (407). This may occur through two mechanisms; stabilizing an active conformation and promoting Raf-1 dimerization (117, 348, 426). Significantly, 14-3-3 binding to S933 does not contribute to similar events in NEK10, and mutation of S933 to alanine did not have any effect on NEK10 catalytic activity or interaction with Raf-1, a known interacting partner (Fig 3.6C, 3.6D).

14-3-3 proteins also act as major modulators of subcellular localization. 14-3-3 binding is predominantly associated with cytoplasmic sequestration as in the case of Cdc25B/C and members of the foxo family of transcription factors (38, 62). However, 14-3-3 binding to other proteins, including telomerase catalytic subunit (TERT) and Chk1, promotes nuclear localization (88, 360). It has been demonstrated that 14-3-3 binding often functions
by masking a subcellular targeting sequence. An example of this is TERT, where 14-3-3 binding obscures a nearby NES (360). While NEK10 does not contain any obvious NES or NLS motifs in close proximity to S933, it is possible that cryptic localization signals exist within NEK10, and are altered by 14-3-3 binding. Alternatively, NEK10 S933 phosphorylation and subsequent 14-3-3 binding may affect its association with proteins that govern its subcellular localization.

The sequence surrounding NEK10 S933 constitutes a consensus target sequence for the AGC kinases and the calcium/calmodulin-dependent kinase (CaMK) families (Scansite). Using a targeted approach, it may be possible to identify the S933 kinase. The ability of candidate kinases to phosphorylate NEK10 in a S933-dependent manner can be explored using recombinant kinases in *in vitro* kinase assays. The *in vivo* relevance of promising candidates may determined by examining the effect that inhibition of these kinases, by RNAi or treatment with chemical inhibitors has on UV-induced NEK10 translocation. The latter approach may be utilized to more broadly identify pathways that regulate NEK10, either dependent or independent of S933 status, using NEK10 subcellular localization as a readout.

The work described here provides the first insight into the importance of phosphorylation for regulation of NEK10. Identification of upstream regulators, as well as NEK10 substrates and substrate recognition sequences will be instrumental in defining the molecular mechanisms of action of this kinase.

**Attributions**

J.Ho purified α-pNEK10 antibodies from rabbit serum. Mass spectral analysis on NEK10 was performed by Protana.
CHAPTER 4

FUTURE DIRECTIONS
The work described in Chapters 2 and 3 constitutes the first characterization of the serine-threonine kinase NEK10. When I began work on NEK10, only a handful of publications focused on mammalian NEK kinases. Since then, NEKs have been more extensively studied and shown to impact cell cycle progression, ciliagenesis and the response to genotoxic stresses. The experiments described in Chapters 2 and 3 link NEK10 to G2/M arrest following UV irradiation. Analogous to the reaction of NEK1 to IR, I found that NEK10 translocates to the nucleus following UV irradiation. Intriguingly, NEK10 was found to promote UV-induced ERK activation. NEK10 interacted with Raf and MEK and enhanced MEK activity through a novel mechanism involving MEK autoactivation. Whether a relationship between other NEKs and the ERK signaling pathway exists is a topic for future study.

Work described in Chapter 3 provides a foundation for further biochemical characterization of NEK10. We have demonstrated that NEK10 is a multiply phosphorylated protein and that the majority of the phosphorylation sites, including S684 and S688 within the activation loop, are likely targets of autophosphorylation. Of significance, five phosphorylation sites that are independent of NEK10-catalytic activity were identified, and represent candidate targets for unidentified upstream kinases. I have shown that one of these sites, S933, is a 14-3-3 binding site and mediates NEK10 nuclear translocation following UV irradiation. Further study of S933 and the other 4 non-autophosphorylation sites likely provides the best opportunity to identify upstream regulators of NEK10.

In addition to the work presented in Chapters 2 and 3, several other lines of evidence will inform future work on NEK10. Namely, a potential role for NEK10 in control of
myogenesis and cell adhesion, preliminary characterization of NEK10-interacting partners, and possible means of NEK10 involvement in tumourigenesis will be discussed below.

