

TNF-alpha Gene Signature in Triple-negative Breast Cancer Cells

by

Ifeoluwa Adewumi

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Biochemistry and Medical Genetics

University of Manitoba

Winnipeg

Copyright © 2017 Ifeoluwa Adewumi

ABSTRACT

Triple-negative breast cancer patients have an increased likelihood of distant recurrence and death when compared with patients with other subtypes of breast cancer. High persistence of tumor necrosis factor- α (TNF α) in breast tumors is linked with local recurrence and new metastasis. TNF α stimulates the RAS-mitogen activated protein kinase and stress kinase pathways; both of which activate the mitogen- and stress-activated protein kinases. We hypothesized that mitogen- and stress-activated protein kinases have a role in the TNF α -response gene signature in triple-negative breast cancer cells.

MDA-MB-231 cells were used to determine the TNF α gene signature in triple-negative breast cancers. RNA-sequencing data revealed that immediate early genes involved in carcinogenesis were strongly induced. Validation studies with the mitogen- and stress-activated protein kinase inhibitor H89 attenuated the induction of these genes, suggesting these kinases play a role in their induction and subsequently may be a potential therapeutic target for triple-negative breast cancers.

ACKNOWLEDGEMENTS

I would like to appreciate my supervisor, Dr. Jim Davie, who took out time to listen and enlighten me whenever I did not understand. It was a great opportunity and privilege to be under your supervision. Your enthusiasm and excitement motivated me to strive to do my best always. I will forever cherish this opportunity.

Additionally, I would like to thank my advisory committee members, Dr. Mark Nachtigal and Dr. Ted Lakwoski. Thank you for your support and guidance throughout my degree. I always felt encouraged after committee meetings. Also, your interest in my success beyond my degree will never be forgotten.

Cathy Webber, you made my graduate school journey less stressful, you always had a solution to every problem a student could encounter during their study. For your resourcefulness, thank you. I would like to thank all the Davie lab members (past and present) for their support throughout my degree. Dr. Kiran Sharma, your continuous willingness to always be of help will forever be appreciated. Veronica Lau and Aleksandar Ilic, I thank you for your support in and outside of the lab.

To my family, my parents Oluwafemi and Oluwaniyi Adewumi, I express my deepest gratitude for all the love, support and words of encouragement, I would have never made it this far without you. Olawale Ayilara for your love and determination to see me succeed, thank you.

Finally, I would like to thank the University of Manitoba, Research Manitoba, CancerCare Foundation and the Canadian Breast Cancer Foundation for their support towards the completion of this work.

TABLE OF CONTENTS

ABSTRACT	I
ACKNOWLEDGEMENTS	II
TABLE OF CONTENTS	III
LIST OF TABLES	VI
LIST OF FIGURES	VII
LIST OF COPYRIGHTED MATERIAL FOR WHICH PERMISSION WAS OBTAINED.....	VIII
LIST OF ABBREVIATIONS	IX
1.0 INTRODUCTION.....	1
1.1 Gene signatures	1
1.1.1 Prognostic signatures	2
1.1.2 Predictive signature.....	3
1.2 The wound response signature	3
1.3 Breast cancer	4
1.4 Triple-negative breast cancer	5
1.4.1 Subtypes of TNBC	5
1.4.2 Treatment of TNBC	6
1.5 TNFα-induced transcriptional program in breast cancer cells	7
1.5.1 TNF α and its receptors.....	7
1.5.2 The TNF α link between inflammation and cancer	8
1.5.3 TNF α -induced transcriptional program in breast cancer cells	9
1.6 Histone post translational modifications	10
1.6.1 Histone methylation	14
1.6.2 Histone acetylation.....	15
1.6.3 Histone phosphorylation	16
1.7 H3S10ph and H3S28ph localization and correlation with H3 acetylation	21
1.8 MSK-induced nucleosomal response.....	24
1.8.1 MSK: Discovery, structure, function and activation	24
1.8.2 MSK-mediated H3 phosphorylation and immediate early gene expression.	27
1.9 MSK as a therapeutic target	29

2.0 RATIONALE, HYPOTHESIS, RESEARCH AIMS AND SIGNIFICANCE	32
2.1 Rationale	32
2.2 Hypothesis.....	33
2.3 Specific Aims	33
2.3.1 Aim 1: To determine the transcriptional response of MDA-MB-231 cells in response to TNF α and the role of MSK	34
2.3.2 Aim 2: Determination of the levels of phosphorylated H3 and acetylated K27 at upstream promoter and enhancer regions of immediate early genes.....	34
3.0 MATERIALS AND METHODS	36
3.1 Cell Culture	36
3.1.1 Cell culture conditions	36
3.1.2 Cell passaging	36
3.1.3 Cell storage and recovery.....	36
3.1.4 Serum starvation and treatment conditions	37
3.2 Ethidium bromide/Acridine orange double staining	38
3.3 Flow cytometric analysis of cells.....	39
3.4 Protein-Based Techniques.....	40
3.4.1 Preparation of total cellular lysate	40
3.4.2 Histone extraction	41
3.4.3 SDS-PAGE and western blotting.....	42
3.4.4 Quantitative western blotting to determine optimal protein concentration	43
3.5 RNA isolation and real time RT-qPCR analysis.....	44
3.5.1 RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR).....	44
3.5.2 cDNA Real Time Polymerase Chain Reaction (RT-qPCR)	45
3.6 Chromatin Immunoprecipitation (ChIP) Assays.....	46
3.6.1 Formaldehyde Crosslinking	46
3.6.2 Chromatin immunoprecipitation.....	46
3.6.3 ChIP qPCR.....	49
3.6.4 ChIP Optimization Steps.....	52
4.0 Results	60
4.1 Synchronization of MDA-MB-231 cells to G₀/G₁ cell cycle phase	60
4.2 TNFα induced the nucleosomal response via the ERK1/2 and p38 MAPK pathways in MDA-MB-231 cells.....	62

4.3 TNFα induced transcriptional response in MDA-MB-231 cells	64
4.3.1 Immediate early genes are amongst the strongest induced genes following TNF inductions	64
4.3.2 Validation of immediate early genes identified from RNA-Seq data	66
4.4 MSK mediated TNF induction of immediate early genes	71
4.4.1 MSK inhibitor H89 attenuated TNF induced genes	71
4.4.2 PKA inhibitor Rp-cAMPs does not attenuate TNF induced genes	73
4.5 TNFα induced H3S10ph at the upstream promoter regulatory of immediate early genes	74
4.6 TNFα induced H3K27ac at the upstream promoter and enhancer region of IL6	77
4.7 Dynamic levels of H3K27ac at a putative enhancer region of IL6	81
DISCUSSION	83
5.1 TNFα induced transcriptional response in MDA-MB-231 cells	83
5.2 TNFα induced nucleosomal response and MSK-mediated induction of immediate early genes in MDA-MB-231 cells	86
5.3 TNFα induced H3K27ac at the upstream promoter region and enhancer region of IL6	88
5.4 Significance	91
5.5 Future Directions	91
REFERENCES	93
APPENDIX	112

LIST OF TABLES

Table 1. Inhibition of protein kinases by H89.	31
Table 2. MSK1/2 specific inhibitors.	31
Table 3. Components of protease and phosphatase inhibitors from Roche.	41
Table 4 List of primers used for cDNA RT-qPCR analyses.....	46
Table 5. List of primers used for ChIP-qPCR analyses	50
Table 6. Top genes induced by TNF α with a fold change greater than 2.0 at 30 minutes.	65

LIST OF FIGURES

Figure 1. PubMed articles on gene signatures per year	2
Figure 2. MSK 1/2, the nucleosomal response and immediate early gene expression.	10
Figure 3. Human core histone amino acid sequences	13
Figure 4. Kinases and phosphatases responsible for H3 N-terminal tail phosphorylation and dephosphorylation during mitosis and interphase.	18
Figure 5. Relative intranuclear localization of H3S10ph and H3S28ph in TPA-treated human primary fibroblasts.	23
Figure 6. The kinase domains of MSK 1 and MSK 2, with their downstream substrates.	25
Figure 7. Fluorescence microscope images of MDA-MB-231 cells stained with ethidium bromide/acridine orange double staining.	38
Figure 8. Protein concentration standard curve using Santa Cruz H3S10ph (8656-X) antibody.	44
Figure 9. Nuclei isolated from MDA-MB-231 cells.	49
Figure 10. Primer location of -80 JUN upstream promoter region.	51
Figure 11. Primer location of IL6 enhancer region.	52
Figure 12. Dot blot assay.	54
Figure 13. Immunoprecipitation efficiency.	56
Figure 14. MNase optimisation.	58
Figure 15. Flow cytometric analysis of serum starved MDA-MB-231 cells.	61
Figure 16. TNF α stimulation correlates with the induction of the nucleosomal response	63
Figure 17. Heat map of TNF α induced MDA-MB-231.	66
Figure 18. Track maps for selected immediate early genes and RNA-Seq validation.	70
Figure 19. mRNA expression of FOS, JUN, IL6 and IER3 genes with or without H89	72
Figure 20. mRNA expression of IL6 and JUN genes with or without PKA inhibitor Rp-cAMPs	73
Figure 21. Levels of H3S10ph at the upstream promoter region of JUN.	75
Figure 22. Levels of H3S10ph at the upstream promoter region of IL6.	76
Figure 23. Levels of H3K27ac at the upstream promoter region of IL6	78
Figure 24. Levels of H3K27ac at a putative enhancer region of IL6 gene	80
Figure 25. TNF α -induced H3K27ac at a putative enhancer region of IL6	82
Figure 26. Approaches leading to clinical validation.	85
Figure 27. Mechanism of MSK-mediated nucleosomal response and induction of immediate early genes.	90

LIST OF COPYRIGHTED MATERIAL FOR WHICH PERMISSION WAS OBTAINED

Figure 3. Human core histone amino acid sequences	13
Figure 5. Relative intranuclear localization of H3S10ph and H3S28ph in TPA-treated human primary fibroblasts.	23

LIST OF ABBREVIATIONS

°C	Degree Celsius
A ₂₆₀	Absorbance at 260nm
ADP	Adenosine diphosphate
AMPK	AMP-activated protein kinase
AP-1	Activated protein 1
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BRCA	Breast cancer gene
CaCl ₂	Calcium chloride
CDK	Cyclin dependent kinase
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CO ₂	Carbon dioxide
CTK	C-terminal kinase
ddH ₂ O	Double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ENCODE	Encyclopedia of DNA elements
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FISH	Fluorescence in situ hybridization
FBS	Fetal bovine serum
FOS	Finkel-Biskis-Jenkins murine osteogenic sarcoma virus

GEO	Gene expression omnibus
HER2	Human epidermal growth factor receptor 2
HDAC	Histone deacetylase
ID	Immunodepleted
IER3	Immediate early response 3
IgG	Immunoglobulin G
IL6	Interleukin-6
IP	Immunoprecipitation
KMT	Lysine methyltransferase
KAT	Lysine acetyltransferase
KCL	Potassium chloride
kDa	Kilodalton
M	Molar
MAPK	Mitogen activated protein kinase
mL	Milliliter
MLL	Mixed-lineage leukemia
mM	Millimolar
MNase	Micrococcal nuclease
mRNA	Messenger ribonucleic acid
MSK	Mitogen- and stress-activated protein kinase
NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
NCBI	National center for biotechnology information
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
nM	Nanomolar
NP-40	Nonyl phenoxy polyethoxy ethanol
NTK	N-terminal kinase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

PCAF	p300/CBP-associated factor
PIPES	Piperazine-N, N'-bis (2-ethanesulfonic acid)
PKA	Protein kinase A
PR	Progesterone receptor
PRMT	Protein arginine methyltransferase
PTM	Post translational modification
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
Rp-cAMPs	Rp-Adenosine 3',5'-cyclic monophosphorothioate
RT-PCR	Reverse transcription polymerase chain reaction
SAGE	Serial analysis of gene expression
SET	Su(var) 3-9, Enhancer of zeste (E[z]) and trx-G Trithorax
SDS	Sodium dodecyl sulfate
TBE	Tris Borate EDTA
TBST	Tris-buffered saline tween 20
TNBC	Triple-negative breast cancer
TNF	Tumor necrosis factor -alpha
TNFR1	Tumor necrosis factor -alpha receptor 1
TNFR2	Tumor necrosis factor -alpha receptor 2
TPA	12-O-tetradecanoylphorbol-13-acetate
UPR	Upstream promoter region
V	Volts
v/v	Volume/volume
w/v	Mass/volume
x g	Centrifugal force
μg	Microgram
μL	Microliter
μM	Micromolar

CHAPTER ONE

1.0 INTRODUCTION

1.1 Gene signatures

A gene signature is defined as a single or combined expression pattern specific in terms of diagnosis, prognosis or prediction of therapeutic response for a medical condition or biological phenotype (Chibon, 2013).

Breast cancer is a heterogeneous disease with highly variable disease outcomes. Understanding the heterogeneity provides significant insight needed to answer underlying questions, such as prediction of progression and treatments to which the tumors will respond (Viale, 2012). Prior to the late 90s, histological and immunohistochemical analysis which failed to accurately classify breast tumors according to their clinical behaviours, were the only techniques available to answer these questions (McGuire, 1991). Two methods, which transformed our understanding of cancer biology, were birthed in 1995. Firstly, a high-throughput method for global gene expression analysis that allows for the quantification and analysis of many transcripts, described as the Serial Analysis of Gene Expression (SAGE) (Hogh and Nielsen, 2008). Secondly, a technique able to quantify the transcriptional programme of samples by analysing the comparative hybridisation of two cDNAs, now described as microarray (Schena, Shalon and Davis, 1995).

The development of the DNA microarray became the game changer in cancer genetics over the last 20 years. In 2002, Veer et al. identified a gene expression signature that could foretell distant metastases in breast tumors, presenting the identification of what was later termed a

‘prognostic expression signature’ (Veer *et al.*, 2002). From this point forward, there was a gain in momentum in this field of investigation, to the point where predictive signatures which identified the likelihood of a patient to respond to a specific drug were developed (Fig. 1).

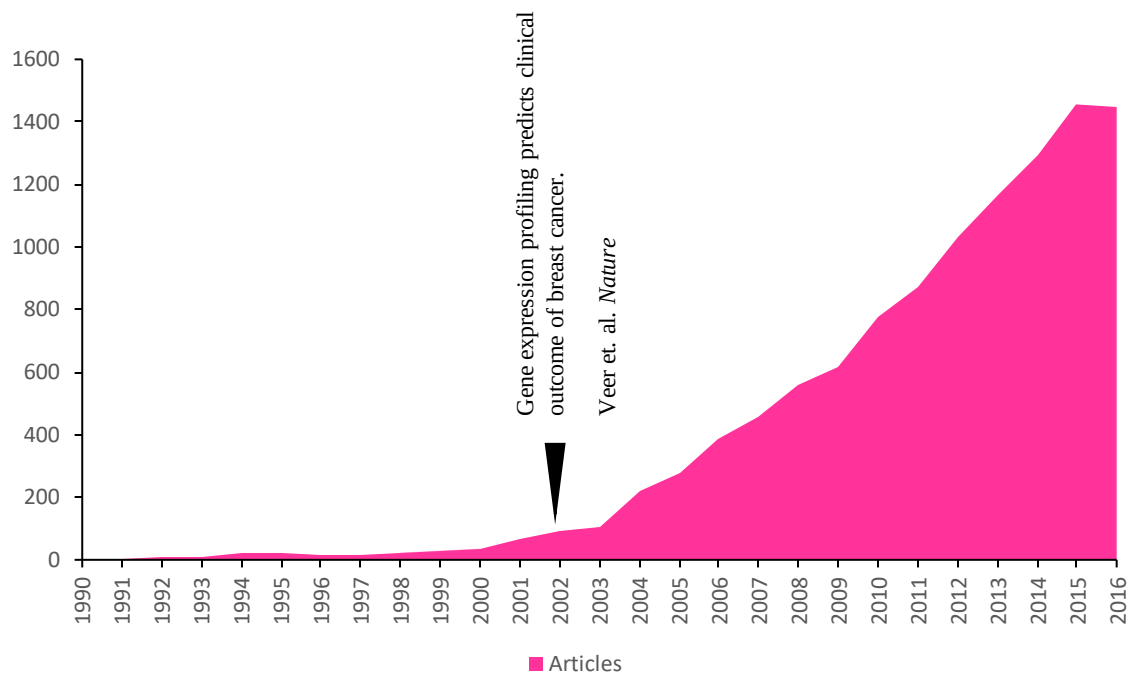


Figure 1. PubMed articles on gene signatures per year

PubMed articles on gene signatures per year, with number of articles on the Y-axis and the year published on the X-axis. Based on (Chibon, 2013).

