Investigation of Inducible Mitogen and Stress Activated Protein Kinase 1 (MSK1) and Histone H3 Phosphorylation by the RAS-MAPK Pathway in Cancer Cells

by

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THESIS ABSTRACT

The RAS-mitogen-activated protein kinase (MAPK) pathway is an essential signaling mechanism that regulates numerous cellular processes and culminates in the activation of specific gene expression programs. Alterations in the RAS-MAPK pathway by receptor over-expression or gain of function gene mutations can modify epigenetic programs and confer cell growth and/ or transformation advantages. In fact, deregulation of the RAS-MAPK signaling cascade is a key event in tumour development and about 30% of human cancers contain *RAS* mutations. In breast and pancreatic epithelial cancers, characterization of an aberrant RAS-MAPK signaling pathway has focused on upstream mediators such as receptors and oncogenic RAS molecules. However, the impact of downstream targets has not been fully explored.

Stimulation of the RAS-MAPK pathway leads to downstream activation of mitogen- and stress-activated protein kinases 1 and 2 (MSK1/2) which are responsible for the phosphorylation of histone H3 on S10 and S28. Herein, we postulate that the deregulation of the RAS-MAPK pathway produced by mitogen stimulation, or that the constitutive activation and/ or over-expression of upstream components consequently leads to enhanced MSK1 activity and elevated histone H3 phosphorylation levels. We further hypothesize that MSK1-mediated H3 phosphorylation is critical for immediate early gene (IEG) expression, *Ras*-driven transformation and associates with regulatory regions upon gene transcription.

Our studies focused on MSK-mediated H3 phosphorylation under the frame of inducible and constitutively activated RAS-MAPK signaling. As model cell systems, we used *Ras*-transformed mouse fibroblasts, human breast cancer cell lines with over-expressed receptors and

human pancreatic cancer cell lines with activating *K-RAS* mutations. We present evidence for the critical involvement of MSK1 and H3 phosphorylation as mediators that bridge the aberrant signals driven by the RAS-MAPK pathway with nucleosomal modifications, chromatin remodeling, IEG expression and malignant transformation in mouse fibroblasts. In order to verify our observations from murine cell model systems, we examined if activation of RAS-MAPK signaling in breast cancer cells elicits similar molecular events. We firstly demonstrate that the RAS-MAPK pathway is induced and consequently enhances the association of MSK1 and H3 phosphorylation on the IEG *Trefoil Factor 1* resulting in transcriptional activation. Secondly, we observed that mutated *K-RAS* expression did not correlate with genomic instability or altered signaling in pancreatic cancer cell lines in comparison to overexpressed HER2 and EGFR breast cancer cell lines which generally exhibit upregulated ERK1/2 and H3 phosphorylation levels.

Taken together, MSK-regulated gene expression may potentially be exploited by epithelial cancerous cells with abnormal MAPK signaling to acquire further growth/ transformation advantages. Our studies also contribute to the further understanding of MSK-mediated transcriptional activation in response to RAS-MAPK signaling in oncogene-transformed and cancer cell lines. As such, inhibition of MSK activity may be an unexplored avenue for combination cancer therapy with abnormal RAS-MAPK signaling pathways.

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A260	Absorbance at 260 nm
Ac	Acetylation
ACF	ATP-dependent chromatin assembly and remodeling factor
ADP	Adenosine diphosphate
α-MEM	Alpha-minimal essential medium
AP-1	Activator protein-1
ATP	Adenosine triphosphate
ATF1	Activating transcription factor-1
BAD	Bcl-2-associated death promoter
BAF155/170	BRG1/BRM-associated factor-155/170
BCEI	Breast cancer estrogen-inducible
BIM	Bcl-2 interacting mediator of cell death
BMK1	Big MAP kinase-1
bp	Base pair
BPTF	Bromodomain and PHD finger-containing transcription factor
BrdUrd	Bromodeoxyuridine
BRM	Brahma
BRG1	Brahma-related gene-1
CARM1	Coactivator-associated arginine methyltransferase-1
CBP	CREB-binding protein
CCA	Clonal chromosome aberrations
cdc25	Cell division cycle
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/Enhancer-binding protein

CHD1	Chromodomain helicase DNA binding protein-1
ChIP	Chromatin immunoprecipitation
Chk1	Checkpoint 1
CHRAC	Chromatin accessibility complex
CIN	Chromosomal instability
CML	Chronic myelogenous leukemia
COMPASS	Complex proteins associated with SET1
COX-2	Cyclooxygenase-2
CREB	cAMP response element-binding protein
CTKD	C-terminal kinase domains
DAPI	4V6-diamidino-2-phenylindole
Dlk	Death-associated protein (DAP)-like kinase
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DOT1	Disruptor of telomeric silencing 1
DRE	Dioxin response element
DUSP	Dual specificity kinase
EBS	Ets binding site
ECM	Extracellular matrix
EDTA	(Ethylenedinitrilo) tetraacetic acid
EGTA	[Ethylenebis (oxyethylenenitrilo)] tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EHMT2	Euchromatic histone-lysine N-methyltransferase-2
eIF-4F	Eukaryotic translation initiation factor 4F

EKLF	Erythroid Kruppel-like factor
ELP3	Elongator protein-3
ER	Estrogen receptor
ERBB	Erythroblastic leukemia viral oncogene
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase
ERRE	Estrogen receptor related element
ESET	ERG-associated protein with a SET domain
ETS	E-twenty six, transcription factor
EZH2	Enhancer of zeste homolog-2
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FLNA	Filamin A
FRA-1	FOS-related antigen-1
FSH	Follicle-stimulating hormone
Fyn	SRC family tyrosine-protein kinase
FU	Fluorouracil
GAP	GTPase activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCN5	General control nonderepressible-5
GDP	Guanine nucleotide diphosphate
GEF	Guanine nucleotide exchange factor
GI	Gastrointestinal
GLC7	GLyCogen-7
GR	Glucocorticoid receptor

GRB2	Growth factor receptor binding protein-2
GSK3β	Glycogen synthase-3β
GTP	Guanine nucleotide trisphosphate
H2BK123ub	Monoubiquitination of H2B at K123
H3 K9me	Histone H3 monomethylation on lysine 9
H3 S10ph	Histone H3 phosphorylation on serine 10
H3 S28ph	Histone H3 phosphorylation on serine 28
H3 T3ph	H3 phosphorylation on Thr3
H3 T11ph	H3 phosphorylation on Thr11
H4 K16ac	Histone H4 acetylation on lysine 16
H4 K20me3	Histone H4 trimethylation on K20
H89	N-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride
Haspin	Haploid germ cell-specific nuclear protein kinase
HD	Huntington's disease
HDAC1	Histone deacetylase-1
HER	Human Epidermal growth factor Receptor
HMGN1	High mobility group nucleosomal binding domain-1
HMGN1S6ph	HMGN1 phosphorylation on serine 6
HP1	Heterochromatin protein-1
HUVEC	Human umbilical vein endothelial cells
hTERT	Human telomerase reverse transcriptase
IEG	Immediate-early gene
IFN-α	Interferon-alpha
IGFR	Insulin receptors/insulin-like growth factor receptors

IKK-α	IkappaB kinase-alpha
IL-2	Interleukin-2
ING2	Inhibitor of growth family, member 2
IPL1	Increase in ploidy-1
ISWI	Imitation switch
JMJD2A	Jumonji domain containing 2A demethylase
JNK1/2/3	c-Jun N-terminal kinase-1/2/3
KAT	Lysine acetyltransferase
KHMTases	Lysine histone methyltransferases
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
LSD1	Lysine-specific histone demethylase-1
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MBT	Malignant brain tumor repeat/domain
Mcl-1	Myeloid cell leukemia 1
MEF2	Myocyte enhancer factor-2
MEK	Mitogen-activated protein kinase/ERK kinase
MEK1/2/5	MAP/ERK kinase-1/2/5
Min.	Minutes
MKK3/4/6/7	MAPK kinase-3/4/6/7
MKP	Mitogen-activated protein kinase phosphatase
MLL	Mixed lineage leukemia-protein complex
MLTK-α	Mixed lineage triple kinase-α
MMLV	Moloney Murine Leukemia Virus

MMTV	Mouse mammary tumour virus
MNase	Micrococcal nuclease
MSK1/2	Mitogen- and stress-activated protein kinase-1/2
MST1	Mammalian Sterile 20-like 1 kinase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MYST	MOZ, Ybf2/Sas3, Sas2 and TIP60
NAD	Nicotinamide adenine dinucleotide
NCCA	Nonclonal chromosomal aberrations
NF-κB	Nuclear factor-kappa B
NGF	Nerve growth factor
NIMA	Never in mitosis gene A
NLS	Nuclear localization sequence
NMDR	*N*-methyl-$_D$-aspartate receptor
NTKD	N-terminal kinase domains
NuRD	Nucleosome remodeling and deacetylase repressor
NURF	Nucleosome-remodeling factor
p90S6K	P90 ribosomal S6 kinases
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly ADP-ribose polymerase
PBS	Phosphate buffered saline
PCAF	P300/CBP-associated factor
PCR	Polymerase chain reaction
Ph	Phosphorylation
PHD	Plant homeodomain

PIM1	Proto-oncogenic serine/threonine kinase 1
PKA	Protein kinase A
PKB	Protein kinase B, Akt1
PKC	Protein kinase C
PP1	Protein phosphatase type 1
PP2	Protein phosphatase type 2
PR	Progesterone receptor
PRK1	Protein kinase C-related kinase 1
PRMT1	Protein arginine methyltransferase-1
PS2	Prenisilin 2
PTM	Post-translational modification
RAR	Retinoic acid receptor
RAS	Rat sarcoma
RB	Retinoblastoma protein
RLPK	RSK-like protein kinase
RNA	Ribonucleic acid
RNAPII S5ph	RNA polymerase II phosphorylated at serine 5
RNase	Ribonuclease
ROCK-II	Rho-dependent protein kinase-II
RSK	Ribosomal S6 kinase
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
RTK	Receptor tyrosine kinase
SAPK2	Stress-activated protein kinase-2
SCN	Suprachiasmatic nucleus

SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
SET1	Supressor of variegation, enhancer of zeste and trithorax-1
SET2	Supressor of variegation, enhancer of zeste and trithorax-2
shRNA	Short hairpin RNA
SKY	Spectral karyotyping
Snf1	Sucrose non-fermented 1
SOS	Son of sevenless
Sp1/Sp3	Specificity protein 1/3
SRC	Sarcoma
SRE	Serum responsive element
STAT3	Signal transducer and activator of transcription 3
SUC2	Sucrase-2 gene (yeast)
SUMO	Small ubiquitin-like modifier
SUV39H1/2	Suppressor of variegation 3-9 homolog-1/2
STAT3	Signal transducer and activator of transcription-3
SWI/SNF	Switch/sucrose non-fermentable
TBP	TATA-binding protein
TBS	Tris buffered saline
TE	Tris-EDTA
TEMED	N,N,N',N-tetramethylethylenediamine
TFF1	Trefoil factor 1
TG2	Tissue transglutaminase 2
TGF-β	Transforming growth factor-beta
TIP60	Tat-interactive protein

TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TNM	Tris-NaCl-$MgCl_2$ buffer
TPA	12-tetradecanoate 13-acetate
TRE	TPA responsive element
Tris	Tris(hydroxylmethyl)aminomethane
TTBS	Tris buffered saline with Tween-20
TSS	Transcription start site
uPA	Urokinase plasminogen activator
UV-B	Ultraviolet B
VDR	Vitamin D receptor
VEGF-A	Vascular endothelial growth factor-A
VRK-1	Vaccinia related kinase -1
ZIP	Zipper interaction protein

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Chapter 1: Introduction

1.1 Cell signaling and the cancer phenotype

Multicellular organisms evolve and maintain homeostasis by responding and adapting to their ecosystem. At the cellular level, neighboring cells require steadfast and efficient communication with each other as well as their microenvironment to enable proper function and constant adjustments to extracellular insults and changes. Cell signaling is thus an essential mechanism that cells use to integrate external cues with physiological responses. Disturbances in this regard lead to abnormalities that can either be advantageous or deleterious to the cell.

Signal transduction is a phrase originally coupled to the discovery of GTP binding proteins in the regulation of metabolism by Martin Rodbell in the 1970s. Generally, these are mechanisms that translate extracellular and/ or intracellular stimulation into a particular cellular or biological response. Classic initiators of signal transduction pathways were primarily ascribed to hormones and second messengers such as calcium and cyclic nucleotides that influence cellular physiology. Since then, diverse molecules and polypeptides such as growth factors, cytokines and reactive oxygen species are but a few of the numerous ligands or stimuli capable of eliciting specific cellular responses through signal transduction pathways. In the 1980s, Nobel laureates John Bishop and Harold Varmus first identified the human oncogene, c-*SRC* that encoded for a tyrosine kinase. Their pioneering work illustrated that retroviral oncogenes have the potential to drive malignant transformation and were capable of growth deregulation. Consequently, their prominent discovery led to the current understanding of cancer and the impact of aberrant signal transduction pathways on the disease.

The Rat sarcoma (RAS)-mitogen-activated protein kinase (MAPK) signal transduction pathway is a signaling mechanism that regulates and influences a wide array of cellular processes such as growth, survival, differentiation, migration and proliferation. Through the signaling cascade, extracellular stimuli are translated into nuclear responses via membrane receptors, effector kinases and downstream chromatin targets. It is therefore not difficult to imagine that homeostatic disruption of this pathway with amplifications, overexpression and mutations of signaling players either independently or concurrently result in changes to cellular processes they control. As such, activation and deregulation of the RAS-MAPK signaling plays a significant role in the oncogenesis and progression of cancer (Dunn et al, 2005).

Cells undergo multiple changes that confer malignant transformation: autonomy from growth stimulatory and inhibitory signals, avoidance of apoptosis, persistent replicative capabilities and acquisition of angiogenic, invasive and metastatic potential (Hanahan & Weinberg, 2000). Cancer cells manipulate various signaling routes like the RAS-MAPK pathway to gain a survival advantage by augmenting proliferation, enhancing migration and evading cell death (Roberts & Der, 2007). As a result, carcinogenesis via the RAS-MAPK pathway is often associated with poor patient prognosis. Increasingly, key members of signaling such as receptor tyrosine and effector kinases have continually been exploited as therapeutic targets and been the focus of studies to develop better diagnostic as well as prognostic strategies (Daub et al, 2004; Roberts & Der, 2007; Sebolt-Leopold & Herrera, 2004).

1.2 RAS-RAF-MEK-MAPK signal transduction pathway

Activation of the RAS signal transduction pathway involves a phosphorylation cascade that couples and amplifies messages received from cell surface receptors to the nucleus. Generally, stimuli such as growth factors, hormones, small molecules or peptides, cytokines and mitogens can bind their respective receptors. Upon ligand binding, the receptor's intrinsic tyrosine kinase activity is activated by autophosphorylation and dimerization. An adaptor complex, Shc/GRB2/SOS, is consequently recruited to specific phospho-tyrosine residues on the receptors which engages an inactive RAS to undergo conformational change and become the activated GTP-bound form (**Figure 1.1**). The activity of RAS proteins are controlled by the balance of GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). As GAP proteins activate the intrinsic GTPase activity of RAS and decreases the pool of GTP-bound form, this class of proteins represent an "off" switch. In contrast, GEF proteins increase RAS activity by facilitating GDP to GTP exchange (Mitin et al, 2005).

RAS enlists downstream effector kinases like RAF to the cell membrane. Upon activation, RAF proteins which are serine/threonine kinases can phosphorylate dual specificity kinases such as MEK (mitogen-activated protein kinase/ERK kinase) that subsequently activate MAPK such as ERK (extracellular-regulated kinases) via phosphorylation. ERK1/2 can phosphorylate another tier of kinases such as p90 ribosomal S6 kinases (p90S6K) and mitogen- and stress-activated protein kinases (MSK) 1/2, cytoplasmic proteins or transcription factors. Thus, this simplistic linear sequence of events underlies an amplification response involving serial phosphorylation, recruitment, protein-protein interactions and activation **(Figure 1.1).**

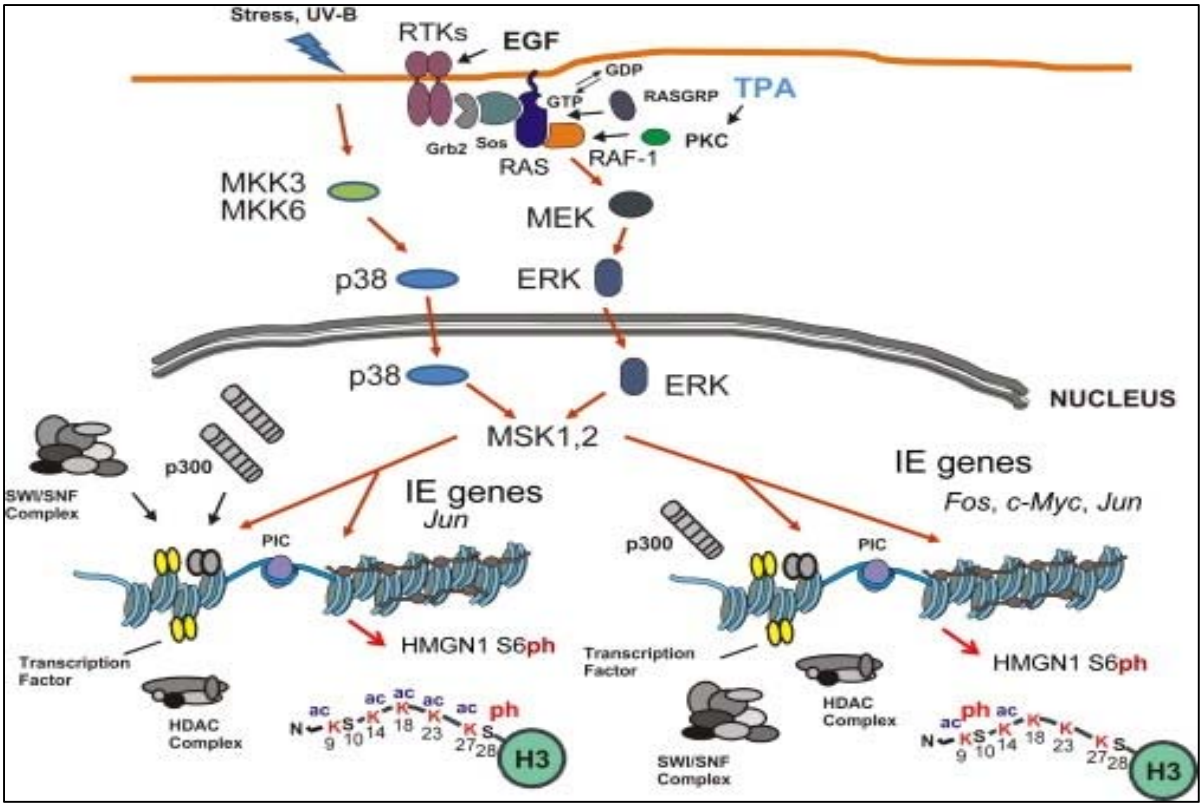

Figure 1. 1. The RAS-RAF-MEK-ERK-MSK Pathway

The RAS-MAPK pathway is activated by epidermal growth factor (EGF) and phorbol ester, 12-tetradecanoate 13-acetate (TPA). TPA acts through Protein Kinase C (PKC) and/or RasGRP. Ultraviolet (UV)-B activates both the RAS-MAPK and the p38 kinase pathways. MSK1/2 is activated and phosphorylates nucleosomal proteins including HMGN1, H3 S10 and H3 S28. Following H3 phosphorylation, chromatin remodelers (SWI/SNF) and lysine acetyltransferases (KATs) (eg., p300) are recruited to promoter and remodel chromatin in preparation for transcription. The result of signaling is an alteration in the pattern of gene expression programs. Immediate early genes (IEG) such as *Fos, Jun, Cox-2* and *Trefoil factor 1* (*TFF1)* are transcribed in response to the RAS-MAPK pathway for growth, proliferation, migration and differentiation. *The figure was reproduced with permission from Figure 1 (Davie et al, 2010).*

Complexity in the cascade occurs when various cytoplasmic kinases such as Sarcoma (SRC) and AGC family kinases or membrane-bound effectors such as adenyl cyclases converge and activate the RAS-MAPK pathway via crosstalk mechanisms (Castoria et al, 2008; Lowes et al, 2002). These crosstalks can elicit a multitude of cellular responses depending on the target proteins that not only demonstrate the diversity but also exemplify the promiscuity of signaling.

1.2.1 RAS

At the forefront of the MAPK pathway is the RAS protein. Initially identified as a rat sarcoma transforming oncogene, RAS is a small 21 kDa guanine nucleotide binding protein with intrinsic GTPase activity. RAS has 3 main members that have cell type-specific expression patterns: H-RAS (Harvey), N-RAS and K-RAS (Kirsten) (Downward, 2003). Mouse knockout studies have shown that *KRas* is essential for development whereas *NRas* and *HRas* are not required (Downward, 2003). K-RAS is expressed in most cell types whereas N-RAS is named after identification of the gene in human neuroblastoma cells. As chief molecular switches, RAS alternates between a GTP-bound active and a GDP-bound inactive form through interactions with GEFs and GAPs respectively (Malumbres & Barbacid, 2003). As such, RAS proteins are capable of producing numerous signaling responses based on stimuli, downstream effectors and cell type. Because of the integral role RAS has in cell signaling networks, it is frequently mutated in cancer. About 30% of all human cancers demonstrate activating mutations in the *RAS* oncogene (Bos, 1989).

1.2.2 ERK1/2 MAP kinases

Downstream of RAS, activation of the pathway converges on MAPK members. These are terminal serine/threonine family of kinases with 4 major groups: ERK1/2, p38 α/β/γ/δ, c-Jun N-terminal kinases 1/2/3 (JNK1/2/3) and atypical MAPKs like ERK5. Of these, ERK1/2 MAPKs respond to growth factors, cytokines, mitogens and tumour promoters (TPA, 12-tetradecanoate 13-acetate). Stress, cytokines and growth factors activate other MAPK family members (Raman et al, 2007). ERK1/2 MAPKs have over 160 target substrates that are cytoplasmic or nuclear

proteins. Consequently, these target proteins promote various cellular functions including proliferation, growth, migration, inflammation and differentiation (McCubrey et al, 2007; Yoon & Seger, 2006). Among the numerous ERK1/2 targets are transcription factors such as cAMP response element-binding (CREB) and Elk-1, steroid receptors such as estrogen receptors and kinases like MSK1/2 and p90S6K or Ribosomal S6 kinase (RSKs). (**Figure 1.2)** Hence, the RAS signal transduction pathway propagate various cellular responses though the action of ERK1/2 MAPKs.

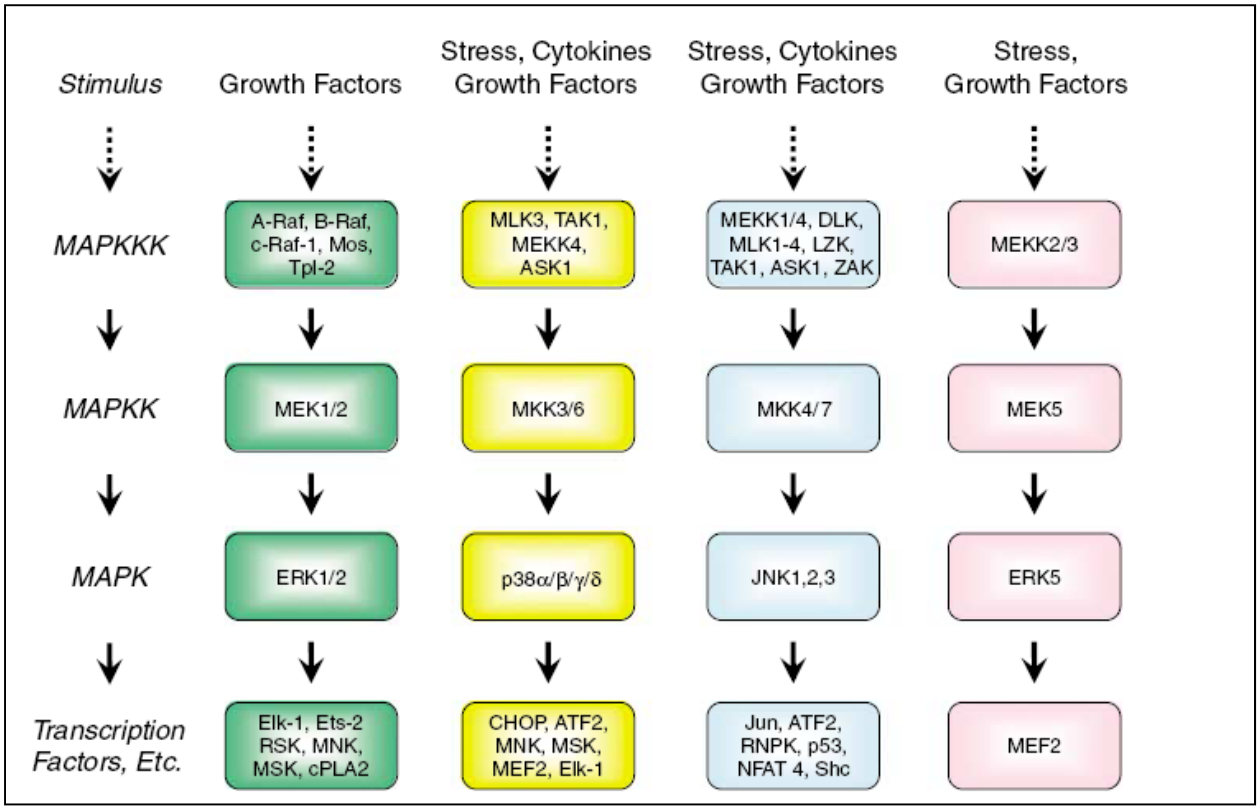

Figure 1. 2. MAPK Cascades in Mammalian Systems

There are four major mammalian MAPKKK–MAPKK–MAPK protein kinase cascades. The ERK pathway is commonly activated by growth factors whereas the JNK, p38 and ERK5 pathways are activated by environmental stress, including osmotic shock, ionizing radiation. *The figure and text were reproduced with permission from Figure 2 (Roberts & Der, 2007)*

1.2.3 Influence of RAS-MAPK pathway on cellular processes

Apart from the classical linear cascade, individual components of the RAS-RAF-MEK-ERK pathway can exert pleiotropic effects on cellular processes that impact biological functions (**Figure 1.1**). These effects can be positive or negative depending on the stimuli used, the cell type and lineage. Consequently, these effects are associated with specific gene expression programs. For example, RAS can regulate proteins such as $p15^{Ink4a}$, $p16^{Ink4b}$ and $p21^{Cip1}$ which are checkpoint and tumor suppressor proteins that control passage through G1 or senescence in cell cycle progression (Chin et al, 1999; McCubrey et al, 2007). In response to growth factors and mitogens, RAS phosphorylates RAF-1 that then activates cyclin-dependent kinases, phosphatases such as "cell division cycle" (cdc)-25 and tumour suppressor retinoblastoma (Rb) proteins which also impact cell cycle progression (Wellbrock et al, 2004). Contingent on the strength and duration of growth factor stimulation, ERK1/2 can either induce proliferation or differentiation in PC12 cells (Marshall, 1995). In apoptosis regulation, RAF proteins are potent effectors of mitochondria-localized proteins such as Bcl-2-associated death promoter (BAD) protein (Wellbrock et al, 2004). ERK1/2 can phosphorylate and target apoptotic components such as myeloid cell leukemia (MCL)-1, Bcl-2 interacting mediator of cell death (BIM) and caspases (McCubrey et al, 2007). Depending on the effects of the target protein, interactions and localization, the RAS-MAPK cascade can impact survival or apoptosis through crosstalks. Stimulation of ERK1/2 to activate downstream kinases such as p90S6K RSKs can trigger cell motility responses through phosphorylation of cytoskeletal proteins such as filamin A (FLNA) (Anjum & Blenis, 2008). Further, p90S6K RSKs phosphorylate substrates that influence cell growth and proliferation such as glycogen synthase-3β (GSK3β) and $p27^{kip1}$ (Anjum & Blenis, 2008; Hauge & Frodin, 2006). ERK1/2 phosphorylation of MSK1/2 kinases has been linked to

long term memory potentiation and stress responses through chromatin remodeling and modulation of gene expression (Borrelli et al, 2008; Brami-Cherrier et al, 2009; Chwang et al, 2007; Reul & Chandramohan, 2007). Further, RAS-MAPK pathway-induced phosphorylation of transcription factors Elk-1, c-Fos, c-Jun and p53 as well as immediate early gene (IEG) expression have been shown critical for cell proliferation and oncogenic transformation (Shaul & Seger, 2007; Yoon & Seger, 2006).

1.2.4 RAS-MAPK pathway in cancer

With considerable focus on the involvement of RAS-MAPK signaling in cellular processes, the contribution of the pathway in malignancies is well recognized. As mentioned earlier, mutations in *RAS* are found in 30% human cancers. The frequency of *RAS* activating mutations, particularly *K-RAS*, has been observed highest in epithelial cancers such as adenocarcinoma of the pancreas (90%), colon (50%) and lung (30%) (Downward, 2003). Likewise, *N-RAS* mutations are frequently observed in melanoma, liver and myeloid neoplasms while *H-RAS* mutations are evident in bladder malignancies (Downward, 2003). Furthermore, persistent activation of the RAS-MAPK pathway not only results from activating mutations in the *RAS* gene but also mutations in *B-RAF* gene or defective or overexpressed cell surface receptors acting through this pathway (Calipel et al, 2003; Dunn et al, 2005). Activating mutations in the downstream effector kinase B-RAF are found in high frequency in melanoma (70%) and thyroid papillary cancer (50%). Also, overexpressed tyrosine kinase receptors are rampant in malignancies of the lung, pancreas and colon (Roberts & Der, 2007). Thus, it is proposed that a great majority of human malignancies have aberrant RAS-MAPK pathway that can be targeted for therapy (Aoki et al, 2008). As an example, tyrosine receptor kinase inhibitors

such as Gleevec or imatinib mesylate are currently in use for treatment of gastrointestinal stromal tumours and chronic myeloid leukemia.

Though the prospect of directed therapy against one signal transduction pathway is pharmacologically attractive, tumorigenesis and subsequent cancer spread is a multi-step process. It has been demonstrated that specific components of the RAS-MAPK pathway are effective initiators of tumorigenesis but insufficient to facilitate complete transformation and metastasis in some models of neoplastic disease (Chambers & Tuck, 1993; Egan et al, 1991). Further, ongoing studies directed at developing inhibitors for tyrosine kinase receptors like epidermal growth factor receptor (EGFR), RAS and RAF have displayed limited efficacy in treatment of various neoplasias (Daub et al, 2004; Roberts & Der, 2007). Hence, there is a great need to elucidate and understand the mechanisms underlying the RAS-MAPK pathway and to expand the current repertoire of biomarkers and therapy modules particularly in cancers.

1.2.5 Role of K-RAS and effectors in pancreatic cancer

Pancreatic cancer has the 4th highest mortality rate with an estimated 3,900 new cases (about 2.2% of all diagnosed cancers) in 2009 (Committee, 2009). It also has the lowest relative survival rate (6%) compared to cancers of the lung, prostate and breast. Patients afflicted with the disease are typically late-presenting and thus have poor prognoses due to the lack of early diagnostic methods. Current treatment modalities for advanced and metastatic pancreatic adenocarcinomas includes gemcitabine monotherapy and/or chemoradiotherapy (Yip et al, 2006). Targeting tyrosine kinase receptors with small molecular inhibitors such as erlotinib have also been used for treatment. However, the scarcity of biomarkers and limited therapeutic

approaches necessitate development of better detection and targeted treatments (He et al, 2008; Yip et al, 2006).

Greater than 90% of pancreatic adenocarcinomas manifest mutations in the *K-Ras* gene, which is among the highest frequency in human cancers (Almoguera et al, 1988; Downward, 2003). The incidence of somatic *K-RAS* mutations accumulate from an initial lesion of pancreatic intraepithelial adenocarcinoma to an invasive disease stage and often observed with other mutations such as for *TP53, P16* and *SMAD4* genes in later histological stages of the disease. Thus, this suggests that the appearance of oncogenic K-RAS occurs early in the disease progression (Bardeesy & DePinho, 2002; Maitra et al, 2006; Yeo et al, 2002). Of note, a substituted glycine to valine residue on codon 12 accounts for the majority of *K-RAS* gene mutations (Aoki et al, 2008; Grunewald et al, 1989; Smit et al, 1988). Further, point mutations on codons 13 and 61 can also lead to amino acid alterations that render the protein constitutively active through inactivation of its GTPase activity (Maitra et al, 2006). In pancreatic cancer, the presence of a *K-RAS* mutation has been associated with adverse effects on patient survival (Lee et al, 2007).

Other components of the RAS-MAPK pathway that can lead to deregulated signaling cascades have been detected in pancreatic cancer. EGFR and tyrosine kinase receptors that bind growth factor ligands are overexpressed in pancreatic adenocarcinoma and may be predictive of sensitivity to monoclonal antibody treatments (Bardeesy & DePinho, 2002; Furukawa, 2008; Huang et al, 2003). However, detection of EGFR gene amplifications and activating mutations in pancreatic cancer showed no significant impact on patient survival (Lee et al, 2007). Further, *B-*

RAF somatic gene mutation V599E was also observed in a proportion of pancreatic cancers carrying *K-RAS* mutations though the functional consequence of harbouring both mutations are unknown (Calhoun et al, 2003; Ishimura et al, 2003). Surprisingly, evaluation of ERK1/2 in pancreatic cancer cell lines harbouring *K-RAS* mutations or expressing ectopic oncogenic K-RAS showed no dramatic increase in activity or expression (Matsuo et al, 2009; Yip-Schneider et al, 1999). This observation has been consequently attributed to an increase in opposing mitogen-activated protein kinase phosphatase-2 (MKP-2) and dual specificity kinase (DUSP-6/ MKP-3) expression in pancreatic cancer cell lines and pancreatic precursor lesions, including dysplastic/*in situ* carcinoma cells respectively (Furukawa et al, 2003; Matsuo et al, 2009; Yip-Schneider et al, 2001). Both MKP-2 and DUSP-6 are negative regulators of ERK1/2 MAPK. Further, ectopic expression of constitutively active K-RAS elevates paracrine factors such as vascular endothelial growth factor (VEGF) and CXC chemokines that promote angiogenesis and invasiveness of pancreatic ductal epithelial cells through the RAS-MAPK pathway (Matsuo et al, 2009). Together, there is evidence to suggest that K-RAS and effectors downstream are important for pancreatic cancer disease progression.

1.2.6 Role of EGFR, HER2 and upregulated effectors in breast cancer

Breast cancer is the most prominent malignancy in Western women today. Among Canadian women, breast cancer leads in incidence and second highest cause of cancer mortality (Committee, 2009). At the earliest stage, estrogens play a critical role in the development and proliferation of the disease mainly through the actions of the estrogen receptor (ER). Hormone-responsive breast cancer cells are dependent on the ER pathway to stimulate the expression of genes important for proliferation and growth (O'Lone et al, 2004). As the disease progresses

towards an aggressive phenotype, breast cancer cells tend to develop hormone independence, acquire resistance to endocrine therapies and display upregulated growth factor signaling pathways. These factors have been shown to correlate to poor patient prognosis. It has been demonstrated that overexpressed growth factor receptors promote degradation of the ER. As such, the RAS-MAPK signaling pathway through overexpressed growth factor receptors may play a role in the development of hormone independence (O'Lone et al, 2004; Oh et al, 2001; Osborne et al, 2005). Current therapies involving autonomous and endocrine resistant tumours exploit the upregulated, cell surface growth factor receptors, by targeting the RAS-MAPK pathway as the primary course of treatment (Daub et al, 2004).

Mutations or alterations in the *RAS* gene rarely occur in breast tumours and represent less than 5% of these malignancies (Bos, 1989; Downward, 2003). In fact, elevated RAS levels in breast tumour samples do not correlate with clinical parameters. Despite this, overwhelming evidence support deregulated RAS signaling in breast neoplasias through the actions of membrane growth factor receptors such as erythroblastic leukemia viral oncogene/ human epidermal growth factor receptors (ERBB/HER/EGFR). EGFRs are cell surface tyrosine kinase receptors containing four closely related members: EGFR (erbB1 or HER1), ERBB2 (HER2/neu), ERBB3 (HER3) and ERBB4 (HER4). Under normal circumstances, when extracellular growth factors such as EGF are bound by these receptors through their extracellular domains, the receptors undergo dimerization and transmit the signals through autophosphorylation of their C-terminal domains. These receptors then activate adaptor proteins and effector kinases downstream which stimulate RAS-RAF-MEK-ERK pathway.

In normal mammary development, the expression and involvement of different EGFR family members dictate their various functional roles in cell growth and development. Among the EGFR receptors, the clinical significance of HER2 is best understood. In breast cancer development, upregulation of the RAS–MAPK signaling cascade occurs as a consequence of HER2 overexpression. HER2 overexpression is found as high as 30% in breast tumours. Further, HER2 overexpression or amplification strongly correlates with a subset of poorly differentiated ductal carcinoma *in situ* (Cooke, 2000). Notably, HER2 does not need ligand stimulation to be in active conformation and forms heterodimers with other ligand-activated members of the EGFR family (Davie et al, 2010). Thus, HER2 overexpression is capable of sustaining an activated RAS-MAPK and has been shown to exhibit an inverse correlation with the expression of ER. Such properties present important implications for patients exhibiting HER2 overexpression as it relates to the elevated oncogenic and chemoresistant features of these tumours. Moreover, HER2 overexpression is characteristic of hormone-independent tumours with poor clinical outcomes and has consequently been useful as a prognostic factor and for the development of targeted therapeutic approaches (Harari & Yarden, 2000).

The insulin receptors/insulin-like growth factor receptors (IGFR) also channel into the RAS-MAPK pathway in breast cancer (Hamelers & Steenbergh, 2003). These receptors respond to insulin and insulin-like ligands. Overexpression of factors involved in IGFR signaling cascade influence breast tumour growth, proliferation, transformation, endocrine resistance as well as patient survival outcomes (Surmacz, 2000). Insulin-like growth factor can also activate the ER via ligand-independent phosphorylation through the RAS-MAPK and enhance the transcriptional potential of the steroid hormone without binding estrogens (Kato et al, 1995).

SRC, an intracellular nonreceptor tyrosine kinase, can also trigger the initiation of the RAS-MAPK pathway in breast cancer through EGFR. Although overexpressed or activated in 70% of breast cancer tumours and cell lines, SRC in itself is not solely responsible for inducing cell transformation events. Instead, SRC can promote tumourigenesis by enhancing signals through the RAS-MAPK pathway (Biscardi et al, 2000). SRC activity often synergizes with HER overexpression in response to growth factors as it can associate with ER to activate RAS signaling upon estrogen stimulation (Migliaccio et al, 1998). Such interactions highlight crosstalk mechanisms that enable multiple pathway stimulation which elevate the risk of breast tumourigenesis.

Another molecule that can sustain an activated RAS-MAPK pathway in breast cancer cells is the adaptor protein, growth factor receptor-bound protein (GRB)-2. Differential expression of GRB2 was observed in normal immortalized breast epithelial compared to breast cancer cell lines suggesting that the adaptor molecule may play a critical role in cancer pathogenesis (Malaney & Daly, 2001).

ERK1/2 MAPK is perhaps the most extensively studied factor involved in the RAS signaling cascade which has been observed to not only regulate the progression, proliferation and hormone hypersensitivity of breast cancer tumours, but also promote the development of hormone-independence. The elevated expression and activity of active ERK in primary breast tumours and tissues is frequently a direct consequence of overexpression or activation of upstream regulators (Adeyinka et al, 2002; Sivaraman et al, 1997; Xing & Imagawa, 1999). For

example, the immortalized estrogen-independent MDA MB 231 cell line which has an amplified HER2 and mutated *K-RAS* gene exhibits a constitutively activated ERK (Malaney & Daly, 2001). Moreover, transient or persistent activation of ERK have been demonstrated in both estrogen-dependent and estrogen-independent breast cancer cells in response to growth factors, insulins, phorbol esters, hormones, estradiol and to long-term estradiol deprivation (Boulton et al, 1991; Hamelers & Steenbergh, 2003; Santen et al, 2002). However, the effects that activated ERK produces in hormone-dependent cells is elicited either by 1) direct phosphorylation and activation of the ER and gene expression, 2) driving constitutive cell production of growth factors that further upregulate the RAS-MAPK pathway or 3) activating membrane associated ER that further activate ERK allowing for nongenomic effects (Santen et al, 2002). In breast cancer cell lines transfected with constitutively active upstream kinases such as RAF-1 and MEK, the hyperactivation of ERK permits the cells to grow in the absence of estrogens and creates an ERα-negative phenotype that is the hallmark of aggressive and hormone-independent malignancies (Oh et al, 2001).

1.2.7 Aberrant characteristics of oncogene-transformed mouse fibroblasts

Much of our current understanding of the role of RAS and oncogenes in neoplastic transformation and metastasis has arisen from studies conducted in mouse models and murine fibroblast cell lines. Introduction of c-*HRas1* oncogene in C3H/10T1/2, NIH 3T3 and primary rat fibroblasts resulted in enhanced transformation, acquisition of tumourigenic potential and anchorage-independent growth (Egan et al, 1987a; Manoharan et al, 1985; Pozzatti et al, 1986; Thorgeirsson et al, 1985). Thus, *H-Ras* expression in these cells correlated with metastatic efficiency and predisposition to malignant phenotypes. Anti-p21 antibodies that target RAS

proteins caused reversion of transformation which indicates that RAS protein is essential in the maintenance of malignancy (Chin et al, 1999; Feramisco et al, 1985; Wong & Chin, 2000). Expression of other oncogenes such as *Mos, Raf, Fms, Src* and *Fes* are also capable of metastatic phenotype induction in these permissive cell lines signifying that the regulation and maintenance of metastasis may occur through growth factor signaling (Egan et al, 1987b).

Cancer cells exhibit abnormalities in nuclear morphology and architecture as well as chromatin structure. These alterations may contribute to cytological identification and diagnosis of some neoplasias (Davie et al, 2008; Zink et al, 2004). Gross and minute alterations in nuclear structure thus reflect the abnormal functional state of the cells. In oncogene-transformed mouse fibroblasts, nuclear changes parallel that of *Ras*-mediated transformation resulting in rigid spherical nuclear shapes, deep nuclear membrane clefts, coarse heterochromatic appearance and altered nuclear matrix profiles (Fischer et al, 1998; Samuel et al, 1997). These observed changes not only correlate with the degree of RAS expression but also with metastatic potential of the transformed fibroblasts. Further, *Ras*-transformed mouse fibroblasts have increased sensitivity to nucleases and exhibit decondensed chromatin structure that may impact gene expression profiles and epigenetic programming (Chadee et al, 1999; Chadee et al, 1995; Laitinen et al, 1990).

Examination of chromatin-associated proteins, nucleosomal targets and karyotypic organization revealed further differences between parental and *Ras*-transformed mouse fibroblasts. Steady-state levels of phosphorylated H3 at serine 10 and 28, $H1^{s}$-3 (formerly H1b) and high-mobility group nucleosome binding domain (HMGN)-1 phosphorylation are greater in *Ras* and oncogene-transformed cells (Chadee et al, 1999; Chadee et al, 1995; Dunn & Davie,

2005; Dunn et al, 2009). Further, the steady state levels of ERK1/2 and MSK1 activity attributed to a constitutively activated RAS signaling are also heightened despite similar phosphatase activities (Chadee et al, 1999; Drobic et al, 2004). In *Ras*-transformed Ciras-3 cells, there is delayed timing of TPA-mediated H3 phosphorylation and higher incidence of chromosomal instability (Dunn et al, 2009; Strelkov & Davie, 2002). As such, the presence of a *Ras* oncogene in immortalized parental 10T1/2 mouse fibroblasts caused an amplification of the signaling cascade that perhaps accounts for the aberrancy in nucleosomal responses, gene expression and epigenetic programming.

