Identification and characterization of a novel CK2-MSK2 interaction in the UV response

By

Kellie Jacks

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

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Doctor of Philosophy 2010

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CK2 is a ubiquitous serine/threonine protein kinase implicated in numerous cellular processes as well as in tumorigenesis. CK2 is composed of two catalytic (αα, αα’, α’α’) subunits and two regulatory (ββ) subunits that assemble to form the active CK2 holoenzyme. CK2 has been shown to phosphorylate, interact with, and regulate other proteins, including other protein kinases. CK2 substrates can be initially bound by the CK2β regulatory subunit, which acts as a docking site to facilitate phosphorylation and mediate CK2 substrate specificity. In a screen to identify novel CK2β interacting proteins, I identified three novel CK2β interactors, including the mitogen- and stress-activated kinase 2 (MSK2), which I pursued for further characterization.

MSK2, and the closely related isoform MSK1, are nuclear kinases that are activated following mitogen stimulation or cellular stress, including UV radiation, by the ERK1/2 and p38-MAPK signaling cascades, respectively. However, factors that differentially regulate MSK1 and MSK2 have not been well characterized. In my thesis, I demonstrate that CK2, which contributes to NF-κB activation following UV radiation in a p38-dependent manner, physically interacts with MSK2 but not MSK1 and that CK2 inhibition specifically impairs UV-induced MSK2 kinase activation. A putative site of CK2 phosphorylation was mapped to MSK2 residue serine-324 and when substituted to alanine (S324A) also compromised MSK2 activity. RNA interference-mediated depletion of MSK2 in human MDA-MB-231 cells, but not MSK1
depletion, resulted in impaired UV-induced phosphorylation of NF-κB p65 at serine-276 in vivo, which was restored by the ectopic expression of MSK2 but not by MSK2-S324A. Furthermore, UV-induced p65 transactivation capacity was dependent on MSK2, MSK2 residue S324, and p65-S276. These results suggest that MSK1 and MSK2 are differentially regulated by CK2 during the UV response and that MSK2 is the major protein kinase responsible for the UV-induced phosphorylation of p65 at S276 that positively regulates NF-κB activity in MDA-MB-231 cells.

Together these findings constitute the body of a thesis submitted as a requirement for the degree of Doctor of Philosophy in the Graduate Department of Medical Biophysics at the University of Toronto.
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<td>AGC</td>
<td>protein kinase A/protein kinase G/protein kinase C</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>ATF-1</td>
<td>activating transcription factor 1</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>Brij35</td>
<td>polyoxyethyleneglycol dodecyl ether</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CLS</td>
<td>Coffin-Lowry syndrome</td>
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<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
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<td>CTKD</td>
<td>C-terminal kinase domain</td>
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<tr>
<td>DKO</td>
<td>double knockout</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DMAT</td>
<td>2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>DUSP1</td>
<td>dual specificity phosphatase 1</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
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<tr>
<td>ELKS</td>
<td>glutamate, leucine, lysine and serine rich protein</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>esiRNA</td>
<td>endoribonuclease-prepared short interfering ribonucleic acid</td>
</tr>
<tr>
<td>FACT</td>
<td>facilitates chromatin transcription</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
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<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
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<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HM</td>
<td>hydrophobic motif</td>
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<td>high-mobility group nucleosome binding domain 1</td>
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<td>HSP90</td>
<td>heat shock protein 90</td>
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<td>IκB</td>
<td>inhibitor of κB</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of κB kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1beta</td>
</tr>
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<td>IL-6</td>
<td>interleukin-6</td>
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<td>interleukin-10</td>
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<tr>
<td>IL-12</td>
<td>interleukin-12</td>
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<tr>
<td>IP</td>
<td>immunoprecipitation</td>
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<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>IR</td>
<td>ionizing radiation</td>
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<td>JNK</td>
<td>c-jun N-terminal kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>KSR</td>
<td>kinase suppressor of Ras</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>murine embryonic fibroblast</td>
</tr>
<tr>
<td>MEK</td>
<td>MAP kinase or ERK kinase</td>
</tr>
<tr>
<td>MOPS</td>
<td>4-morpholinepropanesulfonic acid</td>
</tr>
<tr>
<td>MRN</td>
<td>Mre11-Rad50-Nbs1</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
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<tr>
<td>MSK</td>
<td>mitogen- and stress-activated kinase</td>
</tr>
<tr>
<td>Nbs1</td>
<td>Nijmegen breakage syndrome protein 1</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
</tr>
<tr>
<td>NT</td>
<td>non-targeting</td>
</tr>
<tr>
<td>NTKD</td>
<td>N-terminal kinase domain</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>PDK1</td>
<td>phosphoinositide-dependent kinase-1</td>
</tr>
<tr>
<td>PEST</td>
<td>proline, glutamate, serine, threonine</td>
</tr>
<tr>
<td>PIASy</td>
<td>protein inhibitor of activated STAT Y</td>
</tr>
<tr>
<td>PIDD</td>
<td>p53-induced protein with death domain</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
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PKB  protein kinase B
PML  promyelocytic leukemia protein
PP2A  protein phosphatase 2A
PVDF  polyvinylidene fluoride
qPCR  quantitative real-time polymerase chain reaction
REL  reticuloendotheliosis
RHD  rel homology domain
RIP1  receptor interacting protein 1
RPMI  Roswell Park Memorial Institute
RSK  p90 ribosomal S6 kinase
SDS-PAGE sodium dodecyl sulfate – polyacrylamide gel electrophoresis
siRNA  small interfering RNA
SPL  sphingosine-1-phosphate lyase
SSRP1  structure specific recognition protein 1
TBK1  TANK binding kinase 1
TBP  TATA-binding protein
TNFα  tumor necrosis factor α
TPA  12-O-tetradecanoylphorbol-13-acetate
UV  ultraviolet
WCE  whole cell extract
WT  wild type
Chapter 1: General Introduction
1.1 The UV response

Following exposure to ultraviolet (UV) radiation, mammalian cells mount an immediate and elaborate reaction, termed “the UV response” [1]. The UV response involves the induction of gene expression through the activation of several transcription factors including p53, AP-1 (activator protein-1) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which mediate processes such as cell survival, cell death and inflammation [2, 3]. Interestingly, the UV response is not confined to skin cells but rather is a conserved response elicited by many cell types including fibroblasts, lymphocytes and mammary epithelial cells [1].

UV radiation activates cellular signaling cascades in both the cytoplasm and the nucleus. Nuclear signaling events, which are initiated by UV-induced DNA damage, are required for the activation of p53 [4]. On the other hand, the UV-induced activation of AP-1 and NF-κB relies on cytoplasmic signals that are generated independently of DNA damage [5, 6]. Cytoplasmic signal transduction in response to UV-radiation is largely mediated by members of the mitogen activated protein kinase (MAPK) family [7-10], which include ERK (extracellular signal regulated kinase), p38 and JNK (c-jun N-terminal kinase) [11]. p38 and JNK are more strongly activated by cellular stress such as UV radiation whereas ERK is more strongly activated following mitogen stimulation [11].

1.2 NF-κB

The NF-κB transcription factor is activated by numerous cellular stimuli including pro-inflammatory cytokines, ionizing radiation (IR) and bacterial lipopolysaccharide (LPS) in addition to UV-radiation [12]. NF-κB is involved in inflammation, cell proliferation and cell survival [13]. In addition, aberrant NF-κB activation has been implicated in carcinogenesis [12].
The NF-κB transcription factor family refers to several closely related protein dimers that bind to the common DNA sequence motif κB. There are five mammalian REL (reticuloendotheliosis)/NF-κB family members that are divided into two groups based on their mode of synthesis [14]. The first group consists of p65/RelA, RelB and c-Rel, which are expressed as mature proteins and do not require proteolytic processing. These proteins contain an N-terminal Rel-homology domain (RHD) that mediates dimerization and DNA binding and a C-terminal transcriptional-activating domain [12]. The second group of Rel proteins includes the NF-κB1/p105 and NF-κB2/p100 precursor proteins, which require proteolytic processing to yield mature p50 and p52 proteins, respectively. p50 and p52 contain an N-terminal RHD but lack a transcriptional-activating domain [15]. The p65-p50 heterodimer is the most abundant NF-κB dimer in cells with the p65 subunit possessing the transactivational activity [16].

1.2.1 Activation of the NF-κB p65-p50 heterodimer

The p65-p50 heterodimer is activated by distinct cellular pathways depending on the cellular stimulus encountered. In unstimulated cells, the p65-p50 heterodimer is retained in the cytoplasm, bound to its inhibitor IκB (inhibitor of κB) [17] (Figure 1.1). Pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) are potent activators of NF-κB. This mode of NF-κB activation, known as the classical pathway (Figure 1.1A), is mediated by the IκB kinase (IKK) complex. The IKK complex includes the kinases IKKα and IKKβ and the scaffold protein NEMO (NF-κB essential modulator) also known as IKKγ [18]. The association of NEMO with IKKα and IKKβ is dynamic and is essential for IKKα/β kinase activity [18]. In response to pro-inflammatory cytokines (Figure 1.1A), the IKK complex is activated and phosphorylates IκB at two N-terminal serine residues (serine-32 and serine-36) [19]. This targets IκB for ubiquitin-mediated
Figure 1.1. Classical and atypical activation of NF-κB (p65-p50 heterodimer)

Under unstimulated conditions, NF-κB (p65-p50) is retained in the cytoplasm, bound to its inhibitor IκB. (A) Classical pathway: In response to inflammatory cytokines or bacterial infection, the IKK complex, consisting of the catalytic IKKα and IKKβ subunits and the regulatory NEMO subunit, is activated and phosphorylates IκB on two N-terminal serine residues (S32 and S36), which targets IκB for ubiquitin-mediated proteosomal degradation [20]. The p65-p50 heterodimer can now translocate to the nucleus. (B) Atypical pathway: In response to UV-radiation, CK2 kinase activity towards IκB is stimulated in a p38-dependent manner. CK2 phosphorylates six serine/threonine residues within the C-terminal PEST domain of IκB, which targets IκB for ubiquitin-mediated proteosomal degradation [10]. CK2 also phosphorylates and inhibits β-arrestin, a negative regulator of NF-κB activation [21]. The p65-p50 heterodimer can now translocate to the nucleus.
proteosomal degradation and releases the p65-p50 heterodimer to translocate to the nucleus where it can bind to κB response elements in target gene promoters [20].

In contrast, UV-radiation activates an “atypical pathway” of NF-κB activation (Figure 1.1B). Indeed, initial investigations revealed that the UV-induced degradation of IκB does not require S32 and S36 phosphorylation nor does UV-radiation activate IKK [22], suggesting an IKK-independent pathway mediates the activation of p65-p50 in response to UV [22]. It is now established that following UV radiation the kinase CK2 phosphorylates a cluster of six serine/threonine residues within the C-terminal PEST motif of IκB, targeting it for ubiquitin-mediated proteosomal degradation [10]. Indeed, UV increases CK2 kinase activity towards IκB in a p38-dependent manner [10] and the p38-CK2-NF-κB pathway promotes cell survival under these conditions [10]. In line with this, CK2 also phosphorylates and inhibits β-arrestin2, a suppressor of the UV-induced activation of NF-κB and a promoter of UV-induced cell death [21]. CK2 phosphorylates β-arrestin2 and impairs its ability to bind and protect IκB from degradation [21]. Taken together, CK2 relieves NF-κB suppression by two mechanisms following UV radiation thus acting as a key mediator of the mammalian UV-response.

Although the activation of NF-κB (p65-p50) following IR requires the phosphorylation of IκB on S32 and S36 by the IKK complex, additional factors contribute the IR-induced activation of NF-κB that are not involved in the classical NF-κB activation pathway [23] (Figure 1.2). One factor is the ATM (Ataxia telangiectasia mutated) kinase, which is activated by the MRN (Mre11-Rad50-NBS1) complex in the nucleus in response to the generation of IR-induced DNA double strand breaks [24]. At the same time, IR promotes the nuclear translocation of NEMO, PIDD (p53-induced protein with death domain) and RIP1 (receptor interacting protein 1), which form a complex that promotes the sumoylation of NEMO by the SUMO ligase, PIASy [25-27].
Figure 1.2. Activation of NF-κB in response to ionizing radiation

Two parallel pathways converge on NEMO to activate NF-κB in response to IR. The first pathway involves the activation of ATM by the MRN complex in response to IR-induced DNA double strand breaks [24]. The second pathway involves NEMO shuttling to the nucleus where it associates with PIDD and RIP1 [25-27]. This complex promotes the sumoylation of NEMO. The pathways converge when activated ATM binds and phosphorylates NEMO, which results in the subsequent monoubiquitination of NEMO [26, 28]. The modified NEMO-ATM complex exits the nucleus and associates with IKKα, IKKβ and ELKS to form an activated IKK complex, which phosphorylates IκB at S32 and S36 promoting its ubiquitin-mediated proteosomal degradation [26, 28]. The p65-p50 heterodimer is free to translocate to the nucleus. Figure adapted from [23].
Sumoylation of NEMO induces its nuclear retention [26] and permits activated ATM to bind and phosphorylate NEMO, which promotes the ATM-dependent monoubiquitination of NEMO [26, 28]. The modified NEMO-ATM complex subsequently exits the nucleus and associates with IKKα and IKKβ and another IKK regulator ELKS (protein rich in glutamate, leucine, lysine and serine) to form an activated IKK complex, which phosphorylates IkB at S32 and S36 [26, 28]. Therefore, unlike the UV-induced activation of NF-κB, which is independent of DNA damage signals [5], the IR-induced activation of NF-κB requires nuclear to cytoplasmic signaling. Interestingly, in addition to NF-κB, ATM also activates p53 and inhibits the transcription factor CREB (cAMP response element binding) through phosphorylation following IR [29-31], which links ATM to the regulation of both pro-survival and pro-apoptotic pathways in the IR response.

1.2.2 Post-translational modification of p65-p50

In addition to the phosphorylation and degradation of IkB, several post-translational modifications of the p65-p50 heterodimer are required for optimal activation [14]. Phosphorylation of p65 is a key regulator of p65-p50 transcriptional activity. Several kinases have been implicated in the phosphorylation of p65 in both the cytoplasm and the nucleus and tend to be both stimulus and cell-type dependent [14]. Indeed, phosphorylation of p65 at serine-276, serine-529 and serine-536 have all been reported [32-38]. S276 in located within the RHD while S529 and S536 are located within the transcriptional activating domain of p65. The kinase CK2 has been implicated in the phosphorylation of S529 in response to TNFα and IL-1β stimulation [32, 33]. Several kinases, including IKKα and IKKβ, PKB, RSK1 and TBK1, have been reported to phosphorylate S536 depending on the cellular stimuli examined [14]. The phosphorylation of S529 and S536 have both been reported to enhance p65 transcriptional
activity. However, a study examining p65 transactivation using p65 (-/-) MEFs reconstituted with wildtype p65 or mutant p65 containing either S276A, S529A or S536A point mutants [39] observed that only the S276A mutant displayed impaired p65 transactivation in response to TNFα stimulation. These results suggested that phosphorylation of p65 at S276 is critical for maximal transactivation activity, at least in response to TNFα [39].

The mitogen and stress activated kinases, MSK1 and MSK2, have been implicated in the nuclear phosphorylation of p65 at S276 following TNFα and IL-1β stimulation [37, 38]. Furthermore, the MSK-mediated phosphorylation of S276 was observed to enhance p65 transactivation potential following cytokine stimulation [37, 38]. Phosphorylation of p65 S276 was also reported in the cytoplasm by PKA following LPS stimulation and also enhanced p65 transactivation [34]. p65 S276 phosphorylation has been shown to promote the recruitment of the histone acetyltransferases p300 and CBP (CREB binding protein), which mediate chromatin relaxation through histone acetylation [40], thus facilitating p65-dependent transcription. Furthermore, the recruited CBP and p300 also acetylate p65, which has also been reported to enhance p65 transcriptional activity [41]. Therefore S276 phosphorylation appears to be critical for p65 transactivation potential in response to pro-inflammatory cytokine and LPS stimulation. However, prior to my studies described herein, the role of p65 S276 phosphorylation in the UV response had not been characterized.

1.2.3 NF-κB target genes

In response to cellular stimuli, NF-κB activates the transcription of numerous target genes. NF-κB target genes can be divided into four categories: (1) immunoregulatory and inflammatory genes, (2) anti-apoptotic genes, (3) genes involved in cell proliferation, and (4) genes that encode negative regulators of NF-κB, such as IκB [12]. For the most part, NF-κB is thought to
activate genes from each of these categories following UV radiation [12]. However, whether NF-κB induces or represses target genes in response to UV-radiation is currently a point of controversy in the literature. Indeed, although several studies have observed an activation of NF-κB target genes in response to UV radiation [10, 42, 43], one report claims that NF-κB represses transcription of target genes following UV treatment [44]. This discrepancy may be due, in part, to the cell type examined. For example, studies examining skin cells observed an induction of NF-κB target gene transcription [45], which is consistent with acute inflammation (sunburn) while studies examining T-cells show a suppression of NF-κB transcription, which is consistent with UV-induced immunosuppression [46]. However, discrepancies observed in other cell types are less straightforward and require further investigation. Nonetheless, deregulation of NF-κB-dependent gene expression is linked to carcinogenesis[12].

1.2.4 NF-κB and cancer

Aberrant activation of NF-κB has been implicated in several human cancers [12]. In most cases, constitutive NF-κB activity is not caused by a genetic mutation but rather results from continual exposure to inflammatory stimuli such as pro-inflammatory cytokines [47]. Indeed, chronic infection and inflammation has long been considered a risk factor for several types of cancer [13]. Aberrant activation of NF-κB is associated with cancer promotion, cancer cell survival and increased metastatic potential [13] as several NF-κB target genes stimulate cell proliferation, antagonize apoptosis in response to genotoxic stimuli and induce cell migration [47]. Of importance, the activation of NF-κB in cancer cells by chemotherapy and radiation treatment can impair the ability of these treatments to induce cell death [48-51].