1.1.1 Prognostic signatures

The NCBI dictionary of cancer defines a prognostic factor as “a characteristic of a patient, that can be used to estimate the chance of recovery from a disease or the chance of the disease recurring”. Thus, these factors are useful in selecting patients who are potential candidates for systemic treatments. For a factor to be prognostic, it should correlate with outcomes, be accurate

and reproducible in an independent group of patients and have a prognostic value different from other standard factors in a multivariate analysis (Chibon, 2013). Based on these criteria, several prognostic signatures have since been developed for breast cancers (Perou *et al.*, 2000; Sørli *et al.*, 2001; Veer *et al.*, 2002).

1.1.2 Predictive signature

A predictive factor is defined as “a clinical or biologic characteristic that provides information on the likely benefit from treatment, independently of prognosis” (Italiano, 2011). Thus, these factors are useful in identifying sub-populations of patients that can benefit from a specific therapy. However, predictive signatures are a bit more challenging to develop for several reasons. One of such reasons is the dilemma of how a response should be defined; should it be clinical, biological or histological? Furthermore, the prediction of response to a drug may be specific to that one drug, meaning that more than one signature may exist per drug (Italiano, 2011; Chibon, 2013). Despite this, a predictive signature for the response of breast tumors to Tamoxifen was developed (Xiao-Jun *et al.*, 2004; Jansen *et al.*, 2007).

1.2 The wound response signature

The analysis of the transcriptional response of fibroblasts to serum led to the discovery that many of the serum-induced genes are involved in wound healing and subsequently regulate cell cycle progression, hemostasis, inflammation, angiogenesis and epithelial cell migration (Iyer *et al.*, 1999). Furthermore, since tumor stroma has been linked to the dysregulation of wound healing (Flier, Underhill and Dvorak, 1986), this “wound-response signature” and a subset of these induced genes (the “core serum response”) were found to predict cancer progression and cancer survival in

breast cancer (Chang *et al.*, 2004). A decision tree algorithm that included the "70-gene poor prognosis" and "wound-response" signatures was informative in making clinical decisions (Chang *et al.*, 2005). Moreover, this signature gained recognition from the medical community after validation, triggering the development of a commercialized prognostic test known as MammaPrint (Arranz *et al.*, 2012; Kittaneh and Montero, 2013). Subsequently, other commercially available prognostic tools, such as the Oncotype DX and PAM50 were developed (Kittaneh and Montero, 2013).

1.3 Breast cancer

Breast cancer is the most frequently diagnosed cancer in women, with an estimated 1.68 million new cases per year worldwide (Torre *et al.*, 2015). It is the second leading cause of cancer mortality in women in both developed and developing countries. In Canada, it is estimated that seventy women will be diagnosed with breast cancer every day and thirteen women will die from the disease daily (Canadian Cancer Society 2017).

Clinically, breast tumors are classified based on their endocrine status; either as estrogen receptor positive or negative (ER⁺/ER⁻), progesterone receptor positive or negative (PR⁺/PR⁻), or as human epidermal growth factor receptor 2 positive or negative (HER2⁺/HER2⁻) (Kittaneh and Montero, 2013). The invention of gene expression profiling led to the discovery that breast cancer has at least seven intrinsic biologic subtypes (Sørlie *et al.*, 2001; Kittaneh and Montero, 2013). They include luminal A, luminal B, luminal C, HER2-enriched, claudin-low, normal breast-like and basal-like (Carey *et al.*, 2006; Kittaneh and Montero, 2013).

The basal-like breast tumors are habitually ER⁻, PR⁻ and HER2⁻, and are termed ‘triple-negative’. Nevertheless, triple-negative breast cancers and basal-like breast tumors are not identical. In fact, 30% of triple-negative breast tumors are not basal-like. Furthermore, triple-negative breast tumors represent a more heterogeneous group, than basal-like tumors do (Carey *et al.*, 2006; Kittaneh and Montero, 2013).

1.4 Triple-negative breast cancer

Triple-negative breast cancers (TNBC) are aggressive breast tumors that lack the expression of ER, PR and amplifications of HER2 (Mayer *et al.*, 2014; Lehmann and Pietersen, 2015; Bianchini *et al.*, 2016). They account for about 10-20% of all breast tumors, but are responsible for a disproportionate amount of mortality. TNBCs affect younger patients and are more frequently observed in BRCA1 mutant carriers. There is a higher prevalence of TNBC in women from African and Hispanic descent, as well as women in lower socioeconomic groups (Bianchini *et al.*, 2016). Due to their heterogeneity, there is a lack of recognized molecular targets for therapy and cytotoxic agents which are efficacious remain the standard treatment (Lehmann *et al.*, 2011).

1.4.1 Subtypes of TNBC

Using gene expression analyses, six distinct TNBC subtypes each displaying a unique biology were identified (Lehmann *et al.*, 2011). They include: basal like, immunomodulatory, mesenchymal, mesenchymal stem like, and a luminal androgen receptor subtype. The basal like subtypes are rich in gene ontologies enriched in cell cycle and cell division components and pathways. The immunomodulatory subtype is enriched for gene ontologies in immune cell

processes, such as B cell receptor pathway and cytokine signaling. Gene ontologies enriched in components and pathways involved in cell motility and cell differentiation pathways are representative of the mesenchymal and mesenchymal stem like subtype. However, mesenchymal stem like subtype expresses low levels of proliferation genes. The luminal androgen receptor subtype is composed of androgen receptor (Lehmann *et al.*, 2011; Mayer *et al.*, 2014). These classifications demonstrate the heterogeneity of TNBCs and provides further evidence, that it is inappropriate to treat all TNBCs in a similar fashion. In addition, TNBC subtyping made it possible to predict the best suitable treatment for each subtype. For instance, cell proliferation and DNA damage response are highly expressed in the basal like subtypes, suggesting patients with these basal-like tumors would benefit from agents that preferentially target highly proliferative tumors (Linn and Van 't Veer, 2009).

1.4.2 Treatment of TNBC

The lack of ER, PR and HER2 make it impossible for patients with TNBC to benefit from current viable targeted therapy, such as endocrine or anti-HER2 agents. Consequently, the only option available for TNBC patients is chemotherapy with cytotoxic agents. Currently, chemotherapy such as taxanes, platinum agents, anthracyclines and ixabepilone, as well as selected biologic agents and possibly anti-EGFR drugs are used to treat patients with TNBC (Bianchini *et al.*, 2016).

TNBCs have demonstrated a high response rate to regimens based on anthracyclines or taxanes, such as the taxane-fluorouracil-doxorubicin-cyclophosphamide (T-FAC) regimen or doxorubicin-cyclophosphamide-taxane (Oakman, Viale and Di, 2010). Platinum salts, such as cisplatin, result in DNA intra-strand crosslink and ultimately double strand breaks, replication

lesions and apoptosis in cells unable to repair the lesions (Bosch *et al.*, 2010; Oakman, Viale and Di, 2010; Rakha and Chan, 2011). Clinical and preclinical studies provide evidence of TNBC sensitivity to DNA damage causing agents compared with other subtypes of breast tumors (Kennedy *et al.*, 2004; Rakha and Chan, 2011). Nonetheless, data are still limited and randomized data comparing platinum compound to standard regimen for breast tumors in terms of efficacy and safety are lacking (Bianchini *et al.*, 2016). Seventy-five percent of BRCA1 mutant carriers have TNBCs (Jin *et al.*, 2017). Thus, the use of poly(ADP-ribose) polymerases inhibitors, such as Olaparib is favorable in such tumors. Olaparib resulted in anti-tumor activity in patients with BRCA mutation-associated cancer. Additionally, TNBC patients treated with a new poly(ADP-ribose) polymerases inhibitor BS-201, had a longer overall survival rate compared with women who received chemotherapy alone (Bianchini *et al.*, 2016). Epidermal growth factor receptor (EGFR) overexpression has been habitually seen in TNBCs and has been linked to poor prognosis (De Laurentiis *et al.*, 2010; Bianchini *et al.*, 2016). However, EGFR expression is not a decisive marker of response to EGFR inhibitors, and EGFR status is not yet routinely assayed in breast cancer. In addition, phase II trials demonstrated that no statistically significant improvements were observed in patient outcomes when EGFR inhibitor cetuximab was administered (Bosch *et al.*, 2010; De Laurentiis *et al.*, 2010; Bianchini *et al.*, 2016).

1.5 TNF α -induced transcriptional program in breast cancer cells

1.5.1 TNF α and its receptors

Tumor necrosis factor alpha (TNF α) is a pleiotropic proinflammatory cytokine which is essential to immune protection and homeostasis, but is also involved in tumorigenesis, metastasis, chemoresistance and radioresistance (Aggarwal, 2003). It can signal as a membrane-integrated

protein and as a soluble cytokine released after proteolytic cleavage (Balkwill, 2006). Two TNF receptors exist in the mammalian system; TNFRI, found on most cells in the body, and TNFRII, primarily expressed on haemopoietic cells. TNFRI is activated by soluble ligand, while TNFRII is activated by the membrane-integrated form. The activation of TNF receptors results in the recruitment of intracellular adaptor proteins and downstream activation of various signal transduction pathways (Locksley, Killeen and Lenardo, 2001; Hehlhans and Pfeffer, 2005). Depending on the cellular context, TNFRI activation can lead to two different outcomes. However, the default signaling pathway is in induction of genes involved in inflammation and cell survival. Ligands that bind TNFRI induce inflammatory mediators, such as interleukin 6 (IL6) and growth factors via activation of the AP-1 transcription factors or IK β kinases that, in turn, activate NF- κ B (Balkwill, 2006).

1.5.2 The TNF α link between inflammation and cancer

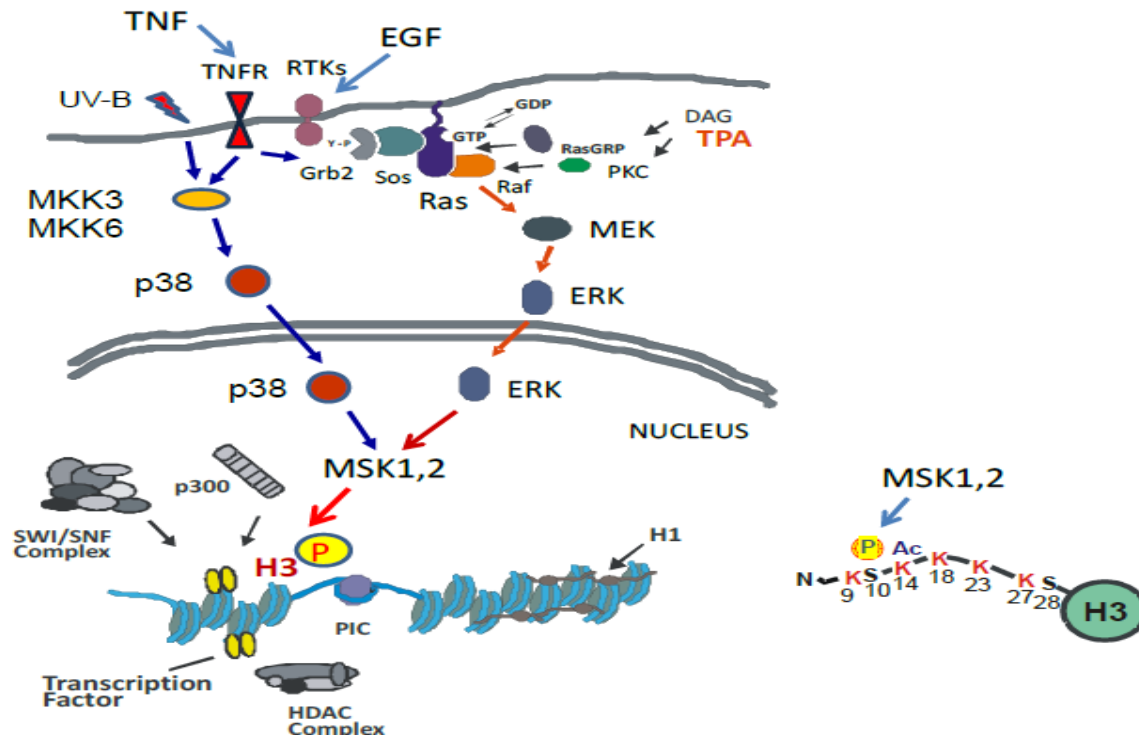
Several lines of evidence support the link between inflammation and cancer. Firstly, many chronic inflammatory diseases have been linked to increased risk of cancer (Coussens and Werb, 2002; Dethlefsen, Højfeldt and Hojman, 2013). Secondly, cancers arise at sites of chronic inflammation (Viatour *et al.*, 2005; Balkwill, 2006; Simpson and Brown, 2013). Finally, chemical mediators of inflammation are found in various cancers and the deletion of these mediators inhibits the cancer spreading (Balkwill, 2006). TNF α is a key chemical mediator of inflammation found in many cancers. It is true that TNF α administered loco-regionally, as demonstrated by William Coley, can cause hemorrhagic necrosis by destroying the tumor vessels (Lejeune, 2002; Aggarwal, 2003). Nonetheless, when produced by the tumor cells and the tumor niche, it acts as an endogenous tumor promoter. In fact, there is substantial evidence that TNF α is involved in

promotion and progression of various cancers, with pathways leading to activation of the NF- κ B and AP-1 transcription factor complexes resulting in the expression of gene linked to the phenotypic events of carcinogenesis (Balkwill, 2006; Ndlovu *et al.*, 2009)

1.5.3 TNF α -induced transcriptional program in breast cancer cells

As mentioned previously, TNF α binds to TNF receptors and stimulates multiple signaling pathways, leading to the expression of pro-cancerous inflammatory and angiogenic chemokines and cytokines, growth factors and matrix metalloproteinases, inducing cell survival, remodeling (epithelial-to-mesenchymal transition), motility and invasion (Aggarwal, 2003). Specifically, TNF α activates the mitogen- and stress-activated protein kinases (MSK 1 and 2) via the extracellular-signal-regulated kinase (ERK) 1/2 and p38 (stress-activated protein kinase 2) mitogen activated protein kinase (MAPK) pathway, resulting in the phosphorylation of the histone H3 at serine 10 or 28 (the nucleosomal response) at the regulatory region of MSK targeted genes (e.g. *IL6*) (Fig. 2) (Vermeulen *et al.*, 2003; Ananieva *et al.*, 2008; Ndlovu *et al.*, 2009).

The nucleosomal response plays a role in the activation of the earliest induced genes termed “immediate early genes”, several of which code for transcription factors. The steady state of these transcription factors is elevated in triple-negative breast cancer cells, which contributes to the gene expression profile of this breast cancer cell type (Ndlovu *et al.*, 2009). TNF α also stimulates the NF- κ B pathway, resulting in the MSK-mediated phosphorylation of serine 276 of the p65 subunit enabling transcriptional transactivation (Vermeulen *et al.*, 2009). NF- κ B is a transcription factor which has a crucial role in inflammation, cell proliferation and apoptosis, and is one of the MSK recruiters at gene regulatory regions upon TNF α stimulation in MDA-MB-231 breast cancer cell line (Vermeulen *et al.*, 2009).