1.3 Mitogen- and Stress-Activated Protein Kinase1/2

1.3.1 Activation, signaling and substrates

Mitogen- and stress-activated protein kinases (MSK)-1/2 are ubiquitously expressed Ser/Thr kinases that lie downstream of MAPK cascade (**Figure 1.1**). These effector kinases are activated by the RAS-MAPK signaling pathway in response to mitogens, growth factors, stresses and phorbol esters. They are members of the AGC kinase superfamily and are primarily localized in the nucleus. Initially identified as a RSK-like protein kinase (RLPK), MSK contains 40% amino acid similarity with RSK and shares several similar structural domains, conserved phosphorylation sites, mechanism of activation and protein targets (Anjum & Blenis, 2008). Unlike RSKs however, MSKs can be activated directly by both ERK1/2 and p38 MAPK upon stimulation. MSKs are fairly conserved proteins. Mammalian MSK1 and MSK2 share approximately 64% identity in amino acid sequence, where human MSK1 and MSK2 exhibit 75% in amino acid sequence identity. Human MSK1 and MSK2 are 802 and 705 amino acids in length respectively whereas mouse MSK1 and MSK2 are 863 and 773 amino acids in length

respectively (Deak et al, 1998). Additional MSK orthologues in various organisms such as *Danio rerio, Xenopus, Caenorhabditis elegans* and *Drosophila melanogaster* have been identified (Arthur, 2008).

Encoded by two genes, MSK1/2 proteins contain two functionally different kinase domains separated by a conserved linker region, namely, the N- and C-terminal kinase domains (NTKD/ CTKD) (Dunn et al, 2005) (**Figure 1.3**). The C-terminal tail has important regulatory functions as it contains the nuclear localization sequence (NLS) and the MAPK docking and autoinhibition sites. Inactivating mutations in the C-terminal domain abolishes MSK function (Deak et al, 1998). Both the RAS-MAPK and p38 stress kinase signal transduction pathways are able to activate MSKs *in vivo*. Depending on the stimuli, activated ERK1/2 or p38 binds MSK via the MAPK docking sequence (D domain) and phosphorylates Ser360, Thr 581 and Thr700 of the CTKD. The direct phosphorylation of Thr581 (mouse Thr645) by either MAPKs is essential for the activation of MSK1. Although both ERK1/2 and p38 are able to phosphorylate MSKs, the relative involvement of each pathway is dependent on the cell type and stimuli (Davie, 2003). The CTKD then autophosphorylates Ser212 in the N-terminal activation loop of the NTKD region as well as Ser376 (mouse Ser375) and Ser381 in the linker region (hydrophobic motif) to fully activate MSK (Hauge & Frodin, 2006; Vermeulen et al, 2009). Consequently, the NTKD autophosphorylates additional serine residues in the C-terminus tail (McCoy et al, 2005) and phosphorylates a plethora of substrates. These substrates include transcription and translation factors, structural, nucleosomal and apoptotic protein targets (Arthur, 2008; Vermeulen et al, 2009) (**Figure 1.3**).

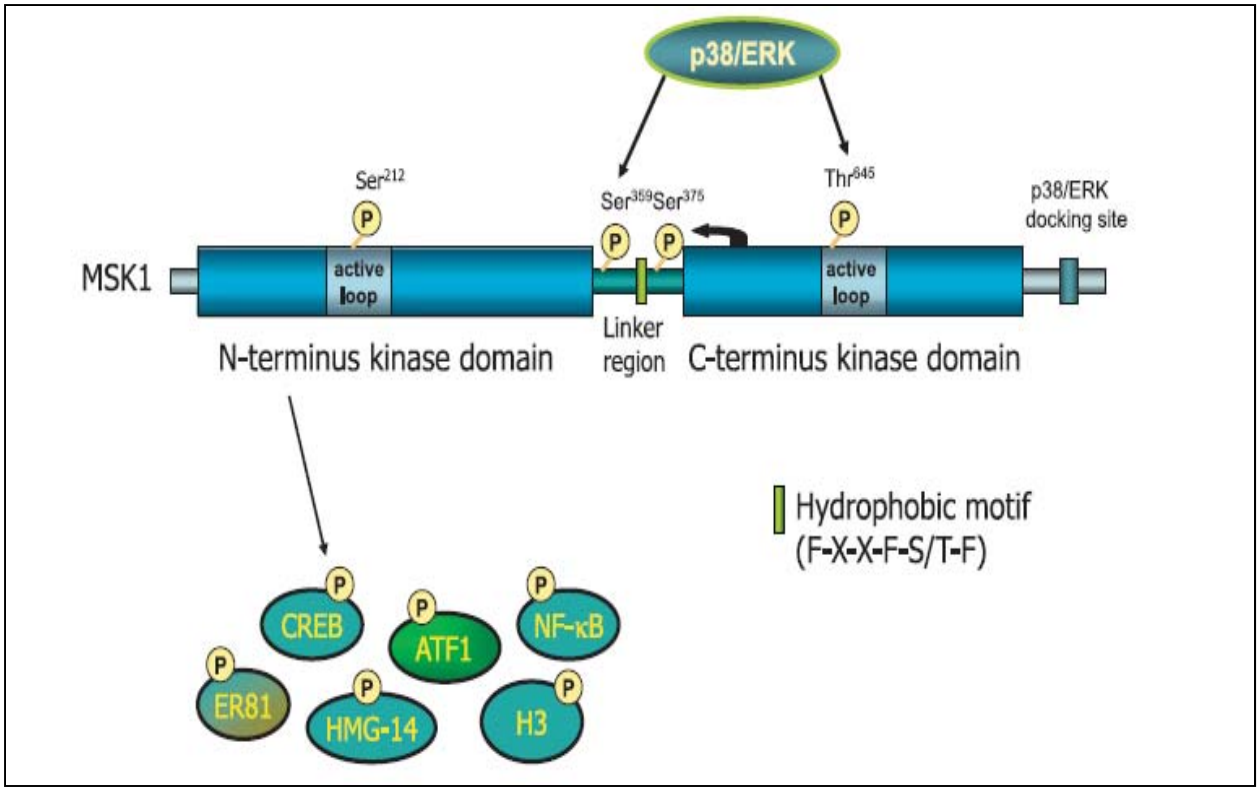

Figure 1. 3. Mouse MSK1 Structure, Activation Sites and Target Substrates

MSK1 is phosphorylated by active ERK and/or p38 stress kinase at threonine 654 and serine 359 residues to promote kinase catalytic activity in response to multiple stimuli. MSK1 then phosphorylates downstream substrates such as transcription factors CREB, ATF1 and p65 subunit of NF-κB aswell as histone H3 at serine 10 and serine 28. *The figure and text were reproduced with permission from (Dunn et al, 2005)*

Although MSK substrates do not contain a specific recognition sequence, the target phosphorylated serine and certain threonine residues in these proteins are generally found within a cluster of basic residues containing a RX/XS consensus. Among the substrates that have been extensively characterized are CREB activated transcription factor (ATF)-1, the p65 subunit of the nuclear factor-kappa B (NF-κB), signal transducer and activator of transcription (STAT)-3, ER81, 4E-BP1, HMGN1, histone H3 and BAD (Vermeulen et al, 2009). Diverse physiological and pathological stimuli have been reported to stimulate the activation of MSKs including

growth factors, progestins, arsenite, anisomysin, UV-B, neuromodulators, light, exercise, cocaine and phorbol esters (Brami-Cherrier et al, 2009; Vermeulen et al, 2009; Yu et al, 2003). Although the identification and use of pharmacological kinase inhibitors and double knockout mice have been invaluable in deciphering the role of MSK1/2 in cellular processes, its role in mediating effector functions is not fully understood. At the very least, the contribution of MSKs in mediating transcription and cellular physiology is achieved by its downstream protein targets.

1.3.2 The role of MSK in gene expression and transcription

The role of MSKs in gene expression and transcriptional regulation was primarily proposed through its kinase activity targeted towards phosphorylation of transcription factor substrates (Deak et al, 1998; Wiggin et al, 2002). Potent selective pharmacological inhibitors H89 and Ro 318220 against MSK1/2 and RSK2 respectively provided the initial evidence that MSK1/2 had a significant function in CREB and ATF1 phosphorylation as well as regulating transcriptional responses (Deak et al, 1998; Thomson et al, 1999). H89, a member of the H-series of protein kinases is a potent inhibitor of MSK but is equally potent against PKA and Rho-dependent protein kinase (ROCK)-II while Ro 218220, a bisindoylmaleimide, is initially developed to inhibit PKC but equally potent against MSK1 and RSK (Davies et al, 2000). Subsequent studies using MSK1/2 double knockout mice identified functional and *bona fide* molecular targets (Arthur, 2008). In fibroblasts, mitogen- and stress-induced activation of MSK1/2 phosphorylates Ser133 of CREB and Ser63 of ATF1, both of which are required for the full activation and expression of target genes such as *c-Fos, Mkp-1, Nur77, Nurr1, Nor1* and *JunB* (Darragh et al, 2005; Hauge & Frodin, 2006; Wiggin et al, 2002). Likewise, MSKs phosphorylate Ser276 of the NF-κB p65 subunit in response to tumor necrosis factor (TNF)-α to

enhance the interaction of NF-κB with KATs such as the CREB-binding protein (CBP) and p300 (Vermeulen et al, 2009). Moreover, MSK1 can phosphorylate the retinoic acid receptor (RAR)-α via the p38 MAPK cascade to enhance RAR-α recruitment and activate gene transcription (Bruck et al, 2009).

To date, the inclusion of nucleosomal proteins histone H3 and HMGN1 among MSK1/2 substrates has validated its role in regulating transcriptional responses (**Figure 1.3**). MSK1/2 phosphorylation of H3 on Ser10 and Ser28 (H3 S10ph and H3 S28ph respectively) is associated with gene activation in response to a multitude of stimuli. Interestingly, stimuli-induced HMGN1 phosphorylation at Ser6 is also catalyzed by MSKs and corresponds to IEG expression (Soloaga et al, 2003). However, the functional consequence by which MSK1/2 phosphorylates HMGN1 to parallel gene induction is contrary to that of histone H3. HMGN1 is a structural protein that binds nucleosomes and consequently reduces histone accessibility without altering higher order chromatin compaction (Hill et al, 2005). The phosphorylation of HMGN1 by MSKs reduces HMGN1 affinity to bind nucleosomes which allows MSK1/2 to further access H3 Ser residues and promote gene activation. The mechanism is possibly through facilitating chromatin remodeling and subsequent recruitment of transcription complexes (Lim et al, 2004).

Studies using the chromatin-incorporated, mouse mammary tumour virus (MMTV) transgene have advanced the current understanding of the role of MSK1 in chromatin remodeling. Upon progestin treatment, ERK phosphorylates both the progesterone receptor (PR) and MSK1 to form an ERK-PR-MSK1 complex which is then recruited to the essential regulatory nucleosome on the MMTV promoter. Chromatin-bound MSK1 is then able to

phosphorylate H3. Chromatin immunoprecipitation (ChIP) assays further demonstrate that following H3 S10ph, heterochromatin protein 1 (HP1) occupancy on the MMTV promoter is lost. Brahma related gene 1 (BRG1), (the ATPase subunit of the SWI/SNF remodeler) and RNA polymerase II are then rapidly recruited. Thus, MSK1 plays a pivotal role in the integration of steroid hormone- and kinase-mediated signaling cascades required for MMTV transcriptional activation (Vicent et al, 2006; Vicent et al, 2008).

1.3.3 The role of MSK in immunological responses

The role of MSK1/2 in modulating immunological responses is attributed largely to its transcriptional regulatory effects on modulating CREB activity and the p65 subunit of NF-κB. MSK activation has been reported to mediate *Cyclooxygenase-2* (*Cox-2*) expression induced by lipopolysaccharide (LPS) stimulation in RAW 264 macrophages (Caivano & Cohen, 2000). In addition, the loss of MSK-induced CREB phosphorylation in MSK1/2 double knockout mice exhibited decreased splenic T cell numbers which correlated with reduced interleukin-2 (IL-2)-dependent T cell proliferation assays. Despite this defect, no deficiencies in T cell development were observed (Kaiser et al, 2007). Investigation into the immune response of MSK1/2 double knockout mice also demonstrated an impaired regulatory response to Toll-like receptor (TLR) ligands. Macrophages derived from these mice had an impaired anti-inflammatory response to LPS, contained elevated levels of proinflammatory cytokines such as TNF-α, IL-6 and IL-12 and had hypersensitive responses to LPS-induced endotoxic shock. Of note, MSK1/2 deficient macrophages had decreased DUSP-1 and IL-10 expression. Both are required for the regulation and resolution of inflammatory responses (Ananieva et al, 2008). In response to glucocorticoids, nuclear MSK1 redistributed in the cytoplasm and exhibited an impaired inflammatory nuclear

factor kappa B (NF-κB)-driven gene profile (Beck et al, 2008). This MSK1 translocation effect is mediated by a glucocorticoid-dependent nuclear export mechanism which counteracts MSK1 function in promoting inflammatory responses. Thus, development of MSK small molecular activators able to mimic the anti-inflammatory effects of glucocorticoids could lead to a viable drug therapy for acute or chronic inflammatory disorders such as septic shock and psoriasis, respectively (Cohen, 2009; Funding et al, 2006).

1.3.4 The role of MSK in neuronal function and behaviour

MSKs are highly expressed in the brain. MSK expression is enriched in neuronal nuclei and in sections of the striatum, amygdala, olfactory tubercle neurons and hippocampal neurons (Brami-Cherrier et al, 2009). Despite the abundance of MSK in these cells and tissues, initial studies characterizing MSK1/2 single and double knockout mice demonstrated no visible phenotypic defects. Further, the MSK knockout mice were fully viable and fertile (Wiggin et al, 2002). Although there was no visible abnormal brain morphology, closer investigation of the mice revealed adverse behavioural deficiencies in adaptation learning, fear conditioning and long term memory potentiation.

MSK1/2 effects on cerebral functions and synaptic neuronal plasticity were attributed to its function in mediating neuronal chromatin remodeling and gene expression responses. For example, the light-induced phosphorylation in the suprachiasmic nucleus (SCN) is mediated by MSK1 (Butcher et al, 2005). The circadian clock, which resides in the hypothalamic SCN, can function in constant darkness. Light, via the retino-hypothalamic tract and glutamate release, can entrain phase resetting of the clock (Davie et al, 2010). Mice exposed to light pulses during the

night results in the concomitant induction of H3 S10ph as well as *c-Fos* and *Per1* genes expression in neurons. These events are important for the control of SCN mammalian circadian rhythm. Thus, the MSK1-mediated H3 phosphorylation is involved in regulation of circadian clock gene expression in the SCN (Crosio et al, 2000).

Interestingly, the lack of MSK1 expression which accounts for the depletion of H3 S10ph and *c-Fos* expression was observed in mouse models of Huntington's disease (HD) and in postmortem brain samples of patients with HD (Roze et al, 2008). These samples demonstrated functional ERK1/2 activity. Overexpression of MSK1 can also rescue striatal neurons from dysfunction and death underlining a protective function for MSK1 (Roze et al, 2008). However, the clear role of MSK in HD pathogenesis remains to be fully understood.

Striatum neurons, a key site of reward in the brain, are responsible for modulating rewarding effects associated with drugs of abuse such as cocaine. The transcriptional activation of *c-Fos* and *dysnorphin* in response to *in vivo* cocaine administration is dependent on MSK1 expression and activation. MSK1deletion disrupts ERK1/2-dependent CREB phosphorylation and H3 phospho-acetylation in striatal neurons. Although mice lacking MSK1 display similar acute and spontaneous short-term responses compared to its wild-type counterparts, knockout mice exhibited altered long term behavioural responses. In response to repeated cocaine administrations, MSK1 knockout mice demonstrated decreased locomotor sensitization attributed to ERK1/2 uncoupling with chromatin remodelers (Brami-Cherrier et al, 2009; Brami-Cherrier et al, 2005). Furthermore, when using the conditioned place preference technique, in which a preference for a context repeatedly associated with cocaine injection is a measure of

reward learning, MSK1 knockout mice appear more sensitive than wild-type mice to the rewarding feeling of cocaine (Brami-Cherrier et al, 2005). Thus, these studies suggest that the role of MSK1 in drug addiction is complex.

MSKs have essential regulatory roles in chromatin remodeling that impact long term memory formation in hippocampal dentate gyrus cells. MSK1/2 knockout mice were found to be defective in stress-related learning and memory process. Neurons re-organize and coordinate into networks in response to new experiences, a process known as neuronal plasticity. Wild-type mice exposed to novelty stresses and forced swimming tests have simultaneous stimulation of the glucocorticoid receptor (GR) and *N*-methyl-$_{D}$-aspartate receptor (NMDR) pathways which signals to ERK-MSK-H3 phospho-acetylation and *c-Fos* expression cascade and translates into an adaptive immobility response. The immobility response to forced swimming was linked to H3 phospho-acetylation (H3 S10ph K14ac) and *c-Fos* induction in specific dentate gyrus granule neurons. As such, this signaling pathway and gene expression profile are concordant with *in vivo* behavioural and neuronal abilities to adapt, learn and recall a series of challenges. In contrast, MSK1/2 knockout mice exhibited depleted H3 S10phK14ac and deficient *c-Fos* induction. Accordingly, these mice also failed to exhibit the behavioural immobility response during the forced swimming tests (Chandramohan et al, 2008; Chandramohan et al, 2007; Reul & Chandramohan, 2007). Further, MSK1 knockout mice exposed to fear training have depleted CREB phosphorylation and H3 phospho-acetylation which was associated with impaired spatial learning, passive avoidance and contextual fear conditioning (Chwang et al, 2007). Overall, these findings support a role for MSK1/2 in modulating neuronal plasticity.

1.4 Genomic and Chromosomal Aberrations in Cancer

Genomic instability is a hallmark of malignancies. Substantial literature supports the progression of specific cancers attributed to accumulating genetic, genomic and chromosomal instability (CIN) in tumours (Fearon & Vogelstein, 1990; Hanahan & Weinberg, 2000; Vogelstein & Kinzler, 2004). For example, chromosomal translocations and inversions produce chimeric lysine acetyltransferases (KATs) that have gain of function in hematological malignancies such as acute myeloid leukemia, therapy-related myelodysplatic syndrome and mixed lineage leukemia. These fusion proteins are consequently mistargeted and acquire new interacting partners that expand their specificity and repertoire of protein as well as promoter targets (Drobic et al, 2006; Iyer et al, 2004). On the other hand, constitutively activated or mutated proteins critical for normal cellular function can promote genomic instability and oncogenesis. For instance, defects in telomerases, tumor suppressors and DNA repair proteins result in cell division abnormalities, breakage-fusion-bridge instability and elevated chromosome mutation rates that lead to aneuploidy (Gisselsson, 2003; Vogelstein & Kinzler, 2004). Thus, accumulation of these genomic and chromosomal aberrations leads to the heterogeneity and stepwise progression of cancer.

Changes in genome content can involve local alterations in DNA sequences. Point mutations can lead to missense and nonsense mutations while DNA deletions can result in frameshift mutations. In solid tumours, complex karyotypes and gross chromosomal aberrations arise from destabilization of chromosome structure or number and exhibit characteristic subsets of aberrations (Hoglund et al, 2002). Despite the understanding that certain cancers such as colorectal cancer can arise from an accumulation of genetic aberrations, the mechanisms that

induce and maintain CIN in solid tumours are complex and involve a plethora of factors (Gisselsson, 2003). Key proteins involved in management of cell cycle checkpoints and telomere length are thought to participate in the mechanism controlling aneuploidy and CIN (Vogelstein & Kinzler, 2004).

Many strategies have been used to measure CIN to understand the acquisition of variations in tumour evolution and progression. Techniques such as multicolor spectral karyotyping (SKY) allow comparison and visualization of genomic changes and composition; therefore, these are invaluable tools in characterization of cancer cell heterogeneity and diversity (Schrock et al, 1996). Chromosomal aberrations are evident as rearrangements, translocations, amplifications or deletions, but more often observed as imbalances in chromosome segments and numbers. In karyotypic analysis, these aberrations are recurrent or nonrandom like clonal chromosomal aberrations (CCA) and can be characteristic particularly of hematological cancers. An example is the clinically used Philadelphia translocation that manifests as the reciprocal translocation between chromosomes 9 and 22 evident in chronic myelogenous leukemia (CML). The translocation produces a fusion gene that expresses an oncogenic Breakpoint Cluster- Abelson (BCR-ABL) tyrosine kinase and can be targeted for therapy. More often, karyotypic analysis of tumours yield random, stochastic and unstable changes often regarded as artifacts. These changes are referred to as nonclonal chromosomal aberrations (NCCA). The frequency of NCCA is not only reflective of the global genetic instability inherent of solid tumours but also representative of the non-linear pattern of tumour initiation and progression (Heng et al, 2006b). Recent studies demonstrate the elegant correlation of previously overlooked NCCA with the dynamic heterogeneity that is typical of most cancer phenotypes especially solid tumours (Heng et al,

2006c). Although much focus has been given on characterizing CCA, current work recognize the contribution of NCCA in understanding neoplastic evolution (Heng, 2007; Heng et al, 2006b; Heng et al, 2006c).

1.5 Chromatin as a substrate for signaling pathways: composition and organization

Chromatin is the cell's intrinsic framework of organization. Both functional and compact, chromatin is composed of DNA and protein that is efficiently packaged within the nucleus of a cell and is the native substrate for DNA-mediated processes. Although compliant and dynamic in events such as mitosis that require accessibility, eukaryotic chromatin is highly organized and repressive in nature. It is divided into functionally and structurally distinct regions known as heterochromatin and euchromatin. Heterchromatin is defined as areas of condensed, transcriptionally inactive DNA whereas euchromatin is separate regions of decondensed, transcriptionally active DNA. A tight balance must exist between these two regions in regulation of many cellular processes.

At its most fundamental level, chromatin is composed of basic repeating units of nucleosomes which contain a core histone H3-H4 tetramer flanked by two histone H2A-H2B dimers at each side (Luger et al, 1997). Approximately 146 base pairs of DNA are wrapped around this complex (Luger et al, 1997) (**Figure 1.4**). Each histone has a basic, unstructured N-terminal tail that protrudes from the nucleosomal core, a globular histone fold domain and an unstructured C-terminal tail. In order to achieve higher level folding and chromatin organization, histones are critical in genomic DNA packaging and hierarchy of compaction. These are accomplished by extensive interactions between linker histones H1 and/or H5 with DNA, and by

inter- and intra-nucleosomal contacts mediated by histone tails and chromatin fiber-fiber interdigitation (Luger et al, 1997) (**Figure 1.4)**. As noted earlier, compact chromatin must also permit local accessibility and dynamic changes for cellular processes such as replication, repair and transcription to occur. Hence, histone post-translational modifications (PTM), recruitment of ATP-dependent remodeling complexes and incorporation of histone variants are important mechanisms that disrupt the chromatin structure and facilitate cellular processes (Wang et al, 2007a; Wang et al, 2007b).

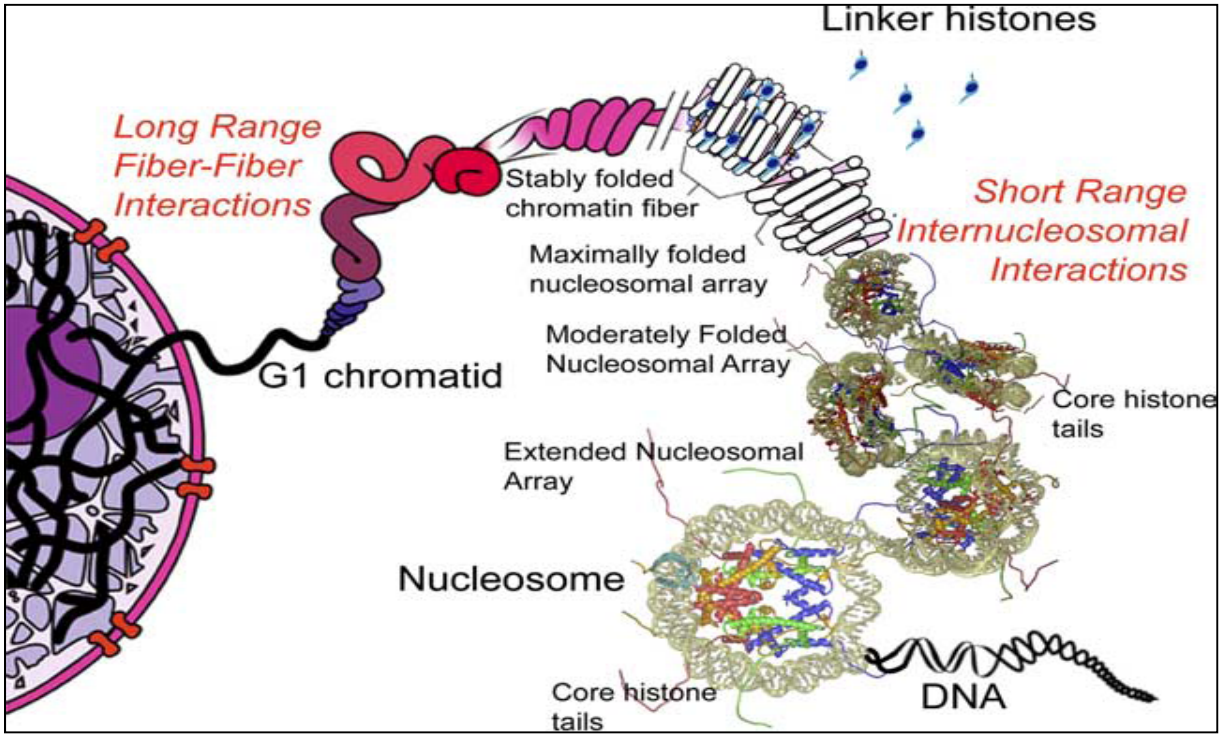

Figure 1. 4. Hierarchy of Chromatin Compaction and the Nucleosome Core Particle

The illustration depicts the multiple levels of chromatin folding from the primary unit of genomic DNA wrapped around nucleosomes to form the 10nm chromatin fiber. Secondary organization involves formation of 30 nm fibers through nucleosome-nucleosome interactions, and further condensation into higher-order fibers which is packaged into the interphase nucleus. *This figure is adapted from (Hansen, 2002).*

1.5.1 Histone modifications

The core histones are subject to a variety of covalent PTMs, all of which are reversible. Catalyzed by histone-modifying enzymes such as acetyltransferases, methyltransferases and kinases, amino acids found in the tail domains predominantly serve as platforms for diverse modifications (Kouzarides, 2007). Although most studies focus on N-terminal tail PTM of histones, there is an increasing body of evidence which supports the existence of modifications in the histone fold domains (Cosgrove, 2007; Cosgrove et al, 2004; Cosgrove & Wolberger, 2005). These PTMs include acetylation, methylation, phosphorylation, ubiquitination, poly ADP ribosylation, sumoylation, carbonylation, glycosylation and biotinylation (Wu et al, 1986) (**Figure 1.5**).

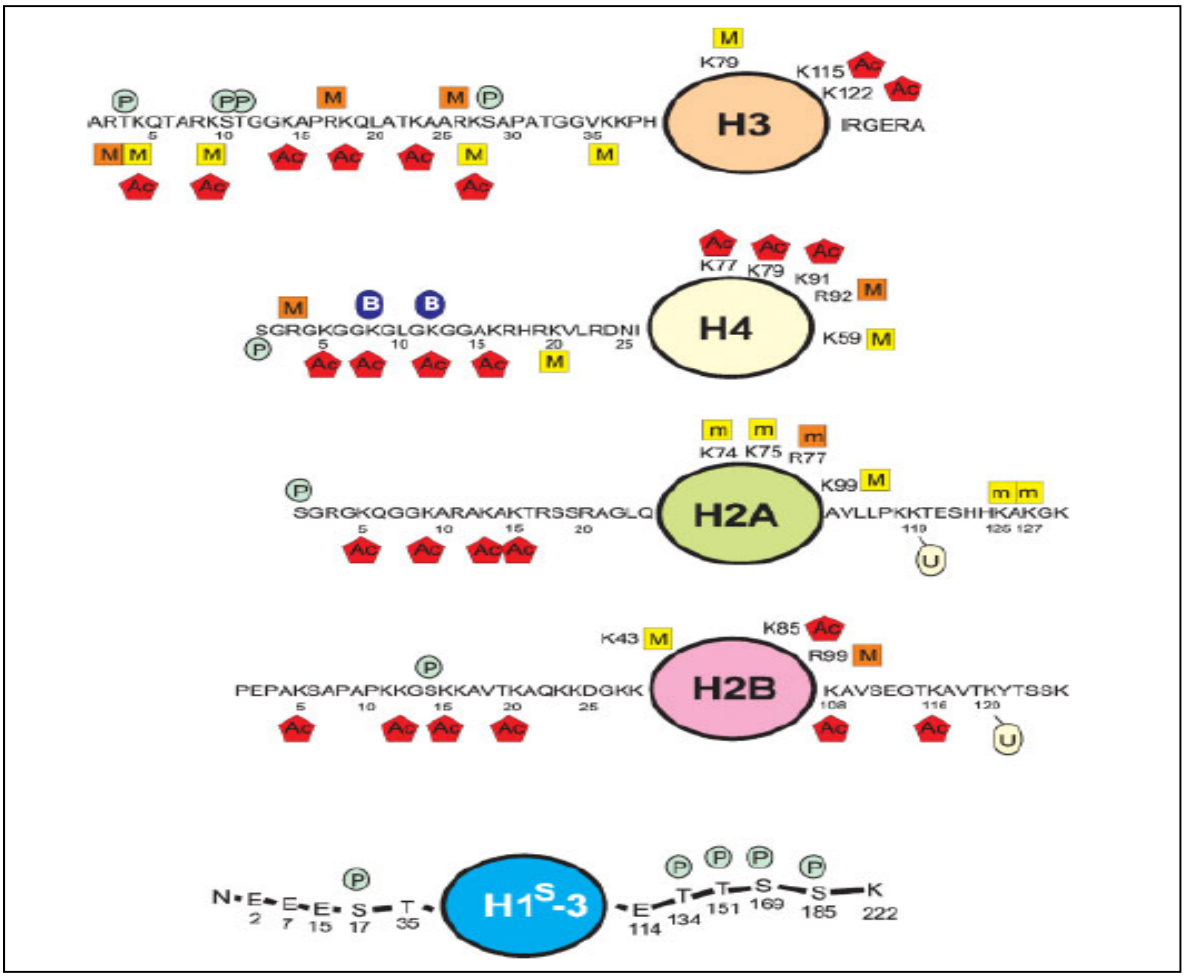

Figure 1. 5. Core and H1 Histone Post-Translational Modifications

Human core histone amino acid sequences are shown. Histone $H1^{S}$-3 is a mouse H1 subtype. The modifications include methylation (M), acetylation (Ac), phosphorylation (P), ubiquitination (U), and biotinylation (B). Methylation sites that are uncertain are denoted as (m). *The figure and text were reproduced with permission from (Espino et al, 2005).*

With pioneering and sensitive techniques such as ChIP assays and mass spectrometric methods, the multifaceted role of histone PTMs in cellular processes and chromatin structure is beginning to be elucidated (Kouzarides, 2007). The functional significance of each modification, particularly histone acetylation, methylation and phosphorylation in facilitating transcription and in dynamically marking global or local regions of active and repressed chromatin are just beginning to come to light (Berger, 2007; Lee & Mahadevan, 2009; Ruthenburg et al, 2007). Certain modifications can directly interfere with histone-DNA contacts and alter chromatin structure or act as sites of recruitment for chromatin-associated proteins. For example, acetylated residues of histones are not only required for interactions with specific protein domains but also disrupt higher order chromatin folding (Bottomley, 2004; Shogren-Knaak & Peterson, 2006). As such, histone modifications have been established as key factors for gene regulation.

As the list of histone PTMs continues to grow, investigators have proposed the existence of a histone code which postulates that sequential modifications of histones are "read" by protein modules and are associated with specific biological responses (Borrelli et al, 2008; Strahl & Allis, 2000). The role of a specific or a combination of histone PTMs may be different depending on the cellular context or gene of interest (Strahl & Allis, 2000). Some PTMs are typical markers of active chromatin while others are coupled with inactive or repressed regions, greatly involved

in the interplay between the transcriptionally permissive state and native inhibitory state of chromatin (Berger, 2007; Fischle et al, 2003b). Along with DNA methylation, RNA interference and nuclear architecture, histone PTM are essential in controlling heritable gene expression profiles in epigenetic mechanisms (Delcuve et al, 2009).

Despite the enormous progress, the association of histone modifications in patterns of gene activity and consequently biological outcomes are far from resolved. Tissue and genome-wide microarray as well as mass spectrometric studies of histone modifications in human tumours have demonstrated correlative relationships between histone PTMs with clinical outcomes of diseases such as cancer (Elsheikh et al, 2009; Fraga et al, 2005). Further, histone methylation patterns in prostate cancer are potentially reflective of diagnostic staging and may have prognostic value in the future (Seligson et al, 2005). Thus, there is evidence of clinical application and value of understanding histone PTMs apart from their mechanistic role in chromatin structure and function.

1.5.2 Histone acetylation

Acetylation is the most extensively characterized modification thus far. Lysine residues of all core histone proteins can undergo acetylation. Newly synthesized histones are readily acetylated during replication but the dynamic addition and removal of acetyl groups on N-terminal tails of H3 and H4 have been broadly linked with transcriptional activation upon gene induction (Annunziato, 2005; Spencer & Davie, 1999). Histone H3 is acetylated at lysines 9, 14, 18 and 23 while histone H4 is acetylated at lysines 5, 8 12 and 16 (**Figure 1.5**). Hyperacetylated histones, particularly on histone H3, are associated with decondensed chromatin allowing

accessibility to DNA binding proteins for transcription. They can act as either recognition docking sites for proteins that contain bromodomains such as KATs and histone deacetylases (HDAC), or directly affect chromatin structure by reducing the affinity towards DNA. This is attributed to the neutralization of the lysine positive charge by the addition of an acetyl group (Cosgrove & Wolberger, 2005; Norton et al, 1990). The balance of KAT and HDAC activities catalyzes reversible histone acetylation. The roles and composition of these histone modifying enzymes are well documented in multiprotein complexes, recruitment mechanisms to regulatory elements of genes and transcription. A recent report demonstrates that H4 K16 acetylation prevents 30-nm chromatin fiber formation and higher level chromatin folding (Shogren-Knaak et al, 2006). Conversely, hypoacetylated histones contribute to chromatin condensation and transcriptional repression (Peterson & Laniel, 2004; Wang et al, 2001).

1.5.3 Histone methylation

Although discovered in the early 1970s by Paik *et al* using biochemical analyses to detect that N-terminal lysine residues undergo methylation (Paik & Kim, 1971), it is not until the past decade that the significance of histone methylation in gene expression regulation has come to light. Methylation occurs on lysine and arginine residues on the N-terminal tails as well as on the histone fold domains of histone H3 and H4 (**Figure 1.5**). These histone PTMs are deposited by specific SET (*Su(var)* 3-9, *Enhancer of zeste* (E[z]) and trx-G *Trithorax*) or non SET domain-containing lysine or arginine histone methyltransferases (KHMTases or PRMT) on the ε-nitrogens or guanido nitrogens, respectively (Bannister & Kouzarides, 2005; Shilatifard, 2006). Lysine residues can be mono-, di- or trimethylated whereas arginine residues are mono- or dimethylated in a symmetrical or asymmetrical arrangement. Once thought to be a stable mark,

enzymatic conversion of methylated arginine histone residues to citrulline and identification of demethylases that can remove mono- and dimethylation have shed light on the possibility that this modification is dynamically regulated (Cuthbert et al, 2004; Schneider & Shilatifard, 2006; Wang et al, 2004). Further, methylated H3 at lysine 9 can be recognized and bound by HP-1 chromodomain-containing proteins and required for formation and maintenance of heterochomatin (Lachner et al, 2001). Di- or trimethylation of specific residues such as K4, K36 and K79 on H3 (H3K4me2, K4me3) and K16 of H4 have been associated to gene activation whereas others such as K9 and K27 of H3 (H3K9me2/3 and H3K27me3) are marks of repressed chromatin (Bannister & Kouzarides, 2005; Peterson & Laniel, 2004).

1.5.4 Histone ubiquitination, biotinylation, ADP-ribosylation

The linkage of a 76-amino acid polypeptide on the C-terminal tails of H2B as a monomer and H2A as a polymer has not only been associated with transcriptional activation but also proteasomal degradation, respectively (Davie & Murphy, 1990; Davie & Murphy, 1994; Fleming & Osley, 2004; Pavri et al, 2006; Shahbazian et al, 2005; Thiriet & Hayes, 2005)(**Figure 1.5**). H2A monoubiquitination has recently been linked to transcriptional silencing and repression (Fleming & Osley, 2004). Studies in yeast have implicated ubiquitination of histone H2B on K123 (H2BK123ub) in trans-tail modification of histone H3 N-terminal methylation (Sun & Allis, 2002). Further, Rad6/Bre1 enzyme-mediated H2BK123ub is necessary to subsequent H3 methylation by the complex of proteins associated with Set1 (COMPASS) or the disruptor of telomeric silencing (DOT)-1 KHMTases and association with elongating RNA polymerase II (Krogan et al, 2003; Shilatifard, 2006). These studies demonstrate the role of H2B monoubiquitination in protein recruitment and transcription. Small ubiquitin-related modifier

(SUMO) can also be covalently attached to lysine residues and sumoylation of histone H4 has been linked to transcriptional repression (Shiio & Eisenman, 2003). Thus, histone ubiquitination tags for opposing enzymatic activities that can either positively or negatively impact transcription. Regardless, the mechanism of how this modification impacts chromatin structure remains unknown (Nathan et al, 2003).

Another modification that has been identified in histones *in vitro* is the covalent attachment of the biotin on lysine residues. The addition and removal of biotin on histone proteins are catalyzed by biotinidase and holocarboxylase enzymes, respectively. Zempleni's group identified multiple biotinylation sites on histones H3, H4 and H2B by mass spectrometry. Further, biotinylated H4 *in vitro* has been abundantly detected in pericentromeric heterochromatin and associated with gene silencing (Hassan & Zempleni, 2006; Zempleni et al, 2009). However, biotin has not been detected in native histone preparations using multiple approaches thus questioning its direct role *in vivo* in gene regulation (Healy et al, 2009).

Histones can also be reversibly modified by covalent ADP-ribosylation. Using radiolabeled NAD^+ and the Edman degradation method, mono-ADP ribosylation has been detected on arginine and glutamate residues of all core histones. Histone poly-ADP-ribosylation is speculated to mediate DNA binding inhibition (Hassa et al, 2006). Exciting work by Poirer and colleagues has renewed interest on enzymes responsible for addition of poly-ADP ribose on diverse proteins including histones (Rouleau et al, 2010). The addition of poly-ADP ribose on histone H1 by poly ADP-ribose polymerases (PARPs) is thought to alter H1 chromatin binding during DNA damage

and transcription. Further, PARP inhibitors as anticancer agents are currently under investigation (Rouleau et al, 2010).

1.6 Histone H3 Phosphorylation

Phosphorylation has been reported on serine and/or threonine residues of the N-terminal tail of histone H3, histones H4, H2A, H2B and linker histone H1 (**Figure 1.5**). Using mass spectrometry, more residues have been identified as phospho-acceptor sites on histone H3 (Cosgrove et al, 2004; Garcia et al, 2005; Thomas et al, 2006). Although not all have been linked to specific cellular consequences, certain phosphorylation events are coupled to processes such as DNA damage, apoptosis, transcription and mitosis (Peterson & Laniel, 2004). Among the four core nucleosomal histones, histone H3 is the most highly modified and abundantly phosphorylated (**Figure 1.5**). The residues of histone H3 are well conserved across species including phosphorylation on Thr3, Ser10, Thr11 and Ser28 (Cerutti & Casas-Mollano, 2009; Cosgrove et al, 2004). While several kinases can target these four sites as global marks that are thought to be integral for chromosome condensation during mitosis, a localized subset of nucleosomes in regions of active chromatin are phosphorylated as a response to transcriptional activation during interphase (Clayton et al, 2000; Johansen & Johansen, 2006; Thomson et al, 1999). As such, evidence points to H3 phosphorylation as fulfilling opposing roles in chromatin function since it is associated to large scale DNA compaction (mitosis) as well as allowing local DNA accessibility (transcription). Further, H3 phosphorylation is the nucleosomal response that links extracellular signal transduction to gene expression (Davie et al, 2010). Although exciting studies explore the role of H3 phosphorylation in mitosis and transcription, the functional contribution and consequences of the modification are still unclear. The influence of H3

phosphorylation in diverse mechanisms is recently highlighted; H3 phosphorylation participates in proper protein recruitment for chromatin remodeling, determines establishment of other PTMs required for transcriptional regulation and is critical for neoplastic transformation.