Both CK2 and MSK1 and MSK2 have been highlighted as key mediators of NF-κB activation. These kinases will be discussed in the following sections.
1.3 CK2

CK2 is an evolutionarily conserved and ubiquitously expressed serine/threonine protein kinase implicated in numerous cellular processes including cell cycle, cellular proliferation, cell survival, and DNA repair [52-54]. Deregulation of CK2 has been implicated in tumorigenesis [55]. Indeed, increased CK2 activity has been observed in many human cancers and transformed cell lines [56]. In addition, transgenic overexpression of the CK2 catalytic subunit, CK2α, in T-cells or in the mammary gland of mice predisposes these animals to lymphomagenesis and mammary tumorigenesis, respectively [57-59]. However, the mechanism by which CK2 kinase activity is regulated still remains poorly understood.

1.3.1 Phosphorylation of CK2 substrates

CK2 is localized to the cytoplasm and the nucleus and phosphorylates substrates in both compartments. Currently, CK2 has been implicated in the phosphorylation of over 300 protein substrates [60]. In general, CK2 is an acidophilic kinase that phosphorylates serine or threonine residues N-terminal to acidic amino acids [61]. The minimal CK2 consensus sequence has been defined as S/T-X-X-D/E/pS/pY [61], however, the presence of additional acidic residues at the n+1 and n+2 positions favour CK2 phosphorylation [60]. Due to the nature of the CK2 consensus sequence, CK2 can phosphorylate clusters of serine/threonine residues [10, 62]. For example, the phosphorylation of one residue increases the acidity of the neighbouring serine/threonine residues thus promoting their subsequent phosphorylation. In contrast, CK2 can efficiently phosphorylate some substrates that do not conform to the established CK2 consensus sequence such as serine-392 of p53, which is efficiently phosphorylated by CK2 in vivo [63] despite its divergence from the CK2 consensus sequence (FKTEGPDS\textsubscript{392}D-COOH).
Some cellular proteins require CK2 “priming phosphorylation” prior to being phosphorylated by other protein kinases. For example, CK2 phosphorylates the CREB transcription factor at serine-108 and serine-111 [64]. These phosphorylation events, in combination with CK1 priming phosphorylation at serine-114 and serine-117, are prerequisites for the IR-induced phosphorylation of CREB at serine-121 by ATM [64]. The phosphorylation of CREB by ATM in response to IR inhibits CREB binding to its co-activator CBP and thus impairs CREB-mediated transcription [30, 31].

1.3.2 Structural features of CK2

CK2 is a heterotetramer composed of two ($\alpha\alpha$, $\alpha\alpha'$, $\alpha'$) catalytic subunits and two $\beta$ regulatory subunits, which assemble to form the $\alpha_2\beta_2$ holoenzyme. CK2$\beta$ dimerization is required for CK2 holoenzyme formation [65, 66]. The X-ray crystal structure of the CK2 holoenzyme revealed that in fact the two catalytic subunits do not contact each other [67], which is contrary to previous reports. In addition, two modes of contact were found to exist between CK2$\alpha$ and CK2$\beta$ within the holoenzyme [67] (Figure 1.3). The primary CK2$\alpha$-CK2$\beta$ interaction occurs between CK2$\alpha$ and a region within the C-terminus of CK2$\beta$ termed the positive regulatory region [67]. This interaction acts to stabilize the CK2 holoenzyme and stimulate its catalytic activity [67]. The secondary CK2$\alpha$-CK2$\beta$ interaction is inhibitory and involves the association of the CK2$\beta$ acidic loop with a basic stretch near the active site of CK2$\alpha$ [67]. Because CK2 phosphorylates serine/threonine residues within acidic regions, it is thought that the acidic loop of CK2$\beta$ competes for binding with CK2 substrates [67]. Interestingly, structural analysis revealed that this interaction must occur between different CK2 tetramers due to the distance separating the acidic loop of CK2$\beta$ and the CK2$\alpha$ catalytic core within a single heterotetramer [67]. In line with this, CK2 has been shown to form higher order complexes [68].
Figure 1.3. Modes of CK2α/α’-CK2β interaction

(A) CK2α/α’ interacts with CK2β via a positive regulatory region near the C-terminus of CK2β [67]. This interaction acts to stabilize the CK2 holoenzyme and stimulates CK2 catalytic activity. Within the CK2 holoenzyme, the CK2β dimerization is mediated by a central zinc finger within each CK2β subunit. The two CK2α/α’ subunits do not contact each other within the CK2 holoenzyme [67]. (B) The CK2 holoenzyme is thought to form higher order structures that mediate the intermolecular secondary interaction between CK2α and CK2β. One proposed higher order structure is a ring-like trimer of CK2 tetramers. This interaction is inhibitory and occurs between the acidic loop of CK2β and the basic stretch of the active site of the neighbouring CK2α/α’ (inset). Figure adapted from [68].
1.3.2.1 The CK2α and CK2α’ catalytic subunits

The CK2α and CK2α’ catalytic subunits are encoded by two distinct genes [69]. They exhibit nearly 90% amino acid sequence identity within their catalytic domains diverging mainly in their C-termini where CK2α contains an approximately sixty amino acid extension that is completely distinct from CK2α’ [70]. The CK2 catalytic subunit contains several conserved basic residues clustered around its active site that facilitate its interaction with the acidic residues present in CK2 substrates [71-73].

1.3.2.2 The CK2β regulatory subunit

Human CK2β is highly conserved between species but shows little resemblance to other kinase regulatory proteins [74]. As mentioned above, CK2β possesses a C-terminal positive regulatory domain that mediates its interaction with CK2α/α’ and enhances CK2 catalytic activity [67] (Figure 1.4). CK2β also contains a central zinc-finger motif, consisting of four cysteine residues, that facilitates CK2β homodimerization, a prerequisite for CK2 holoenzyme formation [66]. Indeed, CK2β mutant proteins lacking a functional zinc finger can no longer interact with CK2α [66, 75]. As mentioned, the acidic loop of CK2β largely resembles the acidic clusters found in CK2 substrates (DLEPDEELED), which is consistent with its autoinhibitory role [76]. CK2β is phosphorylated by CK2α/α’ on serine-2, 3 and 4 through an intermolecular mechanism [77]. These phosphorylation events are thought to enhance the stability of CK2β, potentially by preventing CK2β ubiquitination and proteosomal degradation [78]. CK2β is also phosphorylated on serine-209 by p34cdc2 in a cell cycle dependent manner [79] however, the physiological significance of this phosphorylation remains to be characterized.

A large body of research supports the role of CK2β in the regulation of CK2 kinase activity and substrate specificity [74]. Indeed, CK2β has been reported to mediate the interaction of CK2
Figure 1.4. Important structural elements within the CK2β regulatory protein
Sites of phosphorylation are displayed as black “lollipops”. CK2β is phosphorylated by the CK2 catalytic subunits on Ser2, Ser3 and Ser4 and is phosphorylated on Ser209 by p34cdc2 in cell cycle dependent manner. An auto-inhibitory acidic loop is located N-terminal to the central zinc finger. The zinc finger motif consists of four cysteine residues and mediates CK2β dimerization. The C-terminus of CK2β contains a positive regulatory region through which it interacts with the CK2α/α’ catalytic subunits. Figure adapted from [74].
with more than forty cellular substrates [80]. Furthermore, although CK2α and CK2α’ display catalytic activity in the absence of CK2β [81], the presence of CK2β is required for the phosphorylation of several CK2 cellular targets. For example, in the previous section, I discussed the CK2-mediated phosphorylation of the NF-κB inhibitor, IκB, in response to UV radiation, which promotes its degradation [10]. Interestingly, the UV-induced phosphorylation of IκB is dependent on CK2β as depletion of CK2β results in IκB stabilization [10]. Therefore, CK2β is an important regulator of CK2 holoenzyme activity.

1.3.3 Regulation of CK2 kinase activity

Although CK2β is thought to play a role in mediating CK2 activity and substrate specificity, the exact cellular mechanisms that regulate CK2 activity remain largely uncharacterized. Traditionally, CK2 is characterized as an unregulated constitutively active protein kinase [82]. Indeed, CK2 does not seem to require stimulus-dependent phosphorylation of its activation loop for kinase activity [81] as CK2 exhibits robust kinase activity in the absence of cellular stimuli [83]. One report claimed that CK2α and CK2α’ can undergo autophosphorylation on a tyrosine residue (CK2α Y182) within their activation loop [84]. However, this phosphorylation is counteracted by CK2β making its significance difficult to explain in the context of the CK2 holoenzyme. Therefore, the role of phosphorylation in the regulation of CK2 activity remains unclear.

1.3.3.1 CK2 holoenzyme dynamics

The dynamic formation of the CK2 holoenzyme is another proposed mechanism by which CK2 kinase activity may be regulated. Traditionally CK2 has been regarded as a stable enzyme complex that, once formed, does not dissociate from its heterotetrameric state. However, recent experimental evidence challenges this view in favour of the notion that the CK2 holoenzyme is
a transient complex that readily associates and dissociates. In support of this idea, the CK2 holoenzyme X-ray crystal structure determined that the primary CK2α/CK2β interface was relatively small (832Å²) and flexible [67] compared to the average interface of stable, irreversible protein subunit interactions (1722Å²) [85] suggesting that the CK2α/CK2β contact was more consistent with a transient interaction [67]. Another study using live-cell fluorescence imaging observed the existence of dynamic CK2α and CK2β subpopulations that displayed differential cytoplasmic-nuclear shuttling kinetics [86]. These observations suggested that individual CK2 subunits may perform distinct cellular functions outside of the CK2 holoenzyme. In line with this, the CK2β regulatory subunits has been shown to binds and modulates the activity of other protein kinases including A-Raf, c-Mos and Chk1 independent of CK2α/α’ [87-89] [90]. In addition, the catalytic CK2α subunit was reported to interact with, phosphorylate and activate the protein phosphatase PP2A independent of CK2β [91]. Taken together, it has been proposed that CK2 undergoes regulated assembly and disassembly within the cell, which may serve to regulate CK2 kinase activity.

1.3.4 CK2 in growth factor signaling

The role of growth factors in the regulation of CK2 activity has been a point of controversy in the field. To address conflicting reports in the literature, a thorough analysis examining changes in CK2 activity in response to treatment with various growth factors including epidermal growth factor (EGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) was performed [83]. No reproducible increase in CK2 activity was observed [83]. These finding were consistent with the traditional view that CK2 activity is constitutive and not regulated by growth factors. However, a recent groundbreaking study described a detailed mechanism of a directed increase in CK2 activity following growth factor stimulation [92]. Indeed, EGF
stimulation was shown to enhance CK2 kinase activity toward alpha-catenin [92] in an ERK2-dependent manner. ERK2 was shown to directly interact with and phosphorylate CK2α at threonine-360 and serine-362, which stimulated the CK2-dependent phosphorylation of alpha-catenin at serine-641 and promoted the dissociation of the α-catenin-β-catenin complex [92]. This report presents a model whereby the kinase activity of a subcellular pool of CK2 is enhanced following growth factor stimulation [92]. In addition, it links phosphorylation to the regulation of CK2 catalytic activity.

CK2 has been reported to participate in the Ras-MAPK signaling cascade. Indeed, the CK2 holoenzyme was shown to interact with KSR1 (kinase suppressor of Ras), a scaffold protein that coordinates the assembly of a multiprotein complex containing Raf, MEK and ERK, which are components of the MAPK signaling cascade [93]. CK2 was shown to basally phosphorylate B-Raf on an activating serine residue while CK2-mediated phosphorylation of the equivalent residue on c-Raf was only detected following PDGF stimulation due to the requirement for Src-dependent priming phosphorylations on the n+2 and n+3 tyrosine residues [94]. These CK2-mediated phosphorylation events were found to be required for maximal Raf-MEK-ERK signaling in response to PDGF stimulation [94].

1.3.5 CK2 is a stress-activated kinase

Several studies have also emerged supporting the role of CK2 as a stress activated kinase [10, 95-99]. Indeed, increased CK2 activity has been observed in response to stress stimuli such as UV, arsenite and anisomycin [96]. The stress-induced activation of CK2 is p38-dependent [10, 95, 96]. p38 interacts with CK2 [100] and chemical inhibition of p38 impairs CK2 activation by cellular stress [10, 95, 96]. CK2 has been shown to promote cell survival following cellular stresses such as UV-radiation, in part through its stress-induced phosphorylation of IκB [10],
and the tumour suppressor PML (promyelocytic leukemia protein) [95]. In both cases, CK2 phosphorylates the PEST domains of these proteins, which promotes their ubiquitin-mediated proteosomal degradation [10, 95].

The role of CK2 in the UV-induced degradation of IκB, previously discussed in section 1.2.1, was a key study demonstrating a substrate specific increase in CK2 activity following cellular stress [10]. As NF-κB promotes cell survival following cellular stress [101], the p38-CK2 pathway was established as an important mediator of cell fate following UV radiation [10]. Indeed, inhibition of IκB degradation led to increased UV-induced cell death [10]. The stress-mediated phosphorylation of PML by CK2 promotes its degradation and is p38-dependent [95]. Loss of the PML tumour suppressor through a post-translational mechanism is frequently observed in several human cancers [102, 103]. Increased CK2 activity is proposed to be one mechanism by which PML is lost in tumours [95]. Indeed, examination of a panel of non-small cell lung cancer (NSCLC) cell lines and primary human NSCLC samples found that increased CK2 activity correlated with decreased PML protein levels [95]. Consistent with this idea, aberrant CK2 activity has also been observed in breast cancer [104] and multiple primary breast cancer specimens that display aberrant activation of NF-κB also display increased CK2 activity [105]. Therefore deregulated CK2 activity could also promote NF-κB activation, through IκB degradation, in the absence of stimulation.

On the other hand, CK2 has also been implicated in promoting cell cycle arrest following UV exposure. Indeed, in response to UV radiation, CK2 associates with the FACT complex, (facilitates chromatin transcription), which promotes the regulated phosphorylation of the tumour suppressor p53 at S392 [99]. The CK2-mediated phosphorylation of p53 at S392 activates the DNA-binding and transcriptional activity of p53 [98, 106] leading to attenuated
cell proliferation and increased apoptosis in response to UV-induced DNA damage. Therefore CK2 also plays an important role in the activation of p53 following UV.

In conclusion, CK2 appears to be an important regulator of the cellular response to UV radiation. Indeed, CK2 is a p38-dependent, stress-induced kinase; a group that also includes MSK1 and MSK2.

1.4 The MSK1 and MSK2 kinases

The mitogen- and stress-activated protein kinases, MSK1 and MSK2, are nuclear serine/threonine protein kinases that belong to the MAPK-activated family of kinases [107]. As their name suggests, MSK1 and MSK2 can be activated in response to both mitogens and cellular stress stimuli by the ERK and p38 MAPKs, respectively [108, 109] (Figure 1.5). JNK does not appear to contribute to the kinase activation of MSK1 or MSK2 [108]. The MSKs can also be activated by cytokines, such as TNFα, with both ERK and p38 contributing to full kinase activation [38, 108].

MSK1 and MSK2 are 90 kDa and 84 kDa, respectively, and share 75% amino acid sequence identity [108]. They are most closely related to the MAPK-activated p90 ribosomal S6 kinase (RSK) family sharing approximately 40% amino acid sequence identity [108, 110]. There are four human RSKs isoforms (RSK1, RSK2, RSK3 and RSK4) [110]. In fact, MSK1 and MSK2 were first identified through a genome wide screen for sequences homologous to the N-terminal kinase domain of RSK2 [108]. In addition, MSK2 was concurrently identified through a yeast-two hybrid screen using p38α as bait [109]. Northern blot analysis revealed that MSK1 and MSK2 are ubiquitously expressed with the highest expression detected in the brain, muscle and placenta [108, 111]. The Drosophila kinase JIL-1 is regarded as an MSK1 homologue with 45%
Figure 1.5. Schematic model of MSK and RSK activation

The RSKs (RSK1, RSK2 and RSK3) and the MSKs (MSK1 and MSK2) are activated in response to mitogen stimulation by the Ras-MAPK pathway [110]. The binding of growth factors or phorbol esters to plasma membrane receptors leads to the recruitment of the adaptor proteins Grb2 and SOS, which mediate the activation of the small GTP-binding protein, Ras. Ras stimulates the activation of the Raf-MEK-ERK cascade. ERK1/2 and PDK1 phosphorylate the RSKs and promote their activation [112, 113]. In a similar fashion, ERK1/2 phosphorylates the MSKs and promotes their activation, however, the activation of MSK1 and MSK2 is independent of PDK1 [108, 109, 114]. In response to cellular stress, such as UV radiation, MEK3/6 activate p38, which then phosphorylates and activates MSK1 and MSK2 [108, 109]. Figure adapted from [110]
conserved amino acid identity [115]. JIL-1 mediates histone H3 phosphorylation [116], an evolutionarily conserved function of MSK1 and MSK2 (discussed further in Section 1.4.3.2). In addition, the *C.elegans* hypothetical protein kinase C54G4 shares ~50% amino acid identity with MSK1 though C54G4 remains to be characterized. Currently, no MSK homologues have been identified in yeast or plants. MSK1 and MSK2 are predominately nuclear while the RSKs are localized to both the cytoplasm and the nucleus [108, 110].