Immediate early genes: *FOS*, *JUN*, *FOSL1*, *IL6*

Figure 2. MSK 1/2, the nucleosomal response and immediate early gene expression.

Stimulation of the RAS-MAPK and stress kinase pathways results in the activation of MSK, which phosphorylates histone H3 (at serine 10 and/or 28). Following H3 phosphorylation, there is recruitment of chromatin remodelers (SWI/SNF) and lysine acetyltransferases (KATs) (eg., p300) to the promoter region, which remodel chromatin in preparation for transcription. The immediate early genes such as *FOS/JUN*, as well as TNF responsive gene *IL6* are transcribed in response to the RAS-MAPK pathway for growth and proliferation.

1.6 Histone post translational modifications

Genetic information in the nucleus of cells is organized into a high order structure called chromatin. Chromatin can be categorized into heterochromatin which is densely compacted, and transcriptionally inactive (Trojer and Reinberg, 2007), and euchromatin which is de-condensed

and transcriptionally active (Jenuwein Thomas and Allis C. David, 2001). Different chromatin states dictate biological responses at a cellular level. Thus, variations in chromatin states via the remodeling of chromatin and/or histone modifications is crucial in eukaryotic function.

Histones are a family of evolutionary conserved basic proteins that aid the compaction of DNA. They are made up of the core histones (H2A, H2B, H3 and H4) and the linker histone (H1). A nucleosome, which is the fundamental unit of chromatin consists of eukaryotic DNA wrapped around a histone octamer (Davie and Spencer, 2001; Healy *et al.*, 2012; Thompson *et al.*, 2013). The linker histone H1, ensures the overall stability of the chromatin by binding the linker DNA region between nucleosomes, to form higher order structures. Structural analysis revealed that their N-terminal tails protrudes outwards from the nucleosome, enabling them to interact with other nucleosomes and regulatory factors (Karolin Luger, Armin W. Mader, Robin K. Richmond 1997).

Histones play vital roles in various biological processes, including gene expression and regulation, cell cycle regulation, chromosome segregation and DNA repair (Lee, Smith and Shilatifard, 2010). Their ability to regulate chromatin dynamics, stems from the numerous post-translational modifications (PTM) they undergo, carried out by various modifying enzymes (Fig. 3). These include: methylation, acetylation, phosphorylation, citrullination, ubiquitination, SUMOylation and poly (ADP) ribosylation (Lee, Smith and Shilatifard, 2010; Bannister and Kouzarides, 2011).

The balance of the enzymatic proteins that add (“writers”) or remove (“erasers”) specific histone PTMs regulate the distribution of a given histone PTM. Furthermore, the availability of substrates and protein complexes that interpret (“readers”) the PTMs to elicit specific outcomes also influence the overall abundance of the PTM (Thompson *et al.*, 2013).

The location and type of the histone PTM may or may not dictate the chromatin state

(Jenuwein Thomas and Allis C. David, 2001). For instance, a chromatin region enriched with acetylated lysine 9 on histone H3 (H3K9ac) can result in decondensation of chromatin and transcriptional activation by disrupting the high order chromatin structure (Berger, 2007). Alternatively, Histone H3 phosphorylated at serine 10 was shown to have no effect on the disruption of the high order chromatin structure, even though it is associated with transcriptional activation (Fry, Shogren-Knaak and Peterson, 2004). This would suggest that phosphorylated H3 at serine 10 has an important role in attracting effectors or readers that ultimately allows for transcriptional activation. The ‘histone code’ defined by investigators as the different combinations of histone PTMs, result in distinct regulatory responses and an increase in complexity of signals (Jenuwein Thomas and Allis C. David, 2001). Likewise, the modification of one residue can alter the modification of a neighboring residue, thus dictating its function (Lee, Smith and Shilatifard, 2010). For example, phosphorylation of serine 10 on histone H3 stimulates the ability of the lysine acetyltransferase (Gcn5) to acetylate histone H3 at lysine 14 (Cheung *et al.*, 2000)

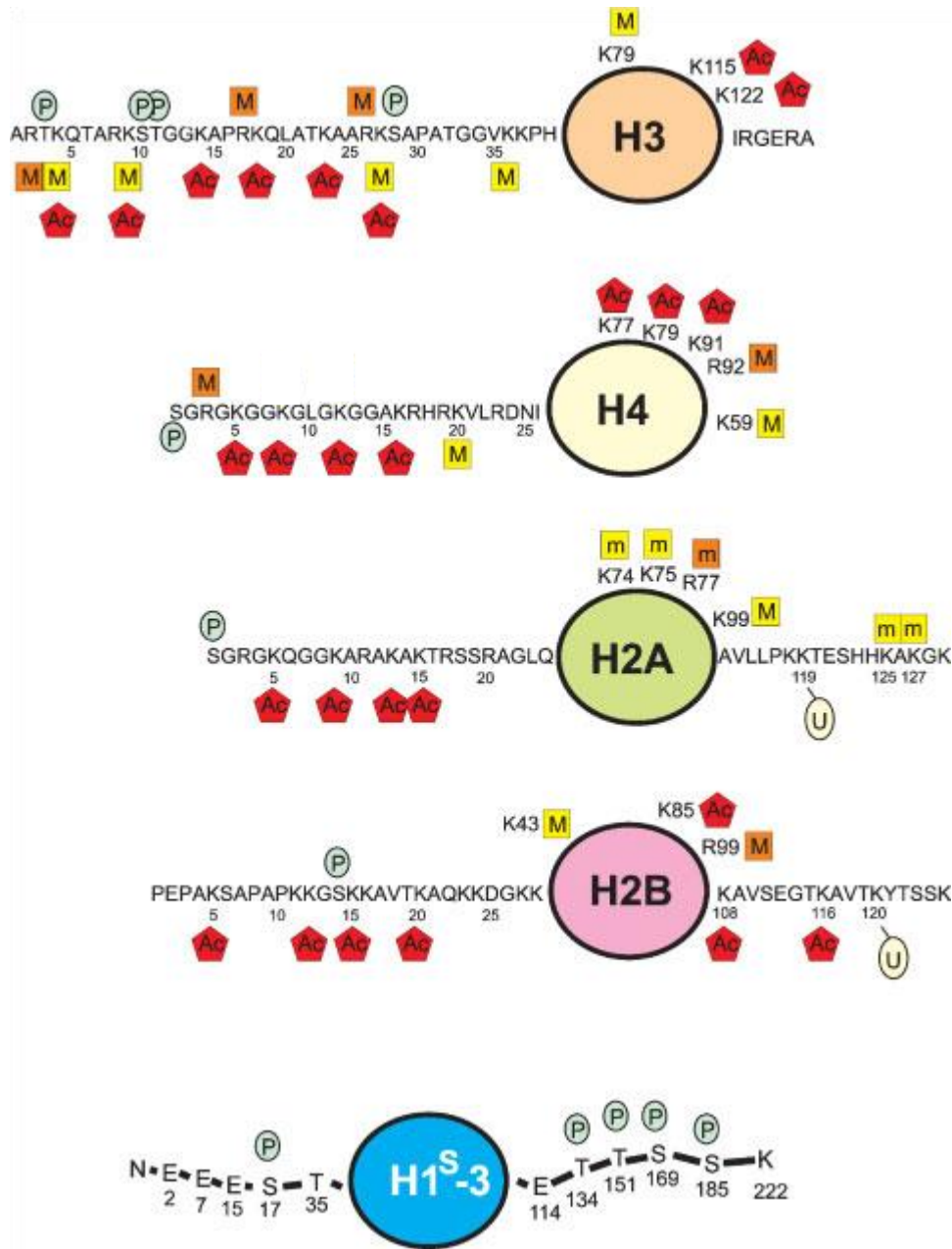


Figure 3. Human core histone amino acid sequences

Histone H1^{S-3} is a mouse H1 subtype. The modifications include methylation (M), acetylation (Ac), phosphorylation (P), ubiquitination (U). Methylation sites that are uncertain are denoted as (m). The figure and text were reproduced with permission from *Journal of Cellular Biochemistry*.

1.6.1 Histone methylation

Using biochemical analyses, methylation of the N-terminal lysine residues was discovered (Woon Ki Paik and Sangduk Kim, 1971). However, the significance of histone methylation in transcriptional regulation was not fully understood until recent years. Although histone lysine methylation is widely studied, histones can be methylated on lysine and arginine residues (Fig. 3). Lysine residues can be mono (me1), di (me2) or tri (me3) methylated, while arginine residues are mono- or di-methylated in a symmetrical or asymmetrical conformation (Bannister and Kouzarides, 2011; Jahan and Davie, 2015). Lysine methylation occurs on the ϵ - nitrogen lysine residues by SET (Su(var) 3-9, Enhancer of zeste (E[z]) and trx-G Trithorax) domain and non-SET domain containing lysine methyltransferases (KMT) (Schneider and Shilatifard, 2006). Lysine methylation marks are recognized by the royal family of domains, consisting of chromodomains and tudor domains (Berger, 2007). Histone lysine methylation is a reversible process, performed by two classes of lysine demethylase; LSD1/BHC110, which removes mono and di-methylation marks of histone H3 at lysine 4 (H3K4me/H3K4me2) and JmjC (jumonji class), which removes di- and tri-methylation marks of histone H3 at lysine 4, 9 and 36 (H3K4me2, H3K4me3, H3K9me2, H3K9me3, H3K36me2 and H3K36me3) (Klose and Zhang, 2007). Furthermore, methylation of histone H3 at lysine 4, 36 and 79 is correlated with euchromatin and transcriptional activation, whereas methylation of histone H3 at lysine 9 and 27 and histone H4 at lysine 20, is associated with heterochromatin and transcriptional repression (Lee *et al.*, 2005; Schneider and Shilatifard, 2006; Berger, 2007). Studies by Pavri *et al.* revealed that H3K4 methylation did not directly affect transcription, rather it recruited additional components required for downstream effects (Pavri *et al.*, 2006). Histone arginine methylation is more complex than lysine methylation, as it can be methylated symmetrically or asymmetrically. Protein arginine methyltransferases

(PRMT) catalyze arginine methylation on histone H2A, H3 and H4. Depending on the symmetry, histone arginine methylation can be associated with transcriptional activation or repression (Jahan and Davie, 2015). Unlike histone lysine methylation, not all arginine methylation is reversible. Peptidyl arginine deaminase 4 and jumonji domain-containing protein 6 catalyze arginine demethylation, however various studies have shown that they are not capable of removing methylation from all methylated arginine sites (Jahan and Davie, 2015).

1.6.2 Histone acetylation

Lysine residues on core histones are reversibly acetylated. Lysine acetyltransferases (KATs), which catalyze acetylation, and histone deacetylases (HDACs), which deacetylate, histones maintain the steady state levels of histone acetylation in the nucleus (Kornberg and Lorch, 1999). The KATs consist of KAT2A/KAT2B (Gcn5- related N-acetyltransferases (GNAT)), KAT3A/KAT3B (cAMP- responsive element binding protein-binding protein (CBP) / E1A-associated protein of 300 kDa (CBP/p300)), and the KAT5/KAT6A/KAT6B/KAT7/KAT8 (MYST) protein families (Lee and Workman, 2007). The GNAT superfamily is the largest group of KATs, which includes KAT2A (Gcn5), KAT2B (PCAF) and ELP3 (Roth *et al.*, 2001). GCN5 has been shown to mediate acetylation at lysine 9 and 14 on histone H3 and CBP/p300 has been shown to mediate acetylation at lysine 18, 23 and 27 of histone H3 (Jin *et al.*, 2011). Histone H3 is acetylated at lysine 9, 14, 18, 23 and 27 while histone H4 is acetylated at lysine 5, 8, 12 and 16. Histone acetylation disrupts the high order chromatin structure, facilitates the solubility of chromatin at physiological strength and maintains the unfolded structure of the transcribed nucleosome; by catalyzing the transfer of an acetyl group to the ϵ -amino group of lysine side chains, thereby neutralizing the lysine's positive charge and subsequently weakening DNA-

histone interactions (Davie and Spencer, 1999). The levels of histone acetylation have long been associated with transcriptional status of many genes. Moreover, it was established over a decade ago that a maximal acetylation of 46% of nucleosomes was sufficient to prevent high order chromatin compaction and facilitate transcription (Davie and Spencer, 1999). Conversely, hypoacetylated histones result in chromatin compaction and gene silencing (Peterson and Laniel, 2004).

1.6.3 Histone phosphorylation

All core histones contain sites that can be phosphorylated by several protein kinases (Fig. 3) and dephosphorylated by phosphatases. Phosphorylation of serine and threonine histone residues play important roles in chromosome condensation/segregation, DNA damage repair, cell cycle progression and transcriptional regulation, depending on the histone tail phosphorylated (Rossetto, Avvakumov and Côté, 2012). For example, serine 139 phosphorylation on the histone variant H2AX plays a major role in DNA damage repair. Furthermore, phosphorylation of H2A and H4 are associated with chromosome condensation. Conversely, the phosphorylation of serine 36 on histone H2B results in the transcriptional activation of AMP-activated protein kinase (AMPK) responsive genes. Likewise, the phosphorylation of histone H3 on serine 10, 28 and threonine 28 have been associated with transcriptional activation of numerous genes (Taverna *et al.*, 2007; Rossetto, Avvakumov and Côté, 2012). Additionally, H3 phosphorylation on serine 10 and 28 is closely associated with condensation of mitotic chromosomes in mammalian cells (Healy *et al.*, 2012).

1.6.3.1 Histone H3 phosphorylation

Histone H3 has the most PTMs of all core histones (Espino *et al.*, 2005). It can be phosphorylated at threonine 3 and 11, as well as serine 10 and 28 (Dunn *et al.*, 2005; Espino *et al.*, 2005). However, the two most studied H3 phosphorylation events are the serine 10 and 28 phospho-modifications, present during mitosis and are minimally present in interphase cells. Since the discovery of AMPK, an increasing number of protein kinases have been reported to phosphorylate H3 (Fig. 4). The phosphorylation of histone H3 at serine 10 and 28 (H3S10ph and H3S28ph) is a unique post translational modification mark, associated with highly condensed chromatin in mitosis and de-condensed chromatin during gene activation in interphase cells (Pérez-Cadahía *et al.*, 2011; Sawicka and Seiser, 2012). Mitotic H3 phosphorylation is fundamental to the formation of the higher-order chromatin structure during cell division (Prigent and Dimitrov, 2003; D'Anello *et al.*, 2010). On the other hand, interphasic phosphorylation is crucial for remodeling the chromatin at the upstream promoter region of a subset of genes, thus initiating transcription (Drobic *et al.*, 2010). This gene specific association restricts the genomic distribution of interphasic H3 phosphorylation to a small fraction of nucleosomes (Sawicka and Seiser, 2012).