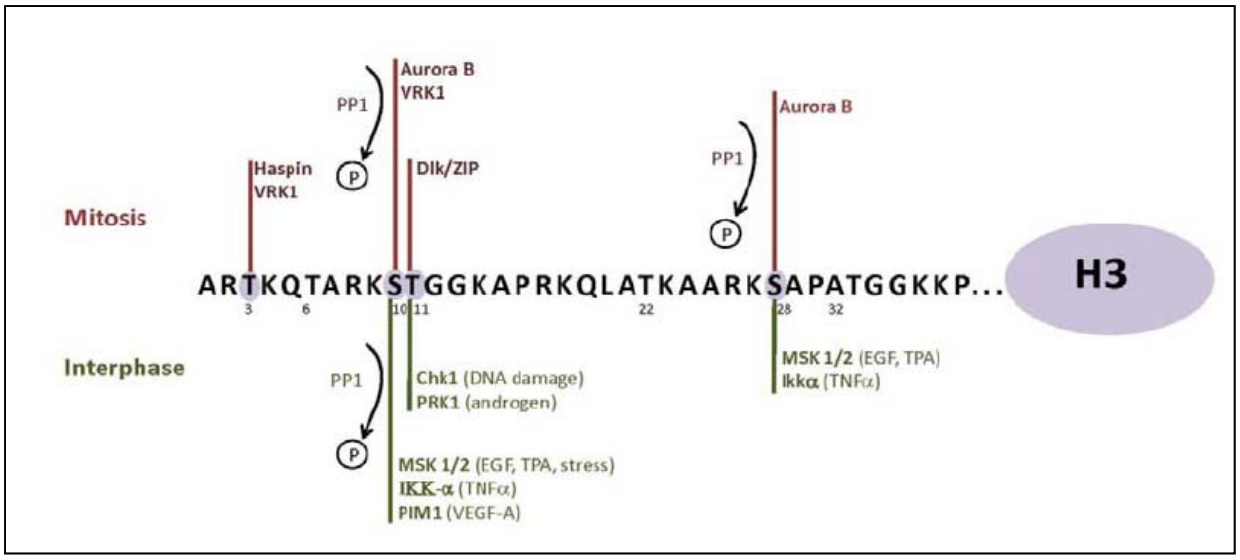

Figure 1. 6. Histone H3 Phosphorylation and Responsible Kinases/ Phosphatases

Kinases and phosphatases responsible for H3 N-terminal tail phosphorylation and dephosphorylation reported to date. Stimuli reported to activate the kinases are indicated in parenthesis. *The figure and text were reproduced with permission from (Perez-Cadahia et al, 2009).*

1.6.1 N-terminal H3 phosphorylation in mitosis: Ser10, Ser28, Thr3 and Thr11

H3 phosphorylation on Ser10 and Ser28 abundantly decorate condensed chromosomes. Both modifications have been used as markers of mitosis and meiosis. Although the onset, abundance and kinases that mediate this mitotic/ meiotic modification may vary in different organisms, H3 S10ph and H3 S28ph are conserved in *Tetrahymena, Drosophila*, plants and

mammalian cells. Studies in various eukaryotes demonstrate that H3 S10ph closely coincides with chromosome condensation events and is required for the initiation and segregation of cell division (Van Hooser et al, 1998; Wei et al, 1999). H3 phosphorylation is thought to be essential for proper chromosome condensation evident in *Drosophila* (Nowak & Corces, 2004) although some systems, such as yeast, display no mitotic defects when H3 Ser10 and/or 28 are mutated (Hsu et al, 2000). Appearance of phosphorylation on serine residues on histone H3 is similar and occurs in a specific spatio-temporal order: phospho-H3 emerges initially on pericentromeric heterochromatin in the late G2 phase, proceeds towards the chromosomal arms during the course of mitosis and peaks in metaphase (Goto et al, 2002; Hendzel et al, 1997). H3 dephosphorylation begins in anaphase and terminates before the decondensation of chromosomes. Mitotic Ser28 disappearance of phosphorylation occurs later and is less abundant than Ser10 (Goto et al, 1999; Hendzel et al, 1997). Paired kinases and phosphatases predominantly responsible for bulk H3 phosphorylation have been identified. In yeast, increase in ploidy-1 (Ipl1) kinase is counteracted by GlyCogen 7 (Glc7) whereas Aurora-B kinase is opposed by protein phosphatase-1 (PP1) in *Drosophila*, *C. elegans, Xenopus* and mammalian cells. In *Aspergillus nidulans*, never in mitosis gene A (NIMA) kinase is the H3 mitotic kinase (Cerutti & Casas-Mollano, 2009).

Although much less studied, conserved Thr3, Thr11 and Thr32 on histone H3 are also phosphorylated in mitosis (Cerutti & Casas-Mollano, 2009; Perez-Cadahia et al, 2009). In mammalian HeLa cells, H3 phosphorylation on Thr3 (H3 T3ph) follows a similar temporal pattern as serine phosphorylations but differs in spatial distribution since H3 T3ph initially follows a speckled pattern to later strongly stain in the inner centromeric region (Dai et al, 2006; Dai et al, 2005). The haploid germ cell-specific nuclear protein kinase (Haspin) and the vaccinia

related kinase (VRK)-1 have been implicated as kinases responsible for mitotic H3 T3ph (Cerutti & Casas-Mollano, 2009). Immunocytological overlap with cohesin proteins and centromeric localization of H3 T3ph implies a role for Thr phosphorylation in chromosome cohesion, alignment and kinetochore assembly (Dai et al, 2006). In contrast to Ser phosphorylation, H3 phosphorylation on Thr11 (H3 T11ph) distribution is solely restricted to centromeric areas in mammals which suggests a role in centromere assembly (Houben et al, 2007). In plants, H3 T11ph intensely decorates entire chromosomes while H3 S10/S28ph is reversibly limited to centromeric regions (Houben et al, 2005). The mitotic kinase in charge of H3 T11ph is reported to be the death-associated protein (DAP)-like kinase (Dlk) or Zipper interaction protein (ZIP) kinase identified in murine fibroblasts (Preuss et al, 2003). Recent studies implicate H3 T11ph during interphase with transcriptional repression. In response to DNA damage, H3 T11ph is catalyzed by the checkpoint (Chk)-1 kinase (Shimada et al, 2008). Upon androgen stimulation of responsive genes, H3 T11ph is catalyzed by protein kinase C-related kinase 1 (PRK1) in prostate tumour cells (Metzger et al, 2008).

The exact role of histone phosphorylation in mitosis/ meiosis is not clear. Some postulate that akin to acetylation, phospho-residues possibly change the nucleosome packing properties due to physiochemical alterations brought about by the negative charges. Another hypothesis is that these modified residues form protein binding sites necessary for mitotic assembly and condensation. Given that mitotic histone H3 phosphorylation is indispensible for chromosome condensation in some but not all organisms, the definitive function of the modifications remains uncertain (Perez-Cadahia et al, 2009).

1.6.2 RAS-MAPK pathway-mediated H3 phosphorylation and gene expression

Apart from its contribution in mitosis/meiosis, phosphorylation of histone H3 has also been extensively linked to transcriptional activation and gene expression. While targeted to a small subset of nucleosomes when compared to that of mitosis, H3 phosphorylation in interphase represents a site of convergence and a direct link between diverse signaling pathways such as the MAPK-MSK1/2 cascade via RAS, or other cell membrane receptors and gene induction responses. The pioneering work by Allfrey and colleagues initially demonstrated that transcriptionally active *c-Fos* is associated to atypical nucleosomal histone H3, and later observed that H3 was modified via acetylation and phosphorylation using Hg-affinity columns which bind exposed sulfhydryl groups (Allegra et al, 1987; Chen & Allfrey, 1987). Mahadevan's group then reported rapid and transient increase of H3 S10ph levels in mouse fibroblasts following stimulation with growth factors and mitogens (Mahadevan et al, 1991). The phosphorylation of H3 Ser10 and HMGN1 Ser6 concurrent with IEG expression is termed the "nucleosomal response." Since then, H3 S28ph has been included in the nucleosomal response. Further, various stimuli in many cell contexts have been reported to stimulate the MAPK-MSK1/2 pathway and augment H3 phosphorylation, particularly on Ser10. The duration, intensity and association pattern of H3 S10ph is dependent on the genes, stimuli and cell type investigated. A general mechanism whereby increased H3 phosphorylation mirrors gene induction was uncovered. Using chromatin immunoprecipitation assays, growth factors (e.g EGF), phorbol esters (e.g. TPA), stress and LPS are observed to increase H3 S10ph which is associated with IEG expression such as *c-Myc, c-Fos, c-Jun, Cox-2* and *Mkp-1* as well as *TFF1* (Chadee et al, 1999; Clayton et al, 2000; Espino et al, 2006; Li et al, 2001; Park et al, 2004). These genes are rapidly transcribed upon stimulation and require no new protein synthesis

(Herschman, 1991). In human fibroblasts and T lymphocytes, MAPK-induced H3 S10ph is associated with human telomerase reverse transcriptase (h*TERT*) gene promoters (Ge et al, 2006). In macrophages, LPS can elicit H3 S10ph on the *Cox-2* gene along with other histone PTMs (Park et al, 2004). In neuronal cells such as hippocampal or striatal neurons, electroconvulsive seizures (Tsankova et al, 2004), neuromodulators (Crosio et al, 2003), glutamate (Brami-Cherrier et al, 2007) stimulate H3 S10ph on *c-Fos* gene or *c-Jun* promoter. In hypothalamic SCN, light-induced H3 phosphorylation is MSK1-mediated and concomitant to *c-Fos* gene induction as well as circadian gene *Per1* (Butcher et al, 2005; Crosio et al, 2000).

The rapid and transient increase in H3 phosphorylation levels in quiescent fibroblasts was observed to be hyperacetylated (Mahadevan et al, 1991). Other studies report concomitant H3 S10ph with acetylation on Lys9 or 14, which provide support for the histone code hypothesis and coupled PTMs on active chromatin (Cheung et al, 2000; Clayton et al, 2000; Lo et al, 2000). However, the loss of H3 phosphorylation in MSK1/2 knockout cells and following H89 inhibitor treatment revealed no significant changes in H3 acetylation levels, especially on immediate early genes (IEG). As such, H3 S10ph and H3 K9ac or K14ac in response to mitogens in mouse fibroblasts appear to be independent of each other (Soloaga et al, 2003; Thomson et al, 2001). Other modifications such as H3 methylation, depending on the residue, have been reported to have either a functional synergistic or antagonistic relationship with H3 phosphorylation (Fischle et al, 2003a). Thus, the examination of co-dependent histone PTMs and the transcriptional outputs they govern is an exciting avenue of research.

Although not as extensively studied as H3 S10ph, RAS-MAPK-MSK1/2-induced H3 S28ph is also linked with transcriptional activation and has recently been shown to associate with *c-Jun* promoter regions (Bode & Dong, 2005; Dunn et al, 2009). Though both phospho-residues have been observed on the same *c-Jun* promoter region, sequential immunoprecipitation and high resolution fluorescence deconvolution microscopy analyses demonstrate that these modifications occur in distinct regions of chromatin independent of each other (Dunn & Davie, 2005; Dyson et al, 2005). It would be of interest to determine how MSK1/2 targets distinct H3 S10 and S28 phospho-residues which do not appear to coexist on the same histone tail, nor on adjacent nucleosomes.

1.6.3 MSK1/2-independent H3 phosphorylation and gene induction: other kinases

Different kinases apart from MSK1/2 have been implicated to phosphorylate histone H3 on serine residues. Prior to generation of MSK1/2 knockout mice, the physiological kinase responsible for mitogen-induced H3 phosphorylation was controversially debated (Davie, 2003). Rapid H3 phosphorylation in response to stress and growth factors was abolished with H89 inhibitor treatment indicating that MSKs are essential kinases (Thomson et al, 1999). However, fibroblasts of patients with the Coffin-Lowry syndrome who lack RSK2, demonstrate impaired EGF-induced H3 phosphorylation (Sassone-Corsi et al, 1999). Since then, several studies have determined that the loss of MSK1/2 abrogates inducible H3 phosphorylation levels which correspond to decreased IEG induction (Arthur, 2008). Thus, MSK1/2 is definitively identified as the key kinases responsible for mediating RAS-MAPK responsive H3 phosphorylation (Davie, 2003).

Other eukaryotes possess H3 kinase homologues that phosphorylate H3 linked with transcriptional activation (**Figure 1.6**). In *Drosophila*, JIL-1 is the major interphase H3 kinase responsible for the dynamic distribution of H3 S10ph along polytene chromosomes which parallel the induction of heat shock gene loci in response to thermal stress (Jin et al, 1999; Nowak & Corces, 2000). In *Saccharomyces cerevisiae,* sucrose non-fermented (Snf)-1 kinase has been shown to mediate the transcriptionally-linked H3 S10ph important for the *INO1* gene promoter (Lo et al, 2001). Although direct evidence is lacking, the activity of the Arabidopsis MSK homologues, ATPK6/19 kinases is enhanced following cold, salt or hormone stress which perhaps impact H3 phosphorylation in activated genes (Houben et al, 2007).

To date, mammalian cells have several inducible H3 kinases responsive to different stimuli that have been identified. Follicle-stimulating hormone (FSH) can bind its receptor and activate cAMP-dependent protein kinase A (PKA); PKA then elicits H3 S10ph on FSH-responsive genes in immature rat ovarian granulosa cells (DeManno et al, 1999; Salvador et al, 2001). Estrogens stimulate the activation of IκB kinase (IKK)-α which interacts with the estrogen receptor and phosphorylates H3 on the chromatin of the transcriptionally active cyclin D1 gene (Park et al, 2005). In addition, TNF-induced H3 S10ph is lost in IKK-α-deficient mouse embryonic fibroblasts which suggest that these events are necessary for the induction of pro-inflammatory genes (Anest et al, 2003; Duncan et al, 2006; Yamamoto et al, 2003). In hepatocellular HepG2 cells, phorbol ester-induced H3 phosphorylation on the *low density lipoprotein* (*LDL)* promoter is mediated by PKC (Huang et al, 2004). In human umbilical vein endothelial cells (HUVEC), the proto-oncogenic serine/threonine kinase 1 (PIM1) responds to vascular endothelial growth factor (VEGF)-A and phosphorylates H3 in the enhancer region of

the *FOSL1* gene that is not affected by pretreatment with H89 (Zippo et al, 2007). Interestingly, H3 phosphorylation was detected at an upstream region of the *FOSL1* enhancer which was susceptible to H89 pretreatment (reduced H3 S10ph). This region did not demonstrate association of the PIM1 kinase implying MSK1/2 as likely kinases that mediate H3 phosphorylation. As such, it appears two kinases can target H3 S10ph at distinct regions of the same gene in response to the same stimuli for activation. Further, it is plausible that within any single organism, many kinases responsive to specific stimuli can phosphorylate H3 at a particular gene during interphase.

Dong and colleagues identified several kinases responding to different stimuli that are able to phosphorylate histone H3 in *in vitro* assays. In HaCaT cells, UV-B exposure induce SRC family tyrosine-protein kinase (Fyn) and mixed lineage triple kinase (MLTK)-α to phosphorylate H3 (Choi et al, 2005b; He et al, 2005b). In response to arsenite, Akt1 or protein kinase B, ERK2 and RSK2 are able to phosphorylate H3 in JB6 C141 mouse epidermal skin cells (He et al, 2003). Further, UV-B can stimulate ERK1/2, p38, JNK1 and MSK1 to phosphorylate H3 in JB6 C141 mouse epidermal skin cells although not via JNK2 (Bode & Dong, 2005; Dong & Bode, 2006). Finally, tissue transglutaminase 2 (TG2) is able to phosphorylate free and nucleosomal histone H3 on serine 10 and is bound to chromatin in MCF-7 breast cancer cells (Mishra et al, 2006). Whether these kinases are able to target histone H3 *in vivo* is currently unknown and remains to be investigated.

1.6.4 Functional role of H3 phosphorylation in transcription: effects on chromatin structure, binding platform for protein modules and histone code hypothesis

To date, the exact role of H3 phosphorylation and its ability to behave in both chromatin condensation and transcriptional activation remains largely unresolved. Innovative approaches attempt to provide insight and elucidate the functional contribution of H3 phosphorylation in these processes. Recently, *in vitro* studies using homogeneously phosphorylated H3 on Ser10 that are ligated and assembled into nucleosomes show that these phospho-modified arrays behave similarly to unmodified H3 (Fry et al, 2004). Further, H3 S10ph nucleosomal arrays do not increase the sensitivity to Switch/sucrose non-fermentable (SWI/SNF)-remodeling outcomes. Thus, H3 S10ph does not appear to have direct effects on internucleosomal interactions involving the N-terminus of H3 which mediates higher order folding. Further, the findings dispute the assumption that H3 phosphorylation alters the biophysical characteristics of nucleosomes by disrupting electrostatic interactions, thereby directly regulating chromatin structure (Fry et al, 2004; Shogren-Knaak et al, 2003). In *Drosophila*, ectopic H3 S10ph by JIL-1 kinase reinforces decondensed and open euchromatic conformation of polytene chromosomes (Deng et al, 2008). Unlike the effects observed in nucleosomal arrays, H3 S10ph appears to be essential in regulating higher order chromatin structures and perpetuating transcriptionally active regions by inhibiting heterochromatinization. Thus, phosphorylation of H3 as a means to directly alter the biophysical composition and influence chromatin structure may prove too simplistic as a mechanism to reconcile the findings in these experimental settings.

Alternatively, H3 phosphorylation is also believed to participate in the modulation of chromatin architecture by creating binding sites important for protein-protein interaction and subsequent effector/ modular protein recruitment. This concept is supported by strong evidence that acetylated tail residues are bound by bromodomains present in many histone-modifying

enzyme complexes such as KATs and HDACs. These modules are designated as "readers" able to distinguish histone modifications as signaling platforms that in turn regulate chromatin structure (Kouzarides, 2007). Recently, 14-3-3 adaptor proteins have been identified as phospho-acetylation recognition modules which are directly recruited to H3S10ph residues during transcriptional activation. 14-3-3 recruitment to H3 S10ph is enhanced by adjacent acetylated residues on H3 tails and facilitates stimuli-induced transcriptional activation of *c-Fos, c-Jun* promoters and the *HDAC1* gene in mammalian cells, as well as *GAL1* in yeast (Macdonald et al, 2005; Walter et al, 2008; Winter et al, 2008a; Winter et al, 2008b). 14-3-3 binding to phospho-H3 could therefore facilitate the recruitment of other modifiers as well as remodelers in a complex that mediates dynamic chromatin architecture at selected genomic loci. Likewise, complexes and proteins necessary for mitotic condensation such as cohesins may have phospho-serine acceptor sites and recognize global H3 phosphorylation which is required for chromatin compaction and cell division.

Modern variations of ChIP assays coupled to microarray and sequencing platforms have uncovered combinatorial relationships between histone modifications that systematically dissect functional correlations. The concept of histone PTM acting as binary switches has given a mechanistic link between enzymatic reactions that cooperate on H3 tails. For example, the "methyl/phos" switch describes H3 S10ph mediating dissociation of HP1 and consequently antagonize H3 K9me by HMTases to prevent heterochromatin formation (Fischle et al, 2005; Hirota et al, 2005). Similarly, H3 S10ph precedes H3 K14ac and appears to be synergistically coupled in order to enhance acetyltransferase binding during transcriptional activation of numerous genes (Cheung et al, 2000; Salvador et al, 2001). Hence, emerging evidence to support

the histone code hypothesis has been pivotal in defining the interplay that occurs between different PTM, not only in demarcating chromatin regions but also in regulating the gene expression profile (Fischle et al, 2003b; Lee & Mahadevan, 2009).

1.6.5 H3 phosphorylation and cancer

Increased H3 phosphorylation as a downstream response to the activated RAS-MAPK pathway is evident in oncogene-transformed mouse fibroblasts (Dunn et al, 2005). This H3 PTM serves as an interesting link between deregulation of RAS signaling and IEG expression profiles. Although the same relationship has not been firmly established in human cancer cell lines or tumours, the stimuli-induced association of H3 phosphorylation is unambiguously observed in various loci frequently linked with oncogenesis such as IEG, inflammatory and angiogenic genes (Davie et al, 2010; Herschman, 1991). Hence, histone H3 phosphorylation patterns on these gene sets may have a significant "signature profile" which may define clinical parameters similar to the global loss of H4 K20me3 and H4 K16ac along repetitive DNA sequences in primary tumours (Fraga et al, 2005).

Mitotic H3 kinases such as Aurora B are linked to cancer development due to its integral role in the regulation of mitotic entry, spindle formation and cytokinesis. Over-expression and anomalous activity of these kinases have not only been observed in many primary tumours, but they have also been demonstrated to unequivocally result in aneuploidy and tumorigenesis (Wang et al, 2007a). Furthermore, the dynamic global H3 S10ph by Aurora-B in mitosis results in the dissociation of HP1 whereas the retention of HP1 due to mutant H3 S10 leads to defective chromosome segregation and chromosome instability (Fischle et al, 2005; Ota et al, 2002). As

such, kinase inhibitors developed against Aurora B have shown promise as a possible approach for chemotherapeutic intervention (Harrington et al, 2004).

Recently, H3 phosphorylation has been directly implicated in neoplastic cellular transformation through a mechanism involving activating protein-1 (AP-1) factors (Choi et al, 2005a). Mouse epidermal skin cells which have elevated levels of H3 phosphorylation upon EGF stimulation form colonies in soft agar reflective of neoplastic transformation unless mutant H3 constructs containing S10A and/or S28A are stably introduced. As such, H3 S10ph is critical in the regulatory process for EGF-induced neoplastic transformation and provides evidence that H3 phosphorylation may be a feasible target for future cancer therapy.

1.7 *Trefoil Factor 1*: model gene to study transcriptional regulation

1.7.1 Implications in breast cancer prognosis and protein function

Human *Trefoil Factor* (*TFF*)-1, formerly known as *prenisilin 2* (*pS2*) or *Breast Cancer Estrogen-Inducible* (*BCEI*) gene, encodes a 6.6 kDa secreted peptide. TFF1 is characterized by a cysteine-rich three-loop trefoil motif which form dimers and protect TFF1 from proteolysis and acid digestion. Initially identified as an estrogen-responsive gene product in the MCF7 breast carcinoma cell line, TFF1 expression was observed to correlate with ERα and PR status though not clinically used (Masiakowski et al, 1982). Although ER and PR status are both currently used in the clinical diagnosis of tamoxifen-responsive breast tumours, TFF1 is thought to be a useful prognostic marker associated with a favourable response to endocrine therapy (Poulsom et al, 1997; Seib et al, 1997; Soubeyran et al, 1996).

Apart from breast carcinoma, many normal and malignant tissues express TFF1 including the gastrointestinal (GI) tract, mucinous respiratory tract epithelia and normal breast luminal ductal cells (Poulsom et al, 1997; Wong et al, 1999). TFF1 knockout mice demonstrate gut maturation abnormalities, gastric tumours and neoplasia which imply a protective role in the integrity of mucosa cells (Lefebvre et al, 1996; Wong et al, 1999). Further, these proteins are able to promote cell migration and stimulate repair processes at sites of mucosal injury (Wong et al, 1999). Although recent findings support an angiogenic and pro-invasive role for TFF1, the definitive physiological function of this protein is unresolved (Rodrigues et al, 2003b). However, due to its motogenic properties, it has been proposed that TFF1 expression leads to efficient cell mobility important for invasive malignancies (Prest et al, 2002; Rodrigues et al, 2003a; Rodrigues et al, 2003b). Further, ectopic expression of TFF1 in mammary carcinoma cells augments the potential for oncogenicity by boosting cell proliferation, survival, anchorage-independent cell growth, tumorigenicity and invasive potential (Amiry et al, 2009).

1.7.2 *TFF1* gene structure and upstream regulatory region

TFF1 is found among a cluster of three human trefoil genes on chromosome 21 and is composed of 3 exons: the first exon encodes for the secretion signal sequence, the second exon encodes for the trefoil domain and the last exon partially encodes for C-terminus (Rio & Chambon, 1990). The 1 kb regulatory region found upstream of the *TFF1* transcription start site (TSS) contains binding sites for ubiquitous transcription factors that respond to diverse extracellular stimuli such as growth factors, hormones, aromatic hydrocarbons and phorbol esters (Chen et al, 1999; Gillesby et al, 1997; Nunez et al, 1989). Initially, DNase footprinting experiments revealed several hypersensitivity sites in the proximal promoter as well as 10.5 kb

upstream of the TSS (Giamarchi et al, 1999). Binding site sequences for AP-1, estrogen receptor related element (ERRE), dioxin response element (DRE) and Sp1 have been shown integral for several specific stimuli-induced transcriptional activation of *TFF1*, particularly near the consensus estrogen response element (ERE) positioned 400 bp upstream of the TSS (Gillesby et al, 1997; Lu et al, 2001; Shang et al, 2000; Sun et al, 2005) (**Figure 1.7**). Mapping studies also demonstrate that nucleosomes found in the proximal promoter, NucT (near the TATA box) and NucE (near the ERE) have altered positioning and assist to regulate transcriptional activation in response to estrogens which is facilitated by histone acetylation (Sewack et al, 2001; Sewack & Hansen, 1997). ChIP coupled with tiled microarrays have also established that distant ER binding sites and perhaps three-dimensional arrangements can impact gene expression responses; regions 10kb upstream of *TFF1* proximal promoter containing ER with close proximity to the FoxA1 binding site can regulate its expression (Carroll et al, 2005).

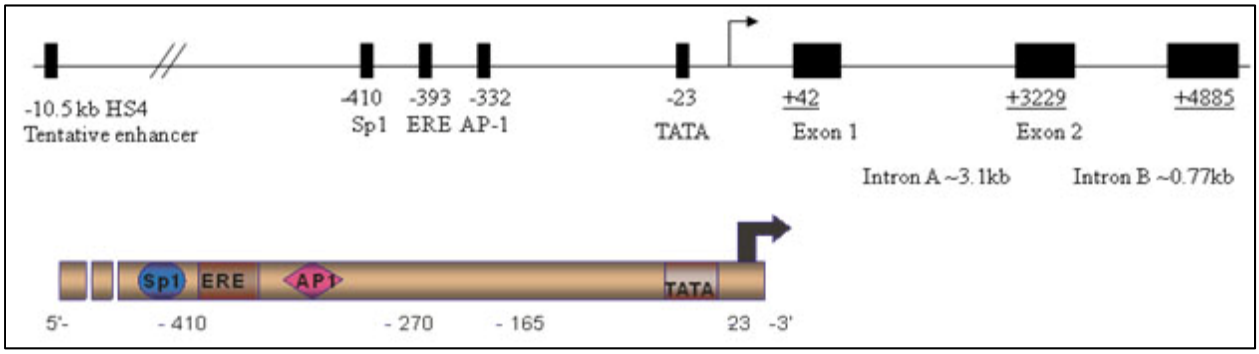

Figure 1. 7. Schematic Representation of *Trefoil Factor 1* Gene Structure

Each region is labeled according to the 5' position of the forward primer relative to the transcription start site. The exons are represented by black boxes, while the binding sites of relevant transcription factors located in the amplified regions of tentative enhancer (-10476) and promoter (-429) are displayed. C/EBP, CCAAT enhancer binding protein; Sp1, GC box that is a binding site for the Sp family of transcription factors; ERE; AP-1, constitutes a combination of

dimers formed of members of the JUN, FOS and ATF families of transcription factors. Asterisk indicates a putative binding site.

1.7.3 Insights of *TFF1* transcriptional regulation

Thus far, estrogen- and growth factor-responsive *TFF1* studies have offered the greatest representation of the mechanisms involved in transcriptional activation. ChIP assays that reveal the temporal, sequential and combinatorial occupancy of hormone receptors, basal and inducible factors, pre-initiation complexes, coactivator complexes, chromatin remodelers, modifying enzymes and histone modifications on the *TFF1* promoter upon activation have shed light into the complexity of the process. Initially, hormone-induced gene activation of *TFF1* uncovered the recruitment of co-activator complexes such as p160, p300/CBP and the estrogen receptors to facilitate histone acetylation (Burakov et al, 2002; Shang et al, 2000). Kinetic studies on *TFF1* gene have increased our knowledge of the elegance and sophistication involved in the dynamic and cyclical nature of gene activation: differential recruitment of ligand-bound and variant forms of estrogen receptors, cooperation and synergism of crosstalk mechanisms between hormone and growth factor signaling pathways, impact of anti-estrogenic treatment and the role of additional histone modifications such as methylation (Baron et al, 2007; Bauer et al, 2002; Liu & Bagchi, 2004; Metivier et al, 2003; Reid et al, 2003). As a consequence of estrogen treatment, topoisomerase II generates DNA strand breaks which is required for the restructuring and transcriptional competence of *TFF1* (Ju et al, 2006). Further, methylation of CpG nucleotides in the *TFF1* promoter alters its competence for activation and also promotes the dynamic and cyclical mechanism of transcriptional repression (Metivier et al, 2008). Thus, it is not only the transient interactions of factors that facilitate the state of transcription but also the primary sequence information that provides an additional level of control.

1.8 Rationale , Objectives and Study Hypotheses

RAS is mutated in 30% of human cancers and the RAS-MAPK signaling cascade is deregulated in many malignancies. The central theme that threads the studies presented in this thesis is that stimulation of the pathway culminates with the activation of MSK1 and H3 phosphorylation which play integral roles in molecular and biological responses such as IEG expression and neoplastic transformation.

In the first two studies (**Chapters 2 and 3**), we examine the role of the H3 kinase, MSK1, as the essential link between a constitutively activated RAS or extracellular stimulation of the pathway and chromatin-mediated responses such as promoter remodeling, IEG expression and anchorage-independent growth. We use the parental (10T1/2) and *Ras*-transformed (Ciras-3) immortalized mouse fibroblasts. Ciras-3 is derived from parental 10T1/2 cells through stable integration of the c-*HRas1* oncogene. The resulting cell line has a highly metastatic and tumourigenic phenotype. As such, both cell systems have been invaluable tools in understanding the role of *Ras*-driven events that differ between a "normal" and "malignant" phenotype. Our lab had previously demonstrated that the persistent activation of the RAS-MAPK signaling in *Ras*-transformed cells caused altered chromatin structure and nuclear matrix profiles. Ciras-3 cells also have aberrant IEG expression with increased steady state levels of phosphorylated histone H1 and MSK1 substrates, histone H3 and HMGN1. However, the definitive role of MSK1 in these cells as a critical mediator that bridges a deregulated pathway with IEG expression and anchorage-independent growth is lacking. We hypothesize that oncogene-mediated activation of the RAS-MAPK pathway promotes increased MSK1 activity

and steady-state levels of phosphorylated H3, which may contribute to the aberrant gene expression and malignant transformation observed in these cells.

In human breast cancer cells, upregulation of the RAS-MAPK pathway can confer advantages in hormone independent growth and endocrine therapy resistance. However, whether the pathway is intact and responsive in hormone-dependent breast cancer cells have not been clearly shown. Further, the mechanism of TFF1 transcriptional activation, an immediate early gene, in response to stimulation of the RAS-MAPK signaling in these cells has not been demonstrated. In the next study (**Chapter 4**), we use an established hormone-dependent MCF7 breast cancer cell line to expand our current understanding of RAS-MAPK signaling and evaluate the contribution of the pathway in estradiol- and TPA-induced TFF1 expression. Although the association of H3 phosphorylation with gene induction is well documented, the role and mechanism of recruitment especially of histone-modifying enzymes such as MSK1 involved for transcription, specifically for the estrogen- and growth factor-responsive gene TFF1, remain elusive. We hypothesize that the irrespective of the stimuli, the RAS-MAPK pathway is induced and intact which consequently enhances association of MSK and H3 phosphorylation on TFF1 gene important for transcriptional activation in breast cancer cells.

We next sought to understand whether the *Ras*-driven MSK1 and H3 phosphorylation responses observed in our mouse fibroblast cell lines are general phenomena and evident in established human cancer cell lines with overexpressed growth factor receptors or activating *RAS* mutations. Characterization of an aberrant RAS-MAPK pathway in human cancer, particularly breast and pancreatic cancers have generally been focused on upstream mediators such as

tyrosine kinase receptors and oncogenic RAS molecules. There are limited studies that establish the contribution and consequences of the downstream factors such as MSK1 activation and histone H3 phosphorylation. In the last two studies (**Chapter 5 and 6**), we examine the relationship of overexpressed receptors or oncogenic K-RAS and elevated H3 phosphorylation levels. We also investigate whether extracellular stimulation of the RAS-MAPK pathway is intact in pancreatic cancer cells. We hypothesize that overexpressed receptors such as EGFR and HER2 or oncogenic K-RAS that results in persistent activation of RAS-MAPK signaling can augment nucleosomal H3 phosphorylation. Understanding the involvement of cytosolic and nucleosomal effectors in an aberrant or activated RAS-MAPK pathway in cancer cells may reveal important factors which may represent and be explored as viable cancer therapy targets.

1.8.1 General bridging hypothesis

Deregulation of the RAS-MAPK pathway produced by constitutive activation or overexpression of upstream components leads to aberrant MSK1 activity and elevated histone H3 phosphorylation levels. In turn, MSK1-mediated H3 phosphorylation (*i*) mediates immediate early gene expression and *Ras*-driven transformation as well as *(ii)* binds and associates with promoters and coding regions upon gene transcription.

1.8.2 Thesis aims

Our objectives are to:

(1) Characterize the activity and subcellular distribution of MSK1 in parental 10T1/2 and *HRas1*-transformed mouse fibroblasts

(2) Describe the importance of MSK1 in steady state levels of IEG-encoded proteins, TPA-mediated IEG expression and anchorage-independent growth in *HRas1*-transformed mouse fibroblasts

(3) Elucidate the molecular mechanism of transcriptional activation of the *TFF1* gene by the RAS-MAPK pathway in MCF7 breast cancer cell line

(4) Determine the steady state and inducible levels of H3 phosphorylation and its correlation with the ERK activity in pancreatic and breast cancer cell lines with overexpressed or mutated upstream components of the RAS-MAPK pathway.

Chapter 2: Mitogen- and Stress-Activated Protein Kinase 1 Activity and Histone H3 Phosphorylation in Oncogene-Transformed Mouse Fibroblasts

2.1 Abstract

Activation of the RAS-RAF-MEK-MAPK signal transduction pathway or the stress-activated protein kinase-2 (SAPK2)/p38 pathway results in the activation of MSK1. This activation of MSK1 leads to a rapid H3 S10ph. Previously, we had demonstrated that Ser10 phosphorylated H3 was elevated in Ciras-3 (c-*HRas1*-transformed 10T1⁄2) mouse fibroblasts and that H3 phosphatase activity was similar in Ciras-3 and 10T1⁄2 cells. Here, we demonstrate that the activities of ERK and MSK1, but not p38, are elevated in Ciras-3 cells relative to these activities in the parental 10T1⁄2 cells. Analyses of the subcellular distribution of MSK1 showed that the H3 kinase was similarly distributed in Ciras-3 and 10T1⁄2 cells, with most MSK1 being present in the nucleus. In contrast to many other chromatin modifying enzymes, MSK1 was loosely bound in the nucleus and was not a component of the nuclear matrix. Our results provide evidence that oncogene-mediated activation of the RAS-MAPK signal transduction pathway elevates the activity of MSK1, resulting in the increased steady state levels of phosphorylated H3, which may contribute to the chromatin decondensation and aberrant gene expression observed in these cells.

This collaborative work was published as:

Bojan Drobic, Paula S. Espino, and James R. Davie.

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Paula S. Espino optimized the protocol used to prepare figures 2.2 and 2.3, collected 20% of the data published (figures 2.1 and p38/p-p38 immunoblot data not shown) in this paper, assisted in the drafting and revisions of the manuscript.

2.2 Introduction

The RAS-MAPK signaling pathway is often deregulated in cancer cells. Mutations in *RAS* are found in about 30% of human cancers. Furthermore, defective or overexpressed cell surface receptors acting through this pathway, overexpressed *RAS* genes and mutations in the *B-RAF* gene result in persistent activation of the RAS-MAPK pathway (Calipel et al, 2003). Activation of the RAS-MAPK signaling pathway results in chromatin remodeling and expression of IEG (Davie et al, 1999; Laitinen et al, 1990). One of the downstream events of this pathway is phosphorylation of histone H3 at Ser10. Mouse fibroblasts transformed with c-*HRas1* have a more decondensed chromatin structure and greater steady-state level of H3 S10ph than that of parental cells (Chadee et al, 1999; Laitinen et al, 1990).

Phosphorylation of H3 at Ser10 occurs rapidly after stimulation of mammalian cells with agents such as EGF, TPA, or UV-B (Chadee et al, 1999; Thomson et al, 1999; Zhong et al, 2001). We and others (Chadee et al, 1999; Cheung et al, 2000; Clayton et al, 2000) have demonstrated that the phosphorylated H3 in EGF- or TPA–stimulated mouse fibroblasts is associated with IEG (c-*Fos*, c-*Myc*, and c-*Jun*). Phosphorylation of the NH2-terminal tail of H3 is thought to contribute to the disruption of IEG chromatin folding and intermolecular fiber-fiber interactions promoting the transcription of the gene. The H3 kinase responding to a stimulated RAS-MAPK pathway is MSK1. MSK1, which belongs to the AGC family of kinases and is related in structure to ribosomal p70 S6 kinase subfamily, is also activated through the p38/SAPK2 signaling pathway after activation by stressful stimuli (Deak et al, 1998).

The elevated steady-state level of H3 S10ph in c-*HRas1*–transformed mouse fibroblasts may be a consequence of increased activity of the H3 kinase and/or decreased activity of the H3 phosphatase. Our previous studies demonstrated that H3 phosphatase activity, which was identified as PP1, was similar in Ciras-3 mouse fibroblasts and in the parental 10T1⁄2 cells, suggesting that the H3 kinase was responsible for the increased steady-state levels of phosphorylated H3.

In this study, we analyzed the H3 kinase activity in Ciras-3 and 10T1⁄2 cells and determined whether the H3 kinase activity was MSK1. Furthermore, we investigated the MSK1 protein level and the subcellular distribution of MSK1 in Ciras-3 and 10T1⁄2 mouse fibroblasts. Evidence is provided that the H3 kinase activity that is elevated in Ciras-3 cells is MSK1. Also, we demonstrate that the subcellular distribution and level of MSK1 protein are similar in Ciras-3 and 10T1⁄2 mouse fibroblasts.

2.3 Materials and Methods

2.3.1 Cell culture.

The cell line Ciras-3 was derived from 10T1/2 cells by transfection with the T-24 c-*HRas1* oncogene (Chadee et al, 1999). Cells were grown in plastic tissue culture plates at 37°C in a humidified atmosphere containing 5% CO2 in α-MEM medium supplemented with 10% (v/v) fetal bovine serum, penicillin G (100 units/mL), streptomycin sulfate (100 μg/mL), and amphotericin B (250 ng/mL). The proportion of cells in the different cell cycle phases was determined by flow cytometry.

2.3.2 Preparation of cell extracts.

Cells were harvested and lysed in 400 µL of ice-cold Nonidet P40 buffer [150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 8.0), 0.5% Nonidet P40, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1.0 mmol/L NaF, 1.0 µg/mL leupeptin, 1.0 µg/mL aprotinin, and 25 µmol/L β-glycerophosphate]. Cell extracts were subjected to centrifugation at 10,000 X *g* for 10 minutes at 4°C, and the supernatant was saved. Protein concentration of the supernatant was determined using the Bio-Rad Protein Assay as per manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

2.3.3 H3 kinase assay.

10T1/2 and Ciras-3 cells were lysed with Nonidet P40 buffer, and the insoluble material was removed by centrifugation for 10 minutes at 10,000 X *g*. Cell cycle-matched total cell extracts (10 µg) were incubated with 2 µg of H3-H4 tetramer fraction isolated from mature chicken erythrocytes as described previously (Strelkov & Davie, 2002), 10 mmol/L $MgCl_2$, 1 µmol/L microcystin-LR and ± 10 µmol/L H89 for 10 minutes at 4°C. Reactions were started with addition of 50 µmol/L ATP and 5 µCi of [γ-^{32}P] ATP (3,000 Ci/mmol/L) and incubation at 30°C for 30 minutes. Reactions were stopped with the addition of SDS-PAGE loading buffer and incubation on ice for 20 minutes. Furthermore, cell cycle-matched total cell extracts (500 µg) were incubated with 3.5 µg of anti-MSK1 antibody coupled to 10 µL of protein G-Sepharose at 4°C for 24 hours. The protein G beads were washed three times with 500 µL of buffer B and twice with buffer C (Thomson et al, 1999). The beads were then resuspended in buffer C containing 10 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L NaF, 1 µg/mL leupeptin, 10 µg/mL aprotinin, 25 µmol/L β-

glycerophosphate, 1 μmol/L microcystin-LR, 5 μg H3-H4 tetramer, and ±10 μmol/L H89. Reactions were carried out as mentioned above. The H3-H4 tetramer and MSK1 immunoprecipitates were analyzed by SDS-15%-PAGE, and visualization and quantification of signals were analyzed by autoradiogram and phosphorimager analysis.

2.3.4 MSK1 kinase assay.

Fifty ng of purified active MSK1 (Upstate Biotechnology, Lake Placid, NY) was incubated with 1 μg of histone H3 (Roche Diagnostics, Mannheim, Germany) or H3-H4 fraction along with Mg^{2+}/ATP solution and 5 μCi [γ-^{32}P] ATP at 30°C for 15 minutes. The reaction was stopped by the addition of SDS-PAGE loading buffer and incubation on ice for 10 minutes. The samples were resolved by SDS-15%-PAGE. The gel was stained with Coomassie Blue stain. Visualization of the phosphorylation signal was detected by phosphorimager analysis and autoradiography.

2.3.5 Cellular fractionation.

Cellular fractionations were carried out with a slight modification as described previously (Sun et al, 2001). In brief, 10T1/2 and Ciras-3 cells were resuspended in Tris-NaCl-Mg^{2+} (TNM) buffer [100 mmol/L NaCl, 300 mmol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4), 2 mmol/L $MgCl_2$, and 1% thiodiglycol] containing all of the inhibitors mentioned in the Nonidet P40 buffer. Lysis of cells was performed by passage through a syringe with a 22-gauge needle. The cytosol and nuclei were isolated from lysed cells by centrifugation at 6000 X*g*. Isolated nuclei were inspected by microscopic analyses. The nuclei were resuspended in TNM buffer, and nuclei

extraction was performed by addition of Triton X-100 to a final concentration of 0.5% and incubation on ice for 5 minutes. After centrifugation at 6000 X *g* for 10 minutes, the supernatant, termed Triton X-100-soluble fraction, was saved. The nuclei pellet was resuspended in TNM buffer with 0.5% Triton X-100, and this fraction was termed Triton X-100-insoluble fraction.

2.3.6 Electrophoresis and immunoblotting.

Proteins were analyzed by SDS-(10% and 15%)-PAGE. The proteins analyzed by SDS-15%-PAGE were visualized by Coomassie Blue staining, and proteins analyzed by SDS-10%-PAGE were visualized by transfer to nitrocellulose membrane and immunochemical staining with various antibodies.

2.3.7 Antibodies.

Anti-phospho-p44/p42 MAPK, anti-phospho-p38, and anti-p38 rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-ERK (sc-93-G) goat polyclonal antibody and anti- Sp3 rabbit polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MSK1 rabbit polyclonal antibody used in immunoblotting was obtained from Sigma (St. Louis, MO). Anti-MSK1 sheep polyclonal antibody used in immunoprecipitation was purchased from Upstate Biotechnology. Anti-HDAC1 rabbit polyclonal antibody was from Affinity Bioreagents, Inc. (Golden, CO).

2.4 Results

Throughout this study we used cell cycle–matched Ciras-3 and 10T1⁄2 cells. Table 2.1 shows that the majority of the 10T1⁄2 and Ciras-3 cells were in G1 phase of the cell cycle. Expression of the c- *HRas1* oncogenes in Ciras-3 mouse fibroblasts is thought to result in the persistent stimulation of the RAS-MAPK pathway although did not cause any significant changes in cell cycle phases. The relative activities of the RAS-MAPK pathways in these cells were evaluated by immunoblot analyses of ERK1 and 2 and their phosphorylated isoforms. Figure 2.1 shows that steady state of activated phosphorylated ERKs was greater (about 6-fold) in Ciras-3 cells. Overexpression of *HRas1* has also been reported to activate the p38 pathway (Deng et al, 2004). However, phosphorylated (activated) p38 was not observed in immunoblot analyses of cell extracts from cell cycle-matched parental and oncogene-transformed cells (Figure 2.1). These data show that the steady-state activities of ERK1 and ERK2 are greater in the Ciras-3 than in the parental 10T1⁄2 cell line.

Table 2.1. Cell cycle distribution of parental and oncogene-transformed mouse fibroblasts

	Cell cycle phase (% distribution)		
Cell line	G_1	G_2-M	S
10T½	62	17	21
Ciras-3	68	13	19

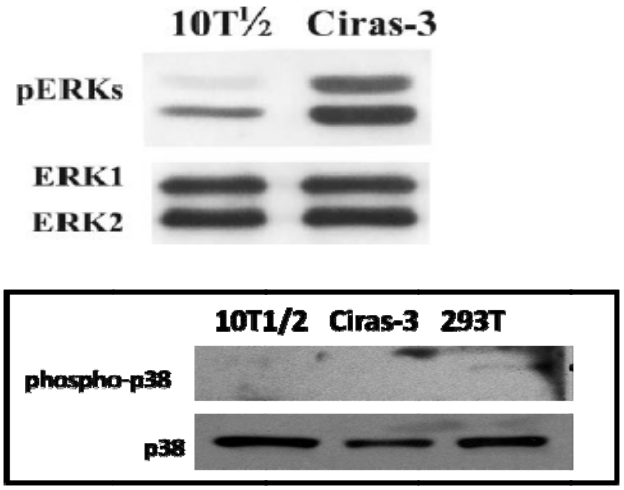

Figure 2.1. Increased level of phosphorylated ERK1/2 in oncogene-transformed mouse fibroblasts.