4.1 Materials and Methods

Cell Culture and Transfection

HEK293 cells were cultured and transfected as in Chapter 2.3. C2C12 cells were cultured in growth media (gm) (DMEM/10% FBS). To induce differentiation, culture medium was changed to differentiation media (dm) (DMEM/2% horse serum) when cells reached confluency. For experiments involving non-adherent and re-plated cells, HEK293 cells were serum-starved overnight. To place cells in suspension, cells were detached by trypsinization (5 min) and collected in DMEM containing 1mg/ml soybean trypsin inhibitor and incubated, with rotation, at 37°C for 90 min. Cells were then maintained in suspension or re-plated onto un-coated or fibronectin-coated plates for 20 min before lysis.

Cell Lysis, Immunoprecipitation and Kinase Assays

Cell lysis, immunoprecipitation and kinase assays were performed as in Chapter 2.3.

Western Blot Analysis and Antibodies

α-NEK10 (sc-100434) was from Santa Cruz, α-MHC ascites fluid from Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa. For list of additional antibodies see Chapter 2.3.
4.2 NEK10 and myogenesis

In the course of characterizing a commercially available antibody against NEK10 (α-NEK10), an immunoblot of mouse tissue lysates demonstrated prominent expression of NEK10 in skeletal muscle (Fig 4.1A). Immunoprecipitated endogenous NEK10, using the same antibody, from a mouse myoblast cell line (C2C12) cultured in growth media (gm) displayed no autophosphorylation activity (Fig 4.1.B). Strikingly, NEK10 immunoprecipitates, from cells grown for 48-96h in differentiation media (dm), readily autophosphorylated (Fig 4.1B). This coincides with increased NEK10 immunoreactivity in lysates from differentiating C2C12 cells (Fig 4.1C). These results raise the possibility that NEK10 is involved in muscle differentiation.

In cell culture, early stages of myogenesis can be studied using a myoblast cell line such as the C2C12 cell line. In the absence of growth factor stimulation, these progenitor cells differentiate and fuse into multinucleated skeletal muscle cells called myotubes (reviewed in (193)). Myotubes can further develop into striated muscle cells called myofibrils, which contain the functional contractile apparatus (Fig 4.2). Commitment to differentiation in this system requires irreversible cell cycle exit during G1. This is accompanied by downregulation of proliferative signaling and is marked by decreased expression of proteins that promote cell cycle progression, such as cyclinD1 and cyclinA (421)(reviewed in (193)). There is a concurrent increase in the expression and activity of proteins that inhibit cell cycle progression, including p27 and pRB, which also contribute to the maintenance of the cell cycle arrest following differentiation (58, 105). Myogenesis is under the control of the muscle specific, MyoD, family of basic helix loop helix (bHLH)
Figure 4.1: NEK10 expression and activity is induced during muscle differentiation.

A) Whole cell protein lysates were prepared from mouse tissues and HEK293 cells. Lysates were separated by SDS-PAGE and immunoblotted with a monoclonal antibody against NEK10 (α-NEK10).

B) α-NEK10 immunoprecipitates were prepared from C2C12 cells grown in growth media (gm; DMEM, 10% FBS) or differentiation media (dm; DMEM, 2% horse serum) and subjected to in vitro kinase assays. Kinase assays were separated by SDS-PAGE and exposed by autoradiography. Whole cell lysates were run as input controls and immunoblotted with α-MHC to monitor cells for differentiation status.

C) Whole cell lysates were prepared from C2C12 cells grown in growth media (gm) or differentiation media (dm) for indicated periods of time. Lysates were separated by SDS-PAGE and immunoblotted with indicated antibodies. A lysate from HEK293 cells transiently transfected with Flag-NEK10 was run as a control.
Figure 4.2: Schematic representation of myogenesis.