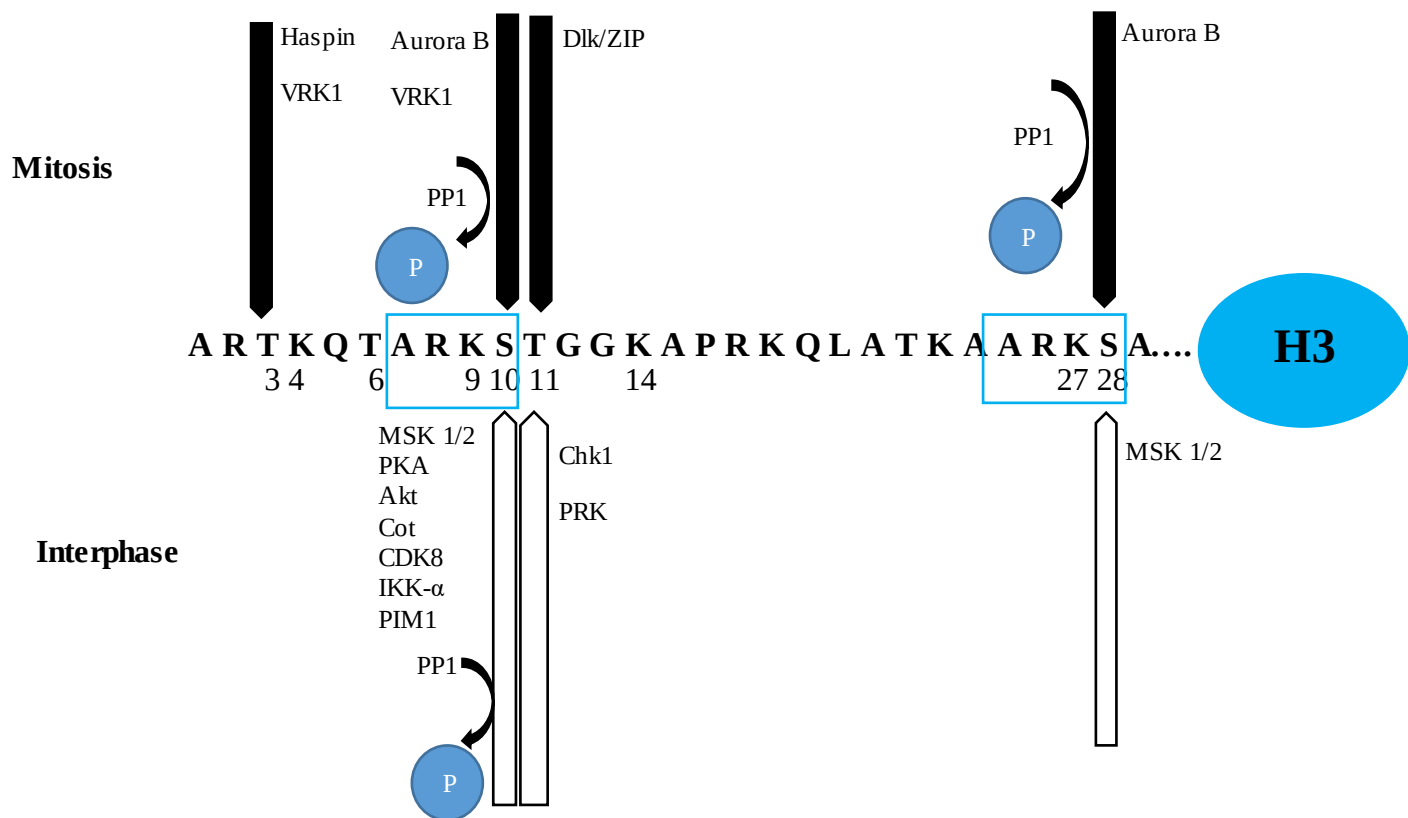


Figure 4. Kinases and phosphatases responsible for H3 N-terminal tail phosphorylation and dephosphorylation during mitosis and interphase.

The ARKS modification boxes are highlighted by a blue frame. Based on (Pérez-Cadahía, Drohic and Davie, 2009; Sawicka and Seiser, 2012).

1.6.3.2 Phosphorylation of histone H3 at serine 10 and 28 during mitosis

Phosphorylation of histone H3 across the different stages of mitosis is conserved in eukaryotes and numerous studies have revealed that this PTM abundantly covers condensed

chromosomes (Sawicka and Seiser, 2012). The importance of H3 phosphorylation in chromosome condensation have been demonstrated. Prior to mitosis, drugs that induced H3 phosphorylation resulted in chromosome condensation (Prigent and Dimitrov, 2003). Likewise, *Drosophila* studies demonstrated the attenuation of H3S10ph which resulted to partially condensed chromosomes during mitosis (Giet & Glover, 2001). Additionally, H3S10A mutant strain in *Tetrahymena thermophile* protozoan showed aberrant chromosome segregation and loss during mitosis (Prigent and Dimitrov, 2003). H3S10ph and H3S28ph are part of the identical sequence motif-ARKS- (Fig. 4) and show only slight differences in their modification patterns during mitosis. In late G2 phase, H3S10ph occurs in pericentromeric heterochromatin and as mitosis proceeds, H3S10ph is completed at anaphase. In contrast, dephosphorylation by protein phosphatase 1 (PP1) begins in late anaphase and is completed in early telophase (Hendzel *et al.*, 1997). Likewise, H3S28ph first appears during prophase with a similar distribution to H3S10ph. Nevertheless, there is evidence of varying levels of the two PTMs on the histone tails. Aurora B kinase is responsible for both PTMs and additionally vaccinia-related kinase-1 (VRK1) was discovered to mediate H3S10ph (Pérez-Cadahía, Drohic and Davie, 2009).

1.6.3.3 Phosphorylation of histone H3 at serine 10 and 28 during interphase

A small portion of H3 can also be phosphorylated in interphasic cycling cells on serine 10 and 28. These phosphorylation events have been linked with gene expression (Davie and Spencer, 1999; Thomson *et al.*, 1999; Dunn and Davie, 2005; Espino *et al.*, 2005). Studies in PC12 cells were among the first to demonstrate that H3 could be a substrate for signaling kinase (Halegoua and Patrick, 1980). In 1991, Mahadevan *et al.* showed that the stimulation of quiescent mouse fibroblast cells with growth factors and mitogens resulted in the phosphorylation of H3 at multiple

serine sites, and ultimately the induction of immediate early genes (Mahadevan, Willis and Barratt, 1991). This phosphorylated H3 was also discovered to be hyperacetylated. The correlation between immediate early gene expression and H3 phosphorylation led to a consensus that these two events are mechanistically linked and was termed the 'nucleosomal response'. Furthermore, chromatin immunoprecipitation (ChIP) assays revealed the presence of phosphorylated H3 at immediate early genes' genomic regions (Drobic *et al.*, 2010; Khan *et al.*, 2013). Moreover, these modifications were observed at the upstream promoter region and not within the coding region of the gene, indicating that they strictly play a role in the regulation of transcription activation and/or initiation.

The functional role of these PTMs at the upstream promoter region, involves a family chromatin binding proteins, known as the 14-3-3 family (Macdonald *et al.*, 2005; Winter *et al.*, 2008; Drobic *et al.*, 2010). This family consists of seven members and specific isoforms have been shown to bind H3S10ph and H3S28ph (Winter *et al.*, 2008). The 14-3-3 ϵ/ξ protein isoforms have a binding affinity for H3S28ph above H3S10ph, and the binding affinity for H3S10ph is increased if lysine 14 or 9 is acetylated (Macdonald *et al.*, 2005; Winter *et al.*, 2008). Thus, the binding affinity for 14-3-3 ϵ/ξ to phosphorylated H3 is, H3S28ph > H3S10ph/K14ac > H3S10ph/K9ac > H3S10ph. The 14-3-3 ϵ/ξ are recruited to promoters of *Jun* and *Fos* with an increase in H3S10ph/K14ac (Macdonald *et al.*, 2005). Subsequently, 14-3-3 ϵ/ξ have been identified as 'readers' of H3S10ph and H3S28ph, that are crucial to the transcriptional activation of immediate early genes. However, the mode of action of these proteins are still being investigated (Rosetto, Avvakumov and Côté, 2012).

1.7 H3S10ph and H3S28ph localization and correlation with H3 acetylation

H3S10ph and H3S28ph in interphasic cells regulate gene expression. However, it was unknown if H3S10ph and H3S28ph differentially affect transcriptional regulation. The differential levels of H3S10ph and H3S28ph in various cell lines, suggests that these phosphorylation marks may have separate roles in chromatin regulation (Steven Z. Josefowicz *et al.*, 2016). H3S28ph but not H3S10ph was shown to make chromatin more permissive to the rate-limiting steps in CBP/p300-dependent transcription (Steven Z. Josefowicz *et al.*, 2016). Conversely, 12-Otetradecanoylphorbol-13-acetate (TPA)-stimulation of MCF 7 cells resulted in the induction of H3S10ph and not H3S28ph. In BxPC-3, Hs766T and Panc-1 pancreatic cells only H3S10ph was induced, while both H3S10ph and H3S28ph were induced in 10T1/2, CCD-1070Sk and Hela cells (Espino *et al.* 2009). In contrast, in HEK 293T cells, H3S28ph but not H3S10ph is associated with the activation of the polycomb-silenced genes (Lau and Cheung, 2011). It is possible that the inducer used in these experiments determines what phosphorylation events will occur. Additionally, factors other than MSK may be responsible for the observed results

Immunocytochemistry studies examining the localization H3S10ph and H3S28ph within the nuclei uncovered that these phosphorylation events are independent and have distinct sub nuclear sites (Fig. 5) (Dunn and Davie, 2005; Dyson *et al.*, 2005; Khan *et al.*, 2017). Most recently, analysis of the nucleosomal response at a single cell level in human primary fibroblast by our group, shows that mitogen-induced H3S10ph and H3S28ph occur in separate pools (Khan *et al.*, 2017). This study, using DNA immuno-FISH, demonstrated that MSK 1/2 phosphorylates H3S10 on one allele and H3S28 on another allele in mouse and human fibroblast cells, thus accounting for the observed absence of H3S10ph and H3S28ph co-localization (Khan *et al.*, 2017). Dyson *et*

al. showed that H3S10 and S28ph phospho-epitopes appeared at the same time on H3 tails in anisomycin induced 10T1/2 cells, suggesting that they occur on the same H3 tail. By testing immunodepleted crosslinked chromatin fragments of one phospho-epitope and asking whether the second phospho-epitope was also co-depleted, they demonstrated that the two phospho-epitopes are not located on the same H3 tail *in vivo* (Dyson *et al.*, 2005). Furthermore, H3S10ph and H3S28ph did not co-localize following TPA stimulation in Cirs-3 cells, suggesting that these PTMs do not occur on the same H3 tail (Dunn and Davie, 2005).

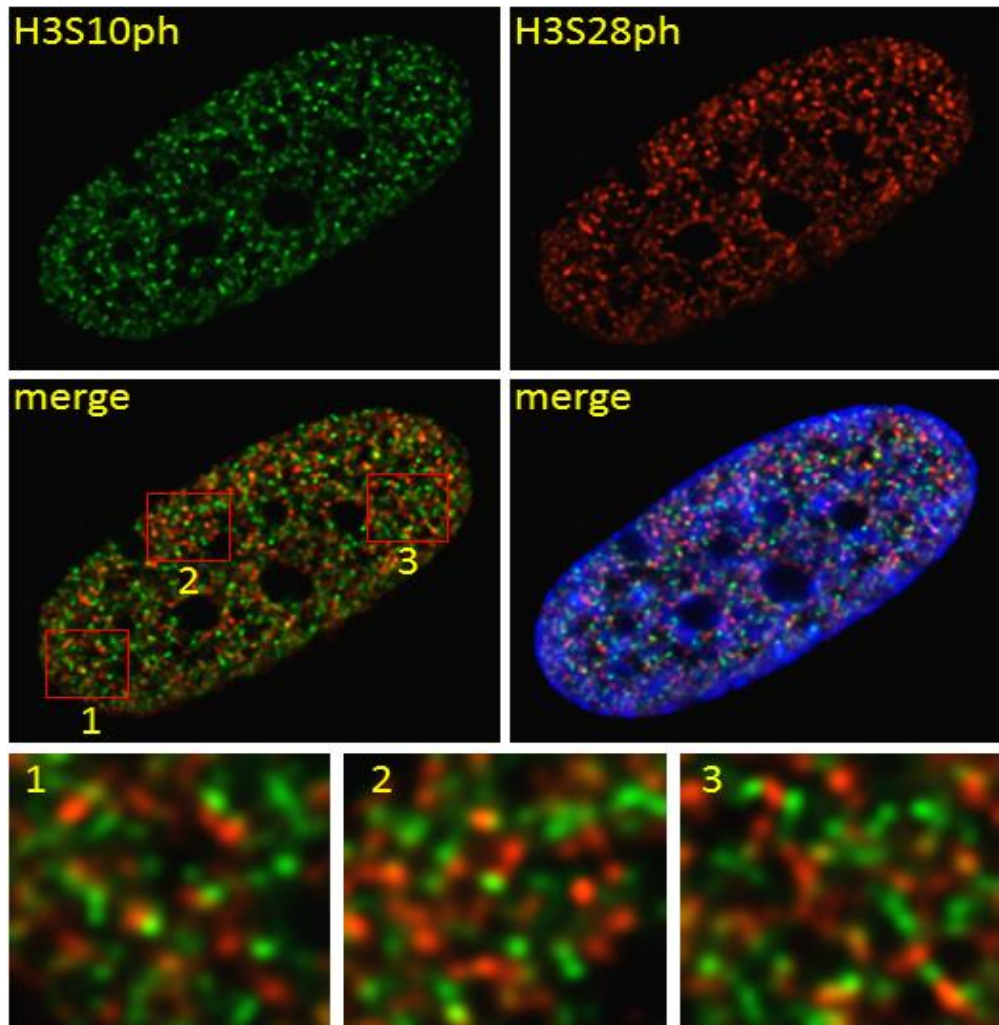


Figure 5. Relative intranuclear localization of H3S10ph and H3S28ph in TPA-treated human primary fibroblasts.

Serum-starved CCD-1070Sk human fibroblast cells treated with 100 nM TPA for 60 min were subjected to immunofluorescent staining using anti-H3S10ph (green) and anti-H3S28ph (red) antibodies, co-stained with DAPI (blue). Cells were digitally imaged. Boxed areas of merged image are shown enlarged in insets. The figure and text were reproduced with permission from *Molecular and Cell Biology Journal*.

Acetylation of H3 at lysine 9 or lysine 14 (H3K9ac/H3K14ac) was shown to be present on the same H3 tail as H3S10ph and enriched at active immediate early gene promoters (Clayton *et al.*, 2000). However, H3S10ph does not appear to depend on H3K9ac (Khan *et al.*, 2017). This H3K9/K14 acetylation is mediated by the transcription co-activator GCN5/PCAF (Jin *et al.*, 2011). Likewise, co-immunoprecipitation studies revealed the co-occurrence of H3S28ph with acetylation of H3 at lysine 27 (H3K27ac), and this acetylation is mediated by CBP/p300 (Jin *et al.*, 2011; Khan *et al.*, 2017).

1.8 MSK-induced nucleosomal response.

1.8.1 MSK: Discovery, structure, function and activation

MSK 1 and 2 are ubiquitously expressed enzymes, first described in 1998 as proteins with similar homology to the ribosomal S6 kinase (RSK) isoforms (Deak *et al.*, 1998; Duda, 2012). Encoded by two genes (RPS6KA5 and RPS6KA4), MSK 1 and 2 have two functional kinase domains separated by a conserved linker region, both of which possess serine/threonine kinase activity (Dunn and Davie, 2005). The N-terminal kinase (NTK) domain belongs to the AGC kinase group and the C-terminal kinase (CTK) domain belongs to the CaMK group, named after its calcium-calmodulin-dependent protein kinase family members (Deak *et al.*, 1998). The CTK domain contains the nuclear localization sequence and the MAPK docking and autoinhibition sites, thus it plays a crucial role in MSK regulation (Fig. 6). Inactivating mutations in the CTK terminates MSK function (Deak *et al.*, 1998; Tomás-Zuber *et al.*, 2001). Due to their nuclear localization sequence, MSKs are predominantly located in the nucleus; nevertheless MSK 1 translocates to the cytoplasm in response to glucocorticoids which contributes to the anti-inflammatory action mechanism of glucocorticoids (Beck *et al.*, 2008). Furthermore, MSK 2 was shown to regulate the

localization of ERK1 and p38 MAPK pathways, indicating that MSK enzymes may control the cellular localization of their upstream kinases (Pierrat *et al.*, 1998).