Total cell extracts (20 μg) were resolved on a SDS-10%-polyacrylamide gel, transferred to a membrane, and stained immunochemically with anti-phospho-p44/42 MAPK (*top panel*), anti-ERK (*middle panel*), anti-phospho-p38 and anti-p38 (*bottom panels*) antibodies. Lysate from 15Gy radiation-treated HEK293T cells were used as a positive control for anti-phospho-p38 antibodies.

Because the relative activity of the H3 phosphatase, PP1, was similar in Ciras-3 and 10T1⁄2, we surmised that an increased H3 kinase activity in the Ciras-3 cells would account for the increased levels of H3 S10ph. *In vitro* H3 kinase assays with equal amounts of cell extracted protein from cell cycle-matched cells were performed. Figure 2.2 shows that H3 kinase activity in Ciras-3 cell extract was greater than that from the 10T1⁄2 cells. We observed an increase of 3-fold in H3 kinase activity in the Ciras-3 cell extracts (average of three separate preparations). Histone H4 was also weakly labeled in this assay. As controls, we did not observe H3 kinase activity when either the histone substrate or cell extract was absent.

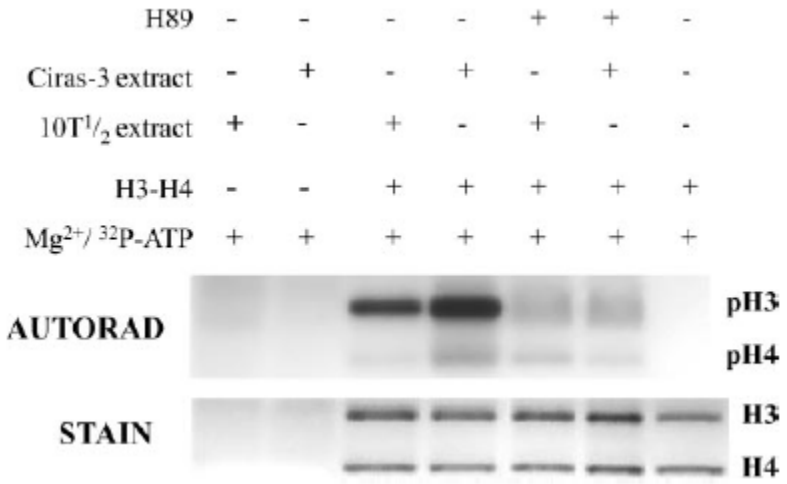

Figure 2.2. Histone H3 kinase activity in parental and oncogene-transformed mouse fibroblasts.

Cell extracts (10 μg) isolated from Ciras-3 and 10T1⁄2 cells were incubated with the H3-H4 fraction (2 μg), Mg^{2+}/ATP/[γ-^{32}P]ATP, and the presence or absence of H89. Samples were resolved by SDS-15%-PAGE. The gels were analyzed by Coomassie Blue staining (*stain*; *bottom panel*) and autoradiography (*autorad*; *top panel*).

H89 is a potent inhibitor of MSK1 (Thomson et al, 1999). To determine whether the H3 kinase activity was due to MSK1, the Ciras-3 and 10T1⁄2 cell extracts were pre-incubated with H89. Presence of the kinase inhibitor significantly reduced the H3 kinase activity in both cell extracts. The remaining H3 kinase activity may be Aurora B, the mitotic H3 kinase. We found that in contrast to MSK1, Aurora B was relatively insensitive to H89 inhibition in *in vitro* kinase assays (data not shown).

The results shown in Figure 2.2 presented evidence that MSK1 activity was greater in Ciras-3 than in 10T1⁄2 cells. However, H89 is also a potent inhibitor of PKA, which may also phosphorylate H3 (Davies et al, 2000; Salvador et al, 2001). To directly test whether the MSK1

H3 kinase activity was greater in Ciras-3 cells, MSK1 was immunoprecipitated from the cell extracts and assayed for H3 kinase activity. Figure 2.3 shows that the MSK1 activity was greater in the immunoprecipitated Ciras-3 fraction than that from the 10T1⁄2 cell extract. The H3 kinase activity of MSK1 in both preparations was inhibited by H89. In addition to radiolabeling of H3, weak labeling of H4 was observed, and H89 suppressed the labeling of this histone. In control experiments when the primary antibody was not included or the immunoprecipitate was not added, H3 kinase activity was not detected. Furthermore, in control experiments, we incubated purified histone H3 with a commercial preparation of MSK1 to show that MSK1 radiolabeled purified H3 (Figure 2.3, *right panel*). Other controls such as kinase assays of MSK1 immunodepleted cell extracts to demonstrate loss of or minimal H3 phosphorylation or use of a competitive MSK1 peptide can be used for future experiments.

The increased MSK1 activity observed in the Ciras-3 sample was not due to an increase in the amount of MSK1 protein immunoprecipitated from the Ciras-3 cell extract, as the stained gel shown in Figure 2.3 (*top panel*) revealed that the MSK1 immunoprecipitates from the cell extracts had similar amounts of MSK1. In repeats of these analyses (n = 4), we observed an average 3-fold increase in the MSK1 activity in the Ciras-3 immunoprecipitates relative to that in the 10T1⁄2 preparations.

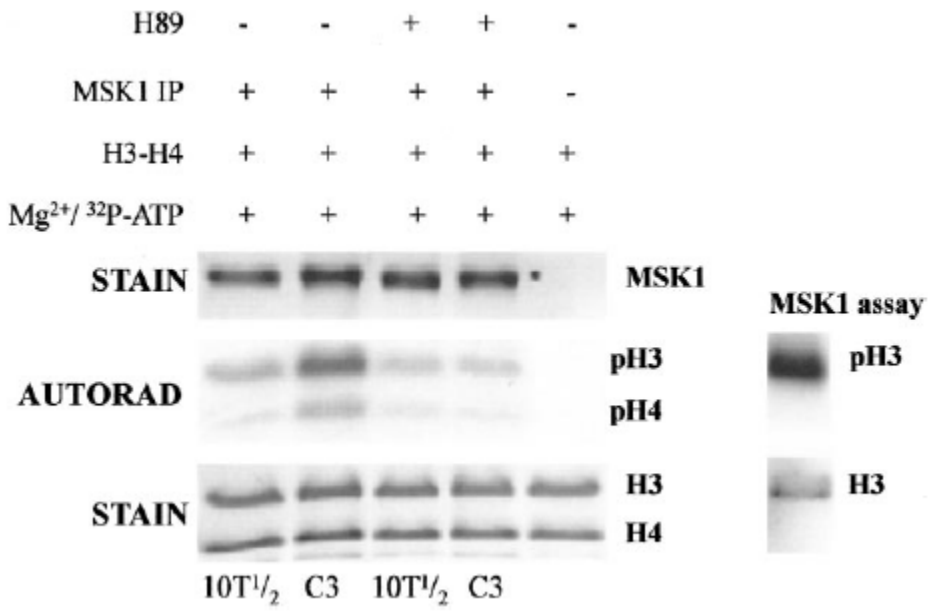

Figure 2.3. MSK1 kinase activity in parental and oncogene-transformed mouse fibroblasts.

MSK1 was immunoprecipitated from cell extracts (500 μg) isolated from Ciras-3 and 10T1⁄2 cells and incubated with the H3-H4 fraction (5 μg) in Mg^{2+}/ATP/[γ-^{32}P]ATP and the presence or absence of H89. The samples were resolved by SDS-15%-PAGE. The gels were analyzed by Coomassie Blue staining and autoradiography. The *top panel* is a coomassie-stained gel showing the amount of MSK1 immunoprecipitated from the cell extracts. The *middle panel* is the autoradiogram of the ^{32}P-labeled histone H3, whereas the *bottom panel* is a coomassie-stained gel showing the amount of H3 present in the kinase assay. The *right panel* referred to as the MSK1 assay is purified MSK1 (50 ng) incubated with H3 (1 μg), with the autoradiogram shown on the *top* and the stained gel shown on the *bottom*.

Constitutive activation of the RAS-MAPK pathway results in the altered expression at the protein level of signaling and cell cycle proteins (Calipel et al, 2003). The previous immunoprecipitation analyses shown in Figure 2.3 provided evidence that MSK1 protein levels were similar in Ciras-3 and 10T1⁄2 cell extracts. To explore this further, we compared the levels of MSK1 with ERKs by immunoblot analyses of cellular extracts from Ciras-3 and 10T1⁄2 cells. Figure 2.4 shows that the protein level of MSK1 in the two cell lines were equivalent in accord

with the immunoprecipitation results. Together, these data demonstrate that the increased phosphorylation of H3 observed in *Ras*-transformed mouse fibroblasts is due to an increase in the activity, but not protein level of MSK1.

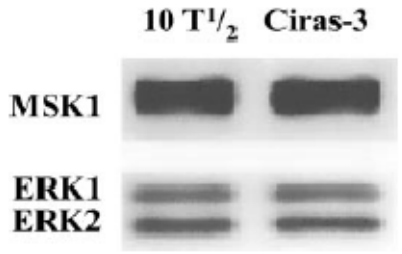

Figure 2.4. MSK1 protein levels in parental and oncogene-transformed mouse fibroblasts

Cell extracts (20 μg) isolated from Ciras-3 and 10T1⁄2 cells were resolved on a SDS-10%-polyacrylamide gel, transferred to a membrane, and immunochemically stained with anti-MSK1 (*top panel*) and anti-ERK (*bottom panel*) antibodies.

MSK1 is located in the nucleus and cytoplasm. Nuclear MSK1 would be responsible for phosphorylating H3 associated with IEG, whereas MSK1 located in the cytoplasm has an interesting role in translation by phosphorylating 4E-BP1 (Liu et al, 2002). To determine whether MSK1 subcellular distribution was altered in the *Ras*-transformed mouse fibroblasts, cell fractions were analyzed by immunoblotting. Cells were lysed in TNM buffer without any detergents to minimize loosely bound nuclear proteins from leaking out of the nuclei. The nuclei were then resuspended in TNM buffer with 0.5% Triton X-100 and incubated on ice to release matin assembly and remodeling factor

bound nuclear proteins, which includes proteins associated with the nuclear matrix (Triton P). As a reference, we compared the distribution of MSK1 with the nuclear transcription factor Sp3 and

chromatin remodeling enzyme, histone deacetylase 1 (HDAC1). Sp3 has three isoforms, the expressions of which are regulated at the level of translation by selection of different translation initiation sites on the Sp3 mRNA. A consistent observation in analyses of these cell fractions was that the relative level of the short Sp3 isoforms compared with the long isoform was greater in the Ciras-3 cells (Figure 2.5). This observation suggests that the translational machinery is altered in the *Ras*-transformed cells. Figure 2.5 shows the distribution of MSK1 among the various cellular fractions was similar for Ciras-3 and 10T1⁄2 cells. In contrast to Sp3 and HDAC1, MSK1 was present in the cytosol fraction. However, most MSK1 was located in the nuclear fractions in Ciras-3 and 10T1⁄2 cells. Also dissimilar from Sp3 and HDAC1, most, if not all, MSK1 was extracted from the nucleus with 0.5% Triton X-100.

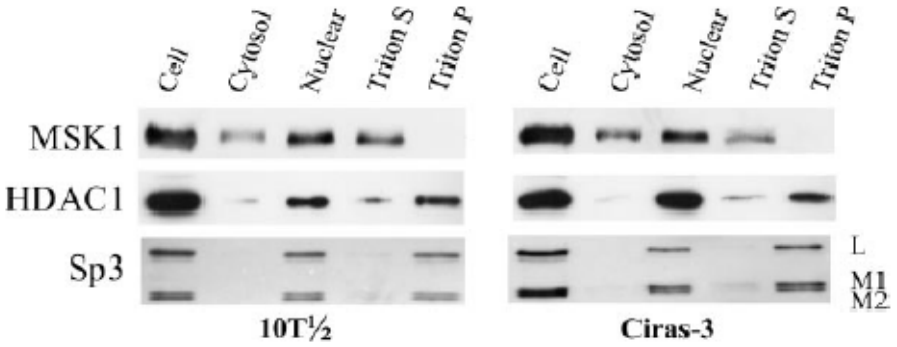

Figure 2.5. Cellular fractionation of parental and oncogene-transformed mouse fibroblasts.

Equal volumes for all fractions isolated from Ciras-3 and 10T1⁄2 cells were loaded and analyzed by SDS-10%-PAGE, transferred to a membrane, and immunochemically stained with anti-MSK1 (*top panel*), anti-HDAC1 (*middle panel*), and anti-Sp3 (*bottom panel*) antibodies. The long isoform of Sp3 is shown as *L*, whereas the short isoforms are denoted as *M1* and *M2*.

2.5 Discussion

Constitutive activation of the RAS-RAF-MAPK pathway in c-*HRas1*-transformed mouse fibroblasts increases the steady-state level of H3 S10ph. Here, we show that the activity of a downstream target of this signal transduction pathway, the H3 kinase MSK1, is increased in the *Ras*-transformed cells. Constitutive activation of the RAS-MAPK pathway did not alter the protein level of MSK1 or the subcellular distribution of the enzyme. Because the level of the H3 phosphatase is not altered in the *Ras*-transformed cells, the net result of this constitutively activated pathway is an increase in the steady-state level of phosphorylated H3.

In our kinase assays with H3 and H4 and immunoprecipitated MSK1, we observed a low level of H4 radiolabeling. There was the possibility that immunoprecipitated MSK1 was associated with another kinase that phosphorylated H4. However, we observed that purified MSK1, when incubated with H3 and H4, would also weakly label H4, with H3 being the preferred substrate. MSK1 phosphorylates H3 at the site RKS. H4 has the sequence RIS47, which may be the site phosphorylated albeit weakly by MSK1. Because this portion of the H4 molecule is in the histone fold, which is in the interior of the nucleosome, it is likely that H4 Ser47 would be inaccessible to MSK1 in chromatin.

Phosphorylated H3 in cycling Ciras-3 cells and in TPA- or EGF-stimulated 10T1⁄2 mouse fibroblasts is associated with relaxed chromatin regions that are located in specific nuclear locations (Chadee et al, 1999). In ChIP assays, we and others (Chadee et al, 1999; Cheung et al, 2000; Clayton et al, 2000) have provided direct evidence that the induced H3 S10ph is bound to the promoter and coding regions of IEG c-*Fos*, c-*Myc*, and c-*Jun*. Pretreatment of the Ciras-3 and

10T1⁄2 cells with the potent MSK1 inhibitor H89 reduced or prevented the transiently TPA-induced expression of IEG such as c-*Fos* and urokinase plasminogen activator (*uPA*), suggesting that phosphorylation of H3 contributed to the induced transcription of these genes (Strelkov & Davie, 2002). Similar results were obtained with MSK1/2 knock out mouse primary embryonic fibroblasts (Soloaga et al, 2003). The elevated activity of MSK1 in the *Ras*-transformed cells may be one of several deregulated chromatin modifying enzymes that through their action of remodeling chromatin lead to aberrant expression of genes in the transformed cells.

MSK1 is located primarily in the nucleus and to a lesser extent in the cytoplasm of Ciras-3 and 10T1⁄2 mouse fibroblasts. Most, if not all, MSK1 is extracted from the nuclei with 0.5% Triton X-100. Thus, the majority of MSK1 is loosely bound to chromatin and other nuclear substructures. This observation suggested that MSK1 was not associated with the nuclear matrix, a conclusion that was confirmed by demonstrating that nuclear matrices did not retain MSK1 (S. He and J. Davie; unpublished observations). MSK1 subnuclear location is in contrast to other chromatin modifying enzymes, such as histone acetyltransferases (CBP and PCAF) and histone deacetylases (HDAC1 and HDAC2), which are tightly bound in the nucleus and associated with the nuclear matrix (Sun et al, 2001).

Mutations in *RAS* are found at high frequency in different types of cancer including adenocarcinomas of the pancreas, colon, and lung. Additional mutations or overexpression of EGF receptors and HER2 receptors that signal through RAS to elevate the activity of the RAS-MAPK pathway are relatively common in breast cancer. Mutations such as those in the *B-RAF* gene and oncoproteins (*e.g.*, SRC) observed in melanoma and breast cancer will also activate the

RAS-MAPK pathway. The wide-spread involvement of the RAS-MAPK pathway in multiple cancer sites suggests that the activation of the H3 kinase MSK1 may be a frequent alteration in neoplasia and, considering the enzymes role in chromatin remodeling, may be a worthy target for therapeutic intervention.

2.6 Acknowledgements

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Chapter 3: Role of Heightened MSK1 Activity in the Malignant Phenotype of *Ras*-transformed Mouse Fibroblasts

3.1 Abstract

MSK1 is activated by the RAS-MAPK signaling pathway and plays a crucial role in the induction of IEG expression, the primary response to extracellular mitogenic and stress stimuli. MSK1 initiates the chromatin remodeling of IEG regulatory regions by mediating the nucleosomal response, including phosphorylation of histone H3 at serine 10 or 28. In metastatic *HRas1*-transformed mouse fibroblast Ciras-3 cells, the RAS-MAPK pathway is constitutively activated, resulting in elevated MSK1 activity mirrored by elevated steady state levels of phosphorylated H3. In this study, we showed that steady state levels of IEG-encoded proteins were also elevated in Ciras-3 cells. Despite these pre-existing upregulated levels of IEG products, AP-1 and COX-2, and heightened MSK1 activity in Ciras-3 cells, the mechanism driving the IEG expression upon extracellular stimulation by a tumour promoter (TPA) was unchanged from that in parental non-tumourigenic 10T1/2 cells. We found that, following MSK1-mediated phosphorylation of nucleosomal H3 at IEG regulatory regions, chromatin remodelers and modifiers were recruited by 14-3-3 proteins, readers of phospho-serine marks, leading to the occupancy of IEG promoters by the initiation-engaged form of RNA polymerase II and the onset of transcription. We demonstrated that, in Ciras-3 stably-expressing MSK1 uthor>Davie</Author><Year>2003</Year><RecNum>81</RecNum><record>well as the steady state levels of COX-2, FRA-1 and JUN were greatly reduced relative to control cells expressing an empty vector. Furthermore, MSK1 knockdown Ciras-3 cells lost their malignant phenotype, as reflected by the absence of anchorage-independent growth.

This collaborative work is submitted as:

Beatriz Pérez-Cadahía, Bojan Drobic, Paula S. Espino and James R. Davie

Role of heightened MSK1 activity in the malignant phenotype of Ras-transformed mouse fibroblasts

Journal of Biological Chemistry (submitted)

Paula S. Espino established the stably-expressing MSK1 knockdown cell lines in mouse fibroblasts and conducted experiments to troubleshoot and optimize cell growth conditions of these cells. She generated 20% of the data presented in the manuscript (specifically Figure 3.5 and part of 3.6), prepared the figures and assisted in the revisions of the paper.

3.2 Introduction

MSK1 is activated by the RAS-MAPK (RAS-RAF-MEK-ERK) and p38 MAPK pathways, and mediates the primary response by connecting mitogenic and stress extracellular stimuli with IEG expression (Deak et al, 1998; Soloaga et al, 2003). IEGs are identified by their rapid and transient transcriptional induction, requiring no new protein synthesis. MSK1 substrates include histone H3, nucleosome-binding protein HMGN1, and transcription factors such as the p65 subunit of NF-κB, ATF1, ER81 and CREB (Arthur, 2008). Stimulation of mouse fibroblasts with phorbol esters such as tumour promoter, TPA or EGF results in the phosphorylation of H3 at serine 10 and serine 28 and HMGN1 at serine 6, events termed the "nucleosomal response" (Dunn et al, 2009)(and references therein). In a recent study, we showed that MSK1 is a component of a multi-protein complex including BRG1, the ATPase subunit of the SWI/SNF remodeler, and phosphoserine adaptor 14-3-3 proteins. This complex is recruited to the upstream promoter elements of target genes by transcription factors, resulting in MSK1-mediated H3 phosphorylation at serine 10 or serine 28. We proposed a model in which 14-3-3 proteins bind to H3S10ph or H3S28ph, and acting as scaffolds, stabilize the SWI/SNF complex at the IEG upstream promoter elements, possibly increasing the residence time of the chromatin remodelers and transcription factors. The recruited SWI/SNF remodels nucleosomes around the promoter, enabling the binding of transcription factors and the onset of transcription (Drobic et al, 2010).

The RAS-MAPK pathway is abnormally active in approximately 30% of human cancers (e.g. colon, pancreatic, thyroid, lung and aggressive breast cancers) (Bos, 1989; Calipel et al, 2003; Dunn et al, 2005). Hence, we aimed to elucidate the physiological role of MSK1, and to

explore the relevance of MSK1 activity to the malignant potential of cells having an overactive RAS-MAPK signaling pathway. As model, we used Ciras-3, an *HRas1*-transformed 10T1/2 mouse fibroblast cell line, which is tumourigenic and metastatic and has a constitutively activated RAS-MAPK pathway (Egan et al, 1987a). Ciras-3 cells also exhibit a higher incidence of chromosomal instability than 10T1/2 cells (Dunn et al, 2009). It was demonstrated that Ciras-3 cells had elevated levels of phosphorylated ERKs, the hallmark of an activated RAS-MAPK pathway, increased MSK1 activity, but not MSK1 protein, and elevated steady state levels of H3S10ph, H3S28ph and HMGN1S6ph compared to the non-tumourigenic parental cell line 10T1/2 (Drobic et al, 2004; Dunn et al, 2009). The H3 phosphatase PP1 activity was similar in both cell lines. As was the case for parental 10T1/2 cells, TPA stimulation of serum-starved Ciras-3 cells resulted in the phosphorylation of H3 S10 or S28 and HMGN1 S6, as well as the IEG induction (Dunn et al, 2009; Strelkov & Davie, 2002). However, it is unknown whether the mechanism by which MSK1-induced chromatin remodeling of IEG regulatory regions is altered in Ciras-3 cells as a consequence of a deregulated RAS-MAPK pathway and genomic instability.

Among the IEGs induced in response to RAS-MAPK signaling, are those coding for the activator protein AP-1, a homodimer or heterodimer consisting of proteins of the JUN, FOS and ATF families. AP-1 regulates the expression of genes involved in proliferation, apoptosis, transformation, and cancer cell invasion (Mariani et al, 2007; Shen et al, 2008). Specifically, JUN and FRA-1 (FOS related antigen 1 encoded by *Fosl1*, the FOS-like antigen 1 gene) play a pivotal role in the RAS-induced transformation process (Mechta et al, 1997; van Dam & Castellazzi, 2001). Besides, amplification or overexpression of genes coding for JUN and FRA-1 induce transformation *in vivo* (Kustikova et al, 1998; Mariani et al, 2007; Shaulian & Karin,

2001; Verde et al, 2007). Another product of the RAS-MAPK pathway activation, COX-2 is highly expressed in different human cancers and enhances angiogenesis and tumor proliferation (Tsatsanis et al, 2006). The regulation of each of these three genes - *Jun*, *Fosl1* and *Cox-2* - is orchestrated by numerous transcription factors, which themselves must be activated. Moreover, transcriptional autoregulation and post-transcriptional stabilization are also involved. Adding to the functional complexities and oncogenic properties of JUN, FRA-1 and COX-2 is the fact that their activity is also tightly regulated. So a legitimate question was what impact MSK1 activity would have on the ability of JUN, FRA-1 and COX-2 to drive the cancer process. If MSK1 activity was knocked down in *Ras*-transformed cells, would JUN, FRA-1 and COX-2 steady state levels be affected, and would the cell malignancy be abolished?

In this study, we provide evidence that, despite deregulation of the RAS-MAPK pathway and genomic instability in Ciras-3 cells, the mechanism of MSK1-induced chromatin remodeling at IEG regulatory elements was similar to that of parental 10T1/2 cells. Furthermore, we demonstrated that the increased activity of MSK1 in *HRas1*-transformed cells resulted in higher steady state levels of IEG products than in parental cells, and conferred to the cells their malignant properties.

3.3 Materials and Methods

3.3.1 Cell culture

Ciras-3 mouse fibroblasts were grown at 37°C in a humidified atmosphere containing 5% CO_2 in α-MEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin G (100 units/mL), streptomycin sulfate (100 μg/mL) and amphotericin B (250 ng/mL). To specifically induce the RAS-MAPK signaling pathway, Ciras-3 cells were grown to 90-100% confluence and serum-starved for 72h in α-MEM medium supplemented with 0.5% FBS. Cells were treated with 100 nM TPA (Sigma, St Louis, MO, USA) from 30 to 240 min for protein levels analyses and for 15 and 30 min for gene expression and ChIP experiments. Pre-treatment with 10 μM H89 (EMD Chemicals, Gibbstown, NJ, USA) for 30 min prior to TPA treatment was included when necessary.

3.3.2 Soft agar colony assay.

Anchorage-independent growth of cells was assayed by estimating cell growth on soft agar (Egan *et al*., 1987). 1×10^4 cells were seeded into 1.5 ml of 10% FBS- α-MEM containing 0.35% agarose, in triplicate. For chemical MSK blockage, 10 μM H89 was added to the medium. Cell suspension in semi-solid medium was placed directly onto a layer of solid base 0.5% agarose containing 10% FBS- α-MEM in a 6-well plate. The plates were allowed to set and 1 ml of 10% FBS-α-MEM with penicillin G (100 units/mL), streptomycin sulfate (100 μg/mL), and amphotericin B (250 ng/mL), and 10 μM H89 when required were added and exchanged every 24 h. The plates were incubated at 37 °C in a 5% CO_2 incubator for 14 or 21 days prior to scoring and documentation. After incubation, the colonies were stained with 0.5 ml of 0.005%

crystal violet for at least 1 h for visualization, and imaged using Olympus SZX12 Dissecting microscope 8X Spot Advanced Camera ver4.0.9. Colonies were scored using the Bio-Rad Versadoc Model 1000 QuantityOne Ver4.6.0 software.

3.3.3 Cell proliferation assay.

Ciras-3 monolayer cell growth on plastic was evaluated using the CellTiter96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA), a colorimetric method for determining the number of viable cells. Cells were counted using a hemocytometer with trypan blue exclusion. $1X10^3$ cells were seeded in triplicate, using 96-well plates, in 100µl medium. Cells, placed at 37°C in a humidified atmosphere containing 5% CO2, were allowed to adhere for 4 h and incubated up to 96 h. At 4 h and every 24 h thereafter, 20 µl/well CellTiter96® Aqueous One Solution was added. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS tetrazolium) compound in the reagent is bioreduced by cells into a colored formazan product. Following addition of the reagent, the plate was incubated for 2 h at 37^0C in 5% CO_2 and absorbance was measured at 490 nm in a 96-well plate reader. To correct for background absorbance of the media, wells containing media alone in each time point were incubated with the solution reagent alongside the cells. The measured values of the time points in each sample were corrected by subtracting the absorbance in media alone and plotted.

3.3.4 Generation and maintenance of MSK1 stable knockdown mouse fibroblasts.

Empty GIPZ lentiviral vector and the GIPZ Lentiviral shRNAmir clones for mouse MSK1 (clone V2LMM_54318- SENSE 2240 (mm1), clone V2LMM_46372 SENSE 1059 (mm2) and clone V2LMM_57259 sense 523 (mm3), Thermo Scientific Open Biosystems, Huntsville, AL, USA) were obtained from the Biomedical Functionality Resource at University of Manitoba. MSK1 stable knockdown Ciras-3 cell lines were obtained as previously described (Drobic et al, 2010).

3.3.5 Chromatin immunoprecipitation (ChIP) assay.

ChIP assays were performed as described in (Drobic et al, 2010).

3.3.6 Preparation of total cell protein extracts.

Cells were harvested and lysed in 400 μL of ice-cold Nonidet P40 (NP-40) buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, 1.0 μg/mL leupeptin, 1.0 μg/mL aprotinin, 25 mM β-glycerophosphate) and centrifuged at 11,950xg for 10 minutes at 4°C. Protein concentration of the supernatant was measured by using Coomassie Plus (Bradford) Assay Reagent (Thermo Scientific, Rockford, IL, USA).

Total cellular protein extracts (20 μg) were resolved by SDS (10%)-PAGE and transferred to nitrocellulose membrane. Immunochemical staining with anti-JUN (Santa Cruz

Biotechnology, Santa Cruz, CA, USA, sc-1694), anti-FRA-1 (Santa Cruz Biotechnology, sc-183), anti-COX-2 (Millipore, Billerica, MA, USA, AB5118) and anti-β-actin (Sigma, A5441) antibodies was performed following the dilutions 1:2000, 1:2000, 1:3000 and 1:5000, respectively. Enhanced chemiluminescence kits were purchased from Perkin-Elmer (Waltham, MA, USA).

3.3.7 RNA isolation and real time RT-PCR analysis.

RNA isolation and real time RT-PCR analysis were done as previously described (Drobic et al, 2010).

3.4 Results

3.4.1 *HRas1*-transformed cells have increased steady state levels of COX-2, FRA-1 and JUN proteins compared to parental cells.

In *HRas1*-transformed, Ciras-3 cells, the elevated MSK1 activity relative to parental 10T1/2 cells results in higher steady state levels of phosphorylated H3 and HMGN1 (Drobic et al, 2004; Dunn et al, 2009; Strelkov & Davie, 2002). It was proposed that these circumstances may contribute to the aberrant gene expression observed in the oncogene-transformed cells (Strelkov & Davie, 2002). Thus, we analyzed the steady state levels of some IEG- encoded proteins. In comparing the steady levels of COX-2, FRA-1 and JUN, we studied parental 10T1/2 and Ciras-3 cells with equal proportions of cells in each phase of the cell cycle (Figure 3.1A). 10T1/2 and Ciras-3 cell extracts were resolved by SDS-PAGE and transferred onto nitrocellulose

for immunoblotting analysis. Figure 3.1B shows that IEG protein levels were higher in Ciras-3 than in 10T1/2 cells; 6 times higher for COX-2, 4 times for FRA-1 and 8 times for JUN. Meanwhile levels of β-actin control were equivalent in both cell lines. Thus, elevated MSK1 activity in Ciras-3 cells is correlated with elevated steady state levels of IEG-encoded proteins.

A

	G_0/G_1	G_2/M	S
10T1/2	70%	12%	18%
Ciras-3	65%	14%	21%

B

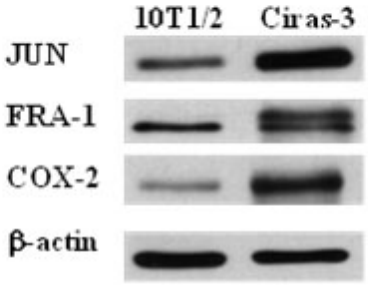

Figure 3.1. Protein levels of COX-2, FRA-1 and JUN in parental and *HRas1*-transformed mouse fibroblasts.

(**A**) Cell cycle distribution of 10T1/2 and Ciras-3 cells as determined by FACS analysis. (**B**) 20μg of cell-cycle matched 10T1/2 and Ciras-3 cell lysates were resolved on SDS-10%-PAGE. Membranes were immunochemically stained with anti-COX-2, anti-FRA-1, anti-JUN and anti-β-actin antibodies.

3.4.2 TPA-stimulation of *HRas1*-transformed cells promotes the MSK1-mediated H3 phosphorylation followed by recruitment of 14-3-3 proteins and chromatin remodelers/modifiers at IEG regulatory regions.

We previously reported that MSK1 activity was required for the establishment of the chromatin environment observed at IEG regulatory regions upon TPA-stimulation of 10T1/2 parental cells. However, it is possible that the chain of events initiated by MSK1 or that the targeting and location of active phospho-marks would not be preserved in a background of genomic instability and constitutive RAS-MAPK signaling, with pre-existing elevated levels of IEG products. Hence, we performed a series of ChIP assays on serum-starved Ciras-3 cells treated with TPA for 0, 15 or 30 min. The genomic structure and regulatory sites of the murine *Fosl1, Jun* and *Cox-2* genes, as well as regions chosen for analysis are displayed in Figure 3.2A (*Fosl1*) and Supplementary Figures 3.7A (*Jun*) and 3.8A (*Cox-2*). The distribution of MSK1, H3S10ph and H3S28ph, along the regulatory and coding regions of these genes was determined, using high resolution ChIP assays, in which nuclei isolated from formaldehyde-treated cells were digested with micrococcal nuclease such that the chromatin was processed down to mononucleosomes. Upon TPA induction, MSK1 associated with the three regulatory regions of the *Fosl1* gene, the 5' distal region (-1113) containing a putative binding site for the C/EBPb protein, the 5' proximal region (-187) with multiple responsive elements and the region (+989) located in intron 1 which has an AP-1 site. There was no TPA-induced association of MSK1 with the *Fosl1* coding region (+2249 and +7676) (Figure 3.2A and B). MSK1 also associated with both upstream regions of the *Jun* gene, the 5' distal region (-711) containing a putative binding site for the ELK-1 transcription factor and the 5' proximal region (-146) with binding sites for several transcription factors including JUN (Supplementary Figure 3.7A and B), and with the 5' distal (-493) and 5' proximal (-111) upstream regions of the *Cox-2* gene (Supplementary Figure 3.8A and B), but not to the coding region of either gene (Supplementary Figures 3.7A, 3.7B, 3.8A and 3.8B). The distribution of H3S10ph and H3S28ph mirrored that of

MSK1 along all three genes (Figure 3.2B and Supplementary Figures 3.7B and 3.8B). TPA-induced occupancy of all three gene regulatory regions by 14-3-3ε and 14-3-3ζ was observed in Ciras-3 cells (Figure 3.2C and Supplementary Figures 3.7C and 3.8C), indicating that the phospho-marks were "read" by 14-3-3 proteins, as in parental 10T1/2 cells (Drobic et al, 2010). Likewise, the H3 K-acetyltransferase PCAF and BRG1, the ATPase subunit of the chromatin remodeler SWI/SNF, were recruited at the three gene regulatory regions upon TPA-stimulation of Ciras-3 cells (Figure 3.2C and Supplementary Figures 3.7C and 3.8C). Further, elevated MSK1 activity in Ciras-3 cells, MSK1 loading and H3S10ph, H3S28ph positioning at IEG upstream promoter elements was not affected. These results show that the mechanism of MSK1-induced remodeling of IEG upstream promoter elements was not altered in *HRas1*-transformed cells, relative to parental 10T1/2 cells.

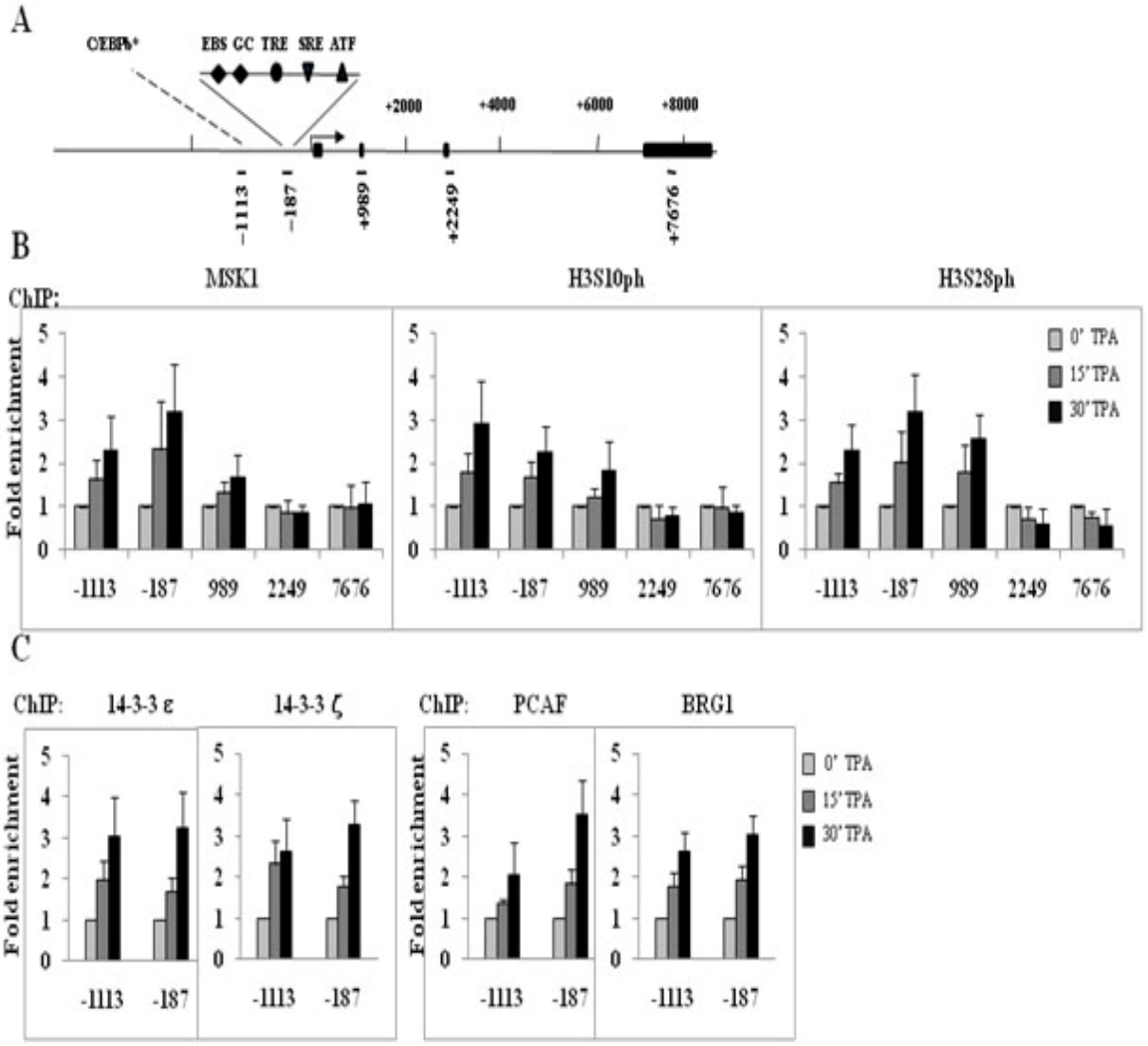

Figure 3.2. TPA-induced nucleosomal response and recruitment of 14-3-3 and chromatin modifiers/remodelers at the *Fosl1* regulatory regions in *HRas1*-transformed mouse fibroblasts.

(**A**) Schematic representation of *Fosl1* gene showing regions amplified in the ChIP assays. Each region is labeled according to the 5' position of the forward primer relative to the transcription start site. Exons are represented by boxes, and binding sites of relevant transcription factors located in the amplified regions are displayed. Abbreviations are: C/EBP,CCAAT-enhancer binding protein; EBS, Ets binding site; GC, GC box which is a binding site for the Sp family transcription factors; TRE, TPA-responsive element; SRE, serum-responsive element; ATF, activating transcription factor. * indicates a putative binding site. ChIP experiments were performed using antibodies against MSK1, H3S10ph, H3S28ph (**B**), 14-3-3ε/ζ, PCAF and BRG1 (**C**) on formaldehyde-crosslinked mononucleosomes prepared from serum-starved Ciras-3 cells

treated with TPA for 0, 15 and 30 min. Equal amounts of input and immunoprecipitated DNA were quantified by real-time quantitative PCR. Enrichment values are the mean of three independent experiments and the error bars represent the standard deviation.

3.4.3 TPA-induced IEG transcription initiation in *HRas1*-transformed cells is abolished by MSK inhibitor H89.

We previously reported that the MSK inhibitor H89 prevented the TPA-induced transcription initiation of the IEGs, *Jun*, *Cox-2* and *Fosl1*(Drobic et al, 2010). To investigate whether TPA-induction of IEGs in *HRas1*-transformed cells is dependent on MSK1 activation, we quantified *Fosl1*, *Jun* and *Cox-2* mRNA levels upon 0, 15 and 30 min TPA-induction of Ciras-3 cells pre-treated or not with 10 μM H89. Figure 3.3A shows that *Fosl1* transcription was not induced when serum-starved Ciras-3 cells were exposed to H89 prior to TPA treatment. Conversely, the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels were not affected by H89 pre-treatment. The TPA-induced transcription of *Jun* and *Cox-2* was also abolished by H89 pre-treatment (data not shown). To test if inhibition of MSK activity prevents the TPA-induced formation of a transcription initiation complex at the 5' end of IEGs, we performed high resolution ChIP assays with antibodies raised against RNA polymerase II phosphorylated at serine 5 (RNAPII S5ph), the initiation-engaged form of RNAPII. We found that upon TPA stimulation of H89 pre-treated Ciras-3 cells, RNAPII S5ph was not associated with the promoter region of *Fosl1* (Figure 3.3B), *Jun* or *Cox-2* (data not shown). These results suggest that MSK activity is required for the formation of an initiation complex at IEG promoters in *HRas1*-transformed cells.

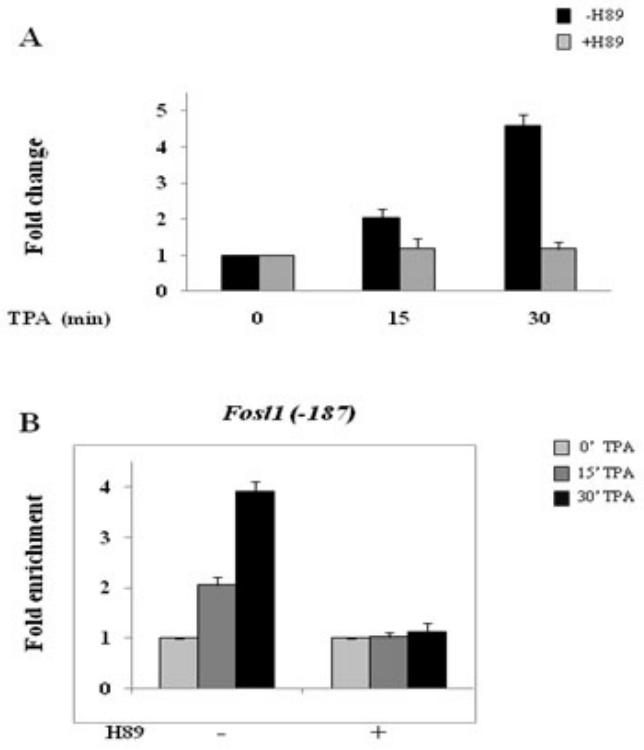

Figure 3.3. TPA induction of *Fosl1* in *HRas1*-transformed cells.

(**A**) Serum-starved Ciras-3 cells were treated or not with H89 prior to TPA stimulation for 0, 15 or 30 min. Total RNA was isolated and quantified by real time RT-PCR. Fold change values were normalized to GAPDH levels and time 0 values. (**B**) Ciras-3 formaldehyde-crosslinked mononucleosomes were prepared and used in ChIP assays with anti-RNAPII S5ph antibodies. Equal amounts of input and immunoprecipitated DNA were quantified by real-time quantitative PCR. The enrichment values of the upstream promoter region of the *Fosl1* gene (-187) are the mean of three independent experiments, and the error bars represent the standard deviation.

3.4.4 TPA-induced MSK1 recruitment, H3 phosphorylation as well as recruitment of 14-3-3 proteins and chromatin remodelers/modifiers at IEG regulatory regions are abolished by MSK inhibitor H89 in *HRas1*-transformed cells.