1.4.1 Structure of MSK1 and MSK2

The MSKs and RSKs are unique in that they possess two distinct functional kinase domains within a single polypeptide chain [108, 109, 117, 118]. The N-terminal kinase domain (NTKD) is part of the AGC-type family of kinases (protein kinase A/protein kinase G/protein kinase C-family of kinases) while the C-terminal kinase domain (CTKD) is a calcium/calmodulin-like kinase domain. A linker region connects the two MSK kinase domains and contains a hydrophobic motif (HM). The HM consists of a serine residue situated within a stretch of hydrophobic amino acids and is common among AGC kinases [119].

Within the C-terminal tail of MSK1 and MSK2 is a MAPK kinase-docking domain (D-domain) that mediates the interaction of the MSKs with their upstream MAPK activators ERK1/2 and p38 [107]. D-domains are common to many MAPK substrates and are thought to be one mechanism by which MAPK signaling specificity is regulated [120]. MSK1 and MSK2 were found to associate with ERK and p38 under unstimulated conditions [109, 121], however, the association of MSK2 with ERK occurred to a lesser extent than with p38 [109]. Neither MSK1 nor MSK2 were found to interact with JNK. The C-terminal tail of MSK1 and MSK2 also contains a putative bipartite nuclear localization signal [107], which is thought to contribute to the predominately nuclear localization of the MSKs.
1.4.2 MSK1 and MSK2 activation

As mentioned, the MSKs and the RSKs belong to the AGC family of kinases [119, 122]. The activation of many AGC kinases requires the phosphorylation of two highly conserved regulatory motifs [119]. Indeed, the phosphorylation of a serine/threonine residue within the activation segment or T-loop of the catalytic kinase domain is essential for kinase activity [123]. In addition, phosphorylation of the serine/threonine residue within the HM is also essential for kinase activity [124]. The phosphorylation of the HM stabilizes of the activation loop of the kinase [124]. The linker region also contains a turn motif, which consists of a serine or threonine residue followed by a proline residue. This site is phosphorylated by the MAPK upstream activator and acts to further stabilize the active conformation of the kinase [125]. Phosphorylation of the turn motif is also thought to protect the HM from dephosphorylation [125].

1.4.2.1 Phosphorylation events mediating MSK1 and MSK2 activation

As with other AGC kinases, the activation of MSK1 and MSK2 requires a multistep phosphorylation cascade. Although the mechanism of MSK1 and MSK2 activation is similar to that of the RSKs, a few key differences exist (Figure 1.6). First, the MSKs are activated by both ERK and p38 while the RSKs are only activated by ERK. Therefore, in response to cellular stimulation, activated ERK/p38 or ERK phosphorylate and activate the CTKD of the MSKs or RSKs, respectively, as well as the turn motif residue within the linker region [109, 121, 126].

The second difference between the activation of the MSKs and the RSKs concerns the mechanism by which the NTKD is activated. In the case of RSK activation, the activated CTKD autophosphorylates the HM within the RSK linker region creating a docking site for the phosphoinositide-dependent kinase-1 (PDK1) [112, 127]. Docked PDK1 subsequently
Figure 1.6. Mechanism of RSK and MSK activation

(1) RSK: In response to mitogen stimulation, ERK phosphorylates and activates the CTKD of RSK. ERK also phosphorylates two residues within the RSK linker region. MSK: In response to mitogen or stress stimuli, ERK or p38 phosphorylates and activates the CTKD of MSK. ERK or p38 also phosphorylate the turn motif within the MSK linker region. (2) RSK: The activated RSK CTKD autophosphorylates the hydrophobic motif which creates a docking site for PDK1. MSK: The activated CTKD autophosphorylates the hydrophobic motif and another residue within the linker region. (3) RSK: Docking of PDK1 to the HM promotes the phosphorylation and activation of the NTKD of RSK. MSK1: The activated CTKD of MSK autophosphorylates and activates of the NTKD of MSK. (4) These phosphorylation events are required for the stabilization of the active enzyme conformation. The NTKD of the RSKs and MSKs phosphorylate downstream substrates. Amino acid numbering corresponds to RSK1 and MSK1. ERK/p38 phosphorylation events are purple (P), CTKD autophosphorylation events are teal (P), PDK1 phosphorylation events are yellow (P). Figure adapted from [128].
phosphorylates the T-loop residue within the NTKD resulting in RSK kinase activation [112]. As PDK1-mediated activation of AGC-family kinase domains is conserved among many AGC kinase family members, it was initially postulated that PDK1 was also required for the activation of the NTKD of the MSKs. However, a few key observations suggested that MSK1 and MSK2 may be activated by a different mechanism. Importantly, while RSK1, RSK2 and RSK3 as well as other AGC kinases showed impaired activation in \( PDK1(-/-) \) cells, the kinase activation of MSK1 was unaffected [114]. It is now believed that MSK1 and MSK2 activate their own NTKD. Indeed, studies suggest that the activated CTKD of MSK1 autophosphorylates both the HM and the T-loop of the NTKD [121]. These phosphorylation events are essential for MSK1 and MSK2 kinase activity [109, 121], and the activated NTKD mediates the phosphorylation of MSK substrates [128].

To identify additional sites of phosphorylation within MSK1, mass spectrometry (MS) analysis was performed [121, 129]. This investigation identified a novel site of autophosphorylation within the linker region of MSK1, which was mediated by the CTKD [121]. This residue, serine-381, is required for maximal MSK1 kinase activation [121]. MS analysis also identified an additional site of ERK/p38 phosphorylation within the C-terminus of MSK1 at threonine-700 [129]. Mutagenesis of T700 increased the basal and stimulated activity of MSK1 [129] and appeared to protect the CTKD from dephosphorylation [129]. Phosphorylation of T700 in response to cellular stimuli is proposed to promote the dissociation of an auto-inhibitory helix from the C-terminal kinase domain [129]. This study also revealed that the activated NTKD of MSK1 phosphorylates three residues proximal to the C-terminal ERK/p38 docking site [121], which is reminiscent of RSK1 [130]. However, unlike RSK1, these phosphorylation events do not promote the dissociation of ERK or p38 from the activated
Finally, several additional sites of phosphorylation were identified by MS within MSK1 that do not seem to be required for kinase activity [129]. Currently, the functional significance as well as the kinase(s) responsible for these phosphorylation events have not been elucidated (Figure 1.7).

A separate study reported an additional site of ERK/p38 phosphorylation within the linker region of MSK2 (serine-347) [131]. MSK2 serine-347 is not conserved in MSK1 but is required for maximal kinase activation of MSK2 [131].

1.4.3 MSK1 and MSK2 substrates

A key cellular role of MSK1 and MSK2 is the regulation of gene expression [132]. Indeed, bona fide MSK substrates include the transcription factors CREB, ATF-1 (activating transcription factor 1) and the p65 subunit of NF-κB as well as histone H3 and the non-histone chromosomal protein, HMGN1 (high-mobility group nucleosome binding domain 1). The MSK1/2 consensus sequence has been defined as R-R-X-S/T [128].

1.4.3.1 Activation of transcription factors by MSK1 and MSK2

CREB and ATF1

CREB was the first substrate identified for MSK1 and MSK2 [108, 109]. CREB is a phosphorylation-dependent transcription factor that mediates cell proliferation, homeostasis, and survival [133]. CREB activates target genes containing cAMP response elements (CRE) in their promoters [134] and requires phosphorylation at S133 for transcriptional activation [135]. MSK1 was found to be a highly efficient CREB kinase in vitro. Indeed, MSK1 phosphorylated CREB at a lower $K_m$ than PKA, a known CREB kinase ($K_m$ of 2μM vs $K_m$ of 17μM) [108]. In the same study, the $K_m$ of RSK2, another previously reported CREB kinase, was too high to be measured [108], thus questioning RSK2 as a bona fide CREB kinase. The role of MSK1 and
Figure 1.7. Schematic showing known phosphorylation sites present on MSK1 and MSK2. Sites of phosphorylation on MSK1 and MSK2 mediated by p38/ERK are represented by green “lollipops”, sites of autophosphorylation by the CTKD are represented by purple “lollipops”, sites of autophosphorylation by the NTKD are shown in blue “lollipops” and sites phosphorylated by unknown kinases are shown as yellow “lollipops”.
MSK2 in the *in vivo* phosphorylation of CREB at S133 was supported by (1) inhibitor studies and (2) analysis of *MSK1(-/-) MSK2(-/-)* double knockout (DKO) mouse embryonic fibroblasts (MEFs). As mentioned, MSK1 and MSK2 are activated by mitogens and cellular stress by ERK and p38, respectively. Inhibition of ERK (using the MEK inhibitor, PD98059) prior to mitogen stimulation or inhibition of p38 (SB203580) prior to UV-radiation in HEK293 cells, largely impaired endogenous CREB S133 phosphorylation [108], which agrees with MSK1 and MSK2 being *in vivo* CREB kinases. Consistent with this observation, the stress-induced phosphorylation of CREB at S133 and the closely related ATF-1 at S63 is absent *MSK1(-/-) MSK2(-/-)* DKO MEFs [136] suggesting that MSK1 and MSK2 are the major stress-induced CREB kinases *in vivo*. On the other hand, the mitogen-induced phosphorylation of CREB and ATF-1 was only partially impaired in the *MSK* DKO MEFs suggesting that although MSK1 and MSK2 play a role in this process, another kinase may also mediate CREB and ATF-1 phosphorylation under these conditions [136]. In line with this, it was previously reported that fibroblasts derived from Coffin-Lowry syndrome (CLS) patients, which express a mutant RSK2 protein, display no EGF-stimulated CREB S133 phosphorylation [137]. However, subsequent studies examining *MSK* DKO MEFs and CLS fibroblasts in parallel demonstrated that the EGF-stimulated phosphorylation of CREB in CLS cells was intact [136]. Furthermore, studies using the specific RSK inhibitor, BI-D1870, also found that the RSKs do not mediate the mitogen-induced phosphorylation of CREB S133 [138]. Analysis of *MSK1(-/-) or MSK2(-/-)* single knockout MEFs show that MSK1 and MSK2 contribute equally to the phosphorylation of CREB and ATF1 and do not appear to compensate for each other when one isoform is absent [136].
CREB plays a role in immediate-early gene expression. Examination of immediate-early gene transcription in the MSK DKO MEFs demonstrated an approximately 50% reduction in *c-fos* and *junB* transcription following cellular stress stimulation while only a minimal reduction was observed following mitogen stimulation [136]. These results suggested that MSK1 and MSK2 regulate immediate-early gene expression in response to cellular stress stimuli but not significantly in response to growth factors.

**NF-κB**

As mentioned above, MSK1 and MSK2 have been shown to mediate NF-κB transcriptional activation through the nuclear phosphorylation of p65 on S276 following pro-inflammatory cytokine stimulation. Indeed, MSK DKO MEFs or cells depleted of MSK1 by siRNA show impaired IL-1β or TNFα-mediated p65 S276 phosphorylation [37, 38]. The phosphorylation of p65 at S276 was shown to be essential for p65 transcriptional activity in response to TNFα-stimulation [38, 39], possibly because p65 S276 phosphorylation is a pre-requisite for its interaction with the histone acetyltransferase CBP [34, 37]. Consistent with this, MSK DKO MEFs show impaired TNFα-mediated NF-κB-dependent transcription of *IL-6* and *IL-8* [38]. However, MSK1 and MSK2 do not appear to be required for the NF-κB-dependent transcription of *NF-κB2* [38], suggesting that the MSKs regulate the transcription of a subset of p65-dependent genes.

**1.4.3.2 Role of MSK1 and MSK2 in the nucleosomal response**

The nucleosomal response has been defined as the rapid phosphorylation of histone H3 on serine-10 and HMGN1 on serine-6 that occurs concurrently with immediate-early gene induction [139]. The mitogen or stress-induced phosphorylation of histone H3 at S10 is associated with gene activation and MSK1 and MSK2 are considered the main histone H3
kinases under these conditions [140, 141]. Similar to CREB, the EGF-mediated phosphorylation of histone H3 at S10 was initially reported to be defective in CLS patient fibroblasts [142]. However, phosphorylation of histone H3 S10 was also absent in MSK DKO MEFs following both EGF and anisomycin stimulation [141], suggesting that RSK2 may not participate in histone H3 S10 phosphorylation. Indeed, re-examination of the CLS fibroblasts found no defect in the EGF-mediated phosphorylation of histone H3 S10 [141]. Examination of MSK1(-/-) or MSK2(-/-) single knockout MEFs suggested that MSK2 contributes to histone H3 S10 phosphorylation to a greater extent than MSK1 [141]. The MSKs are also implicated in the phosphorylation of histone H3 at S28 following both mitogen and stress stimulation [141], which is also associated with immediate-early gene induction.

Another MSK substrate is the nucleosomal associated protein HMGN1 (formerly known as HMG-14) and phosphorylation of HMGN1 at serine-6 also occurs in conjunction with immediate-early gene induction. MSK DKO MEFs showed impaired TPA- or anisomycin-stimulated HMGN1 S6 phosphorylation [141]. The current model proposes that S6 phosphorylated HMGN1 has reduced nucleosomal binding ability thus enabling the MSKs to subsequently access histone H3 [143].

1.4.4 The role of MSK1 and MSK2 in inflammation

As MSK1 and MSK2 phosphorylate and activate NF-κB and CREB/ATF-1, which mediate the expression of inflammatory genes [144], it is not surprising that the MSKs play a physiological role in the inflammatory response. Indeed, while MSK1(-/-)/MSK2(-/-) DKO mice are viable and have no overt phenotype in sterile conditions, they are hypersensitive to LPS-induced endotoxic shock due to an overproduction of the pro-inflammatory cytokines IL-6, IL-12 and TNFα by macrophages [145]. The prolonged inflammation observed in MSK1(-/-)/MSK2(-/-) mice is due
to impaired transcription of the anti-inflammatory cytokine IL-10 and the protein phosphatase DUSP-1 (dual specificity phosphatase-1), a negative regulator of p38 [146]. IL-10 and DUSP-1 are CREB and ATF-1 dependent genes and require MSK1/2 for their activation in macrophages [145]. In line with this, a model of toxic contact eczema showed that MSK1(-/-)/MSK2(-/-) mice displayed prolonged ear inflammation compared to wildtype mice [145], reinforcing the idea that MSK1 and MSK2 are key regulators of a negative feedback loop in the inflammatory response [145]. Based on these findings, it has been proposed that a small molecule activator of MSK1/MSK2 may be an effective strategy to treat inflammatory disorders [147].

On the other hand, other studies have suggested that the role of the MSKs in inflammation may be cell type specific. Indeed, in some cell types, the MSKs mediate the transcription of pro-inflammatory cytokines such as IL-6 and IL-8 via NF-κB activation [38]. In line with this, MSK1 and MSK2 have been linked to psoriasis, an inflammatory skin disorder characterized by epidermal hyperplasia, increased infiltration of T cells and the production of pro-inflammatory cytokines in the skin [148]. Analysis of psoriasis patient skin revealed an increase in activated ERK1/2 and p38, which have both been implicated in inflammation [149]. Furthermore, MSK1 and MSK2 have also been shown to be activated in the keratinocytes of psoriatic skin [150, 151] and are thought to contribute to the production of the pro-inflammatory cytokines IL-6 and IL-8 in the skin. In line with this, treatment of A549 and HEK293 cells with glucocorticoids promoted in the nuclear export of MSK1 to the cytoplasm, which coincided with impaired transcription of NF-κB-dependent inflammatory genes [152]. These observations provided a possible mechanism for the anti-inflammatory effect of glucocorticoid treatment [152].
In summary, these studies demonstrate the important role of MSK1 and MSK2 in the regulation of the inflammatory response through mediating the CREB- and NF-κB-dependent transcription of pro-inflammatory cytokines and negative regulators of inflammation.

**1.4.5 MSK1 and MSK2 in the brain**

MSK1 and MSK2 are highly expressed in the brain [108]. Interestingly, recent studies in MSK1(-/-) mice found that these mice display impaired Pavlovian fear conditioning and spatial learning, which are both associated with memory formation [153]. In addition, MSK1(-/-) mice show impaired CREB and histone H3 phosphorylation in their hippocampus [153]. As CREB and chromatin remodelling play a key role in long-term memory formation [154], MSK1 may be an important regulator of these events during memory formation [153]. Chromatin remodelling is also implicated in the development of drug addiction. Indeed, MSK1(-/-) mice show impaired histone H3 S10 phosphorylation in striatal neurons in response to cocaine stimulation [155]. Interestingly, cocaine strongly activates MSK1 but not MSK2 [155].

Currently, several studies have highlighted the importance of MSK1 in the phosphorylation of CREB and histone H3 in the brain in response to various external stimuli. Indeed, due to the strong phenotypes displayed by the MSK1(-/-) mice, it is possible that MSK1 may be more essential than MSK2 in brain and memory processes. However, studies examining MSK2(-/-) mice have not yet been reported, therefore it is important to establish if MSK2-deficiency phenocopies wildtype mice prior to excluding a role for MSK2 in long-term memory and other histone H3 or CREB-mediated brain processes.

**1.4.6 MSK1 and MSK2 in cancer**

The role of the MSK1 and MSK2 in cellular transformation is largely unexplored. Currently, no studies examining the susceptibility of the MSK1(-/-)/MSK2(-/-) mice to spontaneous or
carcinogen-induced tumour formation have been reported. A connection between MSK1 and MSK2 and cellular transformation has been suggested as increased histone H3 S10 phosphorylation has been observed in ras-transformed mouse fibroblast [156]. One study reported that overexpression of wildtype MSK1 but not kinase dead MSK1 increased TPA- or EGF-induced neoplastic transformation of JB6 CI41 cells as measured by colony forming assay [157]. As mentioned previously, chronic inflammation and aberrant NF-κB activation are implicated in tumorigenesis [13]. Therefore the involvement of MSK1 and MSK2 in the regulation of cytokine production may have relevance in cancer.