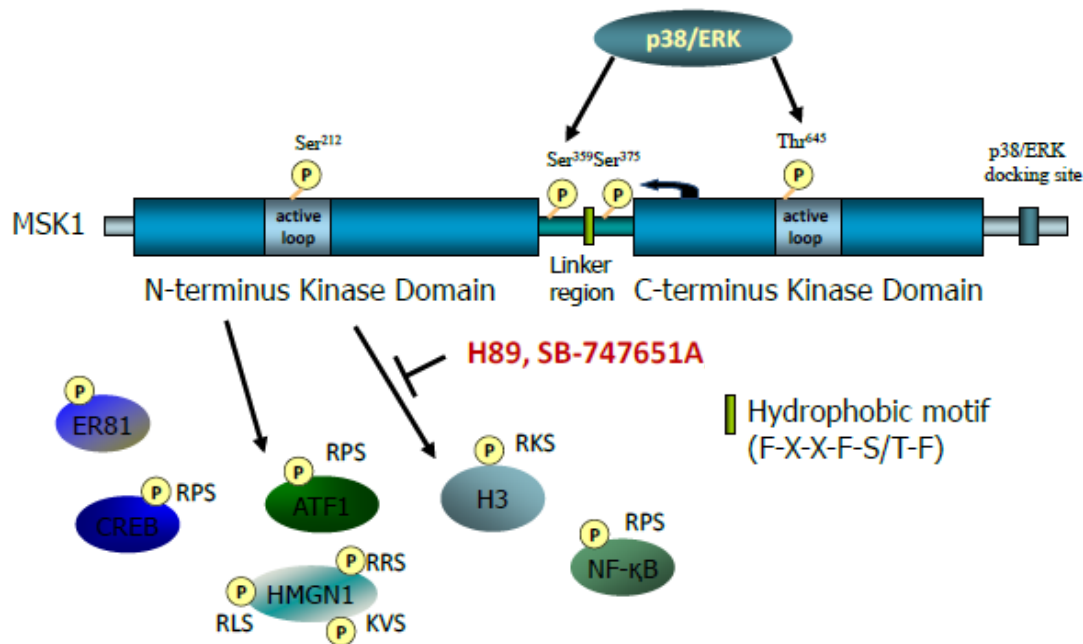


Figure 6. The kinase domains of MSK 1 and MSK 2, with their downstream substrates.

H89 is a potent MSK inhibitor.

Mammalian MSK 1 and MSK 2 share approximately 65% identity in amino acid sequence. Studies in MSK 1/2 double knockout cells, resulted in a consensus that MSK 1 and 2 are functionally redundant (Wiggin *et al.*, 2002; Reyskens and Arthur, 2016). However, there is evidence that they are not functionally redundant. H3 phosphorylation levels upon anisomycin and TPA stimulation was significantly diminished in fibroblasts from MSK 1 and 2 deficient mice. However, ³²P-labelling of these fibroblasts revealed that H3 phosphorylation was completely impaired in MSK 2 and MSK 1/2 double-knockout cells, while phosphorylation was slightly reduced in MSK 1 deficient cells (Sawicka and Seiser, 2012). In contrast, MSK 1 deficient neurons, eliminated brain derived growth factor-induced CREB phosphorylation while no change

was observed in MSK 2 knockdown neurons (Arthur and Cohen, 2000). Data from knockout studies suggest that the individual levels of MSKs plays a crucial role in determining which homolog phosphorylates a given substrate in a particular cell type (Soloaga *et al.*, 2003; Sawicka and Seiser, 2012).

MAPKs are intracellular signal transducers that respond to a plethora of environmental stimuli. Subsequently, they regulate physiological processes by controlling functions of cellular proteins via phosphorylation. MSK 1/2 are activated *in vivo* downstream of the ERK1/2 and p38 MAPK pathways, indicating that they can integrate both mitogenic and stress signals, such as growth factors, neurotransmitters, UV irradiation, oxidative stress and cytokines (Deak *et al.*, 1998; Dunn and Davie, 2005; McCoy *et al.*, 2005). Indeed, the ERK1/2 and p38 inhibitors, PD98059 and SB203580 respectively, blocked MSK 1/2 activation depending on the stimuli used (Dunn and Davie, 2005; Espino *et al.*, 2006). MSKs are regulated by multiple phosphorylation sites. Active ERK1/2 or p38 phosphorylates MSK 1 on two sites, S360 in the linker region, T581 in the activation loop of the CTK and T700 in the autoinhibition site of the CTK (McCoy *et al.*, 2005; Duda, 2012). Phosphorylation of T581 and T700, leads to the autophosphorylation of S376 and -381 in the linker region, as well as S212 in the activation loop of the NTK. Phospho S360, -376, -381 and 212, collectively activates the NTK, which can then phosphorylate exogenous substrates (McCoy *et al.*, 2005; Vermeulen *et al.*, 2009). Upon activation, MSKs efficiently phosphorylate multiple transcription factors and nuclear proteins, thus indicating that MSKs regulates gene expression at multiple levels (Arthur, 2004). The most prominent substrates of MSKs include; p65 subunit of NF- κ B, CREB, ATF1, HMGN1 and histone H3 (Davie, 2003; Arthur, 2004; Dunn and Davie, 2005).

1.8.2 MSK-mediated H3 phosphorylation and immediate early gene expression.

The impact of H3 phosphorylation on gene expression could be explained by the recruitment of factors necessary for transcription, such as chromatin remodelers, co-activators and RNA polymerase II. Sequential co-immunoprecipitation assays and sequential chromatin immunoprecipitation assays on mouse fibroblast 10T1/2 and MSK 1 knockdown 10T1/2 cells showed that H3S10ph/H3S28ph leads to promoter remodeling by recruiting transcription factors, chromatin-remodeling enzymes (BRG1) and RNA polymerase II to target genes (Vicent *et al.*, 2006; Drohic *et al.*, 2010).

The RAS-MAPK signal transduction pathway regulates cellular responses to growth signals and its deregulation by oncogenes (KRAS and BRAF) is predominant in many cancers (Dunn and Davie, 2005). Stimulation by TPA, epidermal growth factor (EGF), serum, and TNF α of mammalian cells results in MSK-mediated phosphorylation of H3 and its variants, at serine 10 and 28 (H3S10ph/H3S28ph), or HMGN1 at serine 6, a phenomenon termed ‘the nucleosomal response’ (Strelkov and Davie, 2002; Soloaga *et al.*, 2003; Dunn and Davie, 2005; Drohic *et al.*, 2010). MSK-mediated H3 phosphorylation elicits rapid expression of immediate-early genes many of which code for transcription factors with major roles in oncogenesis (Drohic *et al.*, 2010). Chromatin immunoprecipitation assays have revealed that the regulatory regions of immediate early genes are associated with H3S10ph and H3S28ph (Drohic *et al.*, 2010; Steven Z. Josefowicz *et al.*, 2016). Studies with MSK 1/2 knockout cells abolish H3S10ph and H3S28ph in response to TPA (Soloaga *et al.*, 2003). While MSK knockout mice are viable, these rodents were observed to have enhanced inflammation and impairments in stress-related learning and memory processes (Chandramohan *et al.*, 2008; Naqvi *et al.*, 2012). Remarkably, a deficiency in MSK 1 expression was used to mimic Huntington’s chorea in a mouse model, due to the lack of induced H3S10ph

and a down regulation of *Fos* transcription in the striatum (Roze *et al.*, 2008). The stimulation of wild-type mouse fibroblasts with anisomycin resulted in a transient increase of *c-fos* and *c-jun* mRNA (Soloaga *et al.*, 2003). Steady state levels of immediate early genes were higher in Ciras-3 cells than in parental 10T1/2 (Strelkov and Davie, 2002). Inhibition and knockdown of MSK 1 in Ciras-3 cells attenuated the levels of immediate early genes, suggesting that MSKs may be involved in their induction (Pérez-Cadahía *et al.*, 2011). Promoter regions of induced *TFF1* in MCF 7 cells and *IL6* gene promoter regions in MDA-MB-231 cells were with H3S10ph and MSK 1 (Espino *et al.*, 2006; Ndlovu *et al.*, 2009). MSK inhibitor H89 prevented TPA-induced expression of *TFF1* and neoplastic transformation in MCF 7 cells (Espino *et al.*, 2006). HER2 overexpression in breast cancer development results in an upregulation of the RAS-MAPK signaling cascade. Steady state levels of H3S10ph were significantly increased in breast cancer cell lines overexpressing ERBB2 (Khan *et al.*, 2013). Dominant-negative MSK 1 knockdown, demonstrated that TPA and EGF induced cell transformation in JB6 C141 cells was via the nucleosomal response (Kim *et al.*, 2008). Furthermore, it was shown that blocking MSK 1 attenuated anchorage-independent transformation of JB6 cells through the inhibition of both phosphorylation of histone H3 and *Fos* and *Jun* activation (Kim *et al.*, 2008). In human gastric cancers, immunohistochemical analysis revealed a distance-dependent relation of H3S10ph with the margin of resection of these tumors, which correlated with prognosis (Khan *et al.*, 2016). MSK 1 is overexpressed in more than 40% of colorectal cancer patients and a positive correlation between MSK 1 and H3 phosphorylation exists. Colorectal cancer patients with high MSK 1 expression had shorter overall survival than those with low MSK 1 expression (Xinhui *et al.*, 2017). Altogether, these studies demonstrate that the MSK-mediated nucleosomal response is involved with the phenotypic events of carcinogenesis.

It is important to note that MSK is not the only kinase responsible for the regulation of H3S10ph (Fig. 4), and various studies have shown that the involvement of more than one kinase helps to achieve proper transcriptional gene activation (Duncan *et al.*, 2006). However, MSK is the only interphase kinase shown so far, to be responsible for H3S28ph (Sawicka and Seiser, 2012).

1.9 MSK as a therapeutic target

MSK 1/2 play crucial roles in the induction of several immediate early genes involved in oncogenesis, such as the AP-1 family members *FOS* and *JUN* (Strelkov and Davie, 2002; Dunn and Davie, 2005; Healy, Khan and Davie, 2012). Thus, targeting MSK 1/2 in cancer may attenuate immediate early gene expression, resulting in a reduction in tumor formation and cell transformation. Commercially available inhibitors such as H89, Ro 318220, SB-747651A are used to study the effects of MSK *in vitro*.

H89 (N-[2-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide) is a potent MSK inhibitor originally marketed as ‘a selective PKA inhibitor’ (Davies *et al.*, 2000; Lochner and Moolman, 2006). However, it was found to inhibit MSK 1/2 with a potency similar to that of PKA inhibition (Table 1) (Davies *et al.*, 2000). H89 inhibits PKA activity competitively with ATP. At a concentration of 10 μ M, H89 has been shown to selectively inhibit the nucleosomal response (Thomson *et al.*, 1999), and studies in TPA-treated MCF 7 cells demonstrated that H89 attenuated TPA-induced recruitment of MSK 1 and MSK 2 at the upstream promoter region of regulatory regions of *TFF1* gene which encodes proteins involved in pro-invasive and angiogenic effects (Khan *et al.*, 2013). The levels of H3S10ph and H3S28ph were also significantly reduced. Similarly, H89 attenuated EGF and TPA-induced *c-fos* in 10T1/2 and Ciras-3 cells (Strelkov and Davie, 2002). Due to the off-target effects of H89, there is a need to control for other kinases.

Ro 318220 due to its low IC₅₀ (Table 2) was believed to be a preferential MSK inhibitor over H89. However, like H89, Ro 318220 inhibits MAPKAPK1, SGK1, GSK3 and RSK protein kinases. Moreover, 1 µM of Ro 318220 inhibited 23 proteins other than MSK1/2 by 90%, thus demonstrating a very low selectivity for MSK (Davies *et al.*, 2000).

SB-747651A is an improved MSK inhibitor that replicates many of the effects of MSK 1/2 knockout cells. Studies have shown that SB-747651A has fewer off targets effects than H89 (Naqvi *et al.*, 2012). However, the Davie group has demonstrated that both SB-747651A and H89 have the same effects on the nucleosomal response (unpublished data). Moreover, SB-747651A inhibits other kinases with a similar potency to MSK 1/2 (PKA, PRK2, RSK1, p70S6K and ROCK-II) (Naqvi *et al.*, 2012).

The NTK domain of MSK 1/2 belongs to the AGC kinase family comprised of more than 50 family members. Due to their shared sequence homology, selectively targeting AGC proteins is challenging, as targeting one family member can lead to off target effects (Duda, 2012). The development of small molecule inhibitors that are specific to MSK 1/2 will be a critical tool necessary to deduce further roles of MSK in the cell transformation as well as for future applications in the clinic.

Table 1. Inhibition of protein kinases by H89.

Adapted from (Bain *et al.*, 2007)

H89 (10 μ M)

Protein Kinase	Activity % of control
MKK1	90 $\pm$ 4
JNK α 1	97 $\pm$ 1
GSK3 β	107 $\pm$ 2
MSK1	3 $\pm$ 1
PKA	2 $\pm$ 1

Table 2. MSK1/2 specific inhibitors.

Adapted from (Healy, Khan and Davie, 2012)

MSK inhibitor	IC ₅₀ values	% MSK activity remaining using 1 μ M inhibitor	Number of known off target effects at similar potency
H89	120 nM	25	8
Ro 318220	8 nM	1	23
SB-747651A	11 nM	5	4

CHAPTER TWO

2.0 RATIONALE, HYPOTHESIS, RESEARCH AIMS AND SIGNIFICANCE

2.1 Rationale

Although multigene profiling has made a major impact in breast cancer treatment, it is important to explore the molecular mechanisms that induce and shut down these genes involved in the cancer cell proliferation and/or metastasis (Chang *et al.*, 2005). Such knowledge will produce the framework for a rational approach to a therapeutic intervention.

The high persistence of TNF α in breast tumors is linked with local recurrence and appearance of new metastasis (Ben-Baruch, 2012; Leibovich-Rivkin *et al.*, 2013). Furthermore, the key role that MSKs play in tumor development was demonstrated in studies showing that MSK 1/2 knockout mice developed significantly fewer skin tumors than wild-type mice when treated with organ specific laboratory carcinogens (Chang *et al.*, 2011). Additionally, a loss of malignant phenotype, demonstrated in the loss of anchorage-independent growth in HCT 116, SKBR3, MDA-MB-231 and Ciras-3 cells was observed with the use of MSK inhibitors (D'Anello *et al.*, 2010; Pérez-Cadahía *et al.*, 2011; Healy, Khan and Davie, 2012; Xinhui *et al.*, 2017).

The characteristic gene expression profile of TNBCs despite their heterogeneity may require continuous active transcription of certain genes, thus they may be more sensitive to drugs that target the transcription of these genes, hence allowing for high selectivity (Hoadley *et al.*, 2014). This was indeed demonstrated in a study that determined the dependency and addiction of TNBCs to CDK7. It was observed that TNBCs were more dependent for proliferation on genes such as *FOS*, and treatment of cells with CDK7 inhibitor THZ1 led to apoptotic cell death in TNBC cell lines, but no cell death was observed in ER+ breast cancer cells (Wang *et al.*, 2015). In MDA-

MB-231 cells, *IL6* transcription is strongly induced by $\text{TNF}\alpha$ and this expression coincides with an increased level of *JUN*, *FOS* and *MSK 1* in MDA-MB-231 cells, which are associated with the regulatory region of *IL6* and is required to elicit selective chromatin relaxation at the *IL6* upstream promoter region (Ndlovu *et al.*, 2009). The increase in *MSK 1* activity and immediate early gene products in TNBC cells suggests that these cells may be dependent on the ERK1/2 and p38 MAPK pathways (Vermeulen *et al.*, 2009).