In the presence of adequate growth factors muscle precursor cells (myoblasts) remain in a proliferative state. Following growth factor withdrawal, cell cycle arrest and myoblast differentiation is initiated. This is accompanied by downregulation of proliferative genes and upregulation of cell cycle inhibitors and muscle-specific genes. Myogenesis involves fusion of myoblasts into multi-nucleated myotubes. Myotubes can develop further into striated muscle cells called myofibrils, which contain the contraction apparatus. The basic unit of the contraction apparatus is the sarcomere which are multi-protein complexes composed of different filament systems.
transcription factors, which contribute to cell cycle withdrawal and stimulate expression of muscle-specific structural genes, such as myosin heavy chain (MHC) and $\alpha$-actin (376) (reviewed in (193, 214, 354, 388)).

The induction of NEK10 reactivity during C2C12 cell differentiation warrants exploration of the impact of its depletion on myoblast differentiation, as well as the status of abovementioned muscle-specific factors in NEK10-depleted cells. NEK10’s involvement in initiating or maintaining cell cycle arrest during differentiation should also be further explored. Work described in Chapter 2 outlines a role for NEK10 in promoting the G2/M checkpoint following UV irradiation, raising an intriguing possibility that cell cycle arrest may be a conserved role for NEK10. In proliferating cells, NEK10 may act at the level of checkpoint control, whereas it may promote cell cycle arrest or withdrawal in the context of cell differentiation. While it is clear that checkpoint arrest and cell cycle withdrawal during differentiation are distinctly regulated, they have partially overlapping molecular signatures (reviewed in (314)). Identification of substrates or proteins that interact with NEK10 during myocyte differentiation would provide a greater understanding of NEK10’s role in myogenesis, and may be instrumental in uncovering the mechanism of NEK10-mediated cell cycle arrest following genotoxic stress.

4.3 NEK10 and cell adhesion

In Chapter 2, NEK10 was shown to enhance MEK autoactivation following UV irradiation. MEK autophosphorylation has also been described in the context of cell adhesion and integrin engagement (303). In adherent cells, Pak phosphorylates MEK1 on S298 and
this is abolished when cells are brought into suspension (369). S298 phosphorylation increases MEK1 activation by enhancing MEK1’s interaction with its upstream activator Raf-1 (109). In addition, S298 phosphorylation of MEK1 may stimulate MEK1 autophosphorylation following adhesion (303). Intrigued by these findings, I pursued NEK10’s effect on MEK activity in the context of cell adhesion, but found that ectopic expression of NEK10 failed to stimulate MEK1 S298 phosphorylation in either adherent cells or cells re-plated onto fibronectin, indicating that NEK10 promotes MEK autophosphorylation by a mechanism independent of Pak and S298 phosphorylation (Fig 4.3).

In the course of these experiments, I also examined the effect of adherence on NEK10 catalytic activity. Endogenous NEK10 was immunoprecipitated from differentiated C2C12 myotubes and subjected to in vitro kinase assays. NEK10 kinase activity decreased when myotubes were placed in suspension and this could be recovered upon re-plating (Fig 4.4A). Interestingly, Flag-NEK10 immunoprecipitated from suspended HEK293 cells exhibited a downward mobility shift in SDS-PAGE, indicative of a lower degree of phosphorylation. Significantly, this mobility shift was abolished upon re-plating (Fig 4.4B), raising several interesting questions regarding possible regulation of NEK10 by cell adhesion.

In tissues, cells are in constant contact with other cells and the extracellular matrix. In 2-D culture, most non-transformed cells require continuous interaction with solid support and loss of adhesion often results in cell cycle arrest, or a form of apoptosis termed anoikis (107, 379). Integrins are one of the key proteins involved in mediating the flow of information between the extra- and intracellular compartments (reviewed in (277)). In response to cell adhesion these transmembrane glycoproteins recruit and activate kinases cascades,
**Figure 4.3: NEK10 does not effect MEK1 S298 phosphorylation.**

HEK293 cells were transfected with the indicated constructs. Whole lysates were prepared from adherent cells (A), cells that had been placed in suspension for 90 minutes (S) or had been placed in suspension and then replated on fibronectin-coated plates for 20 minutes (Fn). Lysates were separated by SDS-PAGE and immunoblotted with indicated antibodies.
Figure 4.4: NEK10 activity in adherent and suspended cells.