1.5 Thesis Objectives

Given the recently established role of CK2 in cellular stress signaling and DNA repair, we were interested in identifying novel CK2 interacting proteins to further characterize CK2 in these processes. CK2 can interact with, phosphorylate and modulate the activity of other proteins, including other protein kinases. Therefore, we hypothesized that CK2 interacts with and modulates the activity of proteins involved in the cellular stress response. In chapter 2, I describe efforts to identify novel CK2β binding partners using a pulldown screen approach. Using this strategy, I identified the mitogen- and stress-activated protein kinase, MSK2, as a novel CK2 interacting protein. As both CK2 and MSK2 are activated in response to UV radiation in a p38-dependent manner, I chose to examine the CK2-MSK2 interaction in the context of the UV response, which is described in chapter 3.
Chapter 2: Identification of MSK2 as a novel CK2β interacting protein

A portion of this chapter is published in the Journal of Biological Chemistry:
2.1 Introduction

CK2 is a highly conserved and ubiquitously expressed serine/threonine protein kinase involved in numerous cellular processes including cell proliferation, cell survival and DNA repair [52-54]. The CK2 holoenzyme is a heterotetramer composed of two catalytic (αα, αα’, or α’α’) subunits and two regulatory (ββ) subunits [67]. The dimerization of the CK2β regulatory subunits is a prerequisite for holoenzyme formation [66, 67]. CK2 has been shown to phosphorylate, interact with and regulate other proteins, including other protein kinases [74, 158]. Several CK2 substrates are bound by CK2β, which is thought to act as a docking site to facilitate substrate phosphorylation and mediate CK2 substrate specificity [74].

Traditionally, CK2 has been described as a constitutively active kinase [158]. However, several studies have demonstrated that CK2 kinase activity is stimulated in response to cellular stress and that CK2 promotes cell survival under these conditions. For example, CK2 displays increased kinase activity toward certain cellular substrates following UV radiation, in a p38-dependent manner [10, 95, 97-99].

In this chapter, I describe the identification of novel CK2 interacting proteins that were identified through a pull-down screen using GST-CK2β as bait. Two novel CK2 interactors were subsequently validated and I chose to further investigate the interaction of CK2β with the mitogen- and stress-activated kinase 2 (MSK2). Like CK2, MSK2 complexes with p38 and is activated in a p38-dependent manner following cellular stress [109, 136]. I show that CK2 physically interacts with MSK2 in a complex that also contains p38. Furthermore, CK2 dissociates from the MSK2-p38 complex following UV-radiation and MSK2 activation.
2.2 Attributions

Protein in-gel digestion and mass spectrometry analysis was a service provided by the Advanced Protein Technology Centre Mass Spectrometry Facility located in the Hospital for Sick Children, Toronto, Ontario.

All other experiments were performed by Kellie Jacks.

2.3 Experimental Materials and Methods

2.3.1 Cloning and plasmid constructions

Human MSK1 cDNA (gift from Dr. Peter Cheung) was obtained in the pcDNA3.1-FLAG/HA vector yielding FLAG-MSK1. To generate a C-terminally tagged MSK1 cDNA construct, the MSK1 cDNA was PCR amplified and cloned into the pcDNA3.1/V5-His mammalian expression vector by directional TOPO® cloning (Invitrogen) yielding MSK1-V5. To obtain the full length human MSK2 cDNA, the following nucleotide sequences were PCR-amplified from human cDNA ESTs (Open Biosystems). PCR-amplified nucleotides 1-1670 (clone ID: 5216639) and nucleotides 1671-2218 (clone ID: 2405246) were each cloned into the pCR®2.1 vector using the TOPO® TA cloning® kit (Invitrogen). PCR-amplified nucleotides 2219-2316 (clone ID: 5763859) were cloned into the pcDNA3.1/V5-His mammalian expression vector by directional TOPO® cloning (2219-2316-pcDNA3.1/V5-His). Enzymatic restriction fragments containing the MSK2 sequences 1-1670 and 1671-2218 were then ligated and subcloned into 2219-2316-pcDNA3.1/V5-His to generate the full length MSK2-containing mammalian expression vector (MSK2-pcDNA3.1/V5-His) or MSK2-V5. MSK2 R730/731A point mutations were generated by QuikChange site-directed mutagenesis (Stratagene) using standard manufacturer protocols. Primer set is listed in Table 3.1. The human CK2β cDNA (Open Biosystems, clone ID:
8327487) was subcloned by enzymatic restriction digestion into (HA)$_2$-pcDNA3.1 (gift from Dr. Lea Harrington) and into pGEX-4T3 (GE Healthcare), yielding (HA)$_2$-CK2β and GST-CK2β, respectively. The human SGPL1 cDNA (Open Biosystems, clone ID: 6150776) was PCR amplified and cloned into the pcDNA3.1/V5-His mammalian expression vector by directional TOPO® cloning (Invitrogen) yielding SPL-V5.

2.3.2 Cell culture and transfections

All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO$_2$. Human embryonic kidney (HEK) 293T cells and MCF7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), and MDA-MB-231 cells in RPMI-1640 medium, both supplemented with 10% fetal bovine serum (FBS) and 50U of penicillin and 50 µg of streptomycin antibiotics per ml. Transient transfection of plasmids into HEK293T cells was performed using the Effectene transfection kit (Qiagen) according to the manufacturer's instructions.

2.3.3 Antibodies

Commercial antibodies used in this study were purchased from Abcam (anti-CK2α rabbit polyclonal, anti-CK2α’ rabbit polyclonal and anti-MSK2 rabbit polyclonal), Cell Signaling Technology (anti-p38 rabbit polyclonal and anti-p38 mouse monoclonal), R&D Systems (anti-MSK1 goat polyclonal and anti-MSK2 rat polyclonal), Santa Cruz Biotechnology (anti-CK2α goat polyclonal, anti-CK2β mouse monoclonal and anti-CK2β rabbit polyclonal), Millipore (anti-HA mouse monoclonal), Sigma (anti-M2 mouse monoclonal) and Invitrogen (anti-V5 mouse monoclonal). Anti-V5 agarose affinity gel was purchased from Sigma.

2.3.4 Expression and purification of fusion proteins

GST and GST-CK2β recombinant proteins were produced in E.coli BL21(DE3)/pLysS (Novagen). For the purification of GST and GST-CK2β, bacteria were grown at 37°C to an
OD$_{600}$ of 0.6-0.8 and expression was induced by the addition of 0.2mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for three hours at 37°C. Cells were pelleted then resuspended in ice cold phosphate buffered saline (PBS), 5mM ethylenediaminetetraacetic acid (EDTA), 1mM dithiothreitol (DTT) and protease inhibitor cocktail (Roche). Cells were lysed on ice by sonication then TritonX-100 was added to a final concentration of 1%. Lysate was centrifuged at 13,200 rpm for thirty minutes at 4°C. Clarified lysates were incubated with glutathione sepharose beads (Pharmacia) for two hours at 4°C with constant neutation to recover GST and GST-CK2β. Beads were washed thrice with ice cold lysis buffer and 1% TritonX-100, aliquoted then snap frozen on dry ice prior to storage at -80°C.

2.3.5 Preparation of cell extracts, immunoprecipitation, pull-downs and immunoblotting

For whole cell extract (WCE) preparation, cells were rinsed once in ice-cold PBS, and lysed on ice in 50mM Tris-HCl pH 7.5, 100mM NaCl, 1% Triton X-100, 0.5mM DTT, 0.5mM EDTA, 1X Complete mini protease inhibitor cocktail (Roche), 1mM sodium orthovanadate, 40mM β-glycerophosphate, and 50mM sodium fluoride. Lysates were cleared at 13,200 rpm for thirty minutes at 4°C. For UV-C (254nm) treatment, cultured cells were washed once with PBS then UV-C irradiated (200 J/m$^2$) in the presence of PBS. Following UV-C irradiation, culture media was replaced and cells were incubated at 37°C for specified periods until cell harvest. For lambda (λ) protein phosphatase treatments (New England BioLabs), cells were lysed on ice in 50mM Tris-HCl pH7.5, 100mM NaCl, 1% Triton X-100, 0.5mM DTT and 1X Complete mini protease inhibitor cocktail (Roche) and lysates were cleared at 13,200 rpm for thirty minutes at 4°C. Lysates were diluted in the protein metallophosphatase reaction buffer (final concentration: 50mM Hepes pH7.5, 100mM NaCl, 2mM DTT, 0.01% polyoxyethyleneglycol dodecyl ether (Brij35), 1mM MnCl$_2$) and incubated in the presence or absence of 2000U lambda protein
phosphatase at 30°C for twenty minutes. Reaction was stopped with 50mM EDTA and samples were immediately placed on ice. All lysates were quantified using Coomassie Plus protein assay reagent kit (Pierce Biotechnology) according to manufacturer’s instructions. For pulldown assays, clarified WCEs were incubated with purified recombinant GST or GST-CK2β immobilized on glutathione sepharose beads at 4°C for two hours with constant neutatation. The complexes were then washed thrice with ice cold cell lysis buffer, and analyzed for associated proteins by immunoblotting as described below. For immunoprecipitations (IPs), clarified WCEs were incubated with 1μg of the relevant antibody on ice for sixty minutes with occasional gentle agitation followed by the addition of 30μl of protein A-coupled sepharose beads (ThermoFisher) and incubated for an additional hour at 4°C with neutatation, or WCEs were incubated with V5-agarose (Sigma) at 4°C for two hours with neutatation. Immunoprecipitates were washed thrice with lysis buffer, resuspended in SDS-PAGE sample buffer, and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) and immunoblotted as indicated. Detection was performed using SuperSignal enhanced chemiluminescence (Pierce).

2.3.6 Mass Spectrometry

Proteins resolved by SDS-PAGE were visualized with GelCode Blue stain reagent (Pierce Biotechnology) and bands were excised from the polyacrylamide gel using a scalpel. Gel pieces were washed in 50mM ammonium bicarbonate then shrunk in 50% acetonitrile/25mM ammonium bicarbonate solution. Samples were incubated for thirty minutes at 56°C in the presence of DTT to reduce disulfide bonds. Cysteine residues were alkylated with iodoacetamide for fifteen minutes in the dark at room temperature. In-gel tryptic digestion of proteins was performed at 37°C for ninety minutes. Peptides were then extracted from the gel
matrix and evaporated down to dryness. For LC-MS/MS analysis, samples were reconstituted in 0.1% Trifluoroacetic acid (TFA) in water and analyzed by an LC-MALDI MS system consisting of an Applied Biosystems/MDS Sciex API QSTAR XL Pulsar MALDI Q-TOF coupled with an Agilent nano HPLC 1100 series. Peptide spectra were searched against NCBI (Bethesda, MD) database with the help of Mascot (Matrix Science, London, UK).

2.4 Results

2.4.1 Identification of novel CK2β interacting proteins

The CK2β regulatory subunit has been suggested to act as a docking site for several CK2 substrates [74]. Therefore I utilized purified recombinant GST-CK2β as bait in a screen to identify novel CK2 interacting proteins. Purified GST and GST-CK2β were immobilized on glutathione–sepharose beads and were incubated with whole-cell extracts (WCEs) from human MCF7 cells in a pulldown assay. Interacting proteins were resolved by SDS-PAGE and stained for visualization. My screen was not intended to be an extensive screen but rather four prominent bands that were present in the GST-CK2β pulldown and not in the GST pulldown were selected for mass spectrometry analysis (Figure 2.1).

Results of mass spectrometry analysis (Table 2.1) show that GST-CK2β selectively retrieved α-6-tubulin, HSP90 and RSK3, which have previously been reported to interact with CK2 [159-161]. I also identified three novel CK2β interactors, including sphingosine-1-phosphate lyase (SPL) and MSK2, two enzymes implicated in cellular stress signaling. Due to large amounts of keratin contamination, peptides corresponding to GST-CK2β interacting proteins could only be identified in samples 1 and 3. Unfortunately, no non-keratin peptides could be elucidated in samples 2 and 4. Sample 1 contained ten peptides corresponding to the
Figure 2.1. Identification of CK2β interacting proteins

Purified recombinant GST and GST-CK2β proteins immobilized on glutathione sepharose beads were incubated with MCF7 whole cell extracts in a pulldown assay. Interacting proteins were resolved by SDS–PAGE and stained with GelCode Blue. Gel bands excised and prepared for mass spectrometry are indicated by the numbered arrows. The identities of the proteins found in gel slices highlighted with an asterisks (*) are found in Table 2.1.

Table 2.1: Summary of mass spectrometry peptide data from samples obtained in Figure 2.1

<table>
<thead>
<tr>
<th>Band</th>
<th>Protein</th>
<th>Molecular Weight</th>
<th>Peptides identified</th>
<th>Function</th>
<th>Previously Identified ?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tubulin-α-6</td>
<td>55 kDa</td>
<td>10</td>
<td>Microtubule component</td>
<td>Yes</td>
</tr>
<tr>
<td>1</td>
<td>Sphingosine-1-phosphate lyase</td>
<td>63 kDa</td>
<td>7</td>
<td>Sphingolipid metabolism</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>Heat shock protein HSP90-alpha4</td>
<td>85 kDa</td>
<td>2</td>
<td>Molecular chaperone</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>p90 ribosomal S6 kinase 3 (RSK3)</td>
<td>84 kDa</td>
<td>1</td>
<td>Growth factor signaling</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>p90 ribosomal S6 kinase 4 (RSK4)</td>
<td>86 kDa</td>
<td>1</td>
<td>Growth inhibition</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>Mitogen- and stress-activated kinase 2 (MSK2)</td>
<td>84 kDa</td>
<td>1</td>
<td>Regulation of gene expression</td>
<td>No</td>
</tr>
</tbody>
</table>

aRefer to Figure 2.1
bAccession number retrieved by the Mascot search engine
known CK2 interactor tubulin-α-6 and 7 peptides corresponding to SPL. Due to keratin contamination, fewer peptides were identified within sample 3. However, HSP90, which yielded 2 peptides, and RSK3, which yielded 1 peptide, are both known CK2 interactors. Therefore, despite the fact that only one peptide was identified for RSK4 and MSK2, the identification of these proteins as CK2β binding partners may be significant.

2.4.2 Validation of the CK2β interacting proteins

To confirm the results obtained by mass spectrometry, I chose to validate the interaction of CK2β with SPL and MSK2, which serve as a representative protein identified in sample 1 and sample 3, respectively, in my screen.

MSK2 and the closely related MSK1 are nuclear protein kinases that mediate gene transcription in response to mitogen or stress stimulation [108, 109, 136, 141]. To validate the CK2β-MSK2 interaction identified by mass spectrometry, MCF7 WCEs were incubated with GST or GST-CK2β in a pulldown assay then immunoblotted with α-MSK1 and α-MSK2 antibodies. As demonstrated in Figure 2.2A, endogenous MSK2 interacts specifically with GST-CK2β but not with GST alone. Interestingly, no interaction could be detected between GST-CK2β and endogenous MSK1. As an additional validation, the GST-CK2β pulldown assay was repeated using ectopically expressed V5-epitope tagged MSK2 and MSK1 fusion proteins. As shown in Figure 2.2B, MSK2-V5 but not MSK1-V5 interacted specifically with GST-CK2β, which is consistent with results obtained for endogenous MSK proteins. To ensure that the C-terminal V5-tag was not interfering with the MSK1-CK2β interaction, I repeated the pulldown assay using an N-terminally-tagged FLAG-MSK1 construct. As shown in Figure 2.2C, an interaction between FLAG-MSK1 and GST-CK2β was not detected. Finally, co-immunoprecipitation analyses also identified an association between MSK2-V5 and ectopically
Figure 2.2. MSK2 but not MSK1 interacts with CK2β in human cells

(A) MCF7 whole cell extracts (WCEs) were incubated with recombinant purified GST or GST-CK2β proteins immobilized on glutathione sepharose beads in pull-down assays, and immunoblotted for endogenous MSK2 or MSK1 as indicated. (B) HEK293T cells ectopically expressing C-terminally V5-tagged MSK2 or MSK1 were harvested and WCEs were incubated with recombinant purified GST or GST-CK2β proteins immobilized on glutathione sepharose beads in pull-down assays and immunoblotted with anti-V5 antibody. (C) HEK293T cells ectopically expressing N-terminally FLAG-tagged MSK1 were harvested analyzed as in (B) and immunoblotted with anti-M2 (FLAG) antibody. (D) HEK293T cells ectopically expressing HA2-CK2β and MSK2-V5, or HA2-CK2β and MSK1-V5 were harvested, subjected to anti-HA immunoprecipitation, then analyzed by immunoblotting with anti-HA (CK2β), and anti-V5 (MSK1, MSK2) antibodies as indicated.
expressed (HA)$_2$-CK2β immunoprecipitated from HEK293T whole cell extracts, but not between (HA)$_2$-CK2β and MSK1-V5 (Figure 2.2D). These data demonstrate that MSK2 but not MSK1 specifically interacts with CK2β.

To validate the interaction of CK2β with SPL identified by mass spectrometry, WCEs from HEK293T cells ectopically expressing V5-epitope tagged SPL or vector control were incubated with GST or GST-CK2β in a pulldown assay then immunoblotted with α-V5 antibody. As demonstrated in Figure 2.3A, SPL-V5 interacts specifically with GST-CK2β but not with GST alone. Furthermore, co-immunoprecipitation analysis in the reverse direction demonstrated that endogenous CK2β co-immunoprecipitated specifically with ectopically expressed SPL-V5 in HEK293T cells. These data suggest that CK2β specifically interacts with SPL.