The Davie lab had previously shown that treatment of cells with the *MSK* inhibitor H89, as well as *MSK* knockdown prevented the EGF- and TPA-induced recruitment of *MSK 1/2* and the induction of immediate early genes in 10T1/2 cells (Drobic *et al.*, 2010; Pérez-Cadahía *et al.*, 2011). Furthermore, they showed that blocking constitutively active *MSK* reduces oncogenic and metastatic phenotypes, such as anchorage independent growth (Pérez-Cadahía *et al.*, 2011). Thus, *MSK* inhibition may provide an effective therapeutic option for patients with this aggressive form of disease. However, *MSK* target genes in TNBC have not been extensively explored. This project aims to determine the role of *MSKs* in mediating the response to $\text{TNF}\alpha$ signaling, that is in maintaining the oncogenic and metastatic phenotypes of TNBC, thereby demonstrating *MSKs* as viable targets for therapeutic development.

2.2 Hypothesis

MSK-mediated nucleosomal response pathway plays a key role in the transcriptional program induced by $\text{TNF}\alpha$ in TNBC cells.

2.3 Specific Aims

2.3.1 Aim 1: To determine the transcriptional response of MDA-MB-231 cells in response to TNF α and the role of MSK

The transcriptional program of TNBC induced by TNF α has not been investigated. Thus, the expression levels of immediate early genes (*FOS*, *JUN* and *IL6*) following TNF α treatment in MDA-MB-231 cells will be monitored. Serum starved cells will be incubated with TNF α (20ng/mL) for 0, 0.25, 0.5, 1 and 2 hours, as induction of the immediate early genes were observed at this concentration. This is a modification in the concentration described by (Ndlovu *et al.*, 2009), where 50 ng/mL of TNF α was used. Expression levels by RT-qPCR of serum starved cells treated with TNF α with or without MSK inhibitor at the above stated time points will be monitored. The addition of MSK inhibitors will provide evidence for which immediate early genes require MSK activity to be induced (Pérez-Cadahía *et al.*, 2011). Although H89 is a potent MSK inhibitor (IC₅₀ of 120 nM), it also inhibits PKA (IC₅₀ of 130 nM). Thus, to control for PKA inhibition, cells will be treated with Rp-cAMPs, which does not inhibit MSK (Pérez-Cadahía *et al.*, 2011; Khan *et al.*, 2013). The TNF α induced transcriptional program in MDA-MB-231 cells will be determined by RNA-Seq and the data analyses (mapping and mining) will be performed by the bioinformatician Dr. Wayne Xu.

2.3.2 Aim 2: Determination of the levels of phosphorylated H3 and acetylated K27 at upstream promoter and enhancer regions of immediate early genes

2.3.2.1 To determine the levels of MSK-induced phosphorylated H3 and enhancers

MSK 1 and H3S10ph are associated with the upstream promoter region of immediate early genes (Drobic *et al.*, 2010). Specifically, Ndlovu *et al.* showed that the MSK 1 and H3S10ph are associated with *IL6* upstream promoter region in MDA-MB-231 cells following *IL6* induction with TNF α (Ndlovu *et al.*, 2009). Also, MSK 1/2 and the nucleosomal response play a pivotal role in the chromatin remodeling of enhancers and upstream promoter region of immediate early genes (Khan *et al.*, 2013; Steven Z. Josefowicz *et al.*, 2016). ChIP assays will be used to determine the levels of MSK-induced phosphorylated H3 and the location of active enhancers at the upstream promoter region of immediate early genes.

2.3.2.2 To determine the dynamic levels of H3K27ac and the role of MSK

In addition to phosphorylation, histone H3 can be acetylated. This acetylation correlates with the transcription status of many genes, including immediate early genes. H3K27ac is present at active upstream promoter regions and at active enhancers (Heintzman *et al.*, 2009). Acetylation of H3K27 is mediated by the transcription co-activator CBP/p300, and studies have demonstrated that MSK is in a multiprotein complex with the lysine acetyltransferase p300 (Drobic *et al.*, 2010; Steven Z. Josefowicz *et al.*, 2016). Additionally, co-immunoprecipitation studies have demonstrated that CBP/p300-mediated H3K27ac is required for MSK 1/2 to phosphorylate S28 on H3 (Khan *et al.*, 2017). ChIP assays will be done for H3K27ac in MDA-MB-231 cells with or without H89 at 0, 0.25, 0.5, 1 and 2 hours, to determine the levels of H3K27ac at the upstream promoter region of genes induced via TNF α stimulation (e.g. *IL6*). These results would reveal the dynamics of H3K27ac and demonstrate if H3K27ac is MSK inhibitor sensitive in MDA-MB-231 cells.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Cell Culture

3.1.1 Cell culture conditions

The mesenchymal stem-like subtype triple-negative breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection. MDA-MB-231 cells were grown at 37°C in a CO₂ free incubator in L-15 medium (Thermo Fisher Scientific) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotic-antimycotic (Thermo Fisher Scientific).

3.1.2 Cell passaging

The cells were passaged in a biological safety cabinet when they were 80-90% confluent in the cell culture flask and placed at 37°C in a CO₂ free incubator. First, the medium was aspirated from the cell culture flask and the cell monolayer was washed with 1X sterile PBS. Subsequently, 1-4 mL of TrypLE Express (Thermo Fisher Scientific) was added to the cell culture flask and incubated for 3 minutes. The detached cells were collected in the L-15 medium and then transferred into 15 mL centrifuge tube (FraggaBio). The cells were centrifuged at 360 x g for 3 minutes. The supernatant was aspirated and the cell pellet was resuspended in 4-7 mL of L-15 medium. The resuspended cells were then seeded to new cultures flasks.

3.1.3 Cell storage and recovery

To store the cells, 80-90% confluent cells were washed with 1X sterile PBS followed by the addition of 1-4 mL of TrypLE Express in a biological safety cabinet. The detached cells were

collected in appropriate media and the cells were counted using an automated cell counter (BIO-RAD) and trypan blue (Sigma) to determine their viability. Once cells were over 75% viable, they were centrifuged at 360 x g for 3 minutes, and the supernatant was aspirated. The cell pellet was resuspended in the cryomedia, which consists of 95% (v/v) L-15 medium and 5% (v/v) DMSO (Sigma). One mL of the resuspended cells was then aliquoted to labelled cryovials (Fisher Scientific), which were placed into a -80°C freezer. For long-term storage, the cryovials were transferred into liquid nitrogen.

To revive cells, the cryovials containing the frozen cells were placed into a 37°C water bath. Once thawed, the cryovials were sprayed with 70% ethanol, and cells were transferred dropwise into a 15 mL centrifuge tube (VWR) which contained 6 mL of prewarmed L-15 medium. The cells were centrifuged at 360 x g for 3 minutes. The supernatant (media, FBS and DMSO) was aspirated without disturbing the pellet, and the cells were resuspended in 5 mL of media. Cells were then transferred to T-25 cell culture flask. The media was changed after 24 hours.

3.1.4 Serum starvation and treatment conditions

Cells were grown in complete L-15 medium (10% FBS and 1% antibiotic-antimycotic). Once cells reached 90% confluency, the cells were washed three times with 1X sterile PBS to remove all traces of the old media. Subsequently, 12 mL of L-15 medium containing 10% (v/v) charcoal-stripped FBS and 1% (v/v) antibiotic-antimycotic was dispensed to the culture flasks and incubated for two days. After two days, the cells were washed three times with 1X sterile PBS to remove all traces of the old media and the 12 mL of L-15 medium containing 3% (v/v) charcoal-stripped FBS and 1% (v/v) antibiotic-antimycotic was added to the culture flask and incubated for three days. Altogether, the cells were serum starved for five days. Following serum starvation, cells were incubated with TNF α (20 ng/mL) (R&D systems) for 0, 0.5, 1 and 2 hours with/without

MSK inhibitor H89 (10 μ M) (Sigma) or PKA inhibitor, Rp-cAMPs (100 nM) (Sigma). H89 was added 30 minutes prior to TNF α treatment, and Rp-cAMPs was added 1 hour prior to TNF α treatment.

3.2 Ethidium bromide/Acridine orange double staining

Cells were detached by the addition of 1-4 mL of TrypLE Express and the detached cells together with the old media were placed in a 15 mL centrifuge tube. The cells were centrifuged at 360 x g for 5 minutes, and the supernatant was aspirated, leaving behind 100 μ L in the tube. The staining solution was prepared by mixing together 100 μ g/mL of acridine orange (Sigma) and 100 μ g/mL of ethidium bromide (Sigma) in 1X PBS in a chemical hood. Two microliters of the staining solution were added to the 15 mL tube, the cells were then mixed with the solution by flicking the tube, and the tube was placed on ice. Subsequently, 10 μ L of the stained cells was placed on a microscope slide with a cover slip and viewed under the fluorescence microscope. Cells that appear orange represent dead cells and cells that appear green indicate viable cells (Fig. 7). Viable cells were counted, while avoiding large clusters of cells.

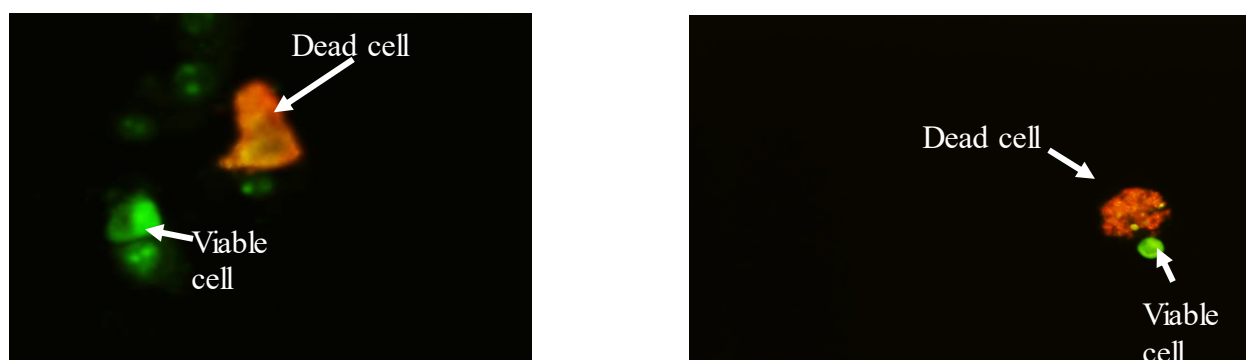


Figure 7. Fluorescence microscope images of MDA-MB-231 cells stained with ethidium bromide/acridine orange double staining.

3.3 Flow cytometric analysis of cells

To determine the stage of the cell cycle serum starved MDA-MB-231 cells were in, propidium iodide staining solution was prepared freshly by adding, 2 mg of DNase-free RNase and 200 ug of 1 mg/mL propidium iodide in water, to 10 mL of 0.1% TritonX-100 in PBS. Seventy % ethanol was prepared freshly and placed in -20°C freezer for 3 hours. Ice-cold ethanol (4.5 mL) was placed in a 15 mL tube and placed on ice always. Cells were detached from the culture flask by the addition of 1-4 mL of TrypLE Express in a biological safety cabinet. The detached cells were collected in appropriate media in a 15 mL centrifuge tube, and the cells were counted using an automated cell counter. After counting and adequately sectioning the cells into 1.5×10^6 cells per 15 mL tube, the cells were centrifuged at 360 x g for 3 minutes. Thereafter, the cells were washed with 1X sterile PBS, followed by a resuspension in 5 mL 1X sterile PBS. The resuspended cells were centrifuged at 360 x g for 3 minutes, and the supernatant was aspirated. The cell pellets were broken by gently flicking the tube and 0.5 mL of 1X PBS was added to completely resuspend the cells. The cell suspension was added to one tube containing 4.5 mL of cold ethanol and was mixed thoroughly. Cells were kept in ice-cold ethanol for at least 2 hours, followed by centrifugation and complete decantation of the ethanol, by touching the edge of the centrifuge tube to a paper towel. The use of a Pasteur pipette to remove the ethanol was avoided. Cells were again suspended in 5 mL 1X of PBS and centrifuged at 360 x g for 3 minutes. The cell pellet was broken up and then resuspended in 1 mL of the staining solution. The cells resuspended in the staining solution, were placed in a 37°C water bath for 15 minutes or at room temperature for 30 minutes. After incubation, the tubes containing the stained cells were covered with foil, placed on ice and sent for cell cycle analysis on a flow cytometer. Flow cytometric analyses were performed by Monroe Chan of the Regen Flow Laboratory.

3.4 Protein-Based Techniques

3.4.1 Preparation of total cellular lysate

Cells (90-95% confluent) were washed two times with cold 1X PBS and harvested in 1X PBS from cell culture flasks, using a cell scraper into 15 mL tubes. The cell pellet was obtained by centrifugation for 3 minutes at 360 x g. The supernatant (1X PBS) was aspirated and the cell pellet was resuspended in cold lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA and 0.5% (v/v) NP-40), containing protease and phosphatase inhibitors (Roche), followed by an incubation on ice for 10 minutes and sonication at a power of 2, three times for 10 seconds each time, with 30-second intervals on ice between sonication cycles (Khan, 2013). Protease and phosphatase inhibitors were used only when ready to lyse the cells. The components of these inhibitors are listed in Table 3. The cell lysate was centrifuged at 13,000 x g at 4°C, and the supernatant was kept while the pellet was discarded. The protein concentration of the total cellular lysate (supernatant) was quantified as per the manufacturer's instructions using the Pierce BCA Protein Assay Kit (Thermo Scientific) and BSA as a standard. Once quantified, the total cellular lysates were stored at -20°C.

Table 3. Components of protease and phosphatase inhibitors from Roche.

Inhibitor	Components
Protease inhibitor	1. 4-(2-Aminoethyl) benzenesulfonyl Fluoride Hydrochloride. 2. 2-Pyrrolidinone, 1-etenyl-, homopolymer. 3. N-Acetyl-L-leucyl-L-leucyl-L-arginial hemisulfate salt, and 4. Aprotinin Trypsin inhibitor
Phosphatase inhibitor	1. 2-Pyrrolidinone, 1-etenyl-, homopolymer. 2. 1-O-n-Dodecyl-beta-D-glucopyranosyl(1-4)-alpha-D-glucopyranosid. 3. Okadaic acid. 4. 4,7-Epoxyisobenzofuran-1,3-dione, hexahydro-3a,7a-dimethyl-, (3ar,4S,7R,7as)

3.4.2 Histone extraction

Serum starved MDA-MB-231 cells were treated with or without $\text{TNF}\alpha$ at 0, 30, 60 and 120 min. Cells were harvested and stored at -80°C or resuspended in 1-5 mL of NP-40 buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.5 mM MgCl_2 and 0.1% (v/v) NP-40) supplemented with protease and phosphatase inhibitors. Resuspended cells were homogenized 10 times, with a Teflon homogenizer. The homogenized sample was transferred into a 15 mL tube. The cell suspension was passed through a syringe with a 22-gauge needle 5 times, cells were then centrifuged at 4000

x g for 4 minutes. The supernatant was discarded, and the pellet containing the nuclei was resuspended in 1 mL of RSB buffer (10 mM Tris-HCl, pH 7.4, 3 mM MgCl₂ and 10 mM NaCl) supplemented with protease and phosphatase inhibitors. Four normal (4N) H₂SO₄ was added to the solution, to a final concentration of 0.4 N. Samples were left on ice for 1 hour, after which they were spun down at 20,000 x g for 20 minutes at 4°C. The pellet was kept and washed 1 time with 1 mL of acetone/HCl (5 mL HCl for every 1 mL acetone). The pellet was then washed again in 1 mL of acetone for 5 times. Samples were spun down, and the pellet was air dried overnight. The next day, the air-dried pellet (histones) was resuspended in 40 uL of ddH₂O. Histones were quantified using the Pierce BCA Protein Assay Kit and BSA as a standard, as per the manufacturer's instructions.