A) NEK10 activity is attenuated in C2C12 cells held in suspension. C2C12 cells were grown in growth media (gm) or in differentiation media (dm) for 96h. α-NEK10 immunoprecipitates from adherent cells (A), cells placed in suspension for 90 min (S) or from replated cells (20 min) (Rp) were subjected to \textit{in vitro} kinase assays. Flag immunoprecipitates from HEK293 cells transfected with Flag-NEK10 were also subjected to kinase assays as a control. Kinase assays were separated by SDS-PAGE and exposed by autoradiography.

B) NEK10 exhibits a downward mobility shift in suspended HEK293 cells. HEK293 cells were transfected with Flag-NEK10 and Flag immunoprecipitates subjected to kinase assays. Kinase assays were separated by SDS-PAGE and exposed by autoradiography. Whole cell lysates were run as input controls.
stimulating downstream signaling (reviewed in (277)). Integrin signaling is critically involved in cell cycle progression, cell shape, tissue organization and cell motility.

To extend on our preliminary observations, NEK10’s regulation by cell adhesion and integrins should be explored. Initially, further insight into the effect of adhesion on NEK10 catalytic activity and/or phosphorylation status will be needed. Identification of adhesion-dependent NEK10 phosphorylation sites would focus the pursuit of upstream regulators of its activity upon adhesion. Candidate approaches aimed at proteins with established roles in mediating signals downstream of cell adhesion/integrins would also be informative. Using chemical inhibitors or RNAi strategies, the effect of inhibition of proteins such as Rac and Rho GTPases and Pak kinases on NEK10 activity/phosphorylation status should be determined. In parallel, contribution of NEK10 to cell adhesion and to the assembly and activation of adhesion-dependent signaling complexes should be examined. The outlined basic experimental strategy will inform subsequent steps in understanding NEK10’s function in cell adhesion.

4.4 NEK10 interacts with tubulin in vivo

To identify interacting partners of NEK10, the protein content of NEK10 immunoprecipitates from transiently transfected HEK293 cells was analysed by mass spectrometry, in collaboration with the laboratory of A.C. Gingras (The Samuel Lunenfeld Research Institute of Mount Sinai Hospital). α- and β-tubulin were isolated as significant binding partners for NEK10. Tubulins are commonly found in mass spectrometry-based interaction screens, and are often considered false-positive hits. However, the number of
NEK10-interacting tubulin peptides was considerably higher than what is considered background. For example, 155 NEK10 peptide events, which covered 39% of the protein were identified. By comparison, the top 4 NEK10-interacting proteins, isoforms of β-tubulin, were each represented by over 250 peptide events providing 60% coverage. In total, over 1000 peptide events corresponding to α- and β-tubulin interacted with NEK10, and were not detected in control immunoprecipitations. The number and variety of identified tubulin peptides provides convincing evidence that tubulin is a true NEK10 interactor.

Significantly, NEK kinases have a role in regulating microtubule (MT) dynamics at the centrosome, cilia and the mitotic spindle. NEK2 activity promotes centrosome separation during early mitosis and deregulation of NEK2 increases the rate of spindle defects (111). Inhibition of either NEK6 or NEK7 leads to fragile spindle formation, activation of the SAC and defects in cytokinesis (292). Interestingly, while both of these kinases are diffusely distributed throughout the cell cycle they also display distinct patterns of subcellular localization. NEK6 localizes to MT-based structures during mitosis and cytokinesis, including the mitotic spindle and the central body respectively, while a small fraction of NEK7 is associated with interphase centrosomes and mitotic spindle poles (292). *In vitro*, both NEK6 and NEK7 interact with and phosphorylate MT preparations (292).

Future work should explore the biochemical properties and the physiological relevance of NEK10 interaction with tubulin. Based on work with NEK6 and NEK7, it would be relevant to confirm a physical interaction between NEK10 and tubulin and/or MT preparations, as well as examine the possibility that NEK10 directly phosphorylates tubulin or a MT-associated protein. The effect of MT stability on NEK10 catalytic activity should
also be determined, for instance in cells treated with microtubule stabilizing or depolymerization agents, paclitaxel and colcemid, respectively.