Taken together, MSK2 and SPL, which were identified as novel CK2β interacting proteins by mass spectrometry, were successfully validated as CK2β binding partners by pulldown assays and co-immunoprecipitation experiments.

2.4.3 The CK2 holoenzyme interacts with MSK2 in human cells

I chose to further characterize the CK2-MSK2 interaction in light of the recent evidence implicating CK2 in the p38-dependent cellular stress response [96]. MSK2, and the closely related MSK1, are stress-activated kinases that interact with and are dependent on p38 for their activation following UV-C radiation [136]. Similarly, CK2 also complexes with p38 and undergoes p38-dependent UV-induced activation [10, 99, 100]. I investigated the physical association of CK2 with the MSK2-p38 and MSK1-p38 complexes under basal conditions and following exposure to UV-radiation. This analysis was performed in the human MDA-MB-231 cell line, which was previously used to examine the role of MSK1 and MSK2 in the TNFα induced phosphorylation of p65 at S276 [38]. I observed that UV treatment induced an upward
Figure 2.3. SPL-V5 interacts with CK2β in human cells

(A) HEK293T cells ectopically expressing SPL-V5 were harvested 48 hours post-transfection and whole cell extracts were incubated with recombinant purified GST or GST-CK2β proteins immobilized on glutathione sepharose beads in pull-down assays, and immunoblotted with anti-V5 antibody. (B) HEK293T cells ectopically expressing vector control or SPL-V5 were harvested, subjected to anti-V5 immunoprecipitation, and analyzed by immunoblotting with anti-V5 (SPL) and anti-CK2β antibodies as indicated.
electrophoretic mobility shift of MSK2, and to a lesser extent MSK1, in the MDA-MB-231 cells (Figure 2.4A). The mobility shift was abolished by lambda protein phosphatase treatment ($\lambda$PPase) suggesting that the shift is largely due to MSK1 and MSK2 UV-induced phosphorylation. Co-immunoprecipitation analysis confirmed an association between endogenous MSK2, but not MSK1, and endogenous CK2β from MDA-MB-231 whole cell extracts (Figure 2.4B). Furthermore, MSK2 co-immunoprecipitated with endogenous CK2α from MDA-MB-231 whole cell extracts under basal conditions, suggesting that MSK2 interacts with the CK2 holoenzyme. Interestingly, following UV-C radiation, I observed a diminished association between MSK2 and CK2, but the MSK2-p38 interaction was maintained. There was no detectable interaction between endogenous MSK1 and p38. To further examine the dissociation of CK2 from MSK2 following UV-radiation, I preformed a GST-CK2β pulldown assay using MDA-MB-231 cells that had been mock or UV-irradiated. As expected, under basal conditions, GST-CK2β was found to specifically interact with endogenous MSK2, but no detectable interaction was observed with MSK1 (Figure 2.4C). I also noted that UV irradiation was associated with a decreased GST-CK2β-MSK2 interaction, and that GST-CK2β appeared to preferentially interact with a faster migrating MSK2 species, which could correspond to an unphosphorylated or lesser phosphorylated form of MSK2. No interaction between GST-CK2β and MSK1 could be detected under UV conditions. Taken together, these data suggest that under basal conditions CK2 complexes with MSK2 and p38 and that UV-irradiation promotes the dissociation of CK2 from the activated MSK2 complex.

Ectopically expressed MSK1 was previously reported to interact with p38 under basal conditions [121]. Indeed, when ectopically expressed MSK1-V5 was immunoprecipitated from HEK293T cells, I detected an interaction with endogenous p38 (Figure 2.5). However, the
Figure 2.4. The CK2 holoenzyme associates with the MSK2-p38 complex in human cells

(A) MDA-MB-231 cells were mock, UV-C irradiated (200 J/m²) or UV-C irradiated and subjected to lambda protein phosphatase (λ) treatment. WCEs were immunoblotted with anti-MSK2 and anti-MSK1 antibodies as indicated. (B) MDA-MB-231 cells were mock or UV-C irradiated and harvested 30 minutes later. WCEs were subjected to anti-MSK1 or anti-MSK2 immunoprecipitation, then immunoblotted with MSK1, MSK2, CK2α, CK2β, and p38 antibodies as indicated. (C) MDA-MB-231 cells were mock or UV-C irradiated (200 J/m²) and harvested 30 minutes later. WCEs were incubated with recombinant purified GST or GST-CK2β proteins immobilized on glutathione sepharose beads in pull-down assays, and immunoblotted for endogenous MSK1 and MSK2 as indicated. * and ** indicate slow and fast migrating MSK2 species, respectively.
Figure 2.5. Analysis of the MSK1/2-p38-CK2 complexes using ectopically expressed MSK1-V5 and MSK2-V5. WCEs and anti-V5 immunoprecipitates from HEK293T cells ectopically expressing empty vector, MSK1-V5 or MSK2-V5 were analyzed by immunoblotting as indicated.
amount of p38 that associated with MSK1-V5 appeared to be less than for MSK2-V5. MSK1 and MSK2 contain a p38 docking site (D-domain) within their C-terminus [107]. Therefore it is possible that p38 mediates the CK2-MSK2 interaction. To investigate this possibility, I mutated the p38 docking site within MSK2 (R730/731A) and examined the ability of this mutant to interact with endogenous CK2 subunits by co-immunoprecipitation. As shown in Figure 2.6, wildtype MSK2-V5 immunoprecipitated both p38 and the CK2 holoenzyme. In contrast, MSK2 R730/731A-V5 failed to interact with p38, as expected, and showed a significantly reduced interaction with the CK2 subunits suggesting that disruption of the MSK2 D-domain impairs the MSK2-CK2 interaction.
Figure 2.6. Mutagenesis of the MSK2 D-domain disrupts the MSK2-CK2 interaction

WCEs and anti-V5 immunoprecipitates (IPs) from HEK293T cells ectopically expressing empty vector, MSK2-V5 or MSK2 R730/731A-V5 were analyzed by immunoblotting as indicated.
2.5 Discussion

As a means to further understand the cellular role of CK2, I utilized a proteomic approach to identify proteins that physically interact with CK2β. I subsequently validated the interaction between CK2β and two novel binding partners, MSK2 and SPL, identified in the screen. Furthermore, I characterized the physical interaction between the CK2 holoenzyme and MSK2 under basal and UV-treated conditions.

2.5.1 CK2 associates with the MSK2-p38 complex under basal conditions

Characterization of the CK2-MSK2 interaction revealed constitutive interactions between endogenous MSK2, CK2 and p38 in human cells, and previous studies have also demonstrated basal interactions between MSK2 and p38, and between CK2 and p38 [100, 109]. MSK1 has also been reported to interact basally with p38 when ectopically expressed [121], but the amount of p38 associated with MSK1 appeared to be less than for MSK2 (Figure 2.5). In keeping with these observations, I was able to detect the association of endogenous MSK2 with p38, but not that of MSK1 and p38 (Figure 2.4B). Whether this discrepancy is significant in vivo or whether the association with CK2 enhances an MSK2-p38 interaction is unclear but is a possibility. Currently, MSK1 and MSK2 are thought to interact with p38 by the same mechanism, through the C-terminal D-domain of MSK1/2. As p38 also binds CK2, it remains unclear how both p38 and CK2 are contained within the MSK2 complex while CK2 is excluded from the p38-MSK1 complex.

2.5.2 MSK1 and MSK2 D-domain interactions

As mentioned, MSK1 and MSK2 contain a conserved ERK and p38 kinase binding site within their C-terminal region termed the D-domain [107]. Indeed, mutagenesis of two critical arginine residues (R742 and R743) within the D-domain of MSK1 was shown to disrupt the MSK1-p38
complex [121]. Furthermore, when I mutated the corresponding arginine residues in MSK2 (R730 and R731) to alanine, the MSK2-p38 interaction was not detected (Figure 2.6). As the binding of p38 to the D-domain of MSK1/2 is considered to be a direct interaction, it is possible that p38 is mediating the CK2-MSK2 interaction. Indeed, disruption of the MSK2-p38 interaction, using the MSK2 R730/731A D-domain mutant, also significantly reduced the MSK2-CK2 interaction (Figure 2.6). However, the interpretation of these results is complicated by the fact that the D-domain of MSK1 and MSK2 overlaps with their bipartite nuclear localization signal sequence. Indeed, the MSK2 truncation mutant lacking its C-terminus (Δ722-772 amino acids) is predominantly cytoplasmic [162]. Therefore the introduction of two point mutations within the D-domain (R730/731A) may disrupt the subcellular localization of MSK2. Currently, the subcellular localization of the MSK1 and MSK2 D-domain mutants (RR/AA) has not been established (Figure 2.6 and [121]). To overcome potential issues concerning the subcellular localization of the MSK2 D-domain mutant when dissecting the MSK2-CK2 interaction, an external NLS sequence could be engineered onto the MSK2-R730/731A mutant. This would permit the examination of the role of p38 in mediating the nuclear CK2-MSK2 interaction. In addition, binding analysis in p38-depleted cells would also facilitate the examination of the CK2-MSK2 complex.

2.5.3 CK2 disassociates from the MSK2-p38 complex following UV-radiation

I observed decreased association of CK2 with the MSK2-p38 complex following UV-radiation (Figure 2.4B). In line with this, other reports have described CK2 dissociation from protein complexes following cellular stimulation. Indeed, CK2 and HSP90 interact under basal condition while thrombin stimulation promotes the dissociation of CK2α from the HSP90 complex [160]. In addition, CK2 binds to the TATA-binding protein (TBP) under undamaged
conditions but dissociates in response to DNA damage [163]. Although it is currently unclear why CK2 dissociates from MSK2 following UV-irradiation, it may result from a conformational change that occurs within MSK2 when transitioning from an inactive to an active kinase state. Indeed, AGC kinases have been shown to undergo significant conformational changes to stabilize the activation loop, which occurs between the NTKD and the HM/linker region [124, 164, 165]. Therefore, it is possible that CK2 dissociates from MSK2 following UV-radiation and MSK2 activation as a result of its binding site being disrupted.

In contrast, I did not observe p38 dissociation from MSK2 following UV-radiation and MSK2 activation. This is consistent with previous reports concerning the MSK1-p38 complex following activation [121]. The nature of the MSK interaction with p38 or ERK differs from that observed for the RSKs and their upstream activator, ERK. Indeed, under unstimulated conditions, inactive RSK associates with ERK through the RSK C-terminal D-domain [130]. However, upon activation, RSK1 autophosphorylates a serine residue within its C-terminus adjacent to the D-domain, which promotes the dissociation of ERK [130]. MSK1 has also been reported to autophosphorylate residues within its D-domain upon activation but unlike the RSKs, these phosphorylation events do not disrupt ERK or p38 binding [121]. Currently, it has not been established if MSK2 undergoes C-terminal autophosphorylation.
Chapter 3: Differential Regulation of MSK1 and MSK2 by CK2 in response to UV-radiation

A version of this chapter is published in the Journal of Biological Chemistry:
3.1 Introduction

In the previous chapter, I identified and characterized the physical association of CK2 with the nuclear kinase MSK2. Interestingly, CK2 interacted with MSK2 but not the closely related MSK1. As MSK1 and MSK2 interact with and are activated by p38 following UV-C radiation, as is CK2, I chose to examine whether CK2 was involved in the differential regulation of the UV-induced activation of MSK1 and MSK2. Furthermore, I explored the role of the p38-CK2-MSK2 pathway in the regulation of NF-κB, a reported MSK substrate, following UV radiation.

In response to UV radiation, mammalian cells modulate the activity of several transcription factors including NF-κB, which is thought to be an important determinant of cellular survival [14]. The most abundant NF-κB complex is the p65-p50 heterodimer with the p65 subunit harboring the transactivation function [16]. Under basal conditions, NF-κB is inactive and predominantly cytoplasmic where it is bound to its inhibitor IκB. Following exposure to cellular stress, IκB is phosphorylated, which promotes its degradation. This results in the nuclear translocation and activation of NF-κB p65-p50 [16].

There are two distinct signaling pathways leading to the phosphorylation and subsequent degradation of IκB: one which is IκB kinase (IKK)-dependent and one which is independent of IKK. The IKK-independent or atypical pathway is unique in that it is activated following UV-C radiation, and IκB is phosphorylated by CK2 [10]. Although CK2 has been traditionally described as a constitutively active kinase [158], several studies have demonstrated that CK2 kinase activity can be stimulated following UV-C radiation in a p38-dependent manner and have shown that CK2 is important in the regulation of cell survival following cellular stress [10, 97-99]. In addition to the degradation of IκB, the phosphorylation of p65 at serine-276 (S276) has been shown to be important for NF-κB transactivation in response to TNFα stimulation [38,
Furthermore, it has been demonstrated that MSK1 and MSK2, are responsible for the TNFα-mediated nuclear phosphorylation of p65-S276 and p65 transactivation potential [38]. MSK1 and MSK2 contain two kinase domains. The C-terminal kinase domain (CTKD) is activated by either p38 or ERK1/2 in response to stimulation. The activated CTKD then serves to activate the N-terminal kinase domain (NTKD), which is responsible for substrate phosphorylation. Specifically, the phosphorylation of the MSK2 NTKD at serine-196 (S196) by the activated MSK2 CTKD is essential for MSK2 activation [131]. Previously, it had not been established if MSK1 and MSK2 kinase activities undergo differential cellular regulation.

In this chapter, I show that MSK2, but not MSK1, undergoes CK2-dependent UV-induced kinase activation. In addition, I have identified a putative site of CK2 phosphorylation at serine-324 (S324) of MSK2, which is required for maximal activation of MSK2 following UV-C radiation. Finally, I demonstrate that MSK2 is the major kinase responsible for p65-S276 phosphorylation and is required for p65 transactivation during the UV response. These results strongly suggest that MSK2 is positively regulated by CK2, and is important for the stimulation of NF-κB activity following UV-C radiation in MDA-MB-231 cells. Significantly, the data also demonstrate for the first time that MSK1 and MSK2 may be activated by distinct signaling pathways.

3.2 Attributions

All experiments were performed by Kellie Jacks
3.3  Experimental Materials and Methods

3.3.1. Cloning and plasmid constructions

Cloning of human MSK1 and MSK2 cDNAs into the pcDNA3.1/V5-His mammalian expression vector is described in section 2.3.1. The MSK2 cDNA was subcloned into pGEX-4T3, yielding GST-MSK2. MSK1 and MSK2 point mutations were generated by QuikChange site-directed mutagenesis (Stratagene) using standard protocols. Primer sets are listed in Table 3.1.

For p65 cloning, RNA was harvested from cycling MDA-MB-231 cells using the RNeasy mini kit (Qiagen), and reverse transcribed to make cDNA using qScript cDNA supermix (Quanta) from which p65 cDNA was PCR amplified (forward primer: 5’-ATGGACGAAGCTGTTCCCTCATC-3’ and reverse primer: 5’-GGAGCTGATCTGACTCAGCAGGGGC-3’). p65 cDNA was TA cloned into the pCR®2.1 vector (Invitrogen) then subcloned into the pM vector (Clontech) in frame with the Gal4-DNA binding domain sequence yielding Gal4-p65. QuikChange site-directed mutagenesis (Stratagene) was performed to generate the p65-S276A mutant yielding Gal4-p65-S276A. Primer set listed in Table 3.1. All of the plasmid constructs were verified by sequence analysis.

<table>
<thead>
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<th>Primer Name</th>
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<td>Reverse</td>
<td>5’-AATCCTACATCTAATTCGAGCTGACTGCTGAGT-3’</td>
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<tr>
<td>MSK2 S196A</td>
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<td>Reverse</td>
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<td>MSK2 R730/731A</td>
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<td>Reverse</td>
<td>5’-GCTCGCAGCTTCTGCTGCGGCCTTGGCGCCAGGGGTGATT-3’</td>
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<tr>
<td>p65 S276A</td>
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<td>Reverse</td>
<td>5’-ACTGAGCTCCGGGTGAGGGGAAGCTGAGG-3’</td>
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3.3.2 Cell culture and transfections

All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO₂. HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) and MDA-MB-231 cells in RPMI-1640 medium, both supplemented with 10% fetal bovine serum (FBS) and 50U of penicillin and 50 µg of streptomycin antibiotics per ml. Transient transfection of plasmids into HEK293T cells was performed using the Effectene transfection kit (Qiagen) according to the manufacturer's instructions. Transient transfection of plasmids into MDA-MB-231 cells was performed using the Lipofectamine™ LTX Plus Transfection Reagent (Invitrogen) according to the manufacturer's instructions. All siRNA (non-targeting (NT) siRNA pool (D-001206-13), human CK2β siRNA pool (L-007679-00), human MSK1 siRNA pool (M-004665-02), and human MSK2 siRNA (J-004664-06)) were purchased from Dharmacon and siRNA transfections were performed using Dharmafect1 reagent (Dharmacon) as per the manufacturer’s instructions.

3.3.3 Production of endoribonuclease-prepared short interfering RNA (esiRNA)

esiRNA against the 3’UTR of MSK2 was generated according to standard protocols [166]. Briefly, the 3’UTR of the MSK2 cDNA was PCR amplified from the pSPORT-MSK2 vector (Open Biosystems) using High Fidelity Taq polymerase (Invitrogen). In vitro transcription was performed using the T7 Megascript Kit (Ambion) as per manufacturer’s instructions. Double stranded RNA (dsRNA) was digested using 5µg of GST-RNaseIII (gift from Dr. Laurence Pelletier) at 22°C for four hours and then at 37°C for two hours. An additional 2µg of GST-RNaseIII was added and digestion was performed for an additional hour at 37°C. dsRNA digestion was monitored by agarose gel electrophoresis until approximately 18-25 base pair fragments were obtained. esiRNA was purified over a Q sepharose column, precipitated then
resuspended in DEPC water and quantified. esiRNA transfections were performed using Dharmafect1 reagent (Dharmacon).