To determine if the absence of TPA-induced IEG transcription initiation in H89-pre-treated Ciras-3 cells was a consequence of a lack of chromatin modification and remodeling at IEG

regulatory regions, we performed the ChIP experiments on H89 pre-treated cells. Figure 3.4 shows that H89 treatment prevented MSK1 recruitment to the three regulatory regions of *Fosl1*. Consequently, phosphorylation of H3 residues S10 and S28 in response to TPA treatment did not occur. In agreement with the proposed model, impairing the setting of the phospho-marks thwarted the recruitment of the phospho-H3 effectors 14-3-3ε and 14-3-3ζ, the chromatin modifier PCAF and the component BRG-1 of the chromatin remodeler complex. H89 pre-treatment of TPA-stimulated Ciras-3 cells similarly blocked this series of events at the regulatory regions of *Jun* and *Cox-2* genes (Supplementary Figures 3.9 and 3.10).

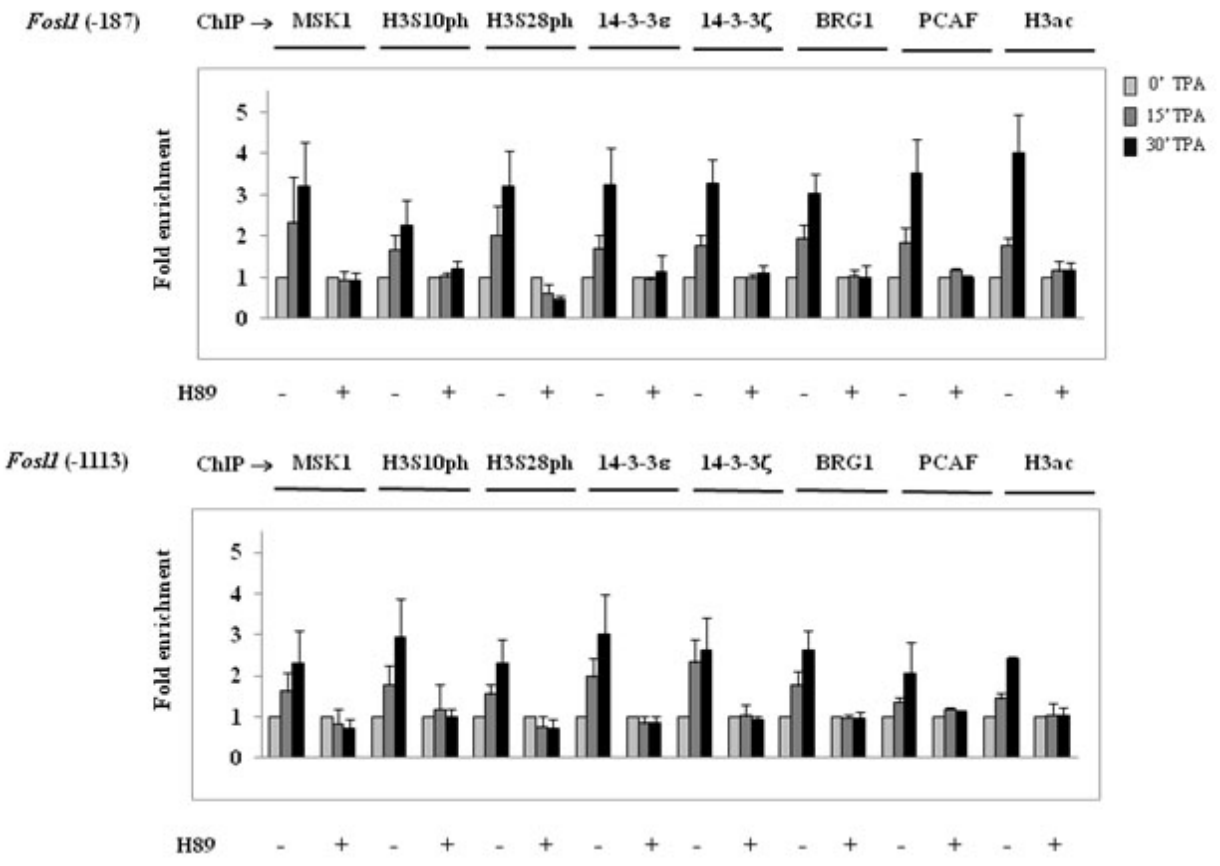

Figure 3.4. H89 inhibition of TPA-induced nucleosomal response and chromatin remodeler/modifier recruitment to *Fosl1* regulatory regions in *HRas1*-transformed mouse fibroblasts.

Serum-starved Ciras-3 cells were treated or not with H89 prior to TPA stimulation for 0, 15 or 30 min. Formaldehyde-crosslinked mononucleosomes were prepared and used in ChIP assays with antibodies as indicated. Equal amounts of input and immunoprecipitated DNA were quantified by real-time quantitative PCR. Enrichment values are the mean of three independent experiments and the error bars represent the standard deviation.

3.4.5 MSK1 activity is crucial to malignant phenotype of *HRas1*-transformed cells.

Ciras-3 cells have metastatic properties as opposed to parental 10T1/2 cells. Ciras-3, but not 10T1/2 cells, readily form colonies in soft agar, exhibiting an anchorage-independent growth that is distinctive of neoplastic transformation (Dunn et al, 2009; Egan et al, 1987a). In order to ascertain whether MSK activity is important to their metastatic potential, Ciras-3 cells were seeded in soft agar and left untreated or treated with 10μM of H89 for 3 weeks, after which the number of colonies was evaluated. Figure 3.5A shows that the MSK inhibitor H89 interfered with the ability of Ciras-3 cells to form colonies in soft agar. To ensure that the lack of growth in soft agar was not a consequence of some other cellular effect of H89 on cell proliferation, we used a lentiviral vector system stably expressing short hairpin RNA (shRNA) to generate MSK1 stable knockdown Ciras-3 cell lines. Ciras-3 cells were transfected with three different shRNA vectors (mm1, mm2 and mm3) targeted to different regions of the MSK1 coding sequence. These multiple targeting vectors were used to control for off-target effects (Echeverri et al, 2006). Controls consisted of mock-transfected and empty vector pGIPZ-transfected cell lines. Immunoblot analysis demonstrated that MSK1 levels in Ciras-3 cells stably-expressing MSK1 knockdown targeting vectors mm1, mm2 or mm3 were, relatively to Ciras-3 cells expressing the pGIPZ control vector, reduced by 60%, 24% or 63%, respectively (Figure 3.5B). When testing for their anchorage-independent growth capabilities in a soft agar assay, Ciras-3 cells expressing

MSK1 knockdown targeting vectors mm1, mm2 or mm3 produced colonies of which numbers represented 34%, 83% and 16%, respectively of the number counted for the empty vector pGIPZ-transfected cell control (Figure 3.5C and D). These numbers inversely correlate with the extents of MSK1 knockdown in the three cell lines. In particular, both mm1 and mm3 vectors, which resulted in at least 60% reduction in MSK1 protein levels, led to only 34% and 16% remaining anchorage-independent growth, respectively when visible colonies were quantified. On the other hand, Ciras-3 cell line expressing the mm2 vector, which exhibited the least MSK1 protein reduction (76% MSK1 remaining protein), behaved similarly to the pGIPZ empty vector Ciras-3 control (83% colonies growing) (Figure 3.5D). In contrast to the anchorage-independent growth, the monolayer cell growth on plastic of the cell line expressing the mm3 vector was not impacted to a significant extent by the MSK1 knockdown (Figure 3.5E). These data demonstrate that the malignant potential of the Ciras-3 cell line, represented by the cell anchorage-independent growth capability, is dependent on MSK1 activity.

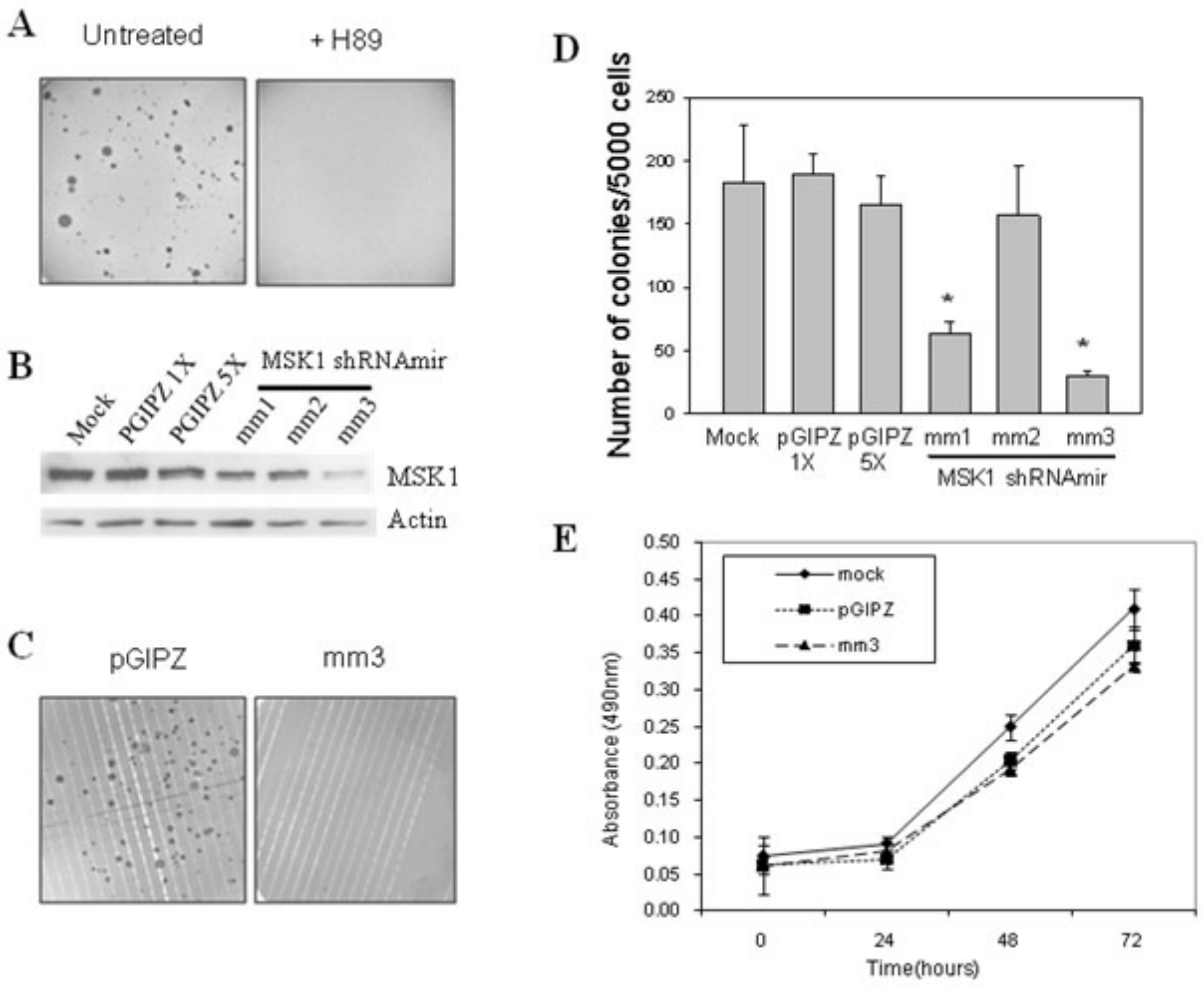

Figure 3.5. MSK1 activity is required for anchorage-independent growth of *HRas1*-transformed mouse fibroblasts.

(**A**) Ciras-3 cells were grown in soft agar with or without H89. (**B**) 20µg of MSK1 knockdown or control cell lysates were resolved on SDS-10%-PAGE and immunoblotted with anti-MSK1 and anti-β-actin antibodies. (**C**) Photographs of growth on soft agar plates are shown for the Ciras-3 cells stably-expressing MSK1 knockdown targeting vectors mm3 or pGIPZ. (**D**) Equal numbers of Ciras-3 stably-expressing MSK1 knockdown targeting vectors mm1, mm2 or mm3 as well as pGIPZ control and mock-transfected cells were grown in soft agar. Numbers of colonies were counted after 14 days and plotted for each cell line. Data are representative of three individual experiments. *Student ttest comparison to pGIPZ control $p<0.005$(**E**) Ciras-3 stably-expressing MSK1 knockdown targeting vector mm3 as well as pGIPZ control and mock-treated cells were seeded in triplicate in a 96-well plate and evaluated for cell proliferation using CellTiter96® Aqueous One Solution reagent. The absorbance readings were measured at 490 nm and measured values were plotted as described in Materials and Methods. Data are representative of three individual experiments. Error bars represent standard deviation.

3.4.6 Levels of MSK1 activity regulate steady state levels of IEG products in *HRas1*-transformed fibroblast cells.

To determine whether heightened activity of MSK1 was responsible for AP-1 and COX-2 protein levels, we compared the TPA-induction of *Fosl1*, *Jun* and *Cox-2* in Ciras-3 expressing MSK1 knockdown targeting vector mm3 or pGIPZ empty vector. Figure 3.6A shows that, in Ciras-3 cells expressing mm3, the TPA-induction of the *Fosl1* gene was reduced by 67% relatively to the control cells. Similar results were obtained with *Jun* and *Cox-2*, their expression being reduced by 58% and 64% in MSK1 knockdown cells compared to control cells (data not shown). We showed in Figure 3.1 that IEG protein steady state levels were higher in Ciras-3 cells than in parental 10T1/2 cells, mirroring the increased MSK1 activity. Hence, we compared IEG protein steady state levels in Ciras-3 expressing MSK1 knockdown targeting vector mm3 or pGIPZ empty vector. In knockdown cells with MSK1 steady state levels reduced by 91% relatively to control cells, steady state levels of COX-2, FRA-1 and JUN, normalized to β-actin levels, were reduced by 34%, 45% and 49%, respectively (Figure 3.6B). Thus, the level of MSK1 activity in *HRas1*-transformed mouse fibroblast cells is critically important as it correlates with the steady state levels of IEG-encoded products.

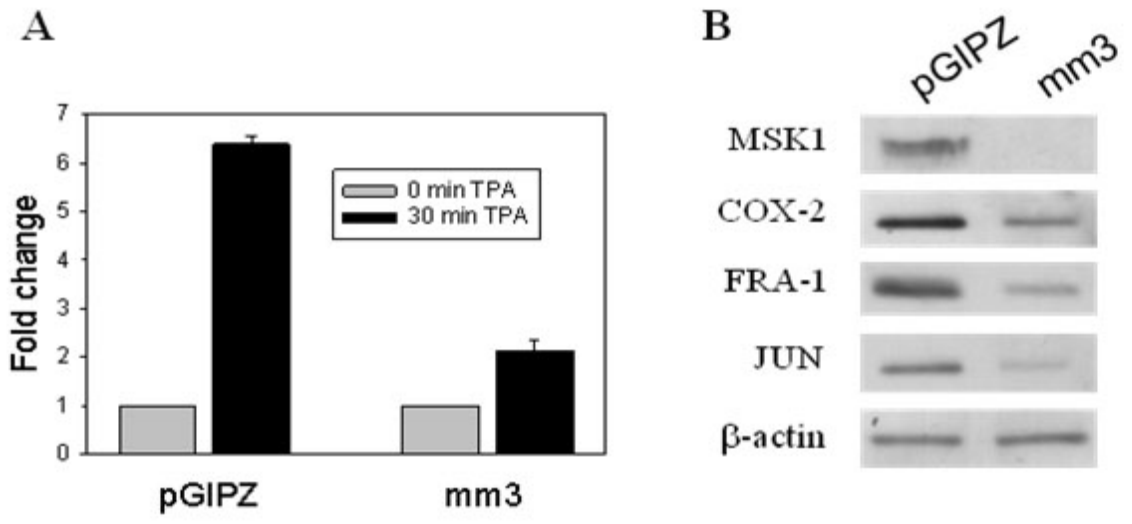

Figure 3.6. : MSK1 activity is required for increased steady state levels of IEG proteins in *HRas1*-transformed fibroblast cells.

(**A**) Serum-starved Ciras-3 stably-expressing MSK1 knockdown targeting vector mm3 and pGIPZ empty vector were left untreated or treated with TPA for 30 minutes. Total RNA was isolated and quantified for *Fosl1* mRNA by real time RT-PCR. Fold change values were normalized to GAPDH levels and time 0 values. (**B**) 20µg of cell lysates from Ciras-3 expressing MSK1 knockdown targeting vector mm3 and pGIPZ control were resolved on SDS-10%-PAGE and immunoblotted with anti-COX-2, anti-FRA-1, anti-JUN and anti-β-actin antibodies.

3.5 Discussion

In this study, we show that *HRas1* transformation leads to elevated steady state levels of JUN and FRA-1, AP-1 family members, and COX-2 in Ciras-3 cells. Further, we demonstrate that MSK1 elevated activity is responsible for the elevated AP-1 and COX-2 levels, and that MSK1 is required for anchorage-independent growth of Ciras-3 cells. Previous studies reported that JUN and FRA-1 were critical to the *Ras* transformation process and that overexpression of JUN and/or FRA-1 in NIH3T3 fibroblast cells induced anchorage-independent growth (Mechta

et al, 1997; van Dam & Castellazzi, 2001). Together, these results demonstrate that RAS oncogene stimulates the MEK-ERK-MSK pathway driving the nucleosomal response and IEG expression, which is responsible for the anchorage-independent growth.

Besides elevated levels of MSK1 and IEG products, *HRas1* transformation results in a more open chromatin structure (Dunn et al, 2009) and increased expression of DNA methyltransferase 1 (Bigey et al, 2000; Patra, 2008), which catalyzes DNA methylation, an epigenetic mechanism altering gene expression. Despite these features distinguishing the cellular environments of *HRas1*-transformed Ciras-3 cells from parental 10T1/2 cells, the mechanism by which MSK1 induces IEG expression is not altered by *HRas1* transformation. The central role played by MSK1 in the nucleosomal response and IEG induction in Ciras-3 cells was made evident by the demonstration that IEG induction and steady state levels of IEG-encoded proteins were attenuated in experiments using H89 or knocking down MSK1. Our studies provide mechanistic understanding to previous data showing the importance of MSK1 and the nucleosomal response in the malignant transformation of mouse epidermal cells and *v-Src*-transformed rat fibroblast cells (Kim et al, 2008; Tange et al, 2009).

Although the mechanism of MSK1-mediated IEG induction is unchanged in *Ras*-transformed cells, the upregulation of MSK1 activity promotes a modified cellular environment and biology with a reprogrammed gene expression profile, causing changes in steady state levels of critical transcription factors, which are mediators of cellular malignancy. Besides AP-1, we studied another MSK1 target, COX-2. COX-2 is an enzyme required for prostaglandin E2

synthesis and a potent stimulator of the apoptosis inhibitor BCL-2. Being highly expressed in several human malignancies, including colorectal, gastric, prostate, breast, lung, and endometrial cancer (Sahin et al, 2009; Tsatsanis et al, 2006), COX-2 has attracted a lot of interest from pharmaceutical companies, which have developed and marketed selective COX-2 inhibitors. While these COX-2 inhibitors might be beneficial in combination with chemotherapy for cancer treatment, their cardiovascular toxicity makes them unsuitable for long-term and/or preventive use (Tsatsanis et al, 2006). Therefore, there is a strong incentive to discover new compounds that would target signaling pathways controlling *COX-2* induction. Other cancer-related genes induced through the MSK1-mediated nucleosomal response are the TPA-induced *TFF1* gene in the human breast cancer cell line MCF-7 and the constitutively active *IL-6* gene in highly metastatic MDA-MB-231 human breast cancer cells (Espino et al, 2006; Ndlovu et al, 2009). As another downstream response to RAS-MAPK sustained activation, the constitutive hTERT expression and telomerase activation, required for cell immortalization, may also contribute to malignancy (Ge et al, 2006). Also induced by RAS-MAPK activation is the gene coding for the *uPA*, involved in extracellular matrix (ECM) degradation, an essential process in the migration, invasion and metastasis of cancer cells (Strelkov & Davie, 2002). It was also shown that *uPA* was induced by tobacco smoke via the activation of the EGFR/ERK1/2/MAPK signaling pathway in the MSK-Leuk1 cell line established from a cancerous lesion of the oral cavity. The *uPA* induction was paralleled with an increased cell migration through ECM, implicating a role of the nucleosomal response in the tobacco smoke-mediated clonal migration of premalignant and malignant lesions in the aerodigestive tract (Du et al, 2007).

The above citations point to only a few of the genes induced downstream of the RAS-MAPK pathway and illustrate the potential consequences of the gene expression reprogramming taking place upon activation of the RAS-MAPK pathway. This reprogramming is rapid and transient, with the activity of IEG-encoded proteins lasting a limited period of time. However, when the RAS-MAPK pathway is constitutively activated, MSK1 activity is continually enhanced, and the resulting continuous IEG induction results, as we demonstrated in this study, in elevated steady state levels of IEG-encoded proteins. This state of deregulation leads to a permanently altered gene expression program; perhaps facilitating the progress of transformed cells towards the next stage of tumour formation or metastasis.

Mutations in RAS are found in approximately 30% of human cancers (Bos, 1989). Furthermore, persistent activation of the RAS-MAPK pathway also results from an overexpressed *RAS* gene, mutations in *B-RAF* gene or defective or overexpressed cell surface receptors acting through this pathway (Calipel et al, 2003; Dunn et al, 2005). For example, overexpression of HER2, belonging to the HER/ERBB family of cell surface receptor tyrosine kinases (RTKs) otherwise known as EGFRs, is found in 25-30% of breast tumours (Slamon et al, 1989). We showed that, in cycling human breast cancer cell lines overexpressing EGFR or HER2/EGFR, increased levels of phospho-ERK1/2 paralleled the increased levels of H3S10ph, but not H3S28ph (Delcuve et al, 2009). Likewise, mutations in *K-RAS* gene occur in most pancreatic cancers. In three human pancreatic cancer cells lines with and without activating mutations in *K-RAS*, an increase in H3S10ph was detected in response to the RAS-MAPK activation (Espino et al, 2009). Thus, it appears that H3 S28ph is not included in the nucleosomal response of all cell types. It is noteworthy that the patterns of RAS-MAPK

stimulation in the three analyzed human pancreatic cancer cells lines appeared to vary with the stimuli and cell contexts, suggesting that each stimulus can dictate diverse responses in different cells. However, the extent of the RAS-MAPK stimulation, as reflected by the levels of phospho-ERKs, and the extent of MSK1-mediated H3 S10 phosphorylation paralleled each other. These data suggest that the MSK1-mediated H3S10ph level might be a better marker for cancer progression than the nature of the K-RAS mutation (Espino et al, 2009).

Considering that MSK1 is the active link between the signaling cascade and the primary response at the gene expression level, it is an ideal candidate for cancer chemotherapy and it has immense potential in the treatment of cancers in which the RAS-MAPK pathway is abnormally active (e.g. colon, pancreatic and aggressive breast cancers). Since MSK knockout mice are viable and fertile (Wiggin et al, 2002), inhibition of MSK1 activity would likely not have significant adverse effects on normal cells and, due to oncogenic addiction of cancer cells, it might result in differentiation or apoptosis of cancer cells (Weinstein, 2002).

3.6 Acknowledgements

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Supplementary Information is available on the Oncogene website (http://www.nature.com/onc)

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Supplementary Figures

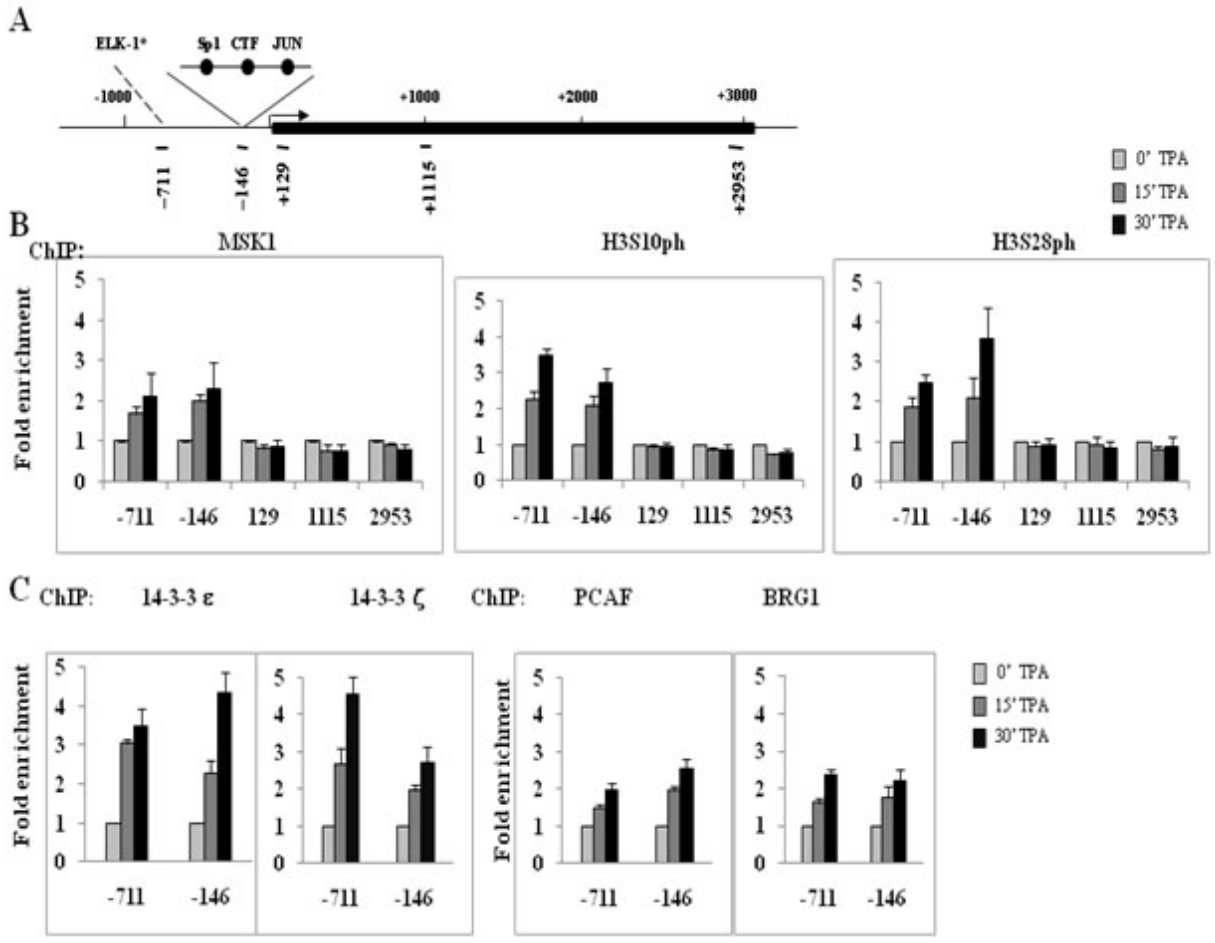

Figure 3.7. TPA-induced nucleosomal response and recruitment of 14-3-3 and chromatin modifiers/remodelers at the *Jun* regulatory regions in *HRas1*-transformed mouse fibroblasts.

(**A**) Schematic representation of *Jun* gene showing regions amplified in the ChIP assays. Each region is labeled according to the 5' position of the forward primer relative to the transcription start site. Exons are represented by boxes, and binding sites of relevant transcription factors located in the amplified regions are displayed. CTF, CCAAT-box-binding protein (also known as NF1, nuclear factor 1); * indicates a putative binding site. ChIP experiments were performed using antibodies against MSK1, H3S10ph, H3S28ph (**B**), 14-3-3ε/ζ, PCAF and BRG1 (**C**) on formaldehyde-crosslinked mononucleosomes prepared from serum-starved Ciras-3 cells treated with TPA for 0, 15 and 30 min. Equal amounts of input and immunoprecipitated DNA were

quantified by real-time quantitative PCR. Enrichment values are the mean of three independent experiments and the error bars represent the standard deviation.

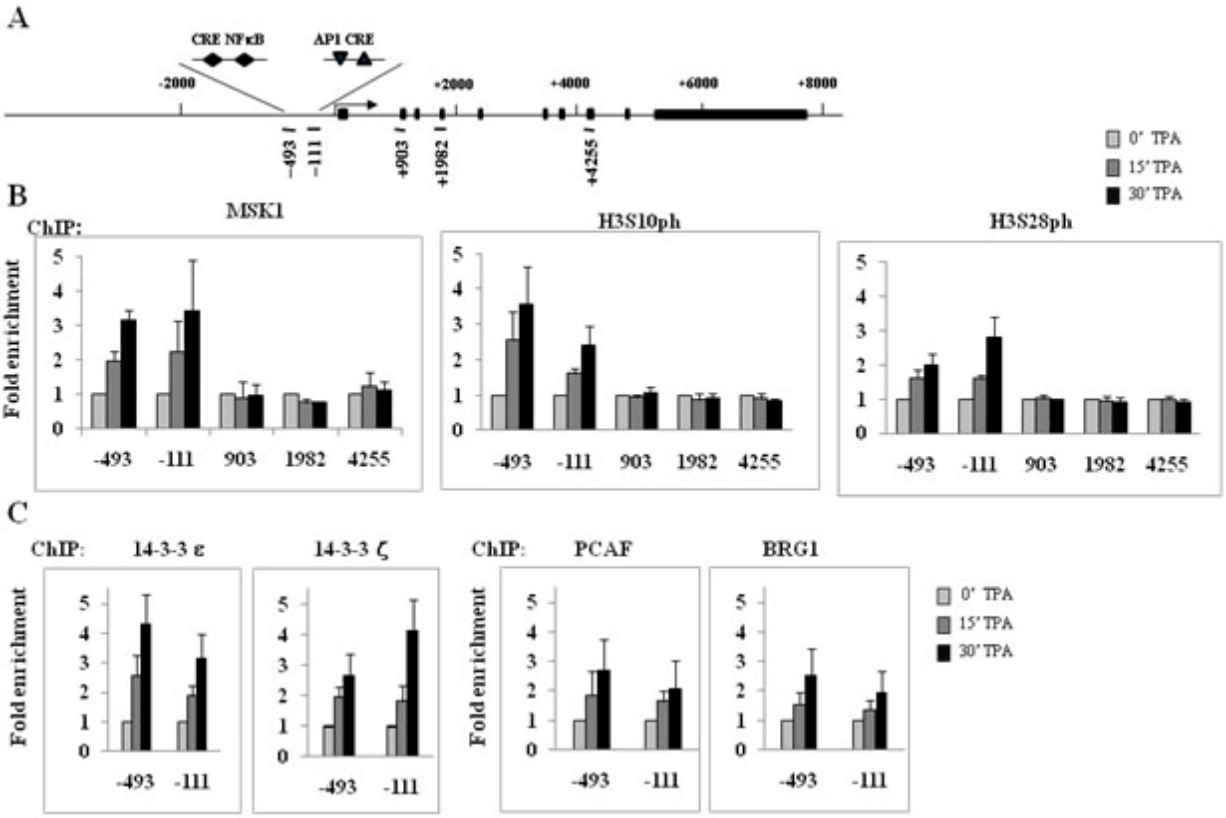

Figure 3.8. TPA-induced nucleosomal response and recruitment of 14-3-3 and chromatin modifiers/remodelers at the *Cox-2* regulatory regions in *HRas1*-transformed mouse fibroblasts.

(**A**) Schematic representation of *Cox-2* gene showing regions amplified in the ChIP assays. Each region is labeled according to the 5' position of the forward primer relative to the transcription start site. Exons are represented by boxes, and binding sites of relevant transcription factors located in the amplified regions are displayed. CRE is the cyclic-AMP responsive element; AP-1 constitutes a combination of dimers formed of members of the JUN, FOS and ATF families of transcription factors. ChIP experiments were performed using antibodies against MSK1, H3S10ph, H3S28ph (**B**), 14-3-3ε/ζ, PCAF and BRG1 (**C**) on formaldehyde-crosslinked mononucleosomes prepared from serum-starved Ciras-3 cells treated with TPA for 0, 15 and 30 min. Equal amounts of input and immunoprecipitated DNA were quantified by real-time quantitative PCR. Enrichment values are the mean of three independent experiments and the error bars represent the standard deviation.

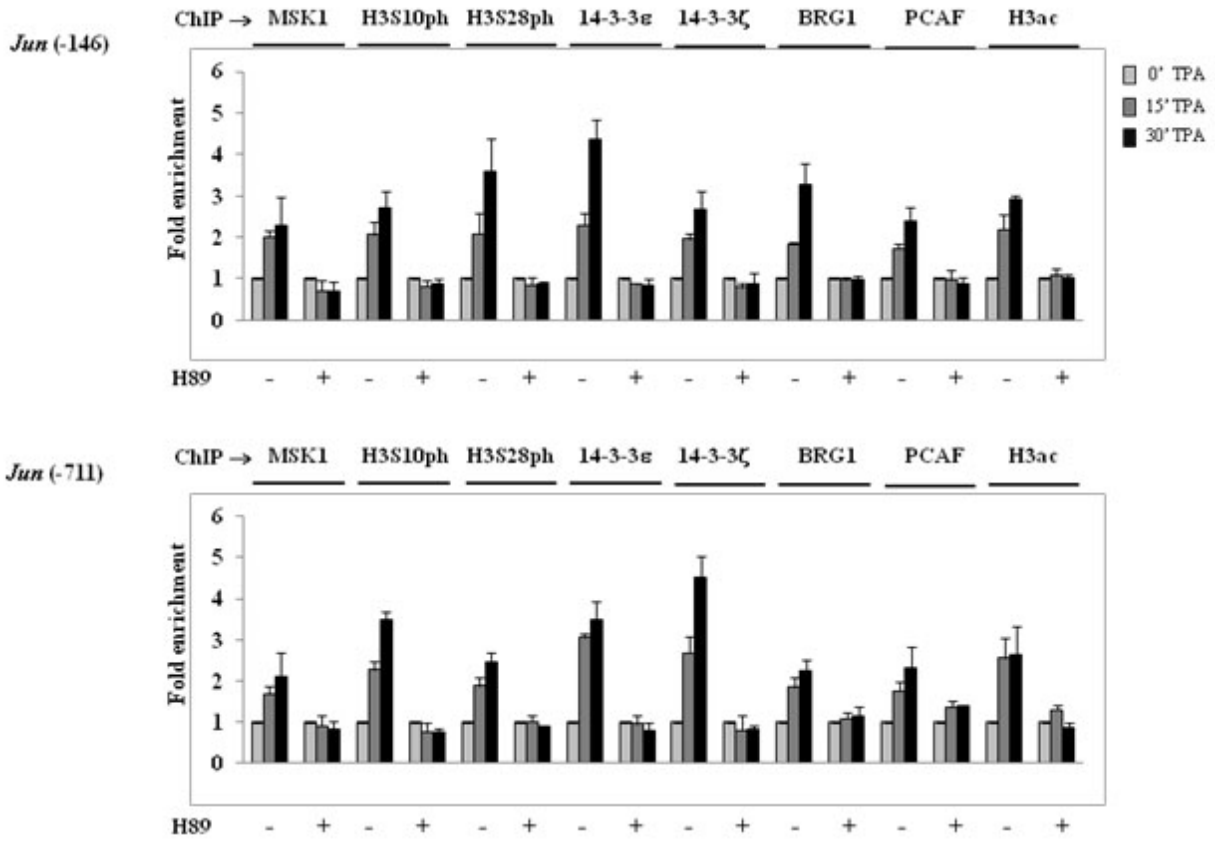

Figure 3.9. H89 inhibition of TPA-induced nucleosomal response and chromatin remodeler/modifier recruitment to *Jun* regulatory regions in *HRas1*-transformed mouse fibroblasts.

Serum-starved Ciras-3 cells were pre-treated or not with H89 prior to TPA stimulation for 0, 15 or 30 min. Formaldehyde-crosslinked mononucleosomes were prepared and used in ChIP assays with antibodies as indicated. Equal amounts of input and immunoprecipitated DNA were quantified by real-time quantitative PCR. Enrichment values are the mean of three independent experiments and the error bars represent the standard deviation.

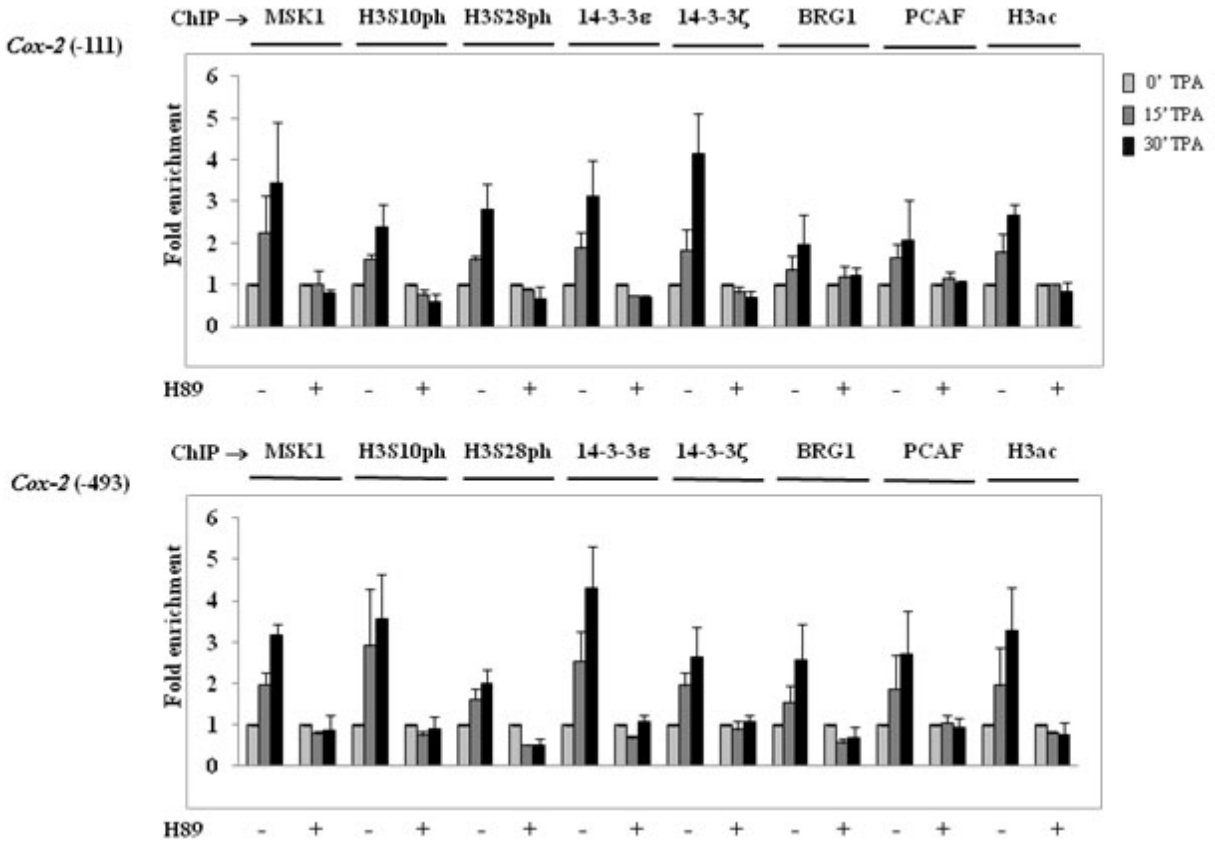

Figure 3.10. H89 inhibition of TPA-induced nucleosomal response and chromatin remodeler/modifier recruitment to *Cox-2* regulatory regions in *HRas1*-transformed mouse fibroblasts.

Serum-starved Ciras-3 cells were pre-treated or not with H89 prior to TPA stimulation for 0, 15 or 30 min. Formaldehyde-crosslinked mononucleosomes were prepared and used in ChIP assays with antibodies as indicated. Equal amounts of input and immunoprecipitated DNA were quantified by real-time quantitative PCR. Enrichment values are the mean of three independent experiments and the error bars represent the standard deviation.

Chapter 4: Chromatin Modification of the Trefoil Factor 1 Gene in Human Breast Cancer Cells by the RAS- Mitogen-Activated Protein Kinase Pathway

4.1 Abstract

Histone H3 phosphorylation is a downstream response to activation of the RAS-MAPK pathway. This modification is thought to have a role in chromatin remodeling and in the initiation of gene transcription. In MCF7 breast cancer cells, we observed that H3 S10ph but not H3 S28ph increased with phorbol ester, TPA treatment. Although phosphorylated ERK 1/2 levels in these cells cultured under estradiol deplete and replete conditions displayed no change, a significant induction was observed after TPA treatment. Furthermore, whereas both estradiol and TPA increased *TFF1* mRNA levels in these cells, only TPA-induced and not estradiol-induced *TFF1* expression was inhibited by the H3 kinase MSK inhibitor, H89 and MAPK kinase inhibitor, UO126, showing the involvement of the RAS-MAPK pathway following TPA induction. Mutation of the AP-1 binding site abrogated the TPA-induced transcriptional response of the luciferase reporter gene under the control of the *TFF1* promoter, showing the requirement for the AP-1 site. In chromatin immunoprecipitation assays, estradiol treatment resulted in the association of ERα and acetylated H3 with the *TFF1* promoter. The levels of phospho-H3 and MSK1 associated with the *TFF1* promoter were moderately increased. In the presence of TPA, whereas ERα was not bound to the promoter, a strong association of acetylated and/or phospho-H3, MSK1, and JUN was observed. These results show that although both stimuli lead to *TFF1* gene activation, estradiol and TPA exert their effects on *TFF1* gene expression by different mechanisms.

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Chromatin Modification of the Trefoil Factor 1 Gene in Human Breast Cancer Cells by the RAS-Mitogen-Activated Protein Kinase Pathway

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Paula S. Espino generated 70% of the data presented in the paper (specifically Figs 4.1, 4.2B and C, 4.4 and 4.6A and B), prepared the figures, drafted the manuscript and assisted in the revisions of the paper.

4.2 Introduction

Many cellular processes fall under the tight regulation of the RAS-MAPK pathway, and it has been reported that its persistent activation can result in the chromatin remodeling and altered gene expression observed in cancer (Davie et al, 1999; Davis, 1993). Growth factors (EGF) and phorbol esters (TPA) activate the RAS-RAF-MEK-ERK pathway. EGF, but not TPA, also weakly activates c-Jun NH2-terminal kinase/stress-activated protein kinases (JNK/SAPK) and p38. Stimulation of the RAS-RAF-MEK-ERK signaling cascade activates MSK1/2, resulting in the phosphorylation of downstream targets, such as transcription factors and nucleosomal proteins. One downstream event is the phosphorylation of the basic NH2-terminal tail of histone H3 at Ser10 and Ser28 (Dunn & Davie, 2005). Both phospho-modified forms of H3 have been shown to have significant roles in chromosome condensation during mitosis in many organisms, and H3 S10ph has been directly associated with the IEG induction in mouse fibroblasts (Chadee et al, 1999; Strelkov & Davie, 2002; Thomson et al, 1999). The investigation of H3 phosphorylation in parental murine fibroblasts is well documented, but few studies (Mishra et al, 2001; Park et al, 2005) have been conducted to ascertain its function in human breast cancer cells. Furthermore, the mechanistic involvement of the RAS-MAPK pathway in H3 phosphorylation in breast cancer has not been shown.