3.4.3 SDS-PAGE and western blotting

Protein samples were reduced and denatured by the addition of the reducing sodium dodecyl sulfate (SDS) loading buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS (w/v), 0.01% bromophenol blue (w/v) and 5% β-mercapthoethanol). Once SDS loading buffer was added, samples were boiled for approximately 5-10 minutes prior to loading onto a polyacrylamide gel. The polyacrylamide gel percentage (10%, 12% or 15%) was chosen depending on the size of the target protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run at 150V for 1-1.5 hour. Using the Trans-Blot Turbo Transfer System (Bio-Rad), as per manufacturer's instructions, the proteins were transferred from the polyacrylamide gel to a 0.45 μm nitrocellulose membrane (Bio-Rad). To test the efficacy of the transfer, the membrane was stained with Ponceau S (0.5% Ponceau S and 5% acetic acid) for 5 minutes. The stained membrane was washed with ddH₂O for 2 minutes and then baked at 65°C for 15 minutes. Antibody manufacturers sometimes specify the blocking buffer to be used. Therefore, depending on the

antibody manufacturer's instructions, the baked membrane was blocked with 5% (w/v) skim milk powder or 5% (w/v) BSA in 0.1% (v/v) TBST (50 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.1% Tween-20) at room temperature for 1 hour on a rocking platform (VWR, Model 200). After blocking, the membrane was incubated with a primary antibody at an optimized dilution in 5% skim milk or 5% (w/v) BSA in 0.1% (v/v) TBST. The primary antibody incubation was usually done overnight at 4°C on a rotator (Boekel Scientific, Model 260200). Alternatively, the primary antibody incubation can also be performed for 3 hours at room temperature. The membrane was then washed three times (10 minutes per wash) at room temperature with 0.1% TBST on a rocking platform. The membrane was then incubated for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated anti-IgG secondary antibody diluted in 5% (w/v) skim milk or BSA. After secondary antibody incubation, the membrane was washed three times (10 minutes per wash) at room temperature with 0.1% TBST on the rocking platform. Lastly, the target protein on the membrane was visualized with Western Lighting Plus-ECL (Perkin Elmer) on Hyperfilm ECL (Amersham) or on a ChemiDoc (Bio-Rad) as per the manufacturers' instructions.

3.4.4 Quantitative western blotting to determine optimal protein concentration

Optimizing protein and antibody concentration reduces background noise due to non-specific binding and significantly reduces cost. Subsequently, an antibody saturation curve helps to produce interpretable western and dot blots. An antibody saturation curve was done for all antibodies used in the experiment. In brief, varying concentrations (ug) of protein were loaded in a 12% or 15% polyacrylamide gel, depending on the size of the target protein. The SDS-PAGE was then run and transferred to a nitrocellulose membrane, as described in section 3.4.3. The target protein on the membrane was visualized with Western Lighting Plus-ECL on the FluorChem HD2 (Alpha Innotech) or Image Lab Software (ChemiDoc, Bio-Rad) and the optical intensity of each

band was measured. A graph of the protein concentration (x axis) against the optical density (y axis) was plotted (Fig. 8). Sample concentrations should not exceed the saturation point, which is observed on the plotted graph. It is important not to visualize the target proteins on a Hyperfilm ECL, as a film's linear dynamic range is very limited.

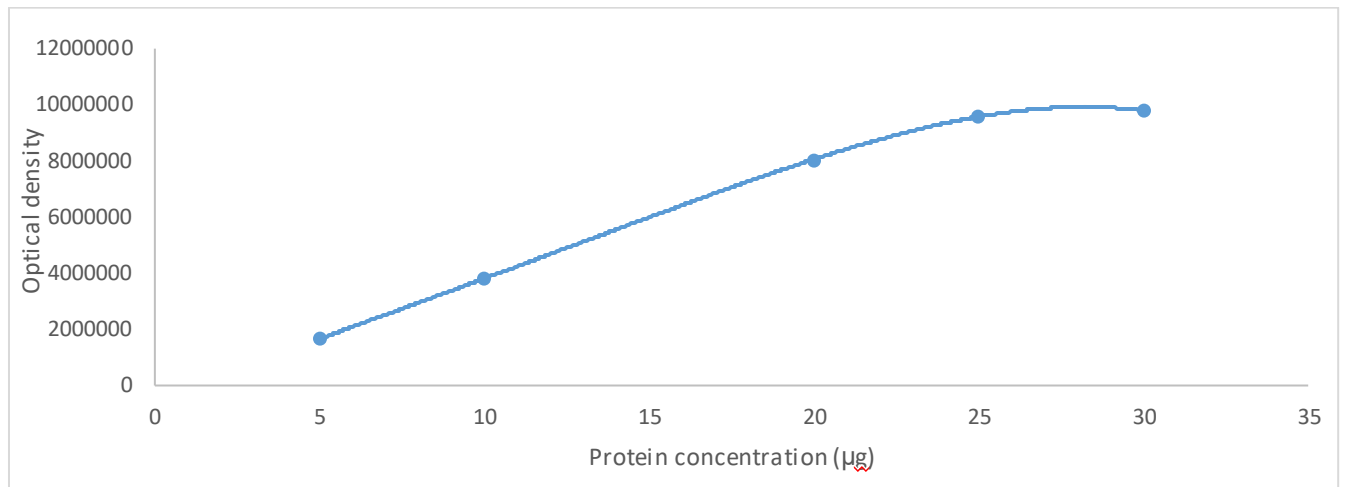


Figure 8. Protein concentration standard curve using Santa Cruz H3S10ph (8656-X) antibody.

3.5 RNA isolation and real time RT-qPCR analysis

3.5.1 RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from MDA-MB-231 cells treated with or without TNF α , with or without H89 or Rp-cAMPs using the RNeasy Plus Mini Kit (Qiagen) as per the manufacturer's instructions. Briefly, once cells were 90% confluent, they were harvested and disrupted in the supplied RLT buffer. Followed by a quick spin through a QIA shredder for 2 minutes at the

maximum speed of the microcentrifuge (42,000 x g). The supernatant fluid was then mixed with 70% ethanol and further taken to run through a RNeasy spin mini-column, supplied in the kit. This step was repeated until all the solution was run through the column. Then the supplied binding buffer was added to the mini column followed by twice washing with supplied washing buffer. At the last step, the RNA was eluted with RNase-free H₂O and stored in -80°C. The RNA concentration and purity based on the A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ ratio was measured by Nanodrop 2000 (Thermo Fisher Scientific). Once RNA was isolated and purified, a DNase 1 treatment to remove any genomic DNA present in the sample was carried out using the Ambion Invitrogen DNA-free kit per manufacturer's instructions. The cDNA synthesis was performed with iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) as per the manufacturer's instructions.

3.5.2 cDNA Real Time Polymerase Chain Reaction (RT-qPCR)

cDNA from RT-PCR was diluted 5X and RT-qPCR analyses were performed using the Bio-Rad CFX96 Real-Time PCR Detection System. The qPCR reactions were prepared in 96-well plates on ice. The reactions were prepared to a total volume of 20 µL by adding 10ul of the SYBR green master mix (Bio-Rad), 5 uL of RNase free water (Fisher Scientific), 1 uL of the forward primer, 1 uL of the reverse primer and 3ul of the 5X diluted cDNA. The primers used for cDNA RT-qPCR analyses are shown in Table 4.

Table 4 List of primers used for cDNA RT-qPCR analyses

Name	Sequences
<i>36B4</i> (Forward)	5'-GATTGGCTACCCAAGTGTG-3'
<i>36B4</i> (Reverse)	5'-CAGGGGCAGCAGCCACAAA-3'
<i>FOS</i> (Forward)	5'-ACTACCACTCACCCGCAAGAC-3'
<i>FOS</i> (Reverse)	5'-GTGGGAATGAAGTTGGACT-3'
<i>JUN</i> (Forward)	5'-CAGGTGGCACAGCTTAAACA-3'
<i>JUN</i> (Reverse)	5'-CCGACGGTCTCTCTTCAAAA-3'
<i>IL6</i> (Forward)	5'-CCCTGACCCAACCACAAA-3'
<i>IL6</i> (Reverse)	5'-GGACTGCAGGAAGTCTTAAA-3'
<i>PTGS2</i> (Forward)	5'-ATCATCAGGCACAGGAGGAA-3'
<i>PTGS2</i> (Reverse)	5'-TCCACCAACTTACAATGCTGA-3'

3.6 Chromatin Immunoprecipitation (ChIP) Assays

3.6.1 Formaldehyde Crosslinking

TNF α treated or untreated MDA-MB-231 cells, with or without H89 were grown in a T-75 cell culture flask to 90-100% confluency. Cells were harvested and resuspended in 1-5 mL of 1X PBS with 1% (v/v) formaldehyde and incubated at room temperature for 10 minutes. Glycine (125 mM) was added and incubated at room temperature for 5 minutes to stop the cross-linking. The cross-linked cells were washed with 1xPBS and centrifuged at 6200 x g for 5 minutes. The cell pellets were either stored at -80°C or used immediately for ChIP.

3.6.2 Chromatin immunoprecipitation

Following formaldehyde crosslinking, the cell pellet was resuspended in cell lysis buffer/ nuclei isolation buffer (10 mM PIPES pH 7.9 at 4°C, 2 mM MgCl₂, 10 mM KCl and 0.5% (v/v) NP-40) supplemented with protease and phosphatase inhibitors. The cells were incubated for 20 minutes at 4°C with rotation followed by centrifugation at 2000 x g for 5 minutes. The cell lysis

step and centrifugation was repeated one more time for 10 minutes to obtain nuclei. A black dot surrounded by a minute bright circle when viewed under a microscope, is indicative of isolated nuclei (Fig. 9). The nuclear pellet was resuspended in 1-2 mL of MNase digestion buffer (10 mM Tris-HCl pH 7.5, 0.25 M sucrose and 75 mM NaCl) supplemented with protease and phosphatase inhibitors. The A_{260} reading of the resuspended nuclei was measured and the total A_{260} in solution was calculated, as mentioned in section 3.4.5. $CaCl_2$ to a final concentration of 3 mM was added. The sample was warmed in a water bath at 37°C for 10 minutes. Then, MNase enzyme was added at a concentration of 2.5 U per 1 A_{260} and incubated at 37°C for 30 minutes. The reaction was stopped with the addition of EDTA pH 8.0 to a final concentration of 5 mM. The nuclei were then lysed by the addition of SDS to a final concentration of 0.5% and incubated with rotation for 1 hour at room temperature. This was followed by centrifugation at 2000 x g for 5 minutes, and the supernatant was diluted 5-fold with RIPA buffer (10mM Tris-HCl pH 8.0, 1% (v/v) Triton-X-100, 0.1% (w/v) SDS, 0.1% (w/v) SDC) supplemented with protease and phosphatase inhibitors. After DNA fragmentation, the nuclear lysate was pre-cleared with the addition of 60 μ L Protein A/G Plus agarose beads (Santa Cruz) per 1 mL of lysate and incubated for 1 hour at 4°C with rotation. This pre-clearing step is optional and can be omitted if using magnetic Dynabeads for immunoprecipitation. The beads were pelleted by centrifugation at 1000 x g for 3 minutes and the A_{260} of the lysate was measured, as described in section 3.4.5. The lysate was aliquoted to several 1.5 mL microcentrifuge tubes (Eppendorf) containing 12 A_{260} per tube. Additionally, 1-2 A_{260} of lysate was also saved as input. H3S10ph (rabbit polyclonal antibody from Santa Cruz, sc-8656-X) or H3K27ac (rabbit monoclonal antibody from Abcam, ab177178) antibody was added to the lysate at 1:1 ratio (μ g antibody: A_{260} of lysate). As a negative control, normal mouse IgG polyclonal antibody was added to the lysate at 1:1 ratio. Also, anti-H3 (rabbit polyclonal antibody

from Abcam, ab1791) was added to the lysate at 1:1 ratio, to assay for nucleosome density. The antibody incubation was carried out overnight at 4°C with rotation. The following day, 7 µL of Dynabeads Protein G (Invitrogen) were added to each A₂₆₀ of the lysate and incubated for 3 hours at 4°C with rotation. The beads were then washed twice with rotation at room temperature for 6 minutes with 1 mL of each of the following buffers: Low Salt wash buffer (0.1% (w/v) SDS, 1% (v/v) Triton-X-100, 2 mM EDTA, 20 mM Tris-pH 8.1 and 150 mM NaCl), High Salt wash buffer (0.1% (w/v) SDS, 1% (v/v) Triton-X- 100, 2 mM EDTA, 20 mM Tris-pH 8.1 and 500 mM NaCl), LiCl wash buffer (250 mM LiCl, 1% (v/v) NP-40, 1% (w/v) deoxycholate, 1 mM EDTA and 10 mM Tris-HCl pH 8.1) and 1xTE buffer (10 mM Tris-HCl pH 7.5 and 1 mM EDTA). After washes, the antibody-chromatin complexes were eluted with the addition of 200 µL of elution buffer (1% (w/v) SDS and 100 mM NaHCO₃) and incubated at room temperature for 30 minutes with rotation. The beads in elution buffer and the input sample were incubated overnight at 65°C to reverse the DNA-protein cross-links. The following day, the samples were treated with RNase A (0.02 µg/mL final concentration for 30 minutes at 37°C) and Proteinase K (0.5 µg/mL final concentration for 1 hour at 55°C). The ChIP and input DNA were purified.



Figure 9. Nuclei isolated from MDA-MB-231 cells.

A black dot surrounded by a minute bright circle is indicate of the nuclei.

3.6.2.1 DNA Purification

ChIP DNA was purified by using the QIAquick PCR Purification Kit as per the manufacturer's instructions (Qiagen). The DNA was eluted with 50 μ L of nuclease free water (Fisher Scientific). The DNA was then used for qPCR, agarose gel or bioanalyzer analyses.

3.6.3 ChIP qPCR

The qPCR analyses were performed using the Bio-Rad CFX96 Real-Time PCR Detection System. The qPCR reactions were prepared in 96-well plates on ice. The reactions were prepared to a total volume of 20 μ L by adding 10 μ L of the SYBR green master mix, 7 μ L of RNase free water, 1 μ L of the forward primer, 1 μ L of the reverse primer and 1 μ L of 0.5-1.0 ng of ChIP DNA.

The primers used for qPCR analyses are shown in Table 2. Figure 10 and 11, show the amplicon location of -80 upstream promoter region of *JUN* upstream promoter region and *IL6* enhancer region used in the ChIP qPCR.