### 3.3.4 Antibodies

Commercial antibodies used in this chapter were purchased from R&D Systems (anti-MSK1 goat polyclonal, anti-pS196-MSK2 rabbit polyclonal), Santa Cruz Biotechnology (anti-CK2α goat polyclonal, anti-CK2β rabbit polyclonal and mouse monoclonal, anti-α-tubulin mouse monoclonal, anti-p65 mouse monoclonal), Abcam (anti-MSK2 rabbit polyclonal, anti-CK2α rabbit polyclonal, anti-CK2α’ rabbit polyclonal and anti-β-actin mouse monoclonal), Oncogene (anti-α-tubulin mouse monoclonal), Cell Signaling Technologies (anti-p38 rabbit polyclonal, anti-phospho-ERK1/2 (Thr202/Tyr204) rabbit monoclonal, anti-ERK1/2 rabbit monoclonal, anti-phospho-p65-S276 rabbit polyclonal) and Invitrogen (anti-V5 mouse monoclonal). Anti-V5 agarose affinity gel was purchased from Sigma.

### 3.3.5 Expression and purification of fusion proteins

Expression and purification of recombinant GST-MSK2 was performed as described in section 2.3.4. GST-MSK2 was eluted from glutathione sepharose beads by the addition of 20mM glutathione (Sigma) then dialyzed into 20mM MOPS, pH 7.2, 5mM EGTA, 1mM DTT, 1% TritonX-100 and 10% glycerol.

### 3.3.6 Preparation of cell extracts, immunoprecipitation, and immunoblotting

For whole cell extract (WCE) preparation, cells were rinsed once in ice-cold PBS, and lysed in 50mM Tris-HCl pH7.5, 100mM NaCl, 1% Triton X-100, 0.5mM DTT, 0.5mM EDTA, 1X Complete mini protease inhibitor cocktail (Roche), 1mM sodium orthovanadate, 40mM β-glycerophosphate, and 50mM sodium fluoride. For inhibition studies, cells were pre-treated with DMSO, DMAT (10μM), SB203580 (10μM), or H89 (10μM) (Sigma) for two hours at 37°C.
For UV-C (254nm) treatment, cultured cells were washed once with PBS then UV-irradiated (40 or 200 J/m² as indicated) in the presence of PBS. Following UV-irradiation, culture media was replaced and cells were incubated at 37°C for specified periods until cell harvest. Pretreatment with 10μM anisomycin was performed for twenty minutes at 37°C. For immunoprecipitations, clarified WCEs were incubated with 1μg of the relevant antibody on ice for sixty minutes with occasional gentle agitation followed by the addition of 30μl of protein A-coupled sepharose beads (ThermoFisher) and incubated for an additional hour at 4°C, or WCEs were incubated with V5-agarose (Sigma) at 4°C for two hours. Immunoprecipitates were washed thrice with lysis buffer, resuspended in SDS-PAGE sample buffer, and resolved by SDS-PAGE. Proteins were transferred to PVDF membrane (Millipore) and immunoblotted as indicated. Detection was performed using SuperSignal enhanced chemiluminescence (Pierce).

3.3.7  In vitro MSK1 and MSK2 IP kinase assays

MDA-MB-231 cells or HEK293T cells ectopically expressing MSK2-V5 or MSK1-V5 were mock or UV-irradiated with 200 J/m² UV-C. Thirty minutes post-treatment, cells were harvested as described above, and WCEs were incubated with antibodies as indicated. Immunoprecipitates were washed thrice with lysis buffer containing 400mM NaCl for ectopic IPs or 100mM NaCl for endogenous IPs, then once with 50mM Tris-HCl pH7.5, 0.1mM ethylene glycol tetraacetic acid (EGTA), 10mM β-mercaptoethanol. IP kinase assays were performed in 10mM MOPS pH 7.2, 12.5mM β-glycerol phosphate, 2.5mM EGTA, 0.5mM sodium orthovanadate, 0.5mM DTT, 6.25mM magnesium acetate, 62.5μM cold ATP, 10μCi γ-[32P]ATP, and 20μM crosstide (GRPRTSSFAEG-KK) (Millipore) harboring two lysine residues (underlined) at the carboxy-terminus to facilitate binding to P81 paper. The reaction mixtures were incubated for exactly ten minutes at 30°C with constant shaking, then terminated by centrifugation and spotting of the
supernatants onto P81 ion-exchange paper (Millipore). P81 papers were washed thrice for five minutes each with 0.75% phosphoric acid then once with acetone for five minutes. Incorporation of radiolabel was measured by scintillation counting.

3.3.8  In vitro CK2 kinase assays

1µg of purified recombinant GST or GST-MSK2 was incubated with 10ng CK2 (Upstate) in 20mM MOPS, pH 7.2, 25mM β-glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM DTT containing 100µM cold ATP and 5µCi γ-[32P]ATP for twenty minutes at 30°C according to the manufacturer's instructions. The reaction was stopped by the addition of Laemmli sample buffer, and phosphorylated proteins were visualized by resolving the samples on SDS-PAGE followed by autoradiography.

3.3.9  Reporter assays

Where indicated, MDA-MB-231 cells were transfected with non-targeting esiRNA or MSK2 esiRNA, and twenty-four hours later cells were transfected with a Gal4-responsive firefly luciferase expression plasmid, pGL2-5xGal4 (gift from Dr. Peter Cheung), the pRL-TK Renilla luciferase normalizing control (Promega), and, as indicated, with pGal4, pGal4-p65, pGal4-p65-S276A, MSK2-V5, MSK2-S324A-V5 or “kinase-dead” (KD) MSK2-S196A-V5. Twenty-four hours post-transfection, RPMI media containing 10% FBS was replaced with RPMI media containing 2.5% FBS. Twenty four hours later, cells were mock or UV-C irradiated (200 J/m²) in PBS, media was replaced and cells were incubated for eight hours at 37°C. Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Assay System (Promega) as per the manufacturer’s instructions. In all experiments, transfections were performed in duplicate and the luciferase values were normalized to Renilla luciferase activity.
3.3.10 Quantitative real-time PCR

Total RNA was extracted from MDA-MB-231 cells using the RNeasy Mini Kit (Qiagen). 5μg of total RNA was reverse transcribed to make cDNA using qScript cDNA SuperMix (Quanta Biosciences). Quantitative real-time PCR (qPCR) was performed with PerfeCTa™ SYBR® Green SuperMix (Quanta Biosciences) as per the manufacturer’s instructions in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Primer sets are listed in Table 3.2. Reactions were performed in triplicate and normalized to TATA-binding protein (TBP) expression.

Table 3.2. Primers used in qPCR analysis of NF-κB dependent genes

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<td>Reverse</td>
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<td></td>
<td>Reverse</td>
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3.3.11 Cell viability assays

MDA-MB-231 cells expressing non-targeting or MSK2-targeted siRNAs were mock or UV-C irradiated (200 J/m²) and cell viability was assessed twenty-four hours later using the colorimetric CellTiter 96® AQueous Assay (Promega) by measuring absorbance at 490 nm as per the manufacturer’s instructions.
3.4 Results

3.4.1 MSK2 activity is regulated by CK2 following UV-C radiation

In light of my observations in Chapter 2, which demonstrated an interaction between CK2 and MSK2, I next examined whether CK2 might regulate MSK2 activity following UV-C radiation. To do so, endogenous MSK2 or MSK1 were immunoprecipitated from MDA-MB-231 whole cell extracts that had been pretreated with or without the CK2 or p38 inhibitors (DMAT and SB203580, respectively) prior to mock or UV-C radiation, and examined in in vitro IP kinase assays with the peptide substrate, crosstide (Figure 3.1). While chemical inhibition of p38 reduced both MSK2 and MSK1 UV-induced activation to baseline values as expected, only UV-induced MSK2 activation was affected by CK2-inhibition, with an approximate 3.5-fold reduction (Figure 3.1). CK2 inhibition partially reduced the UV-induced MSK2 mobility shift, whereas no such effect was noted with MSK1. In contrast, p38 inhibition abolished the UV-induced mobility shifts of both MSK2 and MSK1. CK2 and p38 inhibition had no effect on the migration of MSK2 under basal conditions. Collectively, these data suggested that the CK2-dependent phosphorylation of MSK2 may modulate UV-induced MSK2 activity.

I next examined the role of CK2 in the UV-induced activation of ectopically expressed MSK2-V5 and MSK1-V5 in HEK293T cells by in vitro IP kinase assays (Figure 3.2). Both ectopically expressed MSK1-V5 and MSK2-V5 underwent similar UV-induced kinase activation to that of endogenous MSK1 and MSK2. Indeed, CK2 inhibition impaired MSK2-V5 but not MSK1-V5 UV-induced activation. However, ectopically expressed MSK2-V5 did not undergo a significant electrophoretic mobility shift in response to UV-radiation (Figure 3.2A).

As another means to inhibit CK2 function, I depleted CK2β by siRNA in HEK293T cells, which impairs CK2 holoenzyme formation and activation [10, 66], and found that this was
Figure 3.1. CK2 inhibition impairs endogenous MSK2 but not MSK1 activation following UV-C radiation. MDA-MB-231 cells were pretreated with DMSO, DMAT (10 μM) or SB203580 (10 μM) for two hours prior to mock or UV-C radiation (200 J/m²) and harvested 30 minutes later. WCEs were immunoprecipitated with (A) anti-MSK2 or (B) anti-MSK1 antibodies, and in vitro (A) MSK2 or (B) MSK1 kinase assays were performed with crosstide and [γ-32P]-ATP. Activity was measured by scintillation counting. Data were derived from independent triplicate samples. Error bars represent SEM. Right panels indicate western-blotting loading controls for (A) MSK2 or (B) MSK1 as indicated.
Figure 3.2. CK2 inhibition impairs MSK2-V5 but not MSK1-V5 activation following UV-C radiation. HEK293T cells were transfected with (A) MSK2-V5 or (B) MSK1-V5 constructs. 48 hours post-transfection, cells were pre-treated with DMSO, DMAT (10μM) or SB203580 (10μM) for two hours prior to mock or UV-C radiation (200J/m²). Cells were harvested 30 minutes later and WCEs were immunoprecipitated with anti-V5 agarose and in vitro (A) MSK2-V5 and (B) MSK1-V5 kinase assays were performed with crosstide and [γ-32P]-ATP. Activity was measured by scintillation counting. Data were derived from independent triplicate samples. Error bars represent SEM. Right panels adjacent to histograms indicate western-blotting loading controls.
associated with an approximate 2-fold reduction in MSK2-V5 UV-induced kinase activity (Figure 3.3A), but had no effect on MSK1-V5 kinase activity (Figure 3.3B). I also examined the effect of CK2β depletion on the UV-induced MSK2-V5 kinase activity in MDA-MB-231 cells (Figure 3.4) and similar results to those seen in HEK293T cells were obtained.

It has been previously reported that CK2 is required for the maximal activation of the Raf-MEK-ERK signalling cascade following growth factor stimulation [94]. Although ERK is most potently activated by mitogens, UV-radiation can increase EGFR signalling [167], which can lead to ERK activation in some cell types. Therefore, I wanted to exclude the possibility that the effect of CK2 inhibition on the UV-induced activation of MSK2 was due to impaired ERK activation. As demonstrated in Figure 3.5, western blot analysis of phospho-ERK1/2 revealed a strong activation of ERK following EGF stimulation that was unaffected by DMAT treatment. As expected, stimulation with the p38 activator anisomycin did not increase ERK phosphorylation above baseline levels. In comparison, UV-C radiation resulted in a weak activation of ERK that was also unaffected by DMAT treatment. These results suggest that CK2 inhibition does not affect the UV-induced activation of ERK and therefore is not likely contributing to the observed decrease in the UV-induced activation of MSK2 in the presence of DMAT.

Finally, as an additional control, I examined MSK2-V5 activation following anisomycin treatment, which specifically activates the p38 signaling cascade, and found that siRNA-mediated CK2β-depletion also similarly diminished MSK2-V5 kinase activation (Figure 3.6). Taken together, these data demonstrate that CK2 is required for the maximal activation of MSK2 but not MSK1 following UV radiation.
Figure 3.3. CK2β depletion impairs MSK2-V5 but not MSK1-V5 UV-induced kinase activation in HEK293T cells. HEK293T cells were transfected with non-targeting (NT) or CK2β-specific siRNAs, and 24 hours later, (A) MSK2-V5 or (B) MSK1-V5 constructs were also transfected into the cells. 48 hours later, cells were mock or UV-C irradiated (200J/m²) and harvested 30 minutes later. WCEs were immunoprecipitated with anti-V5 agarose and in vitro (A) MSK2-V5 and (B) MSK1-V5 kinase assays were performed with crosstide and [γ-32P]-ATP. Activity was measured by scintillation counting. Data were derived from independent triplicate samples. Error bars represent SEM. Right panels adjacent to histograms indicate western-blotting loading controls.
Figure 3.4. CK2β depletion impairs MSK2-V5 UV-induced kinase activation in MDA-MB-231 cells. MDA-MB-231 cells were transfected with non-targeting (NT) or CK2β-specific siRNAs, and 24 hours later, MSK2-V5 construct was also transfected into the cells. Cells were treated and in vitro MSK2-V5 kinase assays were performed with crosstide and [γ-32P]-ATP. Activity was measured by scintillation counting. Data were derived from independent triplicate samples. Error bars represent SEM. Right panels adjacent to histograms indicate western-blotting loading controls.
Figure 3.5. Inhibition of CK2 does not impair ERK1/2 activation following mitogen or stress stimulation in HEK293T cells. HEK293T cells were pre-treated with DMSO or DMAT (10μM) for two hours prior to mock, EGF (100ng/ml), anisomycin (10μM) or UV-C radiation (200J/m²) treatment. Cells were harvested and WCEs were resolved by SDS-PAGE and immunoblotted for Thr202/Tyr204 phosphorylated ERK1/2 (pERK1/2) and total ERK1/2.
Figure 3.6 CK2 is required for maximal activation of MSK2 in response to anisomycin stimulation. HEK293T cells were transfected with non-targeting (NT) or CK2β-specific siRNAs, and 24 hours later, the MSK2-V5 construct was also transfected into the cells. 48 hours later, cells were treated DMSO or 10 μM anisomycin for 20 minutes. WCEs were immunoprecipitated with anti-V5 agarose and in vitro MSK2-V5 kinase assays were performed with crosstide and \([γ-^{32}P]\)-ATP. Activity was measured by scintillation counting. Data were derived from independent triplicate samples. Error bars represent SEM. Right panel shows western-blot loading controls.
3.4.2 Serine 324 is required for UV-induced MSK2 kinase activation

The UV-induced MSK2 kinase activity is known to be p38-dependent, and my data suggest that it is also CK2-dependent. I have determined that the UV-induced MSK2 upward electrophoretic mobility shift is largely due to phosphorylation (Figure 2.4A) and that CK2 inhibition partially reduced the UV-induced MSK2 mobility shift, whereas no such effect was noted with MSK1 (Figure 3.1). This suggests that MSK2 may be a CK2 substrate and that the CK2-dependent phosphorylation of MSK2 may modulate UV-induced MSK2 activity. To support this hypothesis, I found that recombinant purified GST-MSK2 was directly phosphorylated by CK2 in vitro (Figure 3.7).

To determine which amino acid residue(s) of MSK2 might be phosphorylated by CK2, I identified several sites possessing the consensus CK2 phosphorylation motif (S/T-x-x-E/D) [60] using Scansite (http://scansite.mit.edu/) and NetPhosK 1.0 Server (http://www.cbs.dtu.dk/services/NetPhosK) predictive software. Of these possible sites of CK2 phosphorylation, only S87, S119, and S324 were found to be unique to MSK2, and not conserved in MSK1. Furthermore, of these residues only S324 was found to possess an additional acidic residue at the n+1 position, which is found in 75% of CK2 sites of phosphorylation (Figure 3.8) [158]. Therefore, I initially examined whether S324 might be involved in the CK2-dependent regulation of MSK2 kinase activity. To more reliably examine this effect in the context of mammalian cells, I performed site-directed mutagenesis to substitute S324 to alanine (S324A) in MSK2-V5 and compared the UV-induced in vitro kinase activity of MSK2-S324A-V5 to that of wild-type (WT) MSK2-V5. In Figure 3.9, in vitro kinase assays of anti-V5 (MSK2) immunoprecipitates from HEK293T cell extracts harvested under basal conditions and following UV-C radiation demonstrate that the MSK2 mutant protein harbouring
Figure 3.7. CK2 phosphorylates MSK2 in vitro

Purified recombinant GST or GST-MSK2 were subjected to CK2 in vitro kinase reactions with \( \gamma^{[32P]} \) ATP. \( [32P] \) ATP incorporation was assessed by autoradiography.
Figure 3.8. Serine-324 in human MSK2 is evolutionarily conserved.
Amino acid sequence alignment of partial protein sequences for MSK1 (human NP_004746, mouse NP_705815, rat NP_001101518, dog XP_547953, chimpanzee XP_001140389, chicken Q5F3L1, cow XP_580522) and MSK2 (human NM_003924, mouse NM_019924, dog XP_854926, chimpanzee XP_508900). Sequences were aligned using Vector NTI software. Human MSK2 S324 and human MSK1 D341 amino acid residues are indicated by the arrow. Acidic residues at positions n+1 and n+3 relative to MSK2 S324 are indicated by asterisks (*).
Figure 3.9. Serine-324 is required for maximal UV-induced MSK2 activation
HEK293T cells ectopically expressing WT MSK2-V5 (WT), MSK2-S324A-V5 (S324A) or MSK2-S324D-V5 (S324D) were mock or UV-C irradiated (200J/m²) and harvested 30 minutes later. WCEs were immunoprecipitated with anti-V5 agarose and *in vitro* kinase assays were performed with crosstide and [γ-32P]-ATP. Activity was measured by scintillation counting. Data were derived from independent triplicate samples. Error bars represent SEM. The right panel adjacent to the histogram indicates western-blotting input loading control.
the S324A substitution was severely impaired for UV-induced kinase activation. To test whether
the phosphorylation of S324 might be an important determinant of MSK2 UV-induced kinase
activation, S324 was also substituted to a phospho-mimicking amino acid (aspartic acid)
(S324D), and compared to WT MSK2-V5 in anti-V5 (MSK2) in vitro kinase assays. As shown
in Figure 3.9, the phospho-mimicking MSK2 mutant did indeed restore UV-induced MSK2
kinase activity to levels that were comparable to that of WT MSK2-V5.