TFF1 expression is high in ERα-positive breast cancer and is a useful prognostic marker associated with a favorable response to primary endocrine therapy (Seib et al, 1997). Although the definitive role of the cysteine-rich secretory TFF1 protein is lacking, it has been reported that TFF1 expression leads to efficient cell mobility that may be important for invasion and metastatic characteristics of aggressive breast cancer (Prest et al, 2002; Rodrigues et al, 2003a;

Rodrigues et al, 2003b). The upstream regulatory region of the *TFF1* gene contains binding sites for different transcription factors that respond to diverse extracellular stimuli, such as growth factors, hormones, and phorbol esters (Nunez et al, 1989). The *TFF1* gene responds to both estrogens and phorbol esters but is thought to follow different mechanisms (Pentecost et al, 2005). Although the chromatin remodeling events taking place during estrogen induction of the *TFF1* are well characterized (Chen et al, 1999; Xu et al, 2004), little is known about the chromatin modifications of the *TFF1* promoter occurring during induction by phorbol esters. Furthermore, although a role for the RAS-MAPK in growth factor- and phorbol ester-induced TFF1 expression may be apparent, the consequences of stimulation of the RAS-MAPK pathway on histone modifications bound to the *TFF1* promoter have not been investigated.

Here, we show that TPA stimulation of the RAS-MAPK pathway results in the recruitment of AP-1, MSK1, but not ERα, and the increased acetylation and S10 phosphorylation of H3 associated with the *TFF1* promoter.

4.3 Materials and Methods

4.3.1 Reagents

17β-Estradiol, TPA, demecolcine, and anti-H3 S28ph rat monoclonal antibody were purchased from Sigma Chemical Co (St. Louis, MO). H89 and PD98059 were purchased from Calbiochem (La Jolla, CA). UO126 was purchased from Promega (Madison,WI). Anti-phospho-p44/p42 MAPK, anti-p38, and anti-phospho-p38 rabbit polyclonal antibodies were purchased from Cell Signaling technologies (Beverly, MA). Anti-ERK goat polyclonal, anti-H3 S10ph, and anti-JUN (H-79) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology

(Santa Cruz, CA). Anti-MSK1 sheep polyclonal, anti-integrinα1 mouse monoclonal, and anti-acetyl H3 at Lys9, 14 rabbit polyclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-ERα mouse monoclonal antibody was purchased from Novocastra (Newcastle upon Tyne, United Kingdom).

4.3.2 Cell culture and treatments.

Hormone-dependent and ERα-positive MCF7 human breast carcinoma cells were maintained in complete culture medium DMEM (Life Technologies, Gaithersburg, MD) as previously described (Sun et al, 2005). Once 60% to 70% confluency was reached, cells were estrogen and serum depleted in phenol red-free DMEM (Sigma, St. Louis, MO) and supplemented with 0.1% (v/v) bovine serum albumin (Sigma) and apo-transferrin (10 μg/mL) to drive the cell population into G0-G1. Cells were either untreated, treated with 10 nmol/L 17h-estradiol, or treated with 100 nmol/L TPA as indicated in the figures. In inhibition studies, cells were pretreated with various inhibitors (50 μmol/L PD98059, 10μmol/L UO126, or 10 μmol/L H89) in DMSO for 30 minutes alone or followed by treatment with estradiol or TPA. To arrest cells in mitosis, MCF7 cells were cultured to 70% confluence in complete medium and treated with 0.06 μg/mL demecolcine by inhibiting spindle formation for 16 hours before harvesting. Cell cycle distribution was monitored by flow cytometry.

4.3.3 Preparation of cell extracts and total histones.

Cell extracts were isolated as described previously (Drobic et al, 2004; Strelkov & Davie, 2002). Acid extraction of histones was done as described previously (Chadee et al, 1999).

Protein concentrations were determined using the Bio-Rad Protein Assay as per manufacturer's instructions (Hercules, CA).

4.3.4 Electrophoresis and immunoblotting.

Proteins were resolved by SDS (10% and 15%)-PAGE and visualized either by Coomassie blue staining or by transfer to nitrocellulose membrane and immunochemical staining with various antibodies as per manufacturers' instructions. Enhanced chemiluminescence kits were purchased from Perkin-Elmer (Boston, MA) or from Amersham Biosciences (Piscataway, NJ) for quantitative analysis using the Storm phosphorimager.

4.3.5 Nascent RNA labeling and fluorescence microscopy.

Active transcription sites were labeled by incorporation of 5-fluorouracil (5-FU) into nascent RNA as previously described (Boisvert et al, 2000; He et al, 2005a). Briefly, MCF7 cells were maintained on coverslips with phenol red-free DMEM for 3 days before 100 nmol/L TPA treatment and 2mmol/L 5-FU (Sigma) labeling for 30 minutes at 37^0C. To detect the labeled nascent RNA, the mouse monoclonal anti-bromodeoxyuridine (anti-BrdUrd; Sigma) was used as primary antibody and was detected by the secondary antibody Alexa 488 anti-mouse IgG (Molecular Probes, Eugene, OR). H3 S10ph was stained with rabbit polyclonal antibody and detected by the secondary Cy3-conjugated goat anti-rabbit IgG antibody (Sigma). Subsequently, the coverslips were mounted onto glass slides using prolong anti-fade (Molecular Probes), and the DNA was counterstained with 4V6-diamidino-2-phenylindole (DAPI). To verify the specificity of our immunodetection of nascent RNA, the following immunostaining control experiments were satisfactorily done: (a) no labeling and no primary antibody, (b) no labeling

but with anti-BrdUrd antibody incubation, (c) with labeling but without anti-BrdUrd incubation, and (d) with labeling followed by RNase A digestion. Fluorescent images were captured on AxioPhot II microscopes with an AxioHRm Camera, and then the stack of images was deconvolved with a Constrained Iterative Algorithm with AxioVision software (Carl Zeiss, Thornwood, NY). Indirect immunofluorescent detection of H3 S28ph in cycling MCF7 cells was carried out as previously described (Dunn & Davie, 2005).

4.3.6 RNA preparation and reverse-transcription PCR.

RNA from MCF7 cells treated as described above was isolated and converted to cDNA as previously described (Sun et al, 2005). PCR reactions with primer sets corresponding to *TFF1* intron A-exon 2and *CYCLOPHILIN 33* exon 2 were carried out as described previously with 1 to 3 μL cDNA template to ensure linear amplification (Sun et al, 2005). Products were resolved on a 1.8% (w/v) agarose gel and stained with ethidium bromide. Intensity analysis was carried out using Kodak Imaging Station 440. TFF1 band intensities were standardized relative to cyclophilin 33 levels for each sample. The un-induced samples were set as one, and ratios for each induction time point were calculated relative to the untreated time point to obtain the fold induction.

4.3.7 ChIP assay.

ChIP assays were done as described previously (Chadee et al, 1999; Spencer et al, 2003; Sun et al, 2005). Input DNA before immunoprecipitation and ChIP DNA were isolated with QIAquick PCR Purification kit (Qiagen, Chatsworth, CA) and analyzed by PCR using primers

for *TFF1* promoter amplifying a 385-bp fragment. PCR reactions were consistently monitored to ensure linearity.

4.3.8 Cloning, transient transfection, and reporter assays.

The pGL3 luciferase reporter plasmid containing a *TFF1* promoter insert (pTFF1-luc) was constructed as described previously (Sun et al, 2005). The AP-1 DNA sequence located upstream of the proximal *TFF1* promoter was mutated using the ExSite PCR-Based Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) following manufacturer's protocol. The modified forward 5'-GGCCATCTCTCACTACTCGAGCCTCCTGCAGTG-3' and reverse 5'-CACTGCAGAAGGCTCGAGGTATGAGAGATGGCC-3' (Barkhem et al, 2002) primers (mutations are underlined) were used to obtain the AP-1-mutated *TFF1* promoter plasmid (pTFF1-ΔAP-1-Luc). The ERE DNA sequence located upstream of the proximal *TFF1* promoter of pTFF1-Luc was mutated with the *Pfu* DNA polymerase (Stratagene) using the primers 5'-TAGAATTCCATATACCCCGTGA-3' and 5'-ATATGGAATTCTATTGCAGGG-3' (mutations underlined) to obtain the pTFF1-ΔERE-Luc plasmid.

MCF7 clone 11 cells were cultured and transfected as previously described with modifications (Sun et al, 2005). Briefly, cells were cultured for 4 days in estrogen-depleted medium and transferred in six-well plates 24 hours before transfection in phenol red–free DMEM with 7% charcoal dextran-treated DMEM. At 60% confluence, cells were transfected using Polyplus transfection reagent (jetPEI) according to manufacturer's instruction using 2.5 µg of reporter plasmid and 0.5 µg of control pCMXbgal. After 24 hours, the cells were washed and given fresh phenol red–free DMEM containing 7% charcoal dextran-treated fetal bovine serum

with either 10 nmol/L estradiol or 100 nmol/L TPA for 24 hours. Luciferase and β-galactosidase assays were done as described previously (Sun et al, 2005).

4.4 Results

4.4.1 TPA but not estradiol activates the RAS-MAPK pathway in MCF7 cells.

To determine the stimulatory effect of estrogens and TPA on the RAS-MAPK pathway in epithelial human breast cancer cells, estrogen-dependent MCF7 cells were cultured under serum and estrogen-depleted conditions. As EGF, but not TPA, weakly activates JNK/SAPKs and p38, we used TPA to exclusively stimulate the RAS-MAPK in MCF7 cells. A time course treatment with estradiol at physiologic concentrations displayed no increase in phospho-ERK1/2 levels, a hallmark of activated RAS-MAPK signaling (Figure 4.1A). Furthermore, no change in total MSK1 protein levels was observed, but phospho-p38 levels displayed an increase in the presence of estradiol (6- to 7-fold at 5 minutes; Figure 4.1A). In contrast, TPA treatment of these cells triggered an immediate and robust elevation in levels of phospho-ERK1/2 (7- to 8-fold at 30 minutes; Figure 4.1B). Activated phospho-p38 was not detected. To verify that TPA upregulation of MAPK occurs through the RAS-RAF-MEK pathway, we used two potent MEK inhibitors PD98059 and UO126 (Davies et al, 2000). Treatment of cells with either agent before TPA stimulation prevented the TPA-induced increase of phospho-ERK, showing that TPA stimulation occurs through the RAS-MAPK pathway in these cells (Figure 4.1C).

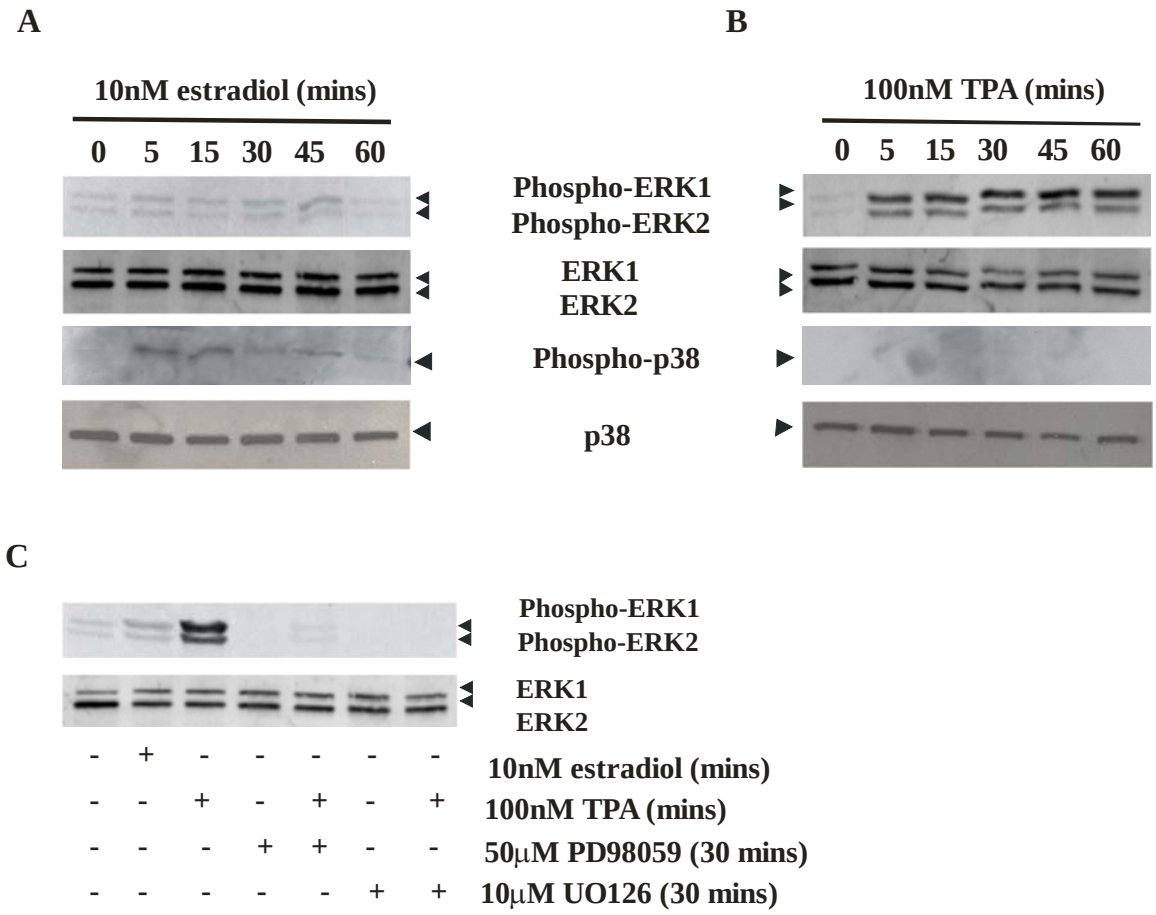

Figure 4.1. TPA but not estradiol stimulates the RAS-MAPK pathway in MCF7 breast cancer cells.

MCF7 cells cultured under estrogen- and serum-deprived conditions were submitted to a 5- to 60-minute time course treatment with 10 nmol/L estradiol **(A)** or 100 nmol/L TPA **(B).** Total cell extracts (25 μg) were then prepared and resolved on a SDS-10% polyacrylamide gel, transferred to a nitrocellulose membrane, and stained immunochemically with antibodies directed against ERK, phospho-ERK, p38, or phospho-p38. MCF7 cells cultured under estrogen- and serum-deprived conditions were incubated for 30 minutes with 50 μmol/L PD98059 or 10 μmol/L UO126 inhibitors before treatment with 10 nmol/L estradiol for 45 minutes or 100 nmol/L TPA for 30 minutes (**C**). Total cell extracts were resolved and analyzed as indicated above.

4.4.2 Histone H3 phosphorylation at Ser10 is increased by TPA.

In mouse fibroblasts, H3 S10ph and H3 S28ph are detected in interphase and mitotic cells (Dunn & Davie, 2005). Using indirect immunolocalization studies, we repeated these analyses with MCF7 cells cultured in complete media. H3 S10ph had a punctate distribution throughout the interphase nuclei of MCF7 breast cancer cells (Figure 4.2A, *grey arrowheads, top inset*) and showed a widespread condensed staining in G2 or mitotic phase (Figure 4.2A, *white arrowheads*) in agreement with previous reports (Hendzel et al, 1997; Li et al, 2005). In contrast, H3 S28ph was not detected in interphase nuclei, although its staining in G2-M cells seemed to be similar to that of H3 S10ph (Figure 4.2A, *bottom inset*). Immunoblot analyses of MCF7 acid-extracted histones isolated from cycling cells confirmed the lack of H3 S28ph in interphase cells (Figure 4.2B). The intense staining observed for H3 S10ph and S28ph in condensed regions of chromatin during mitosis was also confirmed by the elevated levels of both phospho-modified forms in colcemid-arrested cells (Figure 4.2B). These results are in accordance with other studies showing that the staining of nuclei immunochemical stained with antibodies to H3 S10ph or S28ph becomes very intense (Dunn & Davie, 2005; Hendzel et al, 1997). These observations show that unlike mouse fibroblasts, only H3 S10ph is detected in interphase of MCF7 cells.

The effect of TPA treatment of estrogen- and serum-deprived MCF7 cells on H3 S10ph levels was determined in immunoblot experiments. Under these conditions, flow cytometry analyses revealed that 73% of the cells were in G0-G1 phase of the cell cycle, with <9% of the cells being in the G2-M phase. A low level of H3 S10ph was detected before treatment (Figure 4.2C). TPA treatment increased H3 S10ph levels (4.2-fold; Figure 4.2C). This observation shows that stimulation of the RAS-MAPK signaling pathway increases the level of H3 S10ph.

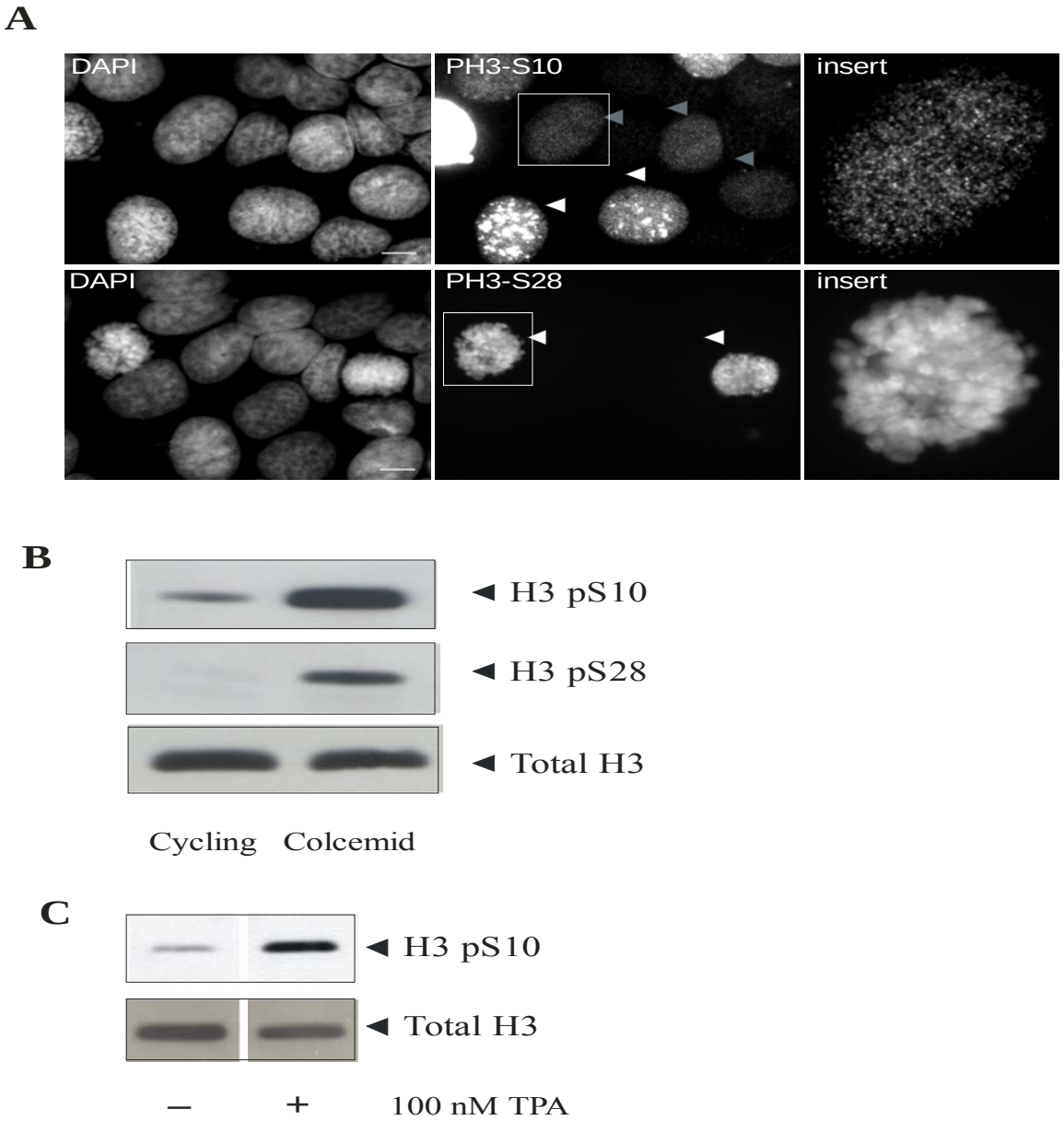

Figure 4.2. Histone H3 phosphorylation at Ser10 is abundant in MCF7 cells and increases after TPA treatment.

(A) Cycling MCF7 cells maintained in estrogen complete DMEM medium were grown on coverslips, fixed, labeled with anti-H3 S10ph or anti-H3 S28ph, and visualized by fluorescence microscopy. DNA was stained by DAPI. *White arrowheads*, cells in late G2 phase or mitosis; *Grey arrowheads*, cells in interphase. Bar, 10 μm. **(B)** Acid-soluble nuclear histones (5 μg) extracted from cycling or colcemid-treated MCF7 cells were resolved on a SDS-15% polyacrylamide gel, transferred to nitrocellulose membrane, and stained immunochemically with anti-H3 S10ph, anti-H3 S28ph, or anti-total H3. **(C)** Serum- and estrogen-depleted MCF7 cells were treated with 100 nmol/L TPA for 30 minutes. Acid-soluble nuclear histones (5 μg) were resolved on a SDS-15% polyacrylamide gel, transferred to a nitrocellulose membrane, and stained immunochemically with anti-H3 S10ph or anti-total H3.

4.4.3 TPA-induced H3 phosphorylation is positioned next to active transcription sites in MCF7 cells.

The TPA-induced stimulation of H3 S10ph was also examined by indirect immunofluorescence microscopy and image deconvolution in MCF7 breast cancer cells. MCF7 cells cultured in estrogen- and serum-deplete conditions displayed a punctate distribution of H3 S10ph. The number of these foci clearly increased upon addition of TPA for 30 minutes (Figure 4.3), consistent with the immunoblot analysis (Figure 4.2C). To determine whether H3 S10ph was positioned next to newly synthesized RNA, 5-FU was incorporated into nascent RNA transcripts of MCF7 cells and detected by anti-BrdUrd antibody. Deconvolved images showed that H3 S10ph foci were situated near or overlapped with active transcription sites as noted by the increase in yellow foci upon TPA stimulation (Figure 4.3, insets). Previous studies have shown that histone modification marks associated with transcription (e.g., acetylated K9 and K14 H3) were positioned next to but did not coincide with the newly synthesized RNA as detected with 5-FU labeling (Hendzel et al, 1998). These results provided evidence that newly phosphorylated S10 H3 was associated with transcriptionally active chromatin.

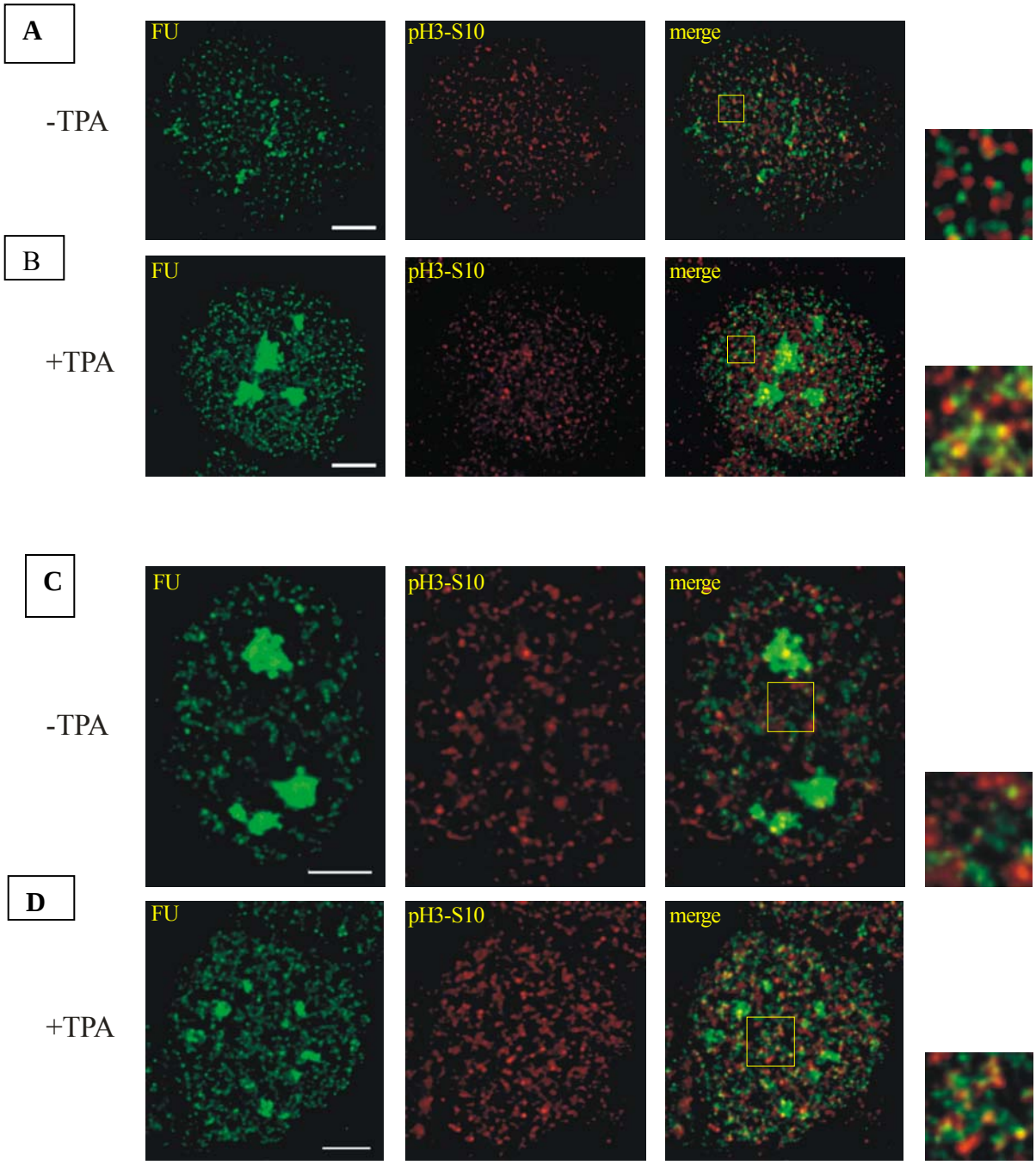

Figure 4.3. Histone H3 phosphorylation at Ser10 colocalizes with active transcription sites in MCF7 breast cancer cells.

MCF7 cells were grown on coverslips in serum- and estrogen-deplete medium (**A and C**), treated with 100 nmol/L TPA for 30 minutes **(B and D)**, fixed, and double labeled with anti-H3 S10ph antibodies and anti-BrdUrd antibodies, which detect nascent RNA after in situ incorporation of 5-FU. Two sets of representative cells: untreated and treated. Spatial distribution was visualized by fluorescence microscopy and image deconvolution as described in Materials

and Methods. Single optical sections. Yellow in merge signifies colocalization. The boxed area in each merged image is shown enlarged. Bar, 5 μm.

4.4.4 TPA-induced *TFF1* expression occurs through the RAS-MAPK pathway.

The association of TPA-induced H3 S10ph with active transcription sites prompted us to investigate whether MAPK inhibitor UO126 and MSK inhibitor H89 affected estradiol or TPA-induced *TFF1* gene expression. To assess the effects of MAPK inhibitors on *TFF1* gene expression, we conducted reverse transcription-PCR (RT-PCR) to determine the levels of *TFF1* transcripts relative to those of *CYCLOPHILIN 33* (Sun et al, 2005; Wang et al, 2005). Treatment of MCF7 cells cultured under serum- and estradiol-depleted conditions with estradiol or TPA resulted in an immediate and sustained increase in *TFF1* gene transcription (3.0-fold for estradiol and for TPA). In the presence of H89, estradiol induction of *TFF1* was unaltered (3.0-fold with H89 versus 3.0-fold at 45 minutes; Figure 4.4A and C). UO126 pretreatment did not prevent the estradiol-induced expression of the *TFF1* gene (3.0-fold; Figure 4.4A and C). However, pretreatment of these cells with H89 reduced the TPA-stimulated *TFF1* expression (1.5-fold with H89 as opposed to 3.0-fold; Figure 4.4B and D). Furthermore, TFF1 transcription was suppressed to basal levels in the presence of UO126 (0.9-fold; Figure 4.4B and D). These results provided evidence that TPA-induced, but not estradiol-induced, expression of *TFF1* occurred through the RAS-MAPK pathway.

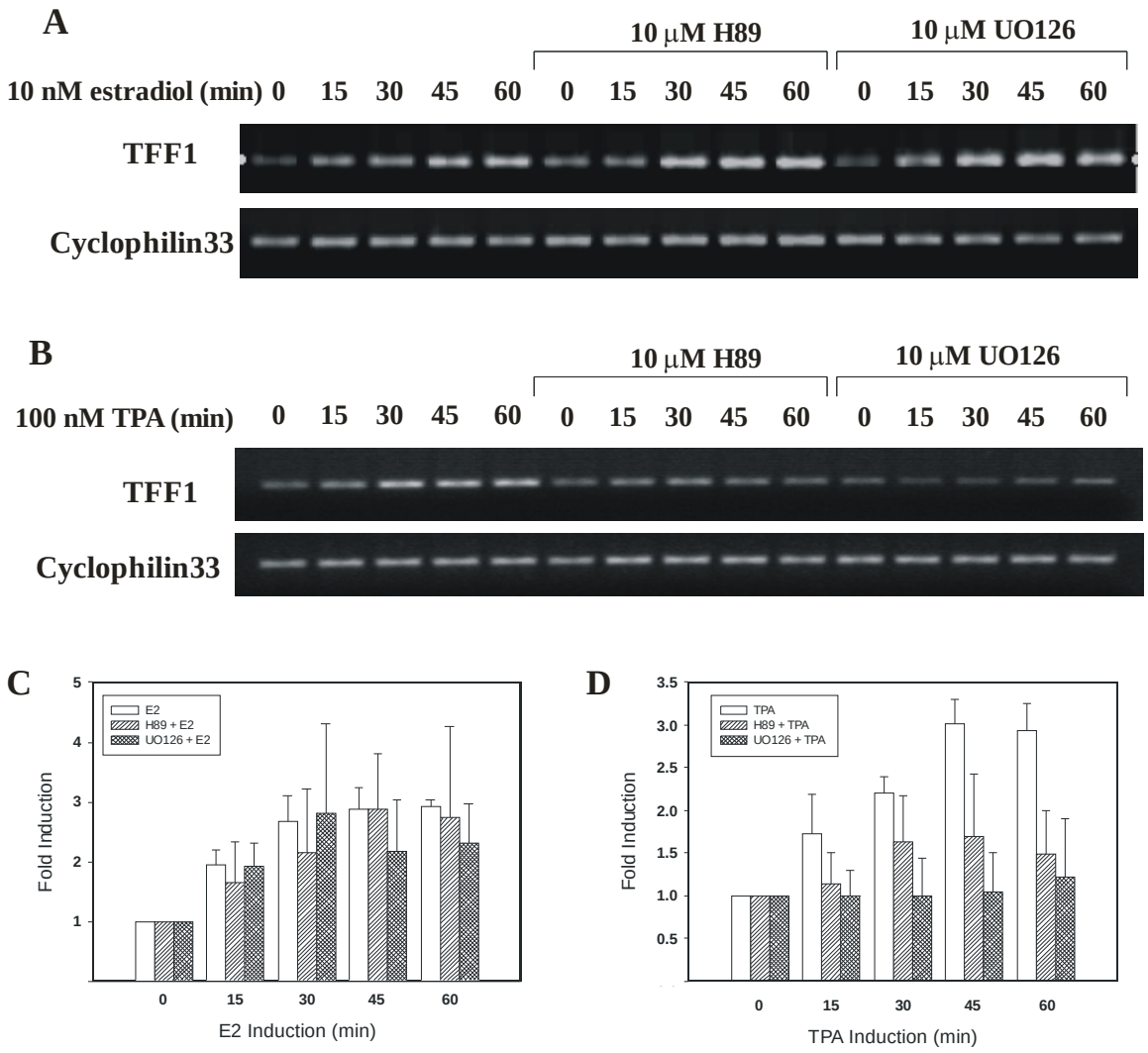

Figure 4.4. Effect of H89 and UO126 inhibitors on *TFF1* expression.

MCF7 cells cultured under estrogen- and serum-deprived conditions were treated for 30 minutes with 10 μmol/L H89 or 10 μmol/L UO126 before a 15- to 60-minute time course treatment with 10 nmol/L estradiol **(A)** or 100 nmol/L TPA **(B).** Cells were harvested at indicated times, and RNA was isolated. One microgram of RNA was converted to cDNA and used for PCR with primer sets corresponding to *TFF1* intron A-exon 2 and *CYCLOPHILIN* 33 exon 2 as loading control. Representative of three separate experiments. Band densitometric analyses and fold induction in triplicate were determined as described in Materials and Methods for estradiol **(C)** and TPA **(D)** time courses. Bars, SD.

4.4.5 AP-1 binding site is important for the TPA-mediated response of *TFF1*.

The *TFF1* proximal promoter contains a specificity protein 1 (Sp1) site, an ERE and an AP-1 site that contribute to conferring estrogen responsiveness (Figure 4.5A; (Barkhem et al, 2002; Gillesby et al, 1997; Sun et al, 2005)). To ascertain the function of the AP-1 and ERE sites in the TPA-induced activation of the *TFF1* promoter, we determined the effect of mutating these elements in the transcriptional activation of *TFF1* promoter. Wild-type and mutated *TFF1* promoter/ luciferase reporter constructs were transiently transfected into MCF7. The transfection efficiencies of the reporter constructs were comparable among the three cell populations. TPA treatment stimulated the activity of the wild-type TFF1 promoter 13-fold (Figure 4.5B). Mutation of the AP-1 site nearly abolished this TPA-mediated response, whereas the ERE mutation had a minor effect (Figure 4.5B). These results suggest that the TPA-induced stimulation of the TFF1 gene requires a functional AP-1 site.

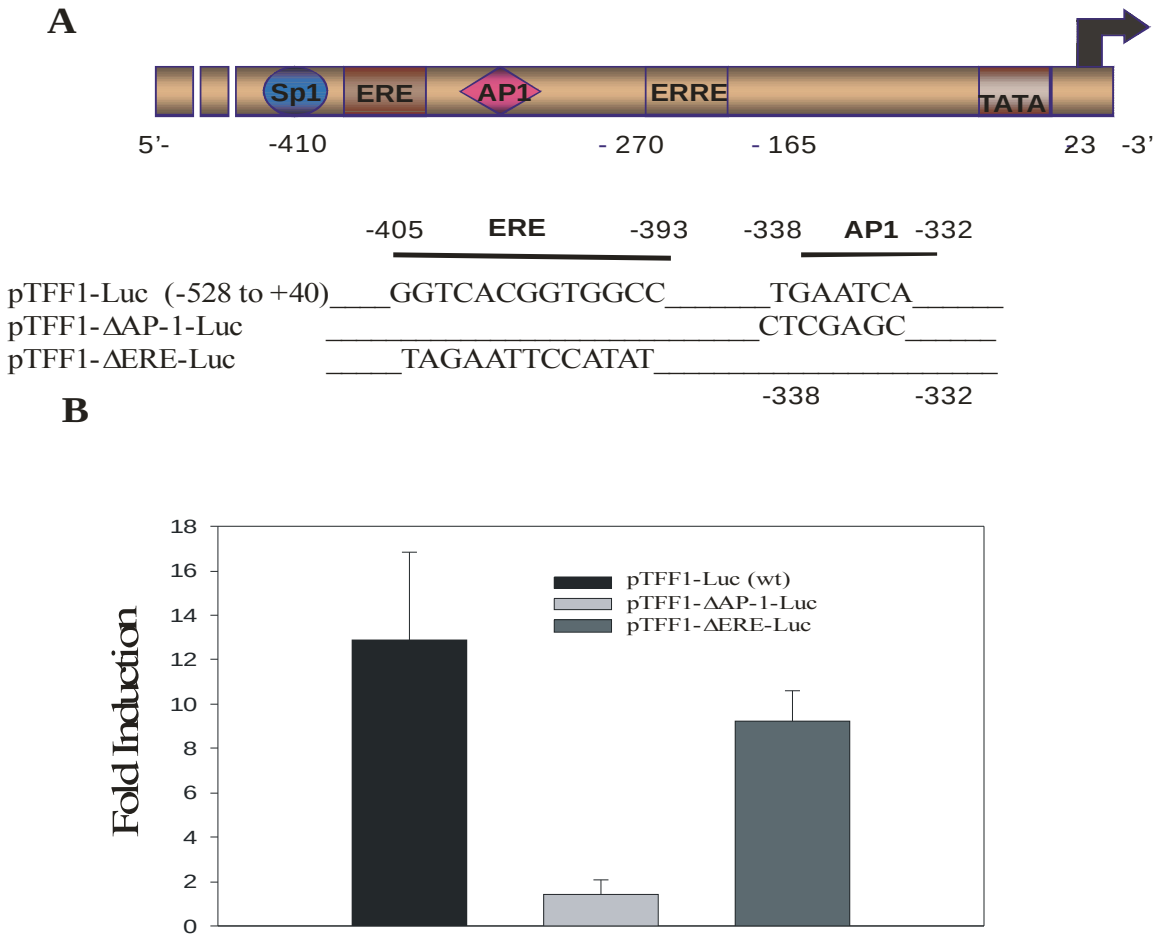

Figure 4.5. AP-1 binding site is important for the TPA-mediated response of the *TFF1* promoter.

(A) Description of the *TFF1* 5'-flanking proximal promoter region and the luciferase reporter plasmids containing either the wild-type *TFF1* promoter, the AP-1 mutant *TFF1* promoter, or the ERE-mutant *TFF1* promoter. **(B)** MCF7 cells were cultured in estrogen-depleted medium for 4 days and transfected with plasmids pTFF1-Luc (wt), pTFF1-ΔAP-1-Luc, or pTFF1-ΔERE-Luc alongside pCMXβgal for 24 hours before incubation in the presence or absence of 100 nmol/L TPA for another 24 hours. Luciferase and β-galactosidase assays were done where luciferase activities were normalized against β-galactosidase activities, and the induction was determined by normalizing against the activities before treatment. Columns, mean of triplicate readings; bars, SD.

4.4.6 H3 S10ph, MSK1, and c-JUN are associated with the *TFF1* promoter upon gene activation in TPA-stimulated cells.

We used the ChIP assay to determine the association of downstream effectors of the RAS-MAPK pathway on the endogenous *TFF1* promoter. From RT-PCR results, we consistently observed that *TFF1* expression peaked at 45 minutes of estradiol treatment and at 30 minutes of TPA treatment of MCF7 cells cultured under serum- and estradiol-deplete conditions. Furthermore, steady-state level of H3 S10ph appeared at its maximum after 30 minutes of TPA induction in our studies (Figure 4.2C). Thus, we assessed the *in situ* association of transcription factors and H3 acetylation and phosphorylation at these time points. As a control for the ChIP assay, we showed that ERα was not associated with the intron A-exon 2 region of the *TFF1* region in cells treated with estradiol (Figure 4.6B). As further controls for the assay, we confirmed the lack of amplification of the *TFF1* promoter fragment in immunoprecipitations using anti-integrin antibodies and when the primary antibody was left out of the assay (Figure 4.6A). Semiquantitative analyses of PCR products from ChIP DNA were normalized against the input, and treated samples (E2 and TPA) were then compared with the untreated to acquire the fold induction. We found that after estradiol stimulation, there was an increase in ERα loading onto the *TFF1* promoter (median 2.1-fold higher than untreated, n = 6), an elevation in H3 acetylation levels (median 1.4-fold than untreated, n = 4), and an enhancement in the levels of JUN (median 1.7-fold than untreated, n = 5) and MSK1 (median 1.2-fold higher than untreated, n = 6), which coincided with an increase in H3 S10ph (median 1.6-fold than untreated, n = 8; Figure 4.6A). Upon TPA stimulation, there was a prominent recruitment of JUN and MSK1 (median 3.6-fold, n = 5 and median 2.1-fold higher than untreated, n = 6, respectively) to the *TFF1* promoter and increased levels of H3 S10ph (median 2.5-fold higher than untreated, n = 6).

These results show that while both estradiol and TPA can activate the same *TFF1* gene, these stimuli follow alternative routes to induce *TFF1* transcription.

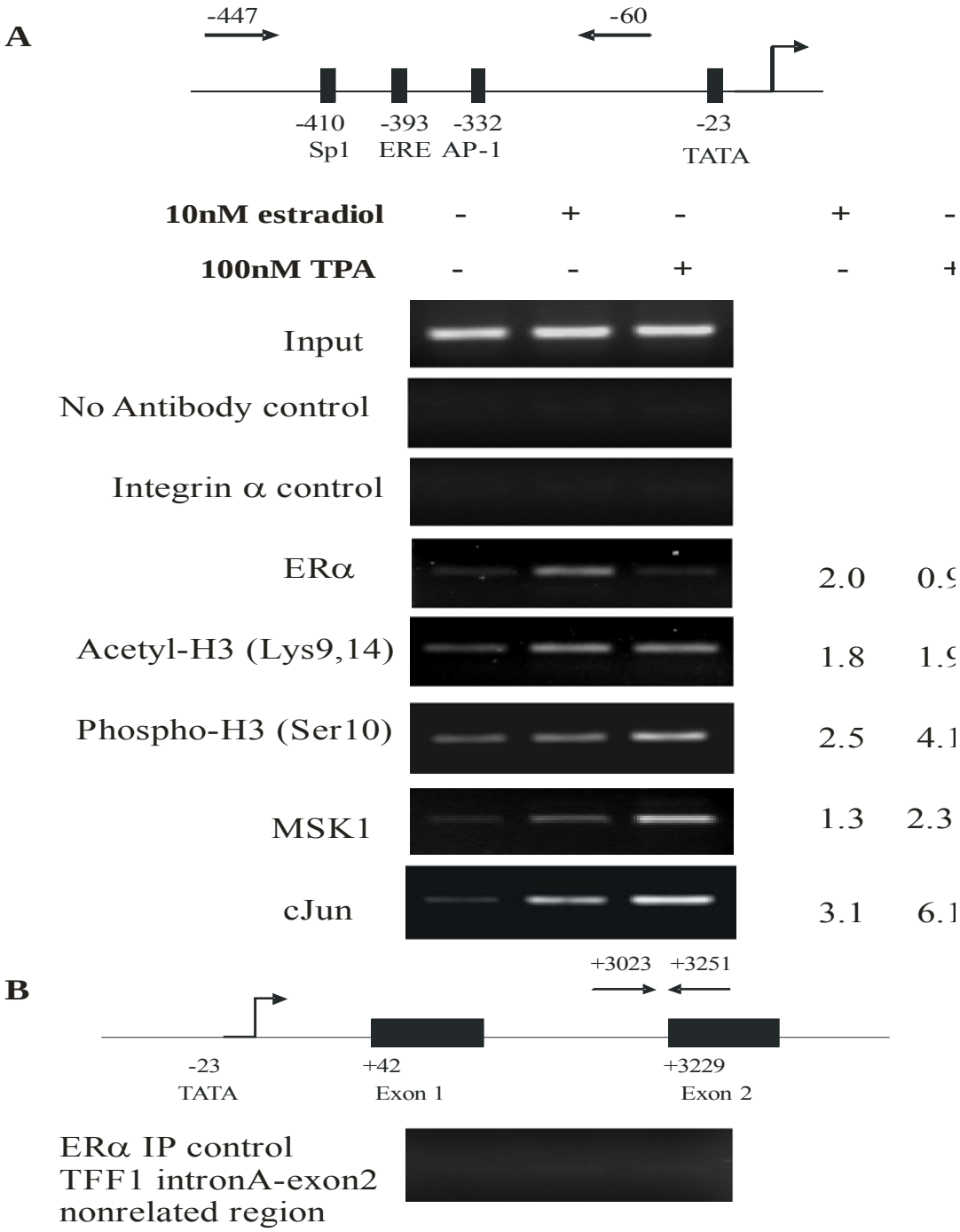

Figure 4.6. H3 S10ph, MSK1, and JUN are associated with the *TFF1* promoter upon gene activation with TPA.