Moreover, a detailed characterization of the subcellular localization of NEK10 should be performed. In particular, localization of NEK10 to tubulin-based structures including MTs, centrosomes and cilia during interphase, and centrosomes, mitotic spindles and the central body during mitosis should be examined. Based on the results of these experiments, further studies could probe the effect of NEK10 on MT organization, stability and dynamics using \textit{in vitro} MT preparations and immunofluorescence-based assays in cultured cells. In case that NEK10 localizes to centrosomes, a role in regulating centrosomal numbers and duplication, separation and maturation should be examined. Finally, possible NEK10 functions in normal spindle assembly, chromosome segregation and cytokinesis should be explored.

In proliferating cells the centrosome nucleates MTs. In most differentiated cells however, MTs are organized by noncentrosomal mechanisms. In these systems MTs often display linear, as opposed to radial, organization and increased stability (reviewed in (20)). Considering our observations that NEK10 may participate in myogenesis, it may be worthwhile to explore a role for NEK10 in noncentrosomal MT organization during myocyte differentiation. Terminal differentiation of muscle cells involves extensive changes in the MT architecture, from a centrosome-bound radial array into parallel fibres that are longitudinally-organized. This may be triggered by a redistribution of centrosomal proteins from the pericentriolar region to the nuclear periphery (394). Significantly, in differentiated myotubes, nuclei gain the ability to nucleate MT assembly while the potential of centrosomes to do so decreases (97). MT organization has been implicated in the process of myofibril generation.
and in maintenance of cell shape, however the mechanism of this is largely unexplored. It is intriguing to speculate that NEK10 may play a role in the reorganization, maintenance or stability of the MT network during differentiation.

4.5 NEK10 in tumourigenesis

Our lab has generated transgenic mouse lines carrying conditionally targeted alleles of NEK10 which upon Cre-recombinase expression are expected to lead to a complete loss of NEK10 function (P.Dutt and V.Stambolic, unpublished). These mice will provide invaluable tools to further explore the physiological roles of NEK10. The course of examination will depend on the viability and/or the phenotype of the mice lacking NEK10. Nevertheless, broad applications for this animal model can be anticipated. MEFs generated from NEK10 knockout mice will provide a powerful model system to study the cellular functions of NEK10 and may be used in place of NEK10 knockdown in previously outlined experiments, as they will feature complete loss of NEK10 and be devoid of potential off-target effects associated with siRNA methods.

The NEK10 knockout mice will also be instrumental in exploring a role for NEK10 in oncogenesis. As outlined in section 1.3.2, there is accumulating evidence that NEK10 may be implicated in breast cancer. Thus, it would be highly informative to conditionally delete NEK10 in the developing mammary gland of the mouse and observe if this event promotes tumourigenesis. Considering that NEK10-deletion alone may not be sufficient to generate mammary tumours, NEK10 deletion can be combined with overexpression of ErbB2, a major transforming oncogene, in the same mammary cells (reviewed in (91)). For example,
MMTV-NIC mice (W.J. Muller), which express the Cre-recombinase and ErbB2/Neu in the same mammary epithelial cells, can be crossed to NEK10 flox/flox mice (367). MMTV-NIC mice develop tumours with 100% penetrance at approximately five months of age. Based upon our data pertaining to the reduced survival of patients with low NEK10-tumour expression it may be predicted that MMTV-NIC;NEK10-/- mice would exhibit reduced tumour latency and/or increased tumour burden. The histopathological properties of tumours should also be examined to determine the impact of NEK10 on the tumour spectrum, possibly associated with the effect of NEK10 on genetic stability. Finally the impact of NEK10 on the metastatic properties of tumours should be examined by monitoring tumour spread to the lungs and liver.

4.6 Concluding Remarks

Impaired checkpoint function can have critical consequences for the cell, leading to genomic instability, transformation and loss of viability. Work described in this thesis provides the first insight into NEK10 regulation and its role in checkpoint control. These studies will serve as a foundation for future studies aimed at understanding the molecular mechanism of NEK10 action and its function in organismal development and tumourigenesis.
5.0 References


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