MSK2 is thought to undergo sequential activation in vivo whereby p38/ERK first
phosphorylates T568 within the CTKD and S343 within the linker region [131]. Based on
mutational analyses, it has been further proposed that the activated C-terminal kinase domain
then autophosphorylates S360 (within the linker region) and S196 (within the activation loop of
the NTKD), which is essential for MSK2 activation [131]. Therefore to monitor the effect of
S324, which lies within the linker region, on MSK2 activation following UV-C radiation, I
examined cell extracts from HEK293T cells expressing WT, S324A or S324D MSK2-V5
proteins subjected to mock or UV-C radiation with phospho-specific pS196 (pS196)
immunoblotting. I found that MSK2-S324A-V5 did not undergo appreciable
autophosphorylation at S196 following UV-C radiation, in contrast to WT MSK2-V5 and
MSK2-S324D-V5 (Figure 3.10A). As a control for the specificity of the pS196 MSK2 antibody,
I also ectopically expressed the mutant V5-tagged MSK2 S196A protein, which lacks the
relevant phospho-acceptor site. When MSK2 S196A-V5 was harvested following mock or UV-
treatment, and subjected to pS196 immunoblotting, no band was detected suggesting this
antibody is specific for the phospho-S196 MSK2 epitope (Figure 3.10B). Therefore, these
results suggest that the phosphorylation of S324 is important for subsequent
autophosphorylation at S196 and activation of MSK2 following UV-C radiation in vivo.
Figure 3.10. MSK2 serine-324 is required for UV-induced phosphorylation of MSK2 serine-196

(A) HEK293T cells ectopically expressing WT, S324A or S324D MSK2 V5-tagged proteins were mock or UV-C irradiated (200J/m²) and harvested 30 minutes later. WCEs were immunoblotted with anti-pS196-MSK2 (pS196) and anti-V5 (MSK2) antibodies. (B) MDA-MB-231 cells ectopically expressing WT or S196A MSK2 V5-tagged proteins were mock or UV-C irradiated (200J/m²) and harvested 30 minutes later. WCEs were immunoblotted with anti-pS196-MSK2 (pS196) and anti-V5 (MSK2) antibodies.
3.4.3 A phospho-mimicking substitution at S324 is resistant to CK2 inhibition

MSK2 kinase activation is dependent on CK2, and the substitution of S324 to a phospho-mimicking amino acid residue relieves MSK2 inhibition following UV-C radiation. Therefore if the phosphorylation of S324 is CK2-dependent, then one would expect that the phospho-mimicking mutant MSK2-S324D would be unaffected by CK2 inhibition. To investigate this possibility further, I compared WT MSK2-V5 and MSK2-S324D-V5 UV-induced activation with or without CK2β-depletion by siRNA by in vitro kinase assays. As shown in Figure 3.11, the ability of WT MSK2-V5 immunoprecipitated from UV-treated cell extracts to phosphorylate crosstide was inhibited by siRNA-mediated CK2β-depletion, whereas the kinase activity of MSK2-S324D-V5 was not appreciably affected. These results are consistent with the notion that the phosphorylation of MSK2 at S324 is dependent on CK2 in response to UV-C radiation.

MSK2 and MSK1 are closely related isoforms, but appear to be differentially regulated by CK2. To further investigate this interesting observation, I noted that S324 at the equivalent position in other mammalian MSK2 proteins was highly conserved, but was an aspartic acid residue in MSK1 proteins (Figure 3.8). Therefore, I substituted the human MSK1 aspartic acid residue (D341), located at the equivalent position to MSK2 S324, to alanine and compared this mutant to wild-type MSK1 in in vitro IP kinase assays. As shown in Figure 3.12, substitution of aspartic acid to alanine at amino acid residue 341 had no effect on MSK1 kinase activity, further suggesting that MSK1 and MSK2 are differentially regulated at this position.

3.4.4 MSK2 is required for the UV-induced phosphorylation of p65 at S276 in vivo.

The MSKs has been shown to phosphorylate the p65 subunit of NF-κB at S276 in response to TNFα stimulation and following IL-1β treatment [37, 38]. Therefore, I wondered whether p65-S276 might be phosphorylated by MSK1 and MSK2 following UV-C radiation in vivo. To
**Figure 3.11. S324D restores MSK2 UV-induced kinase activation in the absence of CK2β.**

HEK293T cells were transfected with NT or CK2β-specific siRNAs (siCK2β), and 24 hours later, WT or S324D MSK2-V5 constructs were also transfected into the cells. 48 hours later, cells were mock or UV-C irradiated (200J/m²) and harvested 30 minutes later. WCEs were immunoprecipitated with anti-V5 (MSK2) agarose and *in vitro* kinase assays were performed with crosstide and [γ-³²P]-ATP. Activity was measured by scintillation counting. Data were derived from independent triplicate samples. Error bars represent SEM. Western-blot input loading controls are shown in right panels and counts were normalized to protein loading levels.
Figure 3.12. Aspartic acid-341 is not required for the UV-induced activation of MSK1.

HEK293T cells ectopically expressing WT MSK1-V5 (WT) or MSK1-D341A-V5 (D341A) were mock or UV-C irradiated (200J/m²) and harvested 30 minutes later. WCEs were immunoprecipitated with anti-V5 agarose (MSK1) and in vitro kinase assays were performed with crosstide and [γ-32P]-ATP. Activity was measured by scintillation counting. Data were derived from independent triplicate samples. Error bars represent SEM. Right panel adjacent to histogram indicates western-blotting input loading control.
address this question, I first examined whether the UV-induced phosphorylation of p65 at S276 in MDA-MB-231 cells (a cell line which has been commonly used in the examination of NF-κB activation[168]) was affected by the inhibition of MSK1/2 (H89), CK2 (DMAT) or p38 (SB203580). Indeed, the immunoblotting of such treated MDA-MB-231 cell extracts with a phospho-specific pS276 antibody demonstrated that the UV-induced phosphorylation of p65 at S276 was undetectable following MSK1/2- or p38-inhibition (Figure 3.13A). In addition, CK2-inhibition led to a substantial reduction in p65-S276 phosphorylation *in vivo*.

Because H89 can inhibit both MSK1 and MSK2, I next examined MDA-MB-231 cells depleted of MSK1, MSK2 or both kinases using RNA interference. I found that MSK2 depletion reduced p65-S276 phosphorylation following UV-C radiation, whereas the depletion of MSK1 had no detectable effect (Figure 3.13B). In addition, when I depleted endogenous MSK2 by esiRNA directed against the 3’UTR, then reconstituted with WT MSK2-V5, MSK2-S324A-V5 or empty vector, I found that only WT MSK2-V5 could rescue the UV-induced phosphorylation of p65 at S276 (Figure 3.13C). Collectively, my results suggest that S324 is important for MSK2 activation and for the *in vivo* phosphorylation of p65 at S276 following UV-C radiation.

### 3.4.5 UV-induced p65 transactivation is dependent on MSK2 and p65-S276

To determine the effect of UV-C radiation on NF-κB activity in MDA-MB-231 cells, genes known to be modulated by NF-κB in response to UV radiation [44] were initially examined using quantitative real-time PCR. I found that UV-C radiation was associated with an increase in the endogenous expression of all such genes examined (Figure 3.14).

To specifically examine the transactivation potential of the NF-κB p65 subunit, I utilized the Gal4-p65 luciferase reporter system in lieu of a general NF-κB response element reporter
Figure 3.13. p65-Ser276 phosphorylation is induced following UV-C radiation in a MSK2-dependent manner. (A) MDA-MB-231 cells were serum-starved for 24 hours, pretreated with vehicle (DMSO), DMAT (10 μM), SB203580 (10 μM) or H89 (10 μM) in serum-free media for two hours, then subjected to UV-C irradiation (40 J/m²) and harvested five hours later. WCEs were immunoblotted for pS276 p65 and total p65 as indicated. (B) MDA-MB-231 cells expressing NT siRNA, MSK1-specific siRNA (siMSK1) and MSK2-specific esiRNA (esiMSK2; alone or in combination) were serum-starved for 24 hours then UV-C irradiated (200 J/m²) and harvested 3 hours later. WCEs were immunoblotted with antibodies as indicated. (C) MDA-MB-231 cells expressing esiMSK2 were transfected with empty vector, MSK2-V5 or MSK2-S324A-V5 plasmids. As controls, MDA-MB-231 cells were also transfected with NT siRNA and siMSK1. 48 hours later, serum-starved cells were mock or UV-C irradiated (200 J/m²) and harvested 3 hours later. WCEs were immunoblotted with antibodies as indicated.
Figure 3.14. Analysis of NF-κB dependent gene expression post-UV.
Quantitative real-time PCR analysis of endogenous A20, Bcl-xL, and X-IAP gene expression (normalized to TBP) in MDA-MB-231 cells 6 hours following mock or UV-C radiation (200 J/m²). Triplicate replicates were performed for each sample and error bars represent SEM.
system, which can be bound by all NF-kB dimers. This system exploits the Gal4 transcription factor from yeast whereby the Gal4 DNA binding domain (Gal4-BD) is fused to p65, which contains a transcriptional activating domain. As the transcriptional activating domain of Gal4 is absent, the transcription of the luciferase reporter is dependent solely on the transactivation activity of p65. Another advantage of the Gal4-p65 system over the general NF-kB reporter system is that it specifically examines the nuclear signaling events that contribute to p65 transcriptional activity. Indeed, the Gal4-BD brings p65 to the promoter of the luciferase reporter plasmid, thus eliminating the requirement for cytoplasmic IkB degradation and p65 nuclear translocation. To investigate the importance of S276 of p65 in UV-induced transactivation, I compared wildtype Gal4-p65 to the mutant Gal4-p65-S276A using this luciferase reporter assays. As demonstrated in Figure 3.15A, UV-C radiation was associated with an increase in p65-dependent transactivation capacity, which was reduced when p65-S276 was substituted to an alanine. To test whether this effect was dependent on MSK2, I depleted MDA-MB-231 cells of MSK2 using esiRNA, and found diminished p65-dependent transcriptional activation following UV-C radiation (Figure 3.15B). Furthermore, expression of WT MSK2-V5 in MDA-MB-231 cells depleted of endogenous MSK2 (by esiRNA directed against the 3’UTR) restored UV-induced p65 transactivation potential, whereas MSK2-S324A-V5 was much less effective (Figure 3.15C).
Relative UV-induced luciferase activity

(A) 

(B) 

(C) 

Gal4-p65 + esiNT  Gal4-p65 + esiMSK2
Figure 3.15. MSK2 contributes to p65 transactivation following UV-C radiation.

(A) Gal4, Gal4-p65, or Gal4-p65-S276A transactivation 8 hours following UV-C radiation (200 J/m²) in MDA-MB-231 cells co-transfected with pGL2-5xGal4 firefly and pRL-TK *Renilla* luciferase plasmids. Firefly luciferase activity was normalized to *Renilla* luciferase internal control. Data is expressed as relative luciferase units normalized to Gal4. Data were derived from independent duplicate samples. (B) MDA-MB-231 cells expressing NT or MSK2-specific esiRNAs were transfected with Gal4 or Gal4-p65, and luciferase plasmids. 48 hours later, UV-induced transactivation was measured and firefly luciferase activity was normalized to *Renilla* luciferase internal control then further normalized to Gal4. Data were derived from independent duplicate samples. (C) MDA-MB-231 cells expressing esiMSK2 were transfected with "kinase-dead" (KD) MSK2-S196A-V5, MSK2-V5 or MSK2-S324A-V5, Gal4-p65 and luciferase plasmids. 48 hours later, cells were mock or UV-C irradiated (200 J/m²), transactivation was measured as in B, and further normalized to MSK2-KD control. Data were derived from independent duplicate samples. Inset: anti-V5 (MSK2) western-blotting controls from aliquots taken prior to UV treatment.
My data suggest that MSK2 is required for the phosphorylation and subsequent transcriptional activation of p65. In addition, UV treatment was found to induce the transcription of NF-κB target genes involved in cell survival (Figure 3.14) suggesting MSK2 may contribute to cell survival in response to UV radiation. Consistent with this idea, I found that the RNAi-mediated depletion of MSK2 from MDA-MB-231 cells reduced cellular viability following UV-C radiation as measured by the MTS assay (Figure 3.16).
Figure 3.16. MSK2 is required for cell survival following UV-C radiation.

MDA-MB-231 cells expressing NT or MSK2-specific siRNAs were mock or UV-C irradiated (200 J/m²) and cell viability was determined 24 hours later. * p < 0.001. All error bars represent SEM derived from triplicate samples from two independent experiments.
3.5 Discussion

The related MSK1 and MSK2 protein kinases are activated by mitogens and cellular stress, and phosphorylate numerous cellular substrates, including the p65 subunit of NF-κB at S276. Indeed following TNFα stimulation, MSK1(-/-)/MSK2(-/-) DKO MEFs have been shown to be defective in the phosphorylation of p65 at S276 [38]. Many other studies have also examined MSK1 and MSK2 in the context of DKO MEFs because of the perceived redundancy of the kinases. While this may be true in certain instances, my results clearly demonstrate that following UV-C radiation MSK2 is the major kinase responsible for p65 phosphorylation at S276, and suggest that this is required for subsequent NF-κB activation. Furthermore, I show for the first time that MSK1 and MSK2 can be differentially regulated, specifically by CK2, following UV-C radiation.

3.5.1 The UV-induced activation of MSK2 is mediated by the p38-CK2 pathway

My results suggest that maximal UV-induced MSK2 activation relies on the activation of a p38-CK2 signaling pathway in mammalian cells. Several studies have suggested that CK2 lies downstream of p38 [10, 95, 96], and that CK2 inhibition (DMAT) does not affect p38 activity [169]. Indeed, my data indicate that the UV-induced activation of p38 is unaffected by CK2 inhibition. These data are consistent with the notion that a p38-CK2-MSK2 pathway regulates UV-induced MSK2 activity in vivo. I observed that CK2 inhibition with DMAT or siRNA-mediated depletion of CK2β similarly reduced the in vitro UV-induced MSK2 activation on the order of 2-3.5-fold. The substitution of serine-324 to alanine (MSK2-S324A), expected to abolish putative CK2 phosphorylation at serine-324, also resulted in an approximate 3-fold reduction in UV-induced MSK2 activity (Figure 3.9). In addition to S324, other residues within MSK2 may be phosphorylated by CK2 in vivo. However, the fact that a phospho-mimicking
substitution at S324 (MSK2-S324D) was resistant to CK2 inhibition suggests that S324 is the major CK2-dependent site of phosphorylation that modulates MSK2 enzymatic function. In comparison to p38 inhibition, the inhibition of CK2 activity or the MSK2-S324A mutant protein were consistently associated with a smaller reduction in UV-induced MSK2 activity. This is likely a result of the p38-dependent phosphorylation of the CTKD. However, while a CK2-dependent pathway of MSK2 activation likely predominates during the UV response, I cannot exclude the possibility that CK2-independent pathways may also operate.

My data support a role for the CK2-dependent phosphorylation of S324, which is consistent with the direct phosphorylation of recombinant purified MSK2 by CK2 in vitro (Figure 3.7). However, it is possible that CK2 may not directly phosphorylate MSK2 in vivo but instead S324 may be phosphorylated by another CK2-regulated kinase. Alternatively, CK2 may regulate MSK2 autophosphorylation of S324. Whether S324 is important for the regulation of MSK2 kinase activity following other stimuli, such as growth factor stimulation, is not known and will be discussed further in Chapter 4. Collectively, my results from Chapter 2 and Chapter 3 are consistent with a model whereby UV-activated p38, contained within a preformed MSK2-CK2-p38 complex, phosphorylates MSK2 and stimulates CK2 kinase activity. CK2 phosphorylates MSK2 at S324, which contributes to maximal MSK2 activation, then subsequently dissociates from the activated MSK2 complex.

3.5.2 MSK2 linker region phosphorylation

T-loop phosphorylation within the N-terminal and C-terminal kinase domains of MSK1 and MSK2 is critical for kinase activity [121, 131]. However, additional phosphorylation events within the MSK1/2 linker region also contribute to maximal MSK kinase activation [121, 128, 131]. In line with this, mutagenesis of the MSK2 linker residue, S324, resulted in impaired UV-
induced kinase activation (Figure 3.9) and reduced UV-induced phosphorylation of the NTKD T-loop residue, S196, (Figure 3.10) thus highlighting the contribution of MSK2 linker region phosphorylation to MSK2 kinase activation.

Although MSK2 S324 is evolutionarily conserved in MSK2 homologues (Figure 3.8), it is not conserved at the equivalent position in MSK1. Notably, while S324 is required for maximal UV-induced activation of MSK2, the acidic residue at that position (MSK1 D341) does not contribute to MSK1 activation following UV-C radiation (Figure 3.12). Recently, a novel site of p38 phosphorylation was identified within the linker region of MSK2 at serine-347 [131]. Like S324, S347 was not conserved in MSK1 and mutagenesis of S347 resulted in impaired but not ablated MSK2 activation [131]. Although the role of the equivalent residue in MSK1 (leucine-364) has not been evaluated, these results collectively indicate that the kinase activation MSK1 and MSK2 are differentially regulated within the linker region by phosphorylation.