A, MCF7 cells cultured under estrogen- and serum-deprived conditions were either untreated, treated with 10 nmol/L estradiol for 45 minutes, or treated with 100 nmol/L TPA for 30 minutes. Soluble chromatin from the samples was released by cell lysis, sheared to an average of 500 bp, and used for immunoprecipitation with antibodies specific to H3 S10, MSK1, acetylated H3, ERa, c-Jun, and integrin α or no antibody for control. Input represents the total chromatin before immunoprecipitation. A primer set spanning nucleotides -446 to -60 of the TFF1 promoter was used for PCR analysis (arrows). Fold induction after treatment. **B**, PCR analysis of the TFF1 intron A-exon 2 region using chromatin immunoprecipitation DNA isolated from anti-ERα immunoprecipitation was used as negative control. Arrows, location of primers in the TFF1 downstream region.

4.5 Discussion

TFF1 is a prognostic marker for breast and other cancers (Ather et al, 2004; Gillesby & Zacharewski, 1999). In agreement with previous studies, we show that the *TFF1* expression is stimulated by estradiol and TPA in MCF7 cells. However, these stimuli act through different pathways. Estrogen acts through the ER, which binds to the *TFF1* promoter, resulting in increased H3 acetylation in agreement with other studies (Metivier et al, 2003; Shang et al, 2000). We observed that MSK1 was associated with the estrogen-induced *TFF1* promoter, resulting in increased H3 phosphorylation. Although ERKs were not activated in the estrogen-induced cells, there was an increase in activated phospho-p38, which will activate MSK (Davie, 2003). However, the enzymatic activity of MSK1 is not required for estrogen-induced expression of the *TFF1* gene as H89, a potent MSK inhibitor, did not reduce *TFF1* expression. In contrast, TPA-induced *TFF1* gene expression was attenuated with H89 and was prevented with the MEK inhibitor. In contrast to events occurring in the estrogen-induced expression of *TFF1*, ERα was not bound to the TPA-induced *TFF1* promoter. Our results provide evidence that TPA activation of the RAS-MAPK pathway results in the activation of MSK1, which is associated with the *TFF1* promoter, leading to the phosphorylation of H3 at S10. The AP-1, which is required in the

TPA-induced expression of the *TFF1* promoter, may be involved in the recruitment of MSK1 to the *TFF1* promoter. A recent study identified two AP-1-like elements in the *Nur77* promoter as the MSK response element, a result that is consistent with AP-1 being involved in the recruitment of MSK (Darragh et al, 2005). These results show that estradiol and TPA elicit transcriptional activation of the *TFF1* gene via alternative routes: estrogen-induced expression occurs through recruitment of ERα, whereas TPA-induced expression requires AP-1 recruitment, leading to MSK1 loading and H3 phosphorylation on the *TFF1* promoter. Similar to our results with *TFF1*, induction of the *Hsp70* gene by heat shock or arsenite operates through different pathways and results in different histone modifications; H4 acetylation in the case of heat shock induction and H4 acetylation and H3 phosphorylation for arsenite (Thomson et al, 2004).

H3 S10ph and H3 S28ph are important not only during mitotic chromosome condensation but also in transcriptional activation of IEG. We and others have previously showed that these two modifications exist independently on separate mouse fibroblast chromatin domains upon activation of the RAS-MAPK pathway, and that MSK1 and MSK2 are the kinases responsible (Drobic et al, 2004; Dunn & Davie, 2005; Dyson et al, 2005; Soloaga et al, 2003). The mechanisms involved in MSK selecting to phosphorylate H3 at S10 or S28 are currently not known (Dyson et al, 2005). In contrast to TPA-induced H3 phosphorylation events in mouse fibroblasts, TPA treatment of MCF7 cells resulted in the increased H3 S10ph but not at H3 S28ph. Human and mouse MSK will phosphorylate H3 at S10 and S28 *in vitro*. Thus, factors other than the MSK are likely responsible for the lack of H3 S28ph in TPA-induced MCF7 cells.

A recent study directly links H3 S10ph to neoplastic cellular transformation through the activation of AP-1 factors (Choi et al, 2005a). Upon TPA stimulation of MCF7 breast cancer cells, we found that the number of H3 S10ph foci increased, and these TPA-induced foci were positioned next to actively transcribed regions in the nucleus. Presumably, these nuclear sites represent the nuclear location of genes that are induced or in a competent state. Thus, growth factors stimulating the RAS-MAPK and increasing H3 S10ph at transcriptionally active loci may contribute to aberrant gene expression and breast cancer progression.

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Chapter 5: Elevated H3 phosphorylation in breast cancer cell lines overexpressing epidermal growth factor receptor genes EGFR or HER2

5.1 Abstract

Overexpression of epidermal growth factor receptors EGFR or HER2 results in the activation of the RAS-MAPK signaling pathway. We studied the level of histone H3 phosphorylation, an epigenetic event responding to this pathway, in four human breast cancer cells lines overexpressing EGFR (MDA MB 231 and MCE5 cell lines) or EGFR and HER2 (SK BR-3 and BT-474 cell lines). In the four cell lines, levels of H3 S10ph, which has a role in neoplastic cell transformation, were markedly increased following the activation of the RAS-MAPK pathway.

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Paula S. Espino generated 100% of the data presented, drafted the materials and methods in the manuscript as well as assisted in the editing of the paper.

5.2 Introduction

HER2 overexpression is found in 25-30% of breast tumors (Slamon et al, 1989). HER2 belongs to the HER/ERBB family of cell surface tyrosine kinase receptors otherwise known as EGFRs consisting of four closely related members: EGFR (ERBB1 or HER1), ERBB2 (HER2/ neu), ERBB3 (HER3) and ERBB4 (HER4). Under normal circumstances, when extracellular growth factors such as EGF are bound by these receptors through their extracellular domains, the receptors undergo dimerization and transmit the signals through autophosphorylation of their C-terminal domains. These receptors then activate adaptor proteins and effector kinases downstream which stimulate the RAS-RAF-MEK-ERK pathway (Bazley & Gullick, 2005; Scaltriti & Baselga, 2006). However, HER2 does not need ligand stimulation to be in an active conformation and forms heterodimers with other ligand-activated members of the EGFR family (Garrett et al, 2003). In breast cancer development, upregulation of the RAS-MAPK signaling cascade occurs as a consequence of HER/ERBB overexpression. Among the ERBB receptors, the clinical significance of HER2 is best understood. Although many human breast tumours express EGFR, the role of EGFR and its value as a prognosis marker are not well established (Chan et al, 2006).

The N-terminal tail of histone H3 has a pivotal role in the three-dimensional structure of chromatin (Zlatanova et al, 1998). Modifications of the H3 tail by phosphorylation and acetylation recruit 14-3-3 proteins, resulting in the transcriptional activation of associated genes (Macdonald et al, 2005; Winter et al, 2008a; Winter et al, 2008b). Stimulation of the RAS-MAPK pathway activates MSK1/2, which results in the phosphorylation of downstream targets such as transcription factors and nucleosomal proteins, including phosphorylation of the N-

terminal tail of H3 at serine 10 or serine 28 (Dunn & Davie, 2005; Soloaga et al, 2003). H3 S10ph has been directly associated with the induction of IEG in mouse fibroblasts (Chadee et al, 1999; Strelkov & Davie, 2002; Thomson et al, 1999). In *HRas1*-transformed mouse fibroblasts, the RAS-MAPK pathway is constitutively activated, MSK1/2 activity is increased (Drobic et al, 2004), and steady-state levels of H3S10ph and H3 S28ph are higher than those in parental cells (Chadee et al, 1999; Chadee et al, 1995; Dunn & Davie, 2005). However, in MCF7 breast cancer cells, activation of the RAS-MAPK pathway leads to H3 S10ph but not at H3 S28ph (Espino et al, 2006).

In this study, we have investigated whether the downstream response to the activation of the RAS-MAPK pathway in HER2 and/or EGFR overexpressing breast cancer cell lines results in H3 S10ph and H3 S28ph.

5.3 Materials and Methods

5.3.1 Reagents

Anti-ERα mouse monoclonal antibody was purchased from Novocastra (UK). Anti-H3 S28ph rat polyclonal antibodies were purchased from Sigma Chemical Co (Saint Louis, MO). Anti-phospho-p44/p42 MAPK (anti-phospho-ERK1/2) and anti-p44/p42 (anti- ERK1/2) rabbit polyclonal as well as anti-histone H3 mouse monoclonal antibodies were purchased from Cell Signaling Technologies (Beverley, MA). Anti-H3 S10ph (sc-8656), anti-EGFR (sc-03) and anti-

HER2/ neu (sc-284) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

5.3.2 Cell lines and cell culture

Human breast cancer cell lines MCF7, MDA MB 231, BT-474 and SKBR-3 were obtained from the American Type Culture Collection (Rockville, MD) and MCF7 stably transfected cell line (overexpressing EGFR) MCE5 cells (a generous gift from Dr. Dorraya El-Ashry's laboratory) were maintained in Dulbecco's Modified Eagle Medium (DMEM, GIBCO) supplemented with 5% (v/v) fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100μg/ml) and 0.3% (v/v) glucose in a 37^0C humified incubator with 5% CO_2. Cells were grown to various confluencies and harvested for cell extracts and histone preparation. Cell cycle distribution was consistently monitored by flow cytometry.

5.3.3 Preparation of total cell extracts and histones

Cell extracts from cell cycle matched cells were isolated as described previously (Espino et al, 2006). Acid extraction of histones was done as described previously (Chadee et al, 1999). Protein concentrations were determined using the Bio-Rad Protein Assay as per manufacturer's instructions (Hercules, CA).

5.3.4 Electrophoresis and immunoblotting

Proteins were resolved by SDS (10% and 15%) - PAGE and visualized either by Coomassie Blue staining or by transfer to nitrocellulose membrane and immunochemical

staining with various antibodies as per manufacturers' instructions. Enhanced chemiluminescence kits were purchased from Perkin Elmer or from Amersham Biosciences (Piscataway, NJ) for quantitative analysis using the Storm phosphorimager.

5.4 Results and Discussion

5.4.1 H3 phosphorylation in breast cancer cell lines overexpressing EGFR

MDA MB 231 and MCE5, an EGFR stable transfectant of MCF7, are human breast cancer cell lines overexpressing EGFR. An immunoblot analysis of extracts from cycling cells detected EGFR in both cell lines (Figure 5.1A). On the other hand, MCF7 cells do not express EGFR but express ERα, and none of these three cell lines expressed HER2 (Figure 5.1A). Increased level of phospho-ERK1/2, the hallmark of the activated RAS-MAPK signaling pathway, is illustrated in Figure 5.1A.

Previously we reported that mouse fibroblasts transformed with oncogenes (*e.g. Fes*, *Mos*, c-*Myc*) whose products stimulate the RAS-MAPK signaling pathway have elevated levels of H3S10ph (Chadee et al, 1999). The relative level of H3S10ph in the MCF7 cells was compared with the H3S10ph levels in the EGFR-overexpressing cell lines. The immunoblot analysis of histone H3 extracted from cycling cells revealed a marked increase in the steady state levels of H3S10ph, and to a lesser extent H3S28ph, in cells expressing EGFR (Figure 5.1B). As mitotic phosphorylation of H3 at serine 10 and serine 28 by Aurora B kinase is elevated at G2/M phase of the cell cycle (Dunn & Davie, 2005), it was necessary to compare the cell cycle distribution of the MCF7, MDA MB 231 and MCE5 cultures. Using flow cytometric analysis of

the cell populations, we found that the distributions of cells in different phases of the cell cycle were not significantly different (Table 5.1). We conclude that the observed differences in H3S10ph levels were not simply due to a higher number of cells in G2/M phase for the EGFR overexpressing cell lines.

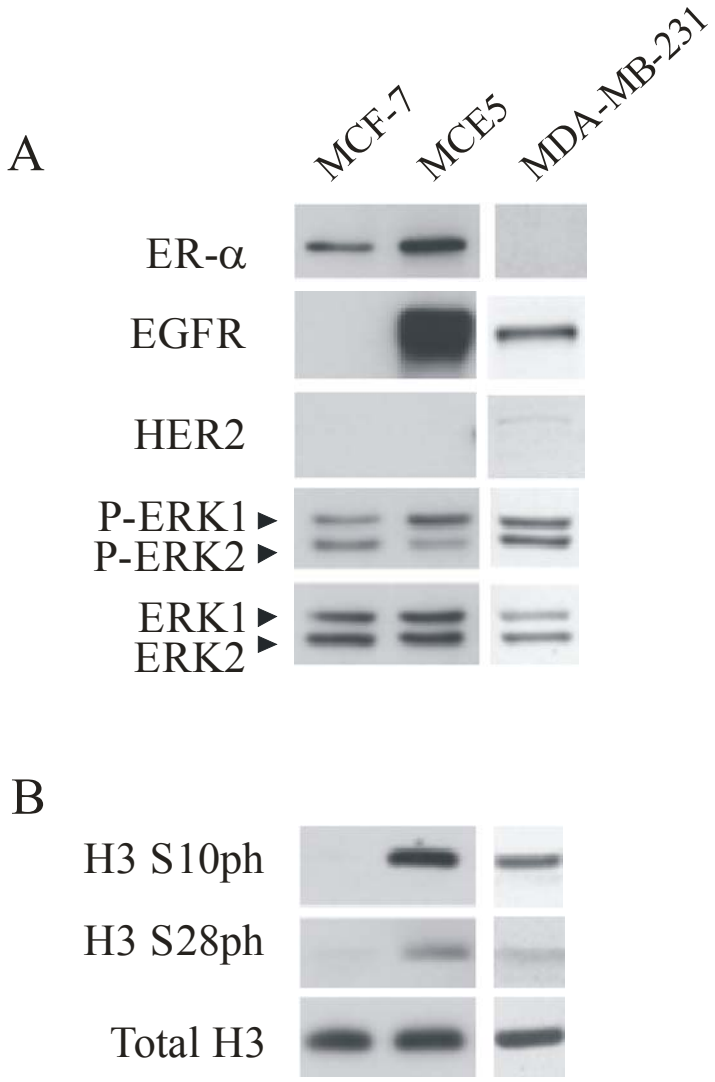

Figure 5.1. H3 phosphorylation levels in EGFR overexpressing human breast cancer cells.

Cell cycle matched MCF7, MDA MB 231 and MCE5 (MCF7 overexpressing EGFR) breast cancer cells were harvested by trypsin. **(A)** Total cell extracts (25 µg) were prepared and resolved on a SDS-10%-polyacrylamide gel, transferred to a nitrocellulose membrane, and immunochemically stained with antibodies directed against ERα , EGFR, HER2, ERK1/2 or

phospho-ERK1/2 as indicated. **(B)** Acid-soluble nuclear histones (5 μg) were resolved on a SDS-15%-polyacrylamide gel, transferred to a nitrocellulose membrane, and immunochemically stained with anti-H3 S10ph, anti-H3 S28ph or anti-total H3 antibodies.

Table 5.1. Cell cycle distribution of breast cancer cell lines

Cell Line	Cell cycle phase (% distribution		
	G1	S	G2/M
MCF-7	59.6	20.1	20.3
MCE5	54.0	29.4	16.6
MDA-MB-231	64.1	16.2	19.6
SK-BR-3	60.7	24.0	15.3
BT-474	69.5	15.1	15.4

5.4.2 H3 phosphorylation in breast cancer cell lines overexpressing HER2 and EGFR

As shown in the immunoblot analysis of cycling cell extracts (Figure 5.2A) and as previously reported (Normanno et al, 2002), human breast cancer cell lines SK BR-3 and BT-474 overexpress HER2 and to a lesser extent EGFR. The stimulation of the RAS-MAPK pathway was shown by the strong immuno-signals obtained with anti-phospho-ERK1/2 antibodies in SK BR-3 and BT-474 cells (Figure 5.2A). These strong signals are in agreement with the fact that EGFR-HER2 heterodimers were shown to lengthen the activation of the downstream RAS-MAPK pathway (Wieduwilt & Moasser, 2008). Again, in comparing steady state levels of H3 S10ph and H3 S28ph, we studied breast cancer cell populations with equal proportions of cells in

G2/M phases (Table 5.1). Thus, elevated levels of H3 S10ph and H3 S28ph would be due to the persistent activation of the RAS-MAPK signaling pathway. Figure 5.2B shows a striking increase in H3 S10ph in response to the RAS-MAPK activation. There was no increase in H3 S28ph.

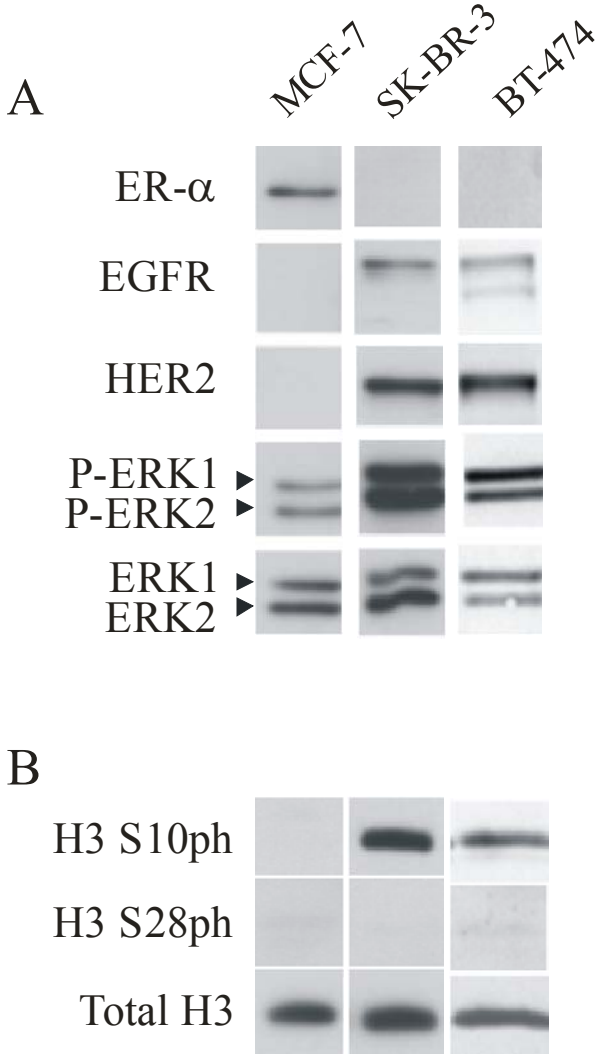

Figure 5.2. H3 phosphorylation levels in HER2 overexpressing human breast cancer cells.

Cell cycle matched MCF7, BT-474 and SK BR-3 breast cancer cells were harvested by trypsin**. (A)** Total cell extracts (25 μg) were prepared and resolved on a SDS-10%-polyacrylamide gel, transferred to a nitrocellulose membrane, and immunochemically stained with antibodies

directed against ER-α, EGFR, HER2, ERK1/2 or phospho-ERK1/2 as indicated. **(B)** Acid-soluble nuclear histones (5 μg) were resolved on a SDS-15%-polyacrylamide gel, transferred to a nitrocellulose membrane, and immunochemically stained with anti-H3S10ph, anti-H3S28ph or anti-total H3 antibodies.

5.4.3 Role of H3 phosphorylation in neoplasia

In breast cancer cell lines, the activation of the RAS-MAPK pathway lying downstream of activated EGFRs results in the H3 S10ph. This MSK1/2-mediated histone H3 modification is associated with the induction of IEG genes including *TFF 1* and protooncogenes *Fos* and *Jun* (Espino et al, 2006; Thomson et al, 1999). In response to a vast array of diverse internal and external stimuli, activation of MSK1/2 results in H3 S10ph and often also H3 S28ph (Dong & Bode, 2006; Dunn & Davie, 2005; Dunn et al, 2005). Interestingly, MSK1/2 will phosphorylate a H3 tail at S10 or at S28, but rarely at both sites *in situ* (Dunn & Davie, 2005; Dyson et al, 2005). We have observed MSK1/2-mediated H3 S10ph or H3 S28ph in mouse fibroblasts and HeLa cells, but in MCF7 cells MSK1/2 will only phosphorylate H3 at S10. In the EGFR and HER2 overexpressing human breast cancer cells analyzed in this study, it is typically H3 S10 that exhibits a high steady state level of phosphorylation. Zigang Dong and colleagues demonstrated that MSK1/2 and H3 S10ph, but not at serine 28, were involved in neoplastic cell transformation (Choi et al, 2005a; Kim et al, 2008). Our results showing that H3S10ph levels are elevated in breast cancer cells overexpressing EGFR and HER2 suggests that chemotherapy approaches to inhibit MSK1/2 activity may be a potential approach to consider in the treatment of breast cancer.

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Chapter 6: Genomic instability and histone H3 phosphorylation induction by the RAS-mitogen activated protein kinase pathway in pancreatic cancer cells

6.1 Abstract

Activating mutations in K-RAS occur in most pancreatic cancers. We investigated whether genetic changes (*K-RAS* mutations) in human pancreatic cancer cell lines altered genomic instability and epigenetic events responding to RAS-MAPK signaling by characterizing 3 human pancreatic cancer cells lines with and without activating mutations in K-RAS. Activation of the RAS-MAPK pathway results in the stimulation of the histone H3 kinase, MSK1, and increased H3 S10ph. MSK1 and H3 S10ph have roles in neoplastic transformation. We demonstrate that the presence of a K-RAS mutation did not correlate with elevated chromosomal aberrations or increased genomic instability. Although the levels of the epidermal growth factor receptors and MSK1 were similar, the RAS-MAPK pathway was differentially induced by phorbol ester (TPA) and epidermal growth factor, with the response of this signaling pathway being cell-type specific. This response corresponded downstream at the level of chromatin where stimuli-induced elevation of H3 S10ph typically paralleled the increase in phospho-ERK1/2. Our results present evidence that nonclonal chromosomal aberrations and epigenetic programming responding to stimulation of the RAS-MAPK pathway may be better markers for cancer progression than the upstream mutated oncogenes.

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Genomic instability and histone H3 phosphorylation induction by the RAS-mitogen activated protein kinase pathway in pancreatic cancer cells

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Paula S. Espino generated 80% of the data presented (specifically Figures 6.2 to 6.5), drafted the manuscript as well as assisted in the editing of the paper.

6.2 Introduction

RAS is one of the most commonly mutated oncogenes found in cancer. Activating mutations in *K-RAS* are most often in codon 12 but also in codons 13 or 61. Expression of the mutated activated K-RAS affects a myriad of downstream targets and lead to the deregulation of cellular signaling pathways as well as transformation, tumourigenesis and metastasis (Dunn et al, 2005; Langst & Becker, 2004). In pancreatic cancer, about 95% of all diagnosed adenocarcinomas have an acquired mutation in the *K-RAS* gene which leads to a constitutively activated oncoprotein (Bos, 1989; Roberts & Der, 2007). Further, the combined activation of proto-oncogene *K-RAS* with inactivating mutations of tumour suppressor genes such as *p53* and *p16* have been linked to progression of pancreatic cancer from pre-invasive lesions to metastatic disease (Maitra et al, 2006). Thus, acquired somatic mutations of the *K-RAS* gene are associated with the early stages of pancreatic oncogenesis. Despite increased understanding of the biology and molecular pathophysiology of pancreatic cancer, the rate of survival among diagnosed patients remain poor and patients continue to be diagnosed at a late incurable state which poses a great urgency in improving strategies for early disease detection and treatment.

RAS elicits a wide array of physiological responses by triggering a protein kinase cascade known as the RAS-MAPK signaling pathway. Stimulation of this pathway by extracellular stimuli such as growth factors, hormones and pharmacological agents (eg., TPA) activates a series of kinases that invoke epigenetic mechanisms such as the modification of chromatin proteins concomitant with the expression of genes coding for immediate early transcription factors and signal transduction proteins (Chadee et al, 1999; Dyson et al, 2005; Espino et al, 2006; Hazzalin & Mahadevan, 2002; Reul & Chandramohan, 2007; Sun et al,

2007). TPA stimulation of mouse fibroblasts results in MSK1/2-mediated phosphorylation of histone H3 at S10 and S28, events that have been named the "nucleosomal response." (Bode & Dong, 2005; Dunn & Davie, 2005; Dyson et al, 2005; Soloaga et al, 2003)

Although it is well documented that K-RAS potentially plays a critical role in initiating pancreatic cancer tumourigenesis and that certain effectors such as B-RAF and HER2 which converge into the RAS-MAPK pathway are deregulated in these cells, there are limited studies that address the downstream events in cells possessing an activated K-RAS protein (Mahadevan & Von Hoff, 2007; Maitra et al, 2006). Our previous findings demonstrate that steady state levels of phospho-ERK 1/2, H3 S10ph and H3 S28ph as well as MSK1 kinase activity are elevated in *HRas1*-transformed mouse fibroblasts (Chadee et al, 1999; Drobic et al, 2004; Dunn & Davie, 2005; Strelkov & Davie, 2002). Thus, the deregulation of upstream effectors by genetic events (mutations) in these cells directly influences the downstream responses in the pathway, including reversible epigenetic events such as histone modifications. Understanding the deregulated downstream targets may facilitate identification of other players in the pathway which may be useful for prognosis, therapeutic targeting and treatment of the cancer. Whether these observations are universal and translate in other cell backgrounds particularly in pancreatic cancer cell lines containing activating *K-RAS* gene mutations have not been demonstrated.

Moreover, recent studies demonstrate that H3 S10ph and MSK1 are critical during neoplastic transformation (Choi et al, 2005a; Kim et al, 2008) and that deregulation of H3 mitotic kinase is linked to genomic instability and oncogenesis (Mahadevan & Von Hoff, 2007; Warner et al, 2006). Hence, there is evidence to warrant examination of downstream players of

the pathway as potential targets for therapy. Nonetheless, H3 phosphorylation as a downstream event in the activation of the RAS-MAPK pathway in human pancreatic cancer cells has not been reported.

In this study, we examined the karyotypic characteristics of 3 human pancreatic cancer cells lines and observed that the presence of a *K-RAS* mutation did not correlate with elevated chromosomal aberrations or increased genomic instability. Further, we investigated whether human pancreatic cancer cell lines containing inherent *K-RAS* mutations have an altered signaling pathway in response to different stimuli when compared to cells with a wild-type K-RAS by evaluating the levels of EGFRs, phospho-ERK1/2, MSK1 and H3 S10ph. We show that stimuli such as epidermal growth factor (EGF) and TPA differentially induce the RAS-MAPK pathway in pancreatic cancer cells, with the response being cell-type specific. This response corresponds downstream at the level of chromatin where stimuli-induced elevation of phospho-ERK1/2 typically parallels an increase in H3 S10ph levels.

6.3 Materials and Methods

6.3.1 Reagents

TPA, EGF and anti-β-actin mouse monoclonal antibodies were purchased from Sigma Chemical Co (St. Louis, MO). Anti-phospho-p44/p42 MAPK, anti-p44/p42 rabbit polyclonal and anti-histone H3 mouse monoclonal antibodies were purchased from Cell Signaling Technologies (Beverley, MA). Anti-H3 S10ph (sc-8656), anti-EGFR (sc-03) and anti-HER2/neu receptor (sc-284) rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MSK1 sheep polyclonal antibodies were purchased from Upstate Biotechnology

(Lake Placid,NY). Anti-K-Ras (ab55391) mouse monoclonal antibodies were purchased from Abcam (Cambridge, MA).

6.3.2 Cell lines, cell culture and treatments

The human pancreatic cancer cell lines BxPC-3, Hs766T and Panc-1 were obtained from the American Type Culture Collection (Rockville, MD) and cultured as recommended by the supplier. Both Hs766T and Panc-1 cell lines were maintained in Dulbecco's Modified Eagle Medium whereas the BxPC-3 cell line was maintained in RPMI 1640 supplemented with 10 mM HEPES and 1.0 mM sodium pyruvate. All 3 cell lines were grown in their respective media supplemented with 10% (v/v) fetal bovine serum, streptomycin/penicillin antibiotics at 37^0C and 5% CO_2. For stimulation studies, cells were cultured until 70% confluency was reached and serum starved (SS) in 0.1% (v/v) fetal bovine serum for 48 hr to drive the majority of the cell population into G0/G1. Cells were either untreated (SS), treated with 100 nM TPA or with 50 ng/ml EGF for the indicated lengths of time in the figures. For SKY analyses, cells were split 24 hr before chromosome preparation as previously described (Mai & Wiener, 2002). Cell cycle distribution was monitored by flow cytometry. The percentage distribution of the SS cells in G0/G1, S, G2/M phases of the cell cycle were typically as follows: BxPC-3—68,12,20; Hs766T—72,13,15; Panc-1—85,5,10.

6.3.3 Preparation of total cell extracts and histones

Cell extracts were isolated as described previously (Espino et al, 2006). Acid extraction of histones was done as described previously (Chadee et al, 1999). Protein concentrations were determined using the Bio-Rad Protein Assay as per manufacturer's instructions (Hercules, CA).

6.3.4 Electrophoresis and immunoblotting

Proteins were resolved by SDS (10% and 15%)-PAGE and visualized either by Coomassie Blue staining or by transfer to nitrocellulose membrane and immunochemical staining with various antibodies as per manufacturers' instructions. Enhanced chemiluminescence kits were purchased from Perkin Elmer or from Amersham Biosciences (Piscataway, NJ) for quantitative analysis using the Storm phosphorimager. To determine the fold induction in response to stimuli, relative protein band intensities were normalized to intensities for the protein loading control such as ERK, β-actin or total H3 and each stimulated time point was normalized against the unstimulated sample.

6.3.5 SKY and analysis

Chromosome spreads for each pancreatic cell line were prepared using a fixation protocol from suspension as previously described (Mai & Wiener, 2002). Briefly, adherent cells grown in log phase were trypsinized, collected and equilibrated in hypotonic solution containing KCl. To carry out fixation, cells were suspended in 3 changes of 3:1 methanol-acetic acid before dropping cell suspension onto slide.

SKY was carried out on metaphase spreads using the (Applied Spectral Imaging, Vista, CA) kit for human according to protocols recommended by the supplier including RNase A and pepsin treatment, denaturation, hybridization and detection. Image Acquisition and analyses were performed using the Spectra CubeTM on a Carl Zeiss Axioplan 2 microscope with a 633 oil

objective and the SKYVIEW 2.0 software (Benedek et al, 2004; Fest et al, 2005). Twenty metaphase spreads were examined per cell line in 3 independent SKY experiments.

6.4. Results

6.4.1 Nonclonal chromosome aberrations reflect the genomic instability of pancreatic cancer cells

Although greater than 95% of pancreatic cancers exhibit genetic mutations in *K-RAS*, the development of pancreatic preinvasive lesions to aggressive carcinomas requires multiple hits in various genes for disease progression (Li et al, 2004; Maitra et al, 2006). As a consequence, gross genome aberrations rather than gene mutations provide an assessment of the cancer chromosome dynamics in pancreatic cancer (Heng et al, 2006a; Heng et al, 2006b; Heng et al, 2006c; Li et al, 2004; Ye et al, 2007). We evaluated the chromosomal organization of the pancreatic cancer cells using SKY. The technique allows for comparative analysis of numerical and structural chromosomal aberrations (Bayani et al, 2007) of individual cells in a population and enables us to assess if there is a correlative relationship between an activated RAS-MAPK pathway signaling and the chromosomal organization of pancreatic cancer cells. The pancreatic cell lines used in this study were BxPC-3, which has a wild-type K-RAS, and cell lines with activating mutations in K-RAS (Hs766T, codon 61 mutation; Panc-1, codon 12 mutation). Aneuploidy and structural aberrations (clonal chromosomal aberrations, CCA) specific to and characteristic of each cell line were evident (Figure 6.1 A-C and Table 6.1). For example, rearrangements such as t(X;3) ($p = 0.0014$) and t(3;6;16;6) ($p = 1 \times 10^{-6}$) characteristic for BxPC-3 were manifested in the respective karyotype and were statistically significant. These pancreatic cancer cell lines displayed specific translocated chromosomes, illustrating that all cell

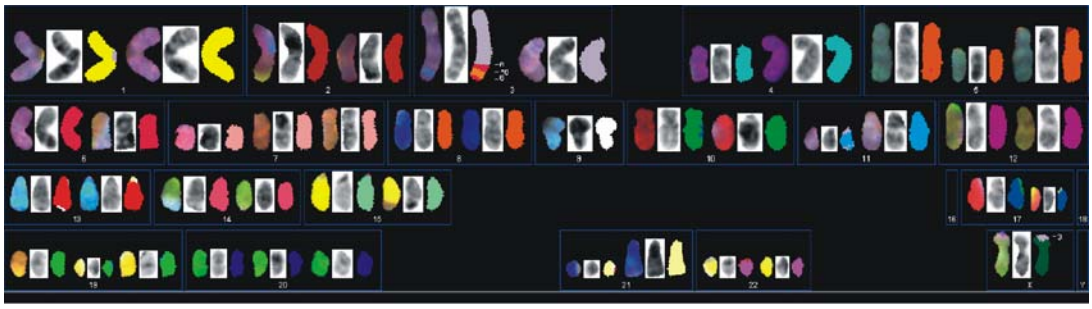
lines were drastically different from each other judging by their genome context (Table 6.1). Further, the observation that all pancreatic cancer cell lines displayed different karyotypes suggested that the altered karyotypes were stochastically achieved. Regardless, we found no striking chromosomal aberrations in the 3 pancreatic cancer cell lines that correlated with an activated K-RAS. Emerging studies reveal that unique aberrations known as nonclonal chromosomal aberrations (NCCAs) more accurately exhibit the heterogeneity and instability observed in cancer evolution (Heng, 2007; Heng et al, 2006b). Some cell lines are more stable than others as illustrated by the different NCCA frequencies detected (Table 6.1). With an NCCA frequency of 60%, Panc-1 cancer cells presented with the highest NCCA frequency (Table 6.1) and thus more genomically unstable when compared to BxPC-3 (20%) and Hs766T (15%).

6.4.2 TPA and EGF differentially stimulate phospho-ERK1/2 levels in pancreatic cancer cells

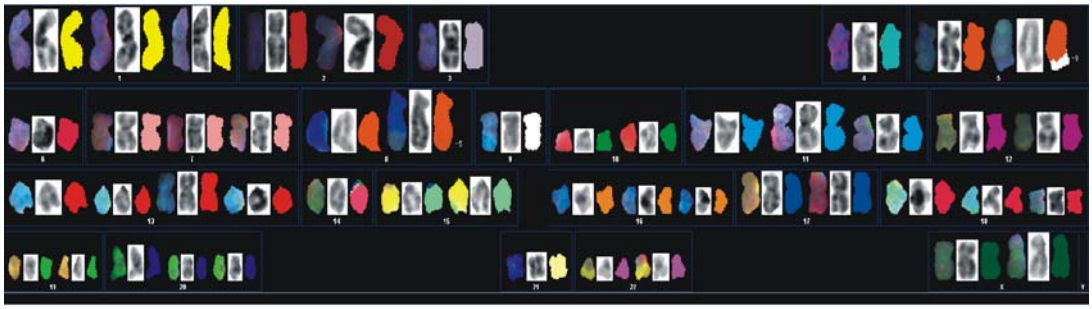
The intensity and duration of the phospho-ERK1/2 induction response varies from transient to sustained depending on the cell context examined and stimuli used (Marshall, 1995). To ascertain whether the presence of activating *K-RAS* mutations confer differences in downstream effector signaling, we compared the extent and strength of phospho-ERK1/2 induction in 3 pancreatic cancer cell lines in response to EGF and TPA. EGF and TPA increased phospho-ERK1/2 levels in all 3 cell lines to different extents, with EGF having lesser effects in Hs766T and Panc-1 cells that have mutated K-RAS (Figure 6.2). BxPC-3 cells, which have a wild-type K-RAS, showed a TPA-induced phospho-ERK1/2 response that was somewhat lower than that with EGF (1.5 ± 0.5 and 2.3 ± 0.8 at 60 min, respectively; n = 3; average ± SEM)

(Figures 6.2A, 6.2D and 6.2E). However, Hs766T, which has a mutated K-RAS, displayed a more robust and sustained phospho-ERK1/2 response to TPA when compared to EGF treatment (3.4 ± 0.3 and 1.1 ± 0.1 at 60 min, respectively; n = 3) (Figures 6.2B, 6.2D and 6.2E). EGF-induced phospho-ERK1/2 levels in these cells exhibited a weak and transient increase (Figures 6.2B and 6.2E). The TPA-induced increase in phospho-ERK1/2 levels in Panc-1 cells, which also have a mutated K-RAS, was typically sustained for longer times than that observed in EGF-stimulated cells (3.6 ± 0.4 and 2.1 ± 0.4 at 60 min, respectively, n = 3) (Figures 6.2C–E).

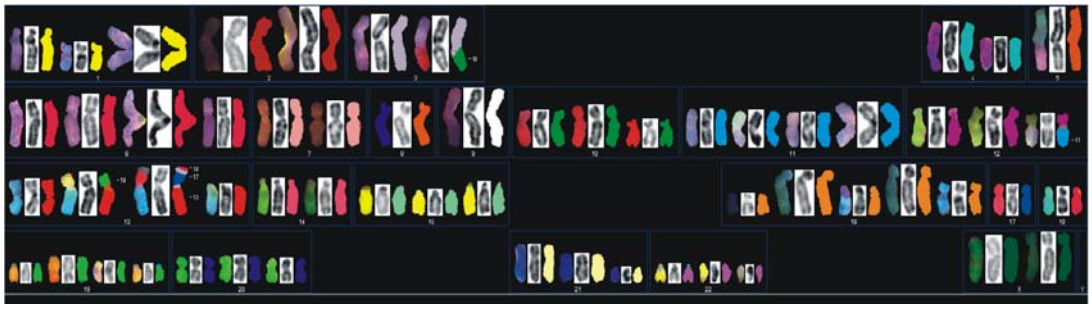
A BxPC-3



B Hs766T



C Panc-1



D

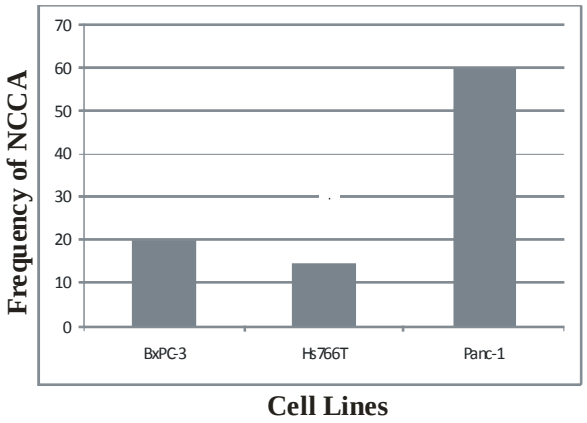


Figure 6.1. Structural and numerical chromosome aberrations exhibited by pancreatic cancer cell lines.

Representative spectral karyotyping (SKY) images of **(A)** BxPC-3, **(B)** Hs766T and **(C)** Panc-1 illustrating their characteristic as well as unique chromosomal aberrations. Twenty mitotic figures were analyzed for each cell line to determine frequency of nonclonal chromosomal aberrations that correlated to relative genomic instability of the cell line **(D).** The majority of structural NCCAs represent random chromosomal rearrangements.

Table 6.1. Nonclonal chromosome aberrations reflect the genomic instability of pancreatic cell lines

Cell Line	Major aberrations	Frequencies
BxPC-3:		
	t(X;3)	35%
	t(3;6;16;6)	35%
	t(X;11)	10%
	t(3;21)	10%
	t(10;11)	10%
	NCCAs	**20%**
Hs766T		
	T(8;5)	40%
	T(13;11)	15%
	NCCAs	**15%**
PANC-1		
	t(3;10)	30%
	t(13;17;18)	20%
	t(13;17)	20%
	t(7;19)	15%
	t(15;19)	10%
	NCCAs	**60%**

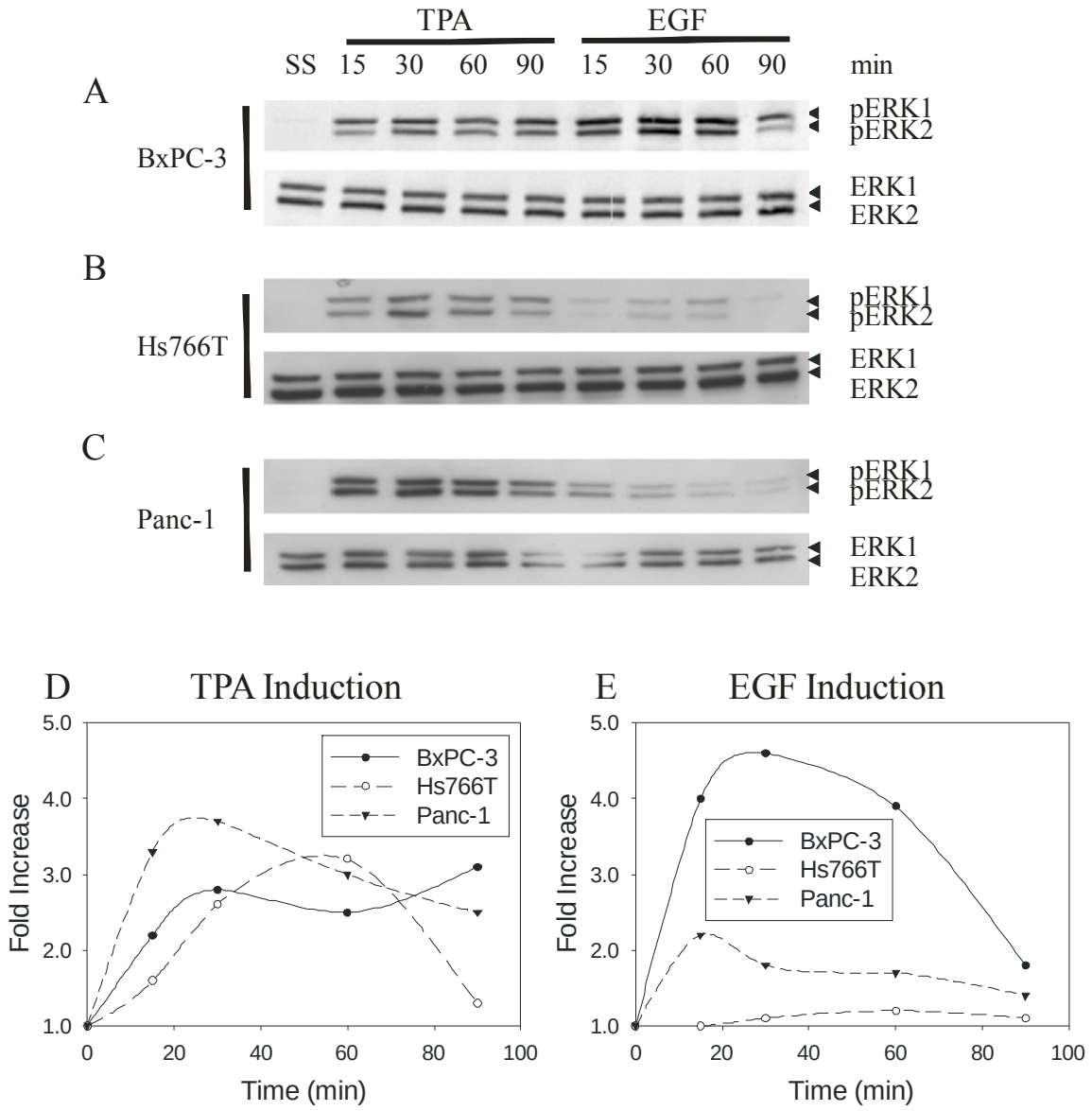


Figure 6.2. TPA and EGF stimulate a differential induction of phospho-ERK levels in pancreatic cancer cell lines

(A) BxPC-3, **(B)** Hs766T and **(C)** Panc-1 pancreatic cancer cells were cultured until 70% confluency and serum-starved for 48 h. Cells were either untreated (SS) or treated for 15 to 90 min with 100 nM TPA or with 50 ng/ml EGF. Total cell extracts (25 μg) were then prepared and resolved on a SDS-10%-polyacrylamide gel, transferred to a nitrocellulose membrane, and stained immunochemically with antibodies directed against ERK and phospho-ERK as indicated. **(D and E)** The fold induction of phospho-ERK in response to stimuli (TPA or EGF) was determined as described under "Materials and methods".