Table 5. List of primers used for ChIP-qPCR analyses

Name	Sequences	Amplicon size (bp)
-80 upstream promoter region of <i>JUN</i> (Forward) -80 upstream promoter region of <i>JUN</i> (Reverse)	5'-AATGGGAAGGCCTTGGG-3' 5'-CCCGAGCTCAACACTTATCT-3'	75bp
<i>IL6</i> upstream promoter region (Forward) <i>IL6</i> upstream promoter region (Reverse)	5'- GCCAAAGTGCTGAGTCACTAATA-3' 5'-GTGCAGCTTAGGTCGTCATT-3'	96bp
<i>IL6</i> Enhancer Region (Forward) <i>IL6</i> Enhancer Region (Reverse)	5'- TCCTTCGCACAAGAAAGATAGAG-3' 5'- CACATGGTAGAGTCCCAGTAAAC-3'	103bp
<i>FOS</i> Enhancer Region(Forward) <i>FOS</i> Enhancer Region (Reverse)	5'-CACAACCCTTCACCACTAGA-3' 5'-GTCTGTACCGCCTACGTAAATG-3'	101bp
<i>IER3</i> Enhancer Region <i>IER3</i> Enhancer Region	5'-GGGAGGAGGAGTTAGAAGGA-3' 5'-GGAGTGTAAGGCCAAGTGAG-3'	89bp

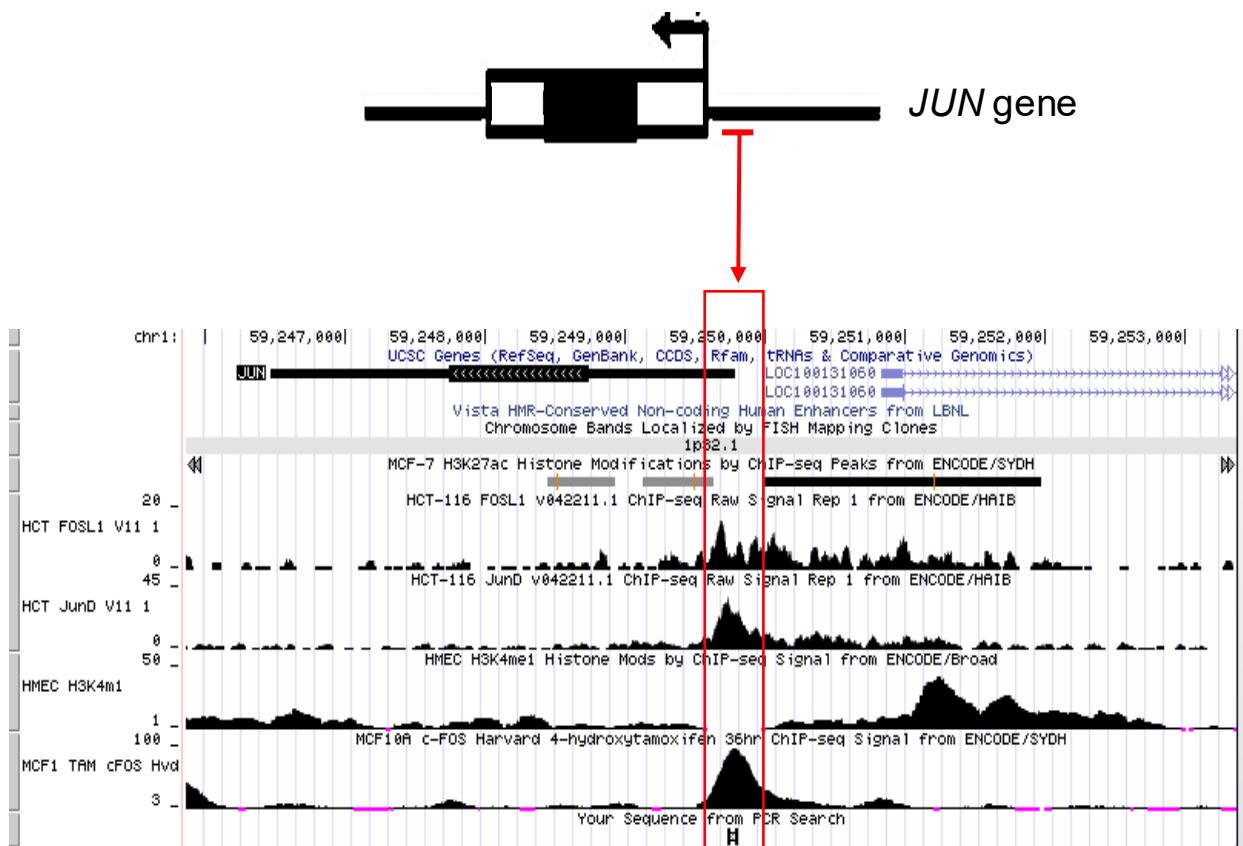


Figure 10. Primer location of -80 *JUN* upstream promoter region.

Primer location of -80 *JUN* upstream promoter region, showing amplicon in region enriched for FOSL2 and JUN, members of the AP-1 factor known to recruit MSK. The red box shows the exact location of the amplicon in ENCODE.

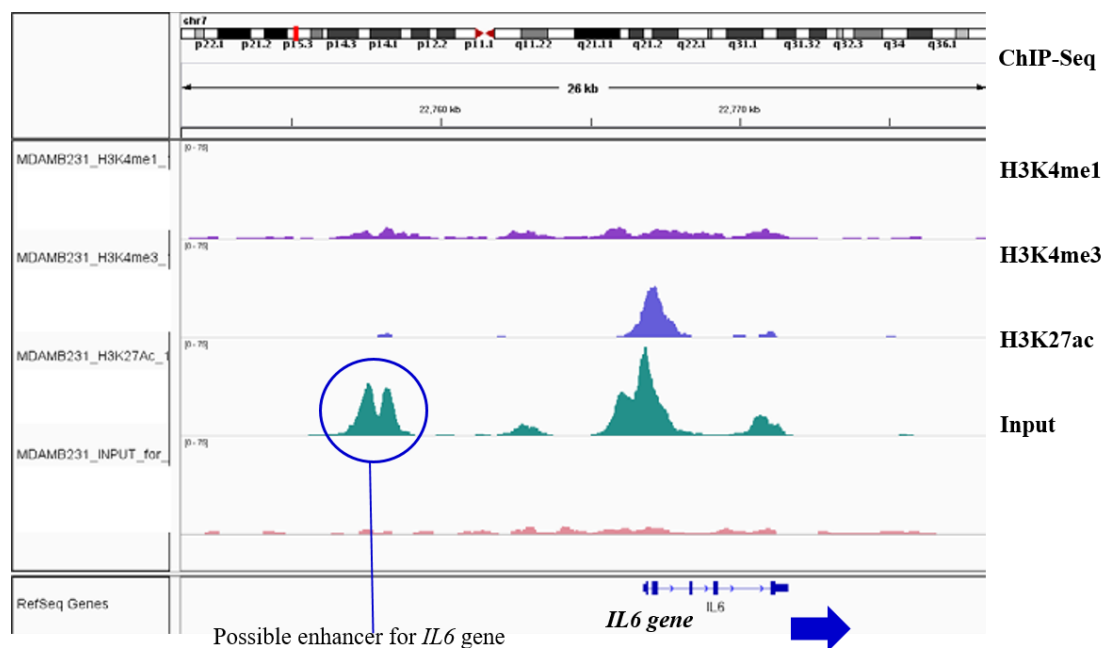


Figure 11. Primer location of *IL6* enhancer region

Location of putative *IL6* enhancer identified by Dr. Wayne Xu based on ChIP-Seq data from GEO on MBA-MB-231 cells (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1204474>).

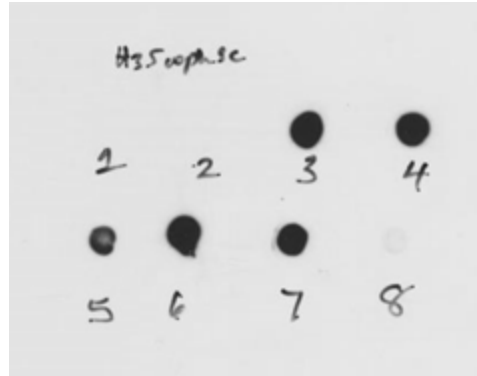
3.6.4 ChIP Optimization Steps

The success of any ChIP assay is dependent, on the quality of the antibody used, as well as proper optimization of conditions. An antibody of good quality should be specific and efficient in the immunoprecipitation of a specific protein (Spencer and Davie, 2002).

3.6.4.1 Determining the specificity of an antibody for its target antigen

Antibody specificity is very crucial, as many commercially available antibodies can bind to non-target protein substrates more efficiently than target proteins. To test for antibody specificity, a dot blot and western blot assay were carried out. In brief for dot blot assay, 0.01-0.4 ug of commercially available peptides (H3 unmodified, H3S10ph, H3K27ac, H3S28ph etc.) were spotted on a nitrocellulose membrane and baked in a 65°C incubator for 15 minutes. The membrane was then blocked in 5% (w/v) skim milk powder or 5% (w/v) BSA in 0.1% (v/v) TBST (50 mM Tris-HCl pH 7.6, 150 mM NaCl and 0.1% (v/v) Tween-20) at room temperature for 1 hour on a rocking platform (VWR, Model 200), depending on the antibody used, followed by incubation with the appropriate primary and secondary antibodies. Ultimately, the target protein on the membrane was visualized with Western Lighting Plus-ECL on Hyperfilm ECL in accordance with manufacturers' instructions. A specific antibody will bind only its target protein.

Antibody occlusion is a phenomenon defined as the failure of an antibody to recognize its epitope resulting from a negative impact of densely distributed multiple modifications (Sawicka and Seiser 2012). Using an antibody that is epitope occluded does not give a full representation of the true events happening in the nucleus. For instance, H3K27ac and H3S28ph have been shown to occur together in 10T1/2 cells (D.H Khan et al. 20117), if an H3S28ph ab that was occluded for the dual mark H3S28ph/H3K27ac is used, we only get results for the single PTM and not the dual PTMs. Antibody occlusion was tested for all antibodies used with a dot blot assay (Fig. 12). Once an antibody showed specificity and no occlusion from dot and western blot assays, it can then be tested under ChIP conditions.



Santa Cruz: H3S10ph. 8656-X
 PEPTIDES: 0.4ug

1. H3 unmodified
2. H3K9ac
3. H3K9ac/S10ph
4. H3K14ac/S10ph
5. H3K9me3/S10ph
6. H3S10ph
7. H3K14/K9ac/S10ph
8. H3K14ac

Figure 12. Dot blot assay.

Test for H3S10ph antibody specificity and epitope occlusion. The antibody identified all its epitope, hence had no epitope occlusion.

3.6.4.2 Determining the efficiency of an antibody to immunoprecipitate its target antigen (IP under ChIP conditions)

Following 5-fold dilution with RIPA of the ChIP lysate and A₂₆₀ quantification (as described in section 3.6.2), 12 A₂₆₀ of total ChIP lysate was aliquoted into 1.5 mL microcentrifuge tubes. Also, 0.6 A₂₆₀ of ChIP lysate was kept as input. Twelve µg of antibody was then added to an aliquot of the ChIP lysate. As described in section 3.6.2, antibody incubation was performed

overnight and magnetic beads were added the following day. After the 3-hour incubation with magnetic beads, an aliquot of the immunodepleted (ID) fractions was kept (equal in volume to the input that was kept earlier in the protocol). Followed by washes with Low Salt wash buffer, High Salt wash buffer, LiCl wash buffer and 1xTE buffer, SDS-PAGE loading buffer was added to the washed magnetic Dynabeads-antibody complex and to the input and ID fractions. Samples were boiled for 10 minutes and loaded onto a polyacrylamide gel. After western blotting, the intensity of the immunoprecipitated and immunodepleted bands were compared to the input band. A 50% enrichment in immunoprecipitated sample, by comparing the intensity of the input versus the immunodepleted, indicated that the antibody was suitable for ChIP. If no enrichment of H3S10ph or H3K27ac was detected compared to the input lane or if the immunoprecipitated lanes were clear, this was an indication that the antibody was not suitable for ChIP and such antibody was not used (Fig. 13).

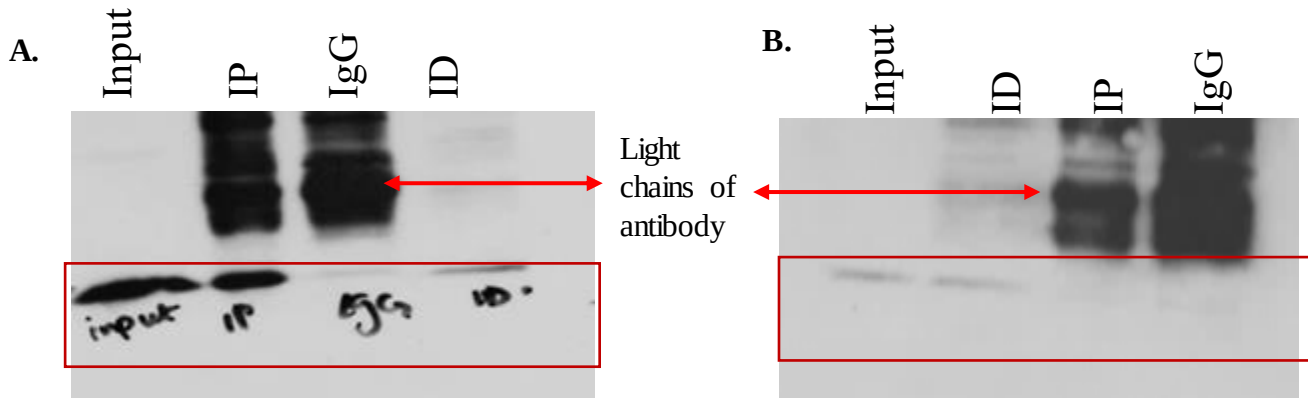


Figure 13. Immunoprecipitation efficiency.

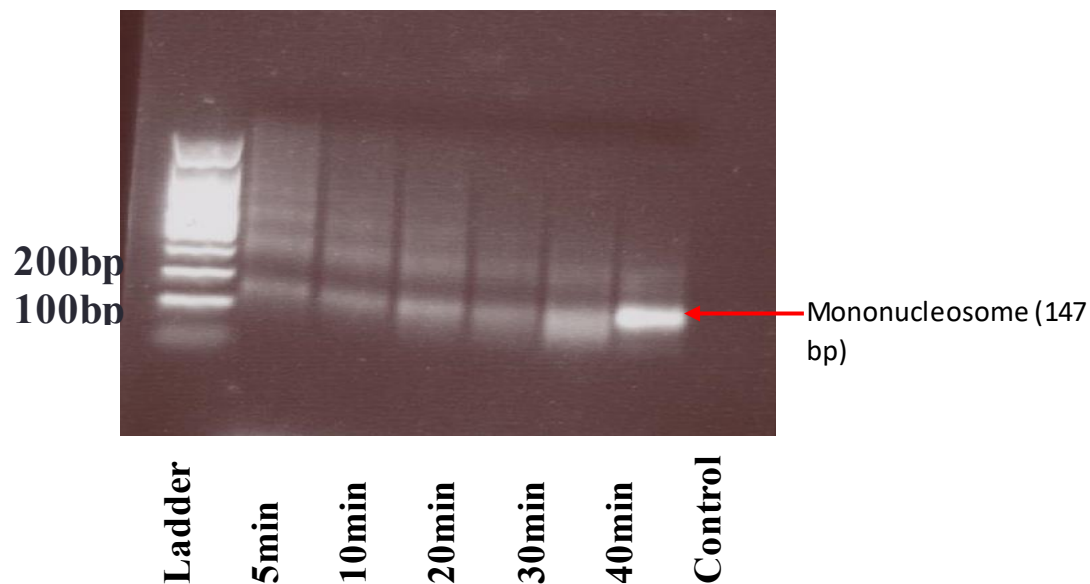
A. Immunoprecipitation with H3K27ac ab showing enrichment for H3K27ac. The red box indicates H3K27ac mark observed at approximately 17kDa. An 80% enrichment in immunoprecipitated was observed by calculating the optical intensity of input versus the immunodepleted (ID) on a Bio-Rad Chemidoc. This antibody is suitable for ChIP. **B.** Immunoprecipitation of H3S10ph ab showing no enrichment for H3S10ph. The red box indicates H3S10ph mark observed at approximately 17kDa. IP represents immunoprecipitated sample, while ID represents immunodepleted samples

3.6.4.3. MNase Digestion

After cellular lysing and pelleting of the nuclei, the nuclear pellet was resuspended in the MNase digestion buffer and the A_{260} absorbance was measured, see section 3.4.5. By quantifying the total A_{260} , it was possible to calculate the amount of MNase enzyme required at a concentration of 2.5 U/ A_{260} . MNase (2.5 U) per A_{260} of nuclear suspension was added to the sample, and equal aliquots were taken for 10, 20, 30, 40 and 50-minute time points. The tubes were then incubated at 37°C. At each time point, the reaction was stopped with the addition of EDTA (5 mM final

concentration) followed by the addition of SDS (0.5% final concentration). Nuclear lysis by SDS was performed at room temperature for 