To date, all sites of phosphorylation identified within MSK1 and MSK2 are due to autophosphorylation or p38/ERK phosphorylation. Therefore, to my knowledge, S324 is the first site of MSK2 phosphorylation required for maximal activation that implicates another kinase.

3.5.3 The CK2-dependent activation of MSK2 following UV radiation is independent of the Raf-ERK signaling cascade

It has been recently shown that the KSR1 scaffold protein constitutively binds to the CK2 holoenzyme and facilitates the CK2-dependent phosphorylation of B-Raf and C-Raf [94]. Interestingly, CK2 appears to constitutively phosphorylate B-Raf, suggesting that the basal phosphorylation of B-Raf by CK2 is required for the subsequent activation of B-Raf kinase activity following PDGF-stimulation, which in turn leads to the activation of ERK. This CK2-
dependent pathway is unlikely to be contributing to my observations herein as UV-C radiation
does not strongly activate the classical MAPK pathway [1], and even if there was a small
contribution, one would expect that both MSK1 and MSK2, operating downstream of this
pathway, would be affected to similar extents. Indeed, pre-treatment with anisomycin, which
stimulates the p38 signaling cascade, activated MSK2 in a CK2-dependent manner, which was
comparable to what was seen with UV-C radiation (Figure 3.6). Furthermore, chemical
inhibition of p38 was found to abolish MSK2 kinase activity to baseline levels (Figure 3.1 and
3.2). Lastly, the weak activation of ERK1/2 following UV-C radiation was unaffected by pre-
treatment with DMAT (Figure 3.5). Collectively, these data suggest that UV-induced and CK2-
dependent MSK2 activation is likely largely attributable to the stimulation of a p38-dependent
pathway rather than the Raf-ERK signaling cascade. However, it is possible that the CK2-
dependent regulation of Raf kinase activity contributes to the ERK-mediated activation of
MSK1 and MSK2 following PDGF stimulation, which was not tested in my studies.

3.5.4 MSK2 but not MSK1 mediates the UV-induced activation of p65

I show that the p65 subunit of NF-κB undergoes UV-induced phosphorylation at S276 in human
MDA-MB-231 cells (Figure 3.13 A, B and C), and surprisingly this appears to be primarily
dependent on MSK2 with very little contribution from MSK1 (Figure 3.13 B and C). In addition
to UV-C radiation, MSK1 and MSK2 may be distinctly regulated following other types of
stimuli erasing the notion that these kinases simply perform redundant functions. In keeping
with this idea, the TPA- and anisomycin-induced phosphorylation of histone H3 at both serine
residues 10 and 28 in primary embryonic fibroblasts has been shown to be largely due to MSK2,
and to a lesser extent MSK1 [141].
3.5.5 NF-κB promotes cell survival in response to UV radiation

I found that UV-C radiation resulted in the upregulation of NF-κB-dependent gene expression and induces NF-κB p65 activation in MDA-MB-231 cells. In contrast, it has been previously suggested that UV-C radiation suppresses NF-κB dependent transcription in human U2OS cells [44]. While this may be a result of cell type specific differences, it is possible that variations in experimental methods may have contributed. For example, I used full length wild-type p65 and p65-S276A fused to the Gal4 DNA-binding domain as previously described [38], whereas the data from U2OS cells were obtained using a Gal4-p65 fusion containing only the minimal p65 transactivation domain (residues 428 to 551) [44]. My data further suggest that p65-S276 phosphorylation is required for full p65 activation following UV and that this in turn depends on MSK2 and MSK2-S324, as MSK2-S324A-V5 did not rescue p65 transactivation potential to wildtype levels (Figure 3.15C). Indeed, the MSK-dependent phosphorylation of p65-S276 following TNFα and IL-1β treatment has been shown to be required and sufficient for NF-κB activation in murine fibroblasts and human lung fibroblasts, respectively [37, 38]. Therefore, it is possible that the phosphorylation of p65-S276 following UV-C radiation is sufficient for the MSK2-dependent activation of NF-κB in MDA-MB-231 cells. However, I cannot exclude the possibility that this depends on the phosphorylation of other p65 sites. The depletion of cellular MSK2 levels by RNAi was associated with decreased cellular viability, which further suggests that the MSK2-dependent activation of NF-κB following UV radiation is important for the promotion of cell survival in MDA-MB-231 cells in vivo.
Chapter 4: Conclusions and Future Directions
4.1 Conclusions

The UV response includes the activation of the transcription factors NF-κB, AP-1 and p53, which mediate UV-induced changes in gene expression and contribute to cell fate following UV radiation [1]. The activation of NF-κB in the UV response is associated with the expression of genes involved in cell survival [170, 171]. My work herein describes the identification of a unique signaling pathway that specifically activates MSK2 and positively regulates NF-κB transcriptional activity in the UV response. I demonstrate for the first time that MSK2 is the major kinase required for the \textit{in vivo} phosphorylation of the p65 subunit of NF-κB at S276 in response to UV radiation. Furthermore, I demonstrate distinct roles for MSK2 and the closely related isoform MSK1 in the UV-induced phosphorylation of p65-S276, erasing any notion that these are redundant kinases. The distinct roles for MSK1 and MSK2 in NF-κB p65 phosphorylation during the UV response may be due to the differential requirement for CK2 as an upstream activator. Indeed, I show that MSK2 but not MSK1 undergoes CK2-dependent activation following UV-C radiation that serves to positively regulate MSK2 kinase activity. The p38-CK2 pathway has previously been associated with cell survival in the UV response by mediating the phosphorylation and degradation of IκB [10]. My results suggest that MSK2, a downstream component of the p38-CK2 pathway, may also contribute to cell survival in the UV response.

The work described in my thesis is the first example of differential regulation of MSK1 and MSK2 in human cells and has identified a novel way in which CK2 may contribute to MSK2 activation \textit{in vivo} (Figure 4.1). Based on my findings, it is intriguing to speculate that MSK1 and MSK2 may have differing roles in response other cellular stimulations such as cytokines, growth factors or ionizing radiation.
Figure 4.1. Proposed model of a novel p38-CK2-MSK2 pathway in the UV response
4.2 Future Directions

4.2.1 Examination of the p38-CK2-MSK2 pathway in the UV response in the skin

Although the UV response is conserved in many cell types [1], skin cells are the primary cell type exposed to environmental UV radiation [171]. Cancers that arise from UV exposure are thought to result from an imbalance in cell survival and cell death signaling [171]. These signals are thought to largely be a balance between the pro-survival signaling of NF-κB and the pro-apoptotic signaling of p53, suggesting that factors that regulate the transcriptional activity of NF-κB and p53 are key players in this process [171]. Therefore, examination of the p38-CK2-MSK2 pathway in the regulation of NF-κB-dependent gene expression in a skin cell system would provide a more physiologically relevant characterization of the p38-CK2-MSK2 pathway in the UV response. Previous studies have observed the p38-CK2 complex in keratinocytes [100] and the CK2-dependence of the UV-induced degradation of IκB has been reported in the A431 epidermal cell line [21]. Based on these observations, keratinocytes or epidermal cell lines would be appropriate systems to further investigate the p38-CK2-MSK2 pathway in the UV response. The p38-CK2-MSK2 pathway may regulate the induction of NF-κB-dependent survival genes in skin cells such as Bcl-χL, A20 and X-IAP, which I previously showed to be induced in response to UV-radiation in the MDA-MB-231 cell line (Figure 3.14). The p38-CK2-MSK2 pathway may also regulate UV-induced inflammatory gene expression such as IL-6, IL-8 or IL-20, which have previously been reported to be MSK-dependent [38, 150, 172]. Changes in gene expression could be examined using a luciferase reporter system where gene specific promoters are employed or by qPCR analysis. In addition, chromatin immunoprecipitation analysis could be used to examine the presence of MSK2 and phospho-S276 p65 at specific NF-κB dependent gene promoters following UV radiation. It is possible that aberrant activation of
the p38-CK2-MSK2 pathway could contribute to the development of skin cancer through the
deregulation of NF-κB target gene expression.

To characterize the p38-CK2-MSK2 pathway in UV-induced disease pathogenesis, animal
models could be employed. The MSK2 (-/-) mice are viable and display no overt phenotype
[145] but their sensitivity to mutagens such as UV radiation has not been established.
Examination of the susceptibility of MSK2 (-/-) mice to UV-induced skin carcinogenesis and
UV-induced inflammation in comparison to wildtype mice would provide further insight into
the role of MSK2 in UV-induced skin pathogenesis.

4.2.2 Characterization of MSK2 S324 phosphorylation in vivo

My results in Chapter 3 demonstrate that serine-324 of MSK2 is a putative site of CK2
phosphorylation that is required for maximal activation of MSK2 in response to UV radiation
(Figure 3.9, 3.10 and 3.11). However, I have not yet characterized MSK2 S324 phosphorylation
in vivo or firmly established if S324 phosphorylation is UV-induced. These questions can be
addressed by several approaches including in vivo incorporation of [32P]-orthophosphate
combined with two-dimensional tryptic mapping analysis of MSK2. This method has
traditionally been considered the gold standard for characterizing protein phosphorylation in vivo. The phosphorylation of residue S324 could also be validated by mass spectrometry (MS)
analysis of ectopically expressed MSK2-V5. Indeed, precursor ion scanning MS analysis was
previously utilized to identify novel sites of phosphorylation in ectopically expressed MSK1
[129]. As a complementary analysis, a phospho-specific antibody against MSK2 S324 could be
generated to examine the phosphorylation of this site by immunoblotting in the presence or
absence of UV radiation. Additionally, an MSK2 S324 phospho-specific antibody would
facilitate future analysis of MSK2 S324 phosphorylation under various cellular conditions, such as in response to growth factor or cytokine stimulation.

4.2.3 Regulation of MSK1 and MSK2 by CK2 in response to growth factor stimulation

MSK1 and MSK2 can be activated by both mitogens and cellular stress [108, 109]. My data presented in chapter 3 show that MSK1 and MSK2 are differentially regulated by CK2 in response to UV radiation. However, it has not been established if CK2 differentially regulates MSK1 and MSK2 following growth factor stimulation. To investigate this possibility, cells pre-treated with DMSO or the CK2 inhibitor DMAT will be stimulated with growth factors, such as EGF or PDGF, and MSK1 and MSK2 in vitro IP kinase assays will be performed. As another means of inhibiting CK2, siRNA-mediated depletion of CK2β could be used as a complimentary analysis. The requirement of MSK2 S324 for the maximal activation of MSK2 following growth factor stimulation could also be examined by in vitro IP kinase assays.

CK2 has been reported to regulate the Ras-MAPK pathway upstream of ERK in response to growth factor stimulation. Indeed, as discussed in Chapter 3, CK2 was observed to interact with the KSR1 scaffold protein, coordinating the CK2-mediated phosphorylation of B-Raf and c-Raf [94]. These phosphorylation events were shown to be required for maximal Raf-MEK-ERK pathway activation following PDGF stimulation in NIH3T3 cells [94]. This same study also reported similar results following EGF stimulation in a Xenopus system [94]. In contrast, a recent study in Drosophila S2 cells observed no defect in KSR-driven Raf activation when CK2 was depleted by siRNA [173]. In line with this, I did not observe a defect in ERK activation in HEK293T cells following EGF stimulation when CK2 was inhibited (DMAT) (Figure 3.5). Collectively these observations raise an interesting question about the role of CK2 in the regulation of the Raf-MEK-ERK pathway in response to different growth factor/mitogen
stimulations and in different cell types/organisms. It is possible that in certain cell types or in response to select mitogens, CK2 operates upstream of ERK. If this is the case, one would expect that CK2 inhibition would affect the mitogen-induced activation of both MSK1 and MSK2. In contrast, if CK2 is acting downstream of ERK and specifically modulating MSK2 activity, one would expect the mitogen-induced activation of MSK1 to be unaffected by CK2 inhibition. Future characterization of the role of CK2 in regulating MSK1/2 activation as well as the activation of Raf-MEK-ERK pathways following various mitogen stimulations and in various cell types would be of interest and of importance.

4.2.4 MSK1 and MSK2 in the IR response

The activation of MSK1 and MSK2 has been examined in response to several stress stimuli such as UV-radiation, anisomycin, TNFα, H₂O₂ and arsenite [108, 109, 131]. However, the role of MSK1 and MSK2 in the cellular response to ionizing radiation (IR) has not previously been examined. As MSK1 and MSK2 are nuclear kinases [108, 109], they may mediate cell survival in response to IR, potentially through the regulation of transcription factors or chromatin remodelling. To initiate the characterization of MSK1 and MSK2 in the IR response, I irradiated HEK293T cells ectopically expressing MSK1-V5 or MSK2-V5 and measured IR-induced kinase activity at various timepoints after IR treatment. IR treatment was associated with an approximately 5-fold increase in MSK1-V5 kinase activity within 15 minutes of irradiation compare to untreated cells (Figure 4.2A). Interestingly, by 30 minutes after IR treatment, MSK1-V5 kinase activity had decreased to approximately 2-fold that of unirradiated MSK1-V5 and remained at this level for the remaining timepoints measured. IR exposure also resulted in the activation of the N-terminal kinase domain of MSK1-V5, which was assessed by phospho-
Figure 4.2. MSK1 but not MSK2 is activated in response to ionizing radiation

HEK293T cells were transfected with (A) MSK1-V5 or (B) MSK2-V5 constructs. 48 hours post-transfection, cells were mock or IR-treated (10Gy) and harvested at the indicated timepoints. WCEs were immunoprecipitated with anti-V5 agarose and \( \textit{in vitro} \) (A) MSK1-V5 and (B) MSK2-V5 IP kinase assays were performed with crosstide and \( [\gamma-^{32}\text{P}]-\text{ATP} \). Activity was measured by scintillation counting. (A) Data derived from independent triplicate samples from two independent experiments. (B) Preliminary analysis from one independent experiment.
Figure 4.3. Analysis of MSK1-V5 N-terminal kinase domain activation following IR
HEK293T cells expressing MSK1-V5 were mock or IR-treated (10Gy) and harvested at the indicated timepoints. WCEs were immunoblotted with anti-pS212 MSK1 and anti-V5 antibodies as indicated. Figure representative of two independent experiments.
S212 MSK1 immunoblotting, and revealed a similar activation profile to that observed by MSK1-V5 IP kinase assays (Figure 4.3). Interestingly, preliminary examination found no significant increase in MSK2-V5 kinase activity in response to IR treatment (Figure 4.2B) suggesting that MSK1 but not MSK2 is activated following IR exposure.

In light of my observation that MSK1 but not MSK2 is activated in response to IR (Figure 4.2), it would be interesting to investigate the role of the MAPK family members in the IR-induced activation of MSK1. ERK, JNK and p38 have all been reported to be activated in response to IR [174]. However, as JNK has not previously been implicated in the activation of MSK1 following other cellular stimulations [108], it is more likely that ERK and/or p38 act as the upstream activator(s) of MSK1 following IR. The role of the MAPK family members in the IR-induced activation of MSK1 could be examined using chemical inhibitors targeting p38 (SB203580), MEK/ERK (U0126) or JNK (SP600125) prior to MSK1 in vitro kinase assay. In the brain, cocaine stimulates MSK1 activity but not MSK2, and this MSK1 activation is mediated by ERK [155, 175]. Therefore, I would hypothesize that ERK is the upstream activator of MSK1 in the IR response. It is also important to ensure that the maximal activation of MSK1 is not occurring prior to 15 minutes after IR. Therefore examination of MSK1 activation at 5 and 10 minutes following IR treatment will help establish when MSK1 is maximally activated in the IR response and facilitate the characterization of the upstream activating kinase for MSK1 following IR.

The IR-induced activation profile of MSK1-V5 (Figure 4.2A) shows a significant reduction in MSK1-V5 kinase activity within 30 minutes of IR treatment. This decrease in MSK1-V5 kinase activity may be due to the dissociation of MSK1 from its upstream activating kinase, a phenomenon that has previously been observed with the ERK-RSK complex following mitogen
stimulation [130], and could be tested by co-immunoprecipitation analysis. Another possibility could be that another IR-activated kinase is phosphorylating and inhibiting MSK1 kinase activity. This could be examined by measuring MSK1-V5 kinase activity at later timepoints following IR (30-120 minutes after IR) in the presence of various kinase inhibitors. If a kinase is involved in the down-regulation of MSK1 kinase activity 30 minutes after IR, one would expect to observe prolonged IR-induced MSK1-V5 activation when this kinase is inhibited.

CREB and NF-κB, established MSK1 substrates, are both associated with cell survival in response to IR [51, 176-178], and importantly, NF-κB activation has been implicated in cancer cell resistance to radiation therapy [51]. Based on these observations, it is interesting to speculate that MSK1 may contribute to cell survival following IR through the regulation of CREB and NF-κB (p65) transcriptional activity.

In conclusion, although MSK1 and MSK2 have traditionally been viewed as redundant kinases, it is clear that MSK1 and MSK2 are differentially regulated by CK2 following UV radiation. In addition, various cellular stimuli, including IR, appear to differentially activate MSK1 and MSK2 [136, 141, 155]. Therefore, the characterization of MSK1 and MSK2 activation profiles in response to growth factor stimulation and IR, as well as examining NF-κB- and CREB-dependent transcription under these conditions, might elucidate further differences between MSK1 and MSK2. It is possible that MSK1 may have a novel role in the DNA damage response that has not yet been elucidated.
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