6.4.3 Levels of EGFRs and MSK1 are unaltered upon induction of RAS-MAPK signaling in pancreatic cancer cells

Phorbol esters (TPA) stimulate the RAS-MAPK pathway using PKC as the major receptor which in turn can phosphorylate RAF kinases and phospho-ERK1/2 whereas EGF elicits its action primarily by binding its respective receptors to activate the same pathway (Blumberg, 1988; Dunn et al, 2005; Lorenzo et al, 2001). Although each stimulus induces the pathway through different upstream effectors, both stimuli converge downstream through activated phospho-ERKs. To determine whether the difference in phospho-ERK1/2 response to EGF in Hs766T and Panc-1 was a consequence of upstream receptor expression, we evaluated the levels of EGFR and HER2/neu in these cells following stimulation. Neither stimulus produced any variation in EGFR or HER2/neu protein levels (Figure 6.3). The K-RAS levels were similar in the 3 cell lines (Figure 6.4D). Thus, the differential response of the 3 pancreatic cancer cell lines to EGF stimulation was not due to EGFR, HER2 or K-RAS levels. As the bulk of studies that examine the impact of the RAS-MAPK pathway on pancreatic cancer development and progression primarily focus on membrane receptors and cytoplasmic effectors, it is of interest to examine the whether there are changes in downstream targets of the pathway particularly protein modifiers at the level of chromatin (De Luca et al, 2008; Friday & Adjei, 2008; Heeger, 2008; Roberts & Der, 2007). MSK1 is activated in response to various stimuli and occurs upon activation of the RAS-MAPK pathway. Despite activation of phospho-ERK1/2 upon EGF and/or TPA treatment, evaluation of MSK1 protein revealed similar levels before and after induction in all cell lines (Figure 6.4). It would be of interest to ascertain whether there is an elevation in MSK1 activity using *in vitro* kinase assays to determine whether there is a stimuli-specific and cell line specific induction.

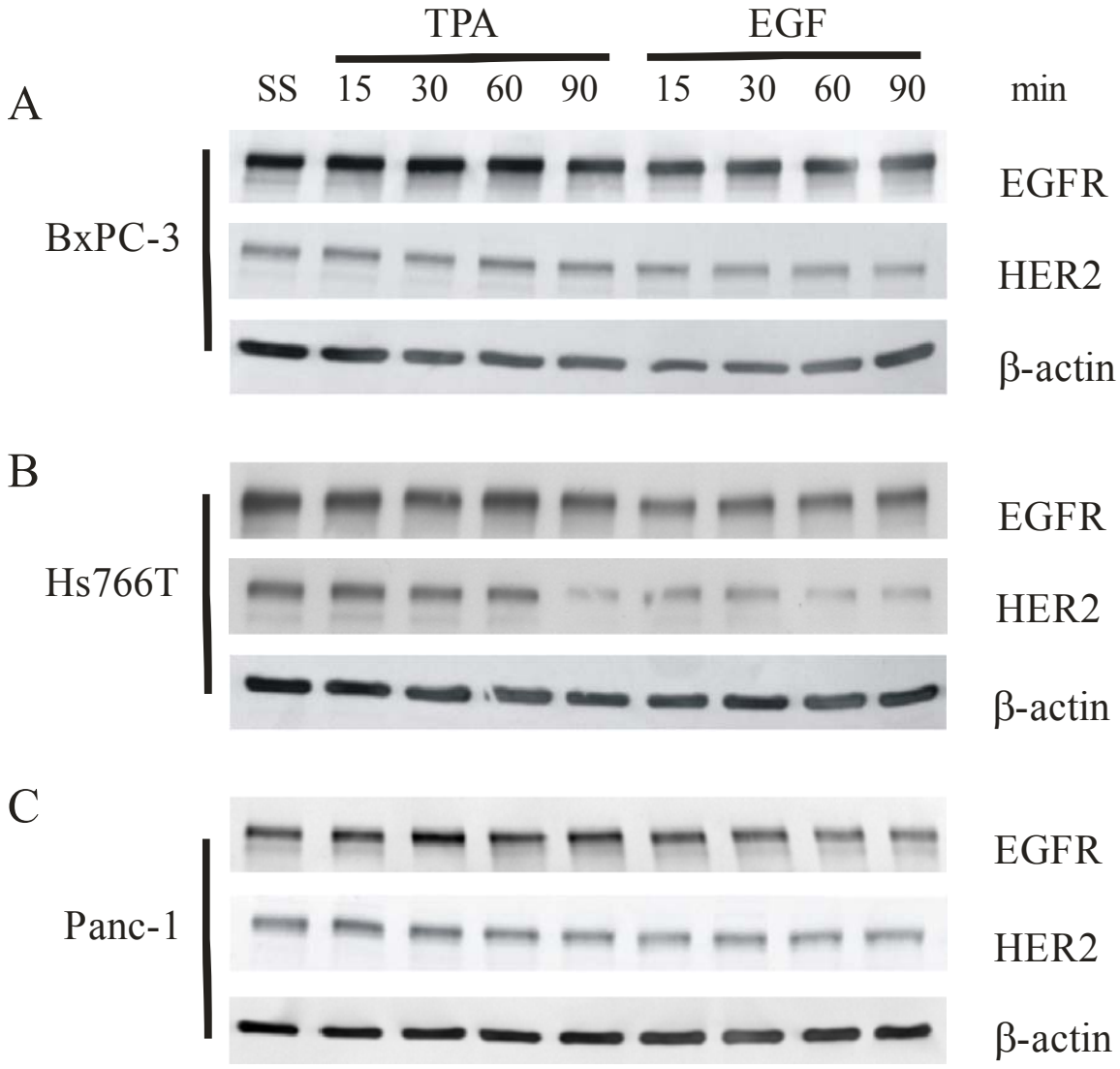


Figure 6.3. Differential response to EGF stimulation is not due to EGFR and HER2 receptor levels.

(A) BxPC-3, **(B)** Hs766T and **(C)** Panc-1 pancreatic cancer cells were cultured, serum-starved and were either untreated (SS) or treated for 15 to 90 min with 100 nM TPA or with 50 ng/ml EGF. Total cell extracts (25 μg) were then prepared and resolved on a SDS-10%-polyacrylamide

gel, transferred to a nitrocellulose membrane, and stained immunochemically with antibodies directed against EGFR, HER2 and β-actin as indicated.

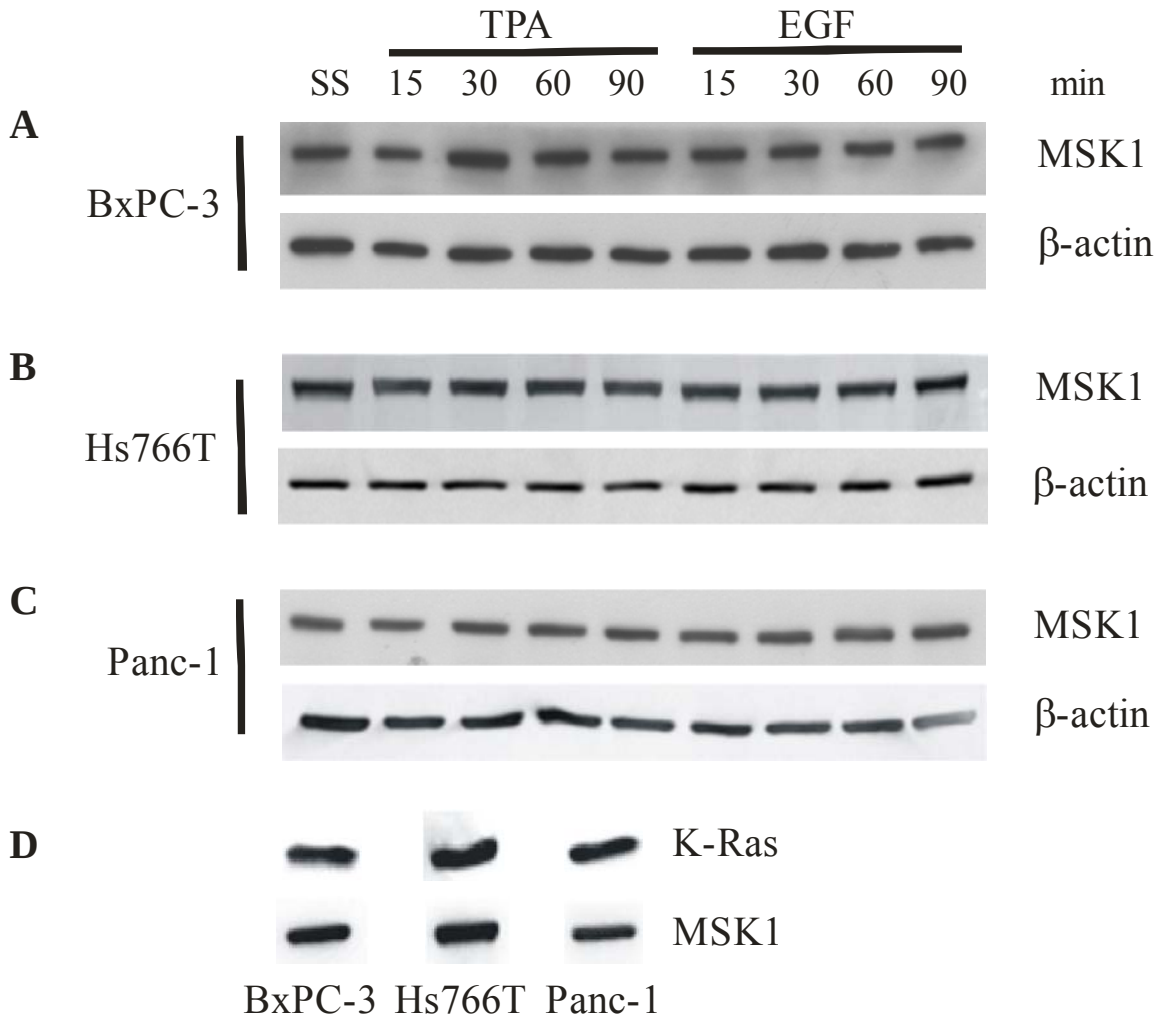


Figure 6.4. MSK1 protein level is unaltered upon induction of RAS-MAPK signaling in pancreatic cancer cell lines.

(A) BxPC-3, **(B)** Hs766T and **(C)** Panc-1 pancreatic cancer cells were cultured, serum-starved and were either untreated (SS) or treated for 15 to 90 min with 100 nM TPA or with 50 ng/ml EGF. Total cell extracts (25 μg) were then prepared and resolved on a SDS-10%-polyacrylamide

gel, transferred to a nitrocellulose membrane, and stained immunochemically with antibodies directed against MSK1 and β-actin as indicated. **(D)** Immunoblots of the resolved total cell extracts (25 ug) from serum-starved cells were stained immunochemically with antibodies directed against K-RAS and MSK1 as indicated.

6.4.4 Induction of H3 S10ph levels parallel phospho-ERK1/2 stimulation in BxPC-3 and Hs766T but differs in Panc-1 cells

Recent studies provide evidence that H3 S10ph is critical during neoplastic transformation (Choi et al, 2005a). To evaluate whether the induction of upstream phospho-ERK1/2 upon stimulation of the pathway can convey a nucleosomal response, we examined the levels of H3 S10ph following stimulation of the RAS-MAPK pathway in the pancreatic cell lines. TPA-induced activation of H3 S10ph in BxPC-3 cells was typically lower than that with EGF (1.4 ± 0.2 and 2.6 ± 0.2 for 60 min, respectively; n= 3; average ± SEM) (Figure 6.5A, D and E). TPA-induced stimulation of H3 S10ph was more robust in Hs766T than in the other cell lines (Figure 6.5B and D). For both cell lines with mutated K-RAS, TPA-induced levels of H3 S10ph were greater than those in EGF-stimulated cells (for Hs766T cells: 4.1 ± 0.3 and 0.9 ± 0.2 for 60 min, respectively; for Panc-1 cells: 2.4 ± 0.7 and 1.3 ± 0.5 for 60 min, respectively; n = 3) (Figure 6.5D and E). However, among the cell lines analyzed, we observed the induction of H3 S10ph in Panc-1 cells to be the most variable from one cell preparation to the next, for example at 90 min of TPA stimulation H3 S10ph levels were 2.0 ± 0.2, 4.1 ± 0.4 and 4.3 ± 2.5 (n = 3) for BxPC-3, Hs766T and Panc-1 cells, respectively (refer to standard error).

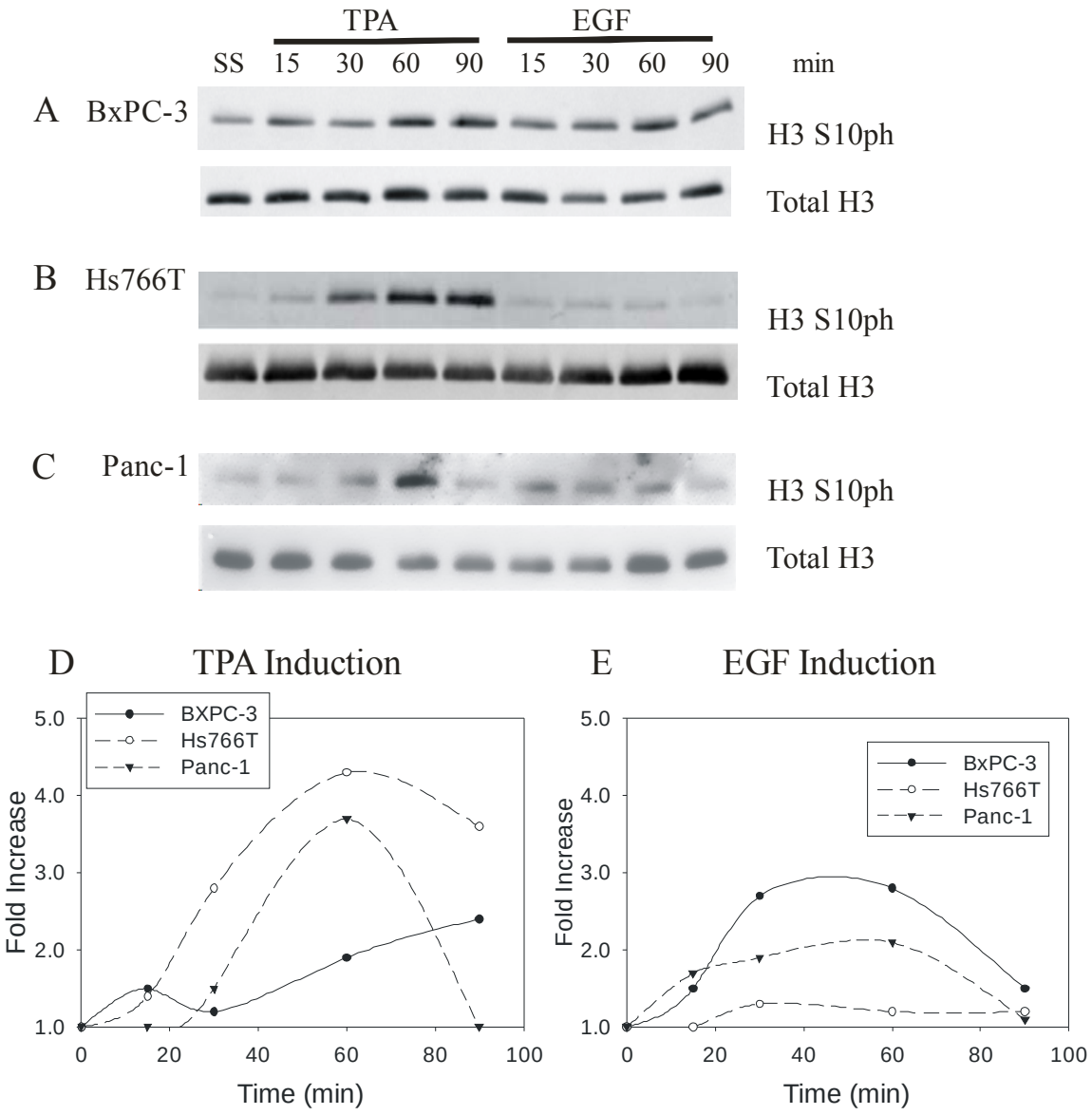


Figure 6.5. Induction of H3 S10ph levels parallel phospho-ERK1/2 stimulation in BxPC-3 and Hs766T but differs in Panc-1 cells.

Serum-depleted **(A)** BxPC-3, **(B)** Hs766T and **(C)** Panc-1 pancreatic cancer cells were treated with 100 nM TPA or 50 ng/ml EGF for 15 to 90 min. Acid-soluble nuclear histones (5 μg) were resolved on a SDS-15%-polyacrylamide gel, transferred to a nitrocellulose membrane, and stained immunochemically with anti-H3 pS10 and anti-total H3. **(D and E)** The fold induction H3 S10ph in response to stimuli (TPA or EGF) was determined as described under "Materials and methods".

6.5 Discussion

In this study, we demonstrate that the RAS-MAPK pathway can be activated by TPA or EGF in human pancreatic cancer cells and lead not only to elevated levels of phospho-ERK1/2 but also to increased downstream levels of H3 S10ph. However, the strength or duration of signaling upon induction did not correlate with the activating *K-RAS* mutation in these cells. Both Hs766T and Panc-1 cells, which contain activating mutations of the *K-RAS* gene, did not display a deregulated RAS-MAPK pathway signaling as measured by irregular augmentation in levels of phospho-ERK1/2, MSK1 or H3 S10ph in comparison to wild-type K-RAS-expressing BxPC-3. Thus, it appears that the constitutive activation of K-RAS does not necessarily convey anomalous downstream signaling events in pancreatic cancer cells as we have repeatedly observed in immortalized mouse fibroblasts (Chadee et al, 1999; Drobic et al, 2004; Dunn & Davie, 2005; Strelkov & Davie, 2002). It is important to note though that while both the cell lines used in this study have constitutively active K-RAS, these cells have different intrinsic genetic backgrounds and properties owing to their distinct source of tumour origin. The same gene mutation can have distinctive functions not only in different cell systems, but also in the same cell type depending on the timing of mutation during tumourigenesis (Vogelstein & Kinzler, 2004). As such, other factors apart from K-RAS status largely contribute to the lack of correlation between RAS and signaling events downstream and will require further study to elucidate. For example, the activity of phosphatases that counteract kinases can significantly

impact signaling pathways and cellular responses (Stephens et al, 2008; Wu et al, 2003). Although we did not detect any change in EGFRs or MSK1 levels, it is possible that there are changes in their activity. Consequently, it is prudent to refrain from drawing correlations and relationships when evaluating other cell backgrounds with Ras mutations such as breast cancer (10%) and colorectal cancer (50%) (Bos, 1989; Roberts & Der, 2007).

Notwithstanding the K-RAS status of the pancreatic cancer cell lines, we observed similarities in the responses to induction between levels of phospho-ERK1/2 and downstream levels of H3 S10ph. In BxPC-3 cells, both EGF and TPA increased phospho-ERK1/2 levels which coincided with the enhanced levels of H3 S10ph particularly for EGF stimulation. A more robust and sustained response of phospho-ERK1/2 and H3 S10ph in Hs766T cells was detected for TPA. As such, the pattern of stimuli-induced increase in these proteins appears to be distinct depending on the stimuli and cell context suggesting that each stimulus can dictate diverse responses in different cells as it is in the case of PC12 cells where depending on the stimuli (EGF versus nerve growth factor, NGF) used to induce a transient or sustained level of phospho-ERK1/2, the cell response can switch from proliferation to differentiation (Marshall, 1995). Regardless, the mirrored responses of phospho-ERK1/2 and H3 S10ph irrespective of stimuli indicate that the signaling pathway remains intact downstream despite the lack of correlation with upstream K-RAS. This study suggests that downstream players such phospho-ERK1/2 and H3 S10ph serve as better markers to gauge the extent and magnitude of cellular stimulation and insults.

In the case of the RAS-MAPK signaling in Panc-1 cells, both EGF and TPA increase phospho-ERK1/2 levels with a strong preference for TPA as a stimulus. However, a much random pattern of induction upon TPA or EGF treatment for H3 S10ph was observed downstream which corresponds to the elevated frequency of NCCA in these cells (60%). In contrast, the lowest NCCA frequency of the 3 cell lines analyzed belongs to Hs766T which also exhibits the most consistent and marked H3 S10ph induction in response to stimuli. Emerging studies reveal that unique aberrations like NCCA more accurately exhibit the heterogeneity and instability observed in cancer evolution (Heng, 2007; Heng et al, 2006a; Heng et al, 2006b). We show that the NCCA frequency of the cells not only reflects the relative genomic instability of the cell line but also appears to correlate with the predictability of induction pattern for H3 S10ph. It would be of interest to investigate whether there are alterations in corresponding MSK1 activity and epigenetic programming of IEG genes in these cells as observed in mouse fibroblasts.

At present, there is no curative treatment for resectable and metastatic pancreatic ductal adenocarcinoma. Current chemotherapy of gemcitabine combined with EGFR tyrosine kinase inhibitor, Tarceva, provides a 4-month survival advantage at best (Mahadevan & Von Hoff, 2007). Further, there is a lack of specific tumour markers for disease staging and early diagnosis. Hence, investigating a vital signaling pathway and its downstream players that can affect pancreatic cancer invasiveness and oncogenic aggressivity is an avenue to explore possible markers or pursue prospects for therapeutic intervention.

6.6 Acknowledgements

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Chapter 7: Discussion and Future Directions

7.1 Summary of findings

Collectively, the manuscripts presented in the previous chapters of this thesis further our understanding of the critical involvement of MSK1 and H3 phosphorylation as mediators that bridge the aberrant signals driven by the RAS-MAPK pathway with nucleosomal modifications, chromatin remodeling, IEG expression and malignant transformation. In order to verify our observations from oncogene-transformed mouse fibroblasts, we examined if the activation of RAS-MAPK signaling in breast cancer cells elicits similar molecular events. We demonstrate that the RAS-MAPK pathway is induced and intact which consequently enhances association of MSK1 and H3 phosphorylation on the *TFF1* gene important for transcriptional activation in breast cancer cells. Although certain breast cancer cell lines with overexpressed HER2 and EGFR exhibit upregulation of phospho-ERK1/2 and H3 S10ph, the presence of an activating mutation in the *K-RAS* gene does not correlate with genomic instability or altered RAS-MAPK signaling in pancreatic cancer cell lines. Given that the RAS-MAPK-MSK1-mediated H3 phosphorylation, chromatin remodeling and IEG activation is unaltered in Ciras-3 cells when compared to parental 10T1/2 cells, it is possible that similar recruitment mechanisms for breast cancer and pancreatic cancer cell lines exist. Taken together, MSK-regulated gene expression may be a mechanism exploited by epithelial cancerous cells with abnormal MAPK signaling to acquire further growth/ transformation advantages.

The first study in this thesis (**Chapter 2**) seeks to address whether MSK1 is the essential link between a constitutively activated RAS or extracellular stimulation of the pathway and elevated nucleosomal responses in mouse fibroblasts. We determined that while MSK1 protein

levels remained the same and maintained its predominantly nuclear localization, MSK1 activity was elevated as a consequence of increased MAPK levels by *Ras* transformation. As the counteracting phosphatase PP1 activity is unaltered (Chadee et al, 1999), we surmised that the **net increase in H3 phosphorylation levels observed in Ciras-3 cells is attributed to elevated MSK1 activity.** Our lab recently demonstrated that TPA induction of MSK1-mediated H3 phospho-acetylation at nucleosomal regulatory regions permitted localized chromatin remodeling and recruitment of 14-3-3, BRG1 and PCAF crucial for IEG expression in parental 10T1/2 cells (Drobic et al, 2010). The second study in this thesis (**Chapter 3**) investigated whether *Ras*-driven MSK1 activity altered the mechanism of IEG expression and investigated the direct contribution of MSK1 in IEG expression and anchorage-independent growth in Ciras-3 cells. Despite the similar mechanism of recruitment necessary for chromatin remodeling and IEG expression, we demonstrated that IEG-encoded proteins such as AP-1 and COX-2 are increased in Ciras-3 cells. Further, diminished MSK1 activity by H89 pretreatment and knockdown in Ciras-3 cells not only reduced IEG-encoded mRNA and protein expression but also abolished anchorage-independent growth. Altogether, **MSK1 plays a central role and is directly involved in RAS-MAPK-induced molecular mechanisms that drive neoplastic transformation in mouse fibroblasts.**

Although our murine cell model system has contributed greatly to our current model and understanding of RAS-MAPK signaling and consequent deregulation, it is of great interest to investigate whether parallel mechanisms exist in established cell line models of human cancers with overexpressed growth factor receptors or overactive RAS molecules. The next study in this thesis (**Chapter 4**) examined the involvement of a stimulated RAS-MAPK pathway, MSK1 and

H3 S10ph in transcriptional regulation and expression of a responsive model gene, *TFF1*. We established that estradiol and TPA can elicit transcriptional activation of *TFF1* via alternative routes in hormone-dependent MCF7 breast cancer cells. Estrogen-induced gene expression occurs through the recruitment of ERα to the *TFF1* promoter which was in line with reports from the literature, whereas TPA-induced expression requires AP-1 factor c-JUN recruitment that leads to MSK1 loading and H3 S10ph on the *TFF1* proximal regulatory region within the promoter. Using an H89 inhibitor that preferentially targets MSK1, site directed mutagenesis of the AP-1 site and ChIP assays on the *TFF1* proximal promoter region, we established that the RAS-MAPK pathway was intact and responsive in these cells. **As such, these findings are in agreement with the TPA-induced RAS-MAPK signaling and MSK1-mediated H3 S10ph on IEG regulatory regions in immortalized mouse fibroblasts.** We speculate that other IEG regulatory regions require MSK1-mediated H3 S10ph in response to the RAS-MAPK pathway which may contribute to hormone-independence and aggressive phenotype changes in breast cancer cells.

The next study presented in this thesis (**Chapter 5**) explored whether overexpressed receptors such as EGFR or HER2 that augment RAS signaling leads to elevated MAPK and H3 phosphorylation in human breast cancer cell lines. We observed that elevated steady state levels of phospho-ERK1/2 and H3 S10ph but not H3 S28ph mirrored the upstream overexpression of HER2 and EGFR (SKBR-3 and BT-474) and to a lesser extent EGFR (MCE5 and MDA MB 231) when compared to MCF7 cells that do not overexpress these tyrosine kinase receptors. Although these breast cancer cell lines are matched for cell-cycle and upregulated receptor status, we recognize that our findings are drawn from cells with inherently distinct breast cancer

epithelial cell backgrounds. Factors such as ERK phosphatases (MKP-1), histone H3 phosphatases (PP1) or crosstalk mechanisms are likely involved. As such, these findings are qualitative and further investigation is required to delineate the contribution of RAS-MAPK signaling and downstream H3 phosphorylation in IEG expression in these cells. **Regardless, we note that there is an intact pathway in these cells with the increased ERK1/2 levels which is a hallmark of upregulated RAS-MAPK pathway mirroring downstream H3 S10ph.** Thus, it would be of interest to investigate whether the status of MSK1 activity in these cells is accordingly elevated.

The last study presented in this thesis (**Chapter 6**) examined whether extracellular stimulation of the RAS-MAPK pathway was intact in pancreatic cancer cell lines. We further explored whether presence of constitutively active K-RAS correlated with genomic instability as measured by NCCA or altered induction of H3 S10ph by EGF or TPA. We revealed that the presence of an activating *K-RAS* mutation in two of the three pancreatic cancer cell lines (Hs766T and Panc-1 compared to wild type K-RAS in BxPC-3) did not correlate with elevated chromosomal aberrations or increased genomic instability as measured by NCCA from karyotypic analyses. Only one of the two cell lines with K-RAS mutation (Panc-1) displayed an elevated NCCA. Interestingly, the same Panc-1 cell line demonstrated the most variable pattern of H3 S10ph of induction in response to TPA or EGF. In the two other pancreatic cancer cell lines with ≤20% NCCA (BxPC-3 and Hs766T), extracellular stimulation of RAS-MAPK pathway resulted in elevated phospho-ERK1/2 which coincided with increased H3 S10ph. Further, qualitative assessment of 2 different pancreatic cell lines with activating *K-RAS* mutations (AsPC-1 and MIA PaCa-2 cells) suggest that there is an apparent disconnect between

the presence of *K-RAS* activating mutations and elevated steady state levels of ERK1/2 and H3 phosphorylation in these cells (unpublished results). **Irrespective of the activating *K-RAS* mutations, it is noteworthy that the ERK1/2 and H3 S10ph responses to TPA and EGF induction parallel each other albeit with differential stimuli and cell-type specificity. It appears that at least for two pancreatic cancer cell lines with lower NCCA frequency, activation of the RAS-MAPK pathway is intact.** These results may suggest that downstream players such as phospho-ERK1/2 and H3 S10ph may serve as better indicators to assess the extent and magnitude of cellular responses. Despite these observations, much work is needed to define the role of RAS-MAPK pathway and MSK1-mediated H3 S10ph in these cells. One approach is to evaluate the contribution of MSK1 in RAS-MAPK signaling in these cells by knockdown or H89 treatment followed by assessment of H3 S10ph levels, IEG expression and loss of anchorage-independent growth. Nevertheless, we demonstrated that pancreatic cancer cell lines do not manifest the causal relationship between an upregulated RAS activity and elevated nucleosomal H3 S10ph observed in mouse fibroblasts.

7.2 Insights from RAS-MAPK-MSK1-H3 phosphorylation in mouse fibroblasts and human epithelial cancer cell lines

MSK1 activity as the critical factor in Ras-driven malignancy

Our work in 10T1/2 and Ciras-3 fibroblasts has provided extensive insight into the distinct mechanisms that allow *Ras*-driven neoplastic oncogenesis and metastasis. Ciras-3 cells are tumourigenic and metastatic exhibiting decondensed chromatin and unstable genomes (Chadee et al, 1999; Dunn et al, 2009; Egan et al, 1987b). Further, steady state levels of nucleosomal proteins such as H3 phosphorylation and HMGN1 are elevated as a consequence of

upregulated RAS-MAPK signaling (Chadee et al, 1999; Dunn et al, 2009). While PP1 activity, subcellular distribution and protein levels of MSK1 remained the same, Ciras-3 cells have augmented MSK1 activity responsible for increased expression of IEG products capable of supporting anchorage-independent growth. In this cellular setting, increased MSK1 activity appears to be the compelling factor in a background of genomic instability which allows for chromatin remodeling and reprogrammed gene expression profile. Accordingly, our work highlights that MSK1 activity promotes the increased steady state levels of IEG products and modifies the cellular environment important for *Ras*-driven malignancy in mouse fibroblasts.

Mouse Fibroblasts and Human Epithelial Cancer Cell Lines as Model Systems

Although fibroblasts are indispensable structural and functional cells that have pleiotropic roles in mammalian systems, they are one of the many cellular components that make up the stromal compartment in solid tumour tissues. Fibroblasts are vital in wound healing, extracellular matrix remodeling, cytokine production, inflammatory responses and cell mobilization characteristic of neoplastic oncogenesis and metastasis. Neoplastic transformation by means of loss of anchorage-dependence permits cell mobility crucial for tumourigenesis as well as metastasis and involves complex gene expression signature profiles (Mori et al, 2009). However, the transition from a normal to a malignant phenotype is a progressive process which necessitates acquisition of multiple genetic and epigenetic hits in most solid tumours (Vogelstein & Kinzler, 2004). It is well recognized that apart from stromal cells, epithelial cells and their interactions with the supporting environment are important in dissemination of neoplasms. As fibroblasts are often regarded as a component of stroma in tumour tissues, epithelial cells are the cancer cells. An upset in the RAS-MAPK pathway either through overexpressed receptors or activating

mutations in the players (e.g., RAS or BRAF) of epithelial cells thus has great implications in the biology of the tumour. Our work in breast cancer cell lines and pancreatic cancer cell lines, both of which are epithelial in origin, expands and contributes to our current understanding of the RAS-MAPK-MSK-H3 phosphorylation beyond the pathway's impact in fibroblasts.

Our present work has greatly capitalized on established cell lines as *in vitro* cancer models. In particular, we have extended our studies in breast cancer and pancreatic cancer cell line models that have overexpressed receptors or activating mutations. Though we are mindful that primary tumours may be regarded as better models and that they may more closely mimic *in vivo* pathobiology, established cell lines have advantageous features that make them ideal in our studies. Cell lines have relatively simple handling methods and display a high degree of cellular homogeneity. Further, cell lines have unlimited self-replicating potential and represent an unlimited resource. Unlike primary cultures, cell lines are not prone to senescence, finite population doubling or heterogeneous contamination of normal cells like primary cultures (Burdall et al, 2003). Despite their immortalized nature and tendencies towards genotypic and phenotypic changes under continual culture, cell lines are invaluable in facilitating the characterization of the impact of the RAS-MAPK pathway on biological responses.

Distinct pools of H3 S10ph/ H3 S28ph and MSK1 targeting specificity

Another noteworthy observation from our studies which is corroborated by other groups is the existence of MSK1-mediated H3 S10ph and H3 S28ph in separate pools. Using high-resolution microscopy analyses, previous work demonstrated that the bulk of H3 S10ph foci marking sites of active transcription observed upon RAS-MAPK pathway stimulation did not

coincide with H3 S28ph foci in mouse fibroblasts (Dunn & Davie, 2005). Furthermore, sequential immunoprecipitation and re-ChIP assays revealed that individual histone tails or adjacent nucleosomes contain H3 S10ph or H3 S28ph but not both (Dunn & Davie, 2005; Dyson et al, 2005). Moreover, both phospho-H3 modified forms do not co-occupy the same MSK1 targeted regulatory region (Drobic et al, 2010). We observed that in some cellular settings, only H3 S10ph but not S28ph occurs when the RAS-MAPK-MSK pathway is stimulated. We highlight in our studies that **TPA stimulation of MCF7 cells resulted in the induction of H3 S10ph but not H3 S28ph.** In cycling human breast cancer cell lines overexpressing EGFR or HER2/EGFR, **increased levels of phospho-ERK1/2 paralleled the increased levels of H3S10ph, but not H3S28ph**. Notably, this difference is not due to the genetic background of human or mice cells as both phospho-H3 PTMs are stimulated in HeLa cells (unpublished results). Why certain cell types induce H3 phosphorylation at S10 and S28 and others only at S10 is currently unknown. It is possible that different MSK complexes exist and are favored depending on the stimuli and specific cell type. Another possibility is that the PTM environment surrounding S10 and S28 residues such as methylation or acetylation can dictate the preference for H3S10ph for MCF7 cells. In any case, MCF7 provides an excellent system to determine the preferential targeting of MSK towards S10 to promote gene expression. A strategy to test this hypothesis is to compare genes like *COX-2* or *FOSL1* that are both EGF and TPA-responsive in two cellular backgrounds (MCF7 and 10T1/2 cells). High resolution ChIP and re-ChIP analyses on the promoter regulatory regions of these genes using antibodies against H3 PTMs, PTM readers, transcription factors such as AP-1 or NF-kB and MSKs will determine the mechanism by which MSK selects these residues upon gene induction.

In any event, several mechanisms have been suggested to explain the MSK targeting specificity towards either phosphorylation of S10 and S28 on regulatory regions of particular genes. One possibility is that the environment of nucleosomal PTM surrounding S10 and S28 such as adjacent acetylation or methylation on K9 and K27 or the presence of nearby bulky proline residues can either promote or deter MSK1 from recognition or binding (Perez-Cadahia et al, 2009). Another explanation argues that the dynamic preference of the corresponding phosphatase (PP1 or DUSP-1) for H3 S10ph and higher sensitivity to dephosphorylation may permit residue exposure and consequent kinase targeting (Goto 2002). As suggested earlier, differential specificity may also be achieved depending on the complex of transcription factors that recruit and direct MSK1 to either residue. It is possible that any or all of these mechanisms take place depending on the gene, enzymes present and cell type. **Addressing these outstanding questions may further clarify the means and context by which MSK1 is recruited to its nucleosomal environment and confirm specificity towards a particular H3 serine in a distinct cell type.**

Cautionary Note with the Use of Pharmacological Kinase Inhibitors

In defining the role of MSK1 kinase activity in IEG expression (*Jun, Fosl1, Cox-2* and *TFF1)* and anchorage-independent growth, we used a member of the H7 series of inhibitors H89 in our studies. Although H89 preferentially targets MSK1 and 2 at 10 mM, it equally targets PKA, S6K1, and ROCK-II (Davies et al, 2000; Thomson et al, 1999). Thus, it is pertinent to verify systematically that the effects we observe upon treatment with pharmacological kinase inhibitors such as H89 are attributed to impedance of MSK activity. Arthur and colleagues evaluated the effect of MSK1/2 in nucleosomal responses and IEG expression by comparing

their findings from H89 use with subsequent knock-out studies (Soloaga et al, 2003). As we did not have the MSK1/2 knock-out mice at our disposal, validation of H89 results were conducted using different approaches. Contrary to the reduced IEG expression with H89, treatment with PKA specific inhibitor Rp-cAMP did not alter the expression of the IEGs we studied. MSK1 shRNA knockdown in 10T1/2 and Ciras-3 replicated the diminished IEG expression with H89 treatment. Further, H89 did not interfere with the formation of MSK1/14-3-3/ SWI-SNF complex in co-immunoprecipitation experiments of parental 10T1/2 mouse fibroblast (Drobic et al, 2010). Lastly, we observed that the hindered Ciras-3 anchorage-independent growth with H89 treatment is comparable to the reduced soft agar colony formation with MSK1 shRNA knockdown. Taken together, we confirmed that the lack of factor recruitment and consequent promoter remodeling, abrogation of TPA-induced IEG expression and diminished anchorage-independent growth with H89 are due to the inhibition of MSK1 kinase activity.

Differential cell-type specific responses to stimuli

With our work in pancreatic cancer cell lines, we observed that TPA and EGF can stimulate phospho-ERK1/2 and H3 S10ph differentially and exhibit cell-type specificity. As examples, BxPC-3 cell line displayed enhanced phospho-ERK1/2 and H3 S10ph with both TPA and EGF while Hs766T cell line exhibited a more robust and sustained response for TPA. Another important corollary is that an activated mutated *K-RAS* does not translate into a higher steady state of H3 S10ph or altered signaling in these cells. What aspects of the RAS-MAPK signaling cascade determine how a specific cell type responds differently to stimuli? This is a complex question with many variables that our studies were unable to address and will require further investigations. We speculate that the downstream players present in these cells may be

critical factors (e.g., phosphatases) and may ultimately determine the responses and gene expression programming. It is also possible that depending on whether a stimulus can exert a transient or sustained level of phospho-ERK1/2 response, unique outcomes can result such as the switch between proliferation and differentiation observed in PC12 cells in response to nerve growth factors (Marshall, 1995). It is known that phorbol esters such as TPA can activate phospho-ERK1/2 via PKC and RAF kinase whereas EGF can trigger either p38 MAPK or phospho-ERK1/2 MAPK pathways via EGF receptors (Blumberg, 1988; Dunn et al, 2005; Lorenzo et al, 2001). As such, underlying cross-talks stimulated by EGF may impact end-of-the-line players and their activities that ultimately dictate cell-specific responses.

The Role of MSKs in cancer

The physiological role of MSKs is beginning to be unraveled. MSKs have been implicated in diverse biological functions such as long-term memory potentiation, immunological responses and transcriptional activation (Arthur, 2008). However, the contribution of MSKs in cancer is ill-defined despite MSKs playing a key mediator role downstream of the RAS-MAPK pathway. In lung adenocarcinoma cells, MSK1 regulates inflammatory genes such as *IL-6*, *CXCL-3* and *COX-2* in response to farnesol (Joo & Jetten, 2008). Further, MSK1 impacts adhesion and cell proliferation of colon carcinoma cells through the vitamin D receptor (VDR) and Wnt signaling pathways (Ordonez-Moran et al, 2008). In JB6 mouse epidermal cells, Dong and colleagues demonstrated that MSK1 and H3 phosphorylation are indispensable for EGF-induced cell transformation (Choi et al, 2005a; Kim et al, 2008) which supports our findings of MSK1 knockdown in Ciras-3 cells. It is tempting to speculate that targeting of MSK1 alone can have therapeutic implications to avert metastasis in RAS-driven

oncogenesis due to its involvement in neoplastic transformation. However, we are mindful of the presence of other kinases such as TG2 or MSK2 (Mishra et al, 2006; Soloaga et al, 2003). TG2 has been demonstrated to phosphorylate H3 *in vitro* as well as in nucleosomes of MCF7 cells. TG2 is also associated with chromatin in MCF7 cells and inferred to have a role in regulation of gene expression. As a MSK1 functional homologue, murine MSK2 shares about 65% sequence identity and similar transcription factor targets with MSK1 (Arthur, 2008). Further, MSK2 is reported to be a stronger H3 kinase in studies comparing single and double MSK knockouts in mouse embryonic fibroblasts (Soloaga et al, 2003). Also, the lack of effect in response to cocaine or neutrophin stimulation in cortical neurons implies functional non-redundancy of MSK2 from MSK1 (Brami-Cherrier 2005, Arthur 2008). **Thus, it would be integral to examine the extent and specific contribution of MSK2 in the RAS-MAPK pathway and decipher the mechanism of their involvement not only in our mouse fibroblast cell lines but also in breast and pancreatic cancer cell lines.**

7.3 Conclusions and Significance

Overall, the studies presented in this thesis support our hypothesis that MSK1 activity has a role in mediating downstream effects of RAS-MAPK signaling. We demonstrate the critical role of MSK1 as a mediator between extracellular stimulation to IEG expression and neoplastic transformation under the frame of RAS-MAPK pathway activation. We provide evidence that MSK1 activity is required for the TPA-induced nucleosomal response and promoter remodeling of IEG. We further demonstrate that MSK1 activity has a direct contribution in the RAS-driven malignant phenotype of mouse fibroblasts. These findings define an integral role for MSK1 in RAS-induced oncogenesis as well as in epigenetic programming in mouse fibroblasts. As

such, it is possible that modulation of MSK1 activity may have an impact in cancer cells with deregulated RAS-MAPK signaling.

We also highlight that MSK1-mediated H3 phosphorylation in response to the RAS-MAPK pathway is intact in breast cancer cells. Our findings that MSK1-mediated H3 phosphorylation stimulated by the RAS-MAPK pathway is associated with promoter regulatory regions of the *TFF1* gene and results in transcriptional activation validate our hypothesis. Nevertheless, much work needs to be done to demonstrate that similar mechanisms of RAS-MAPK signaling to MSK1-mediated H3 phosphorylation in pancreatic cancer cells exist. These findings broaden our understanding of how diverse stimuli such as growth factors, phorbol esters and hormones can work in concert to activate mechanisms that mediate gene expression and further our awareness of the underlying cross-talks that occur between signaling pathways.

Chapter 8: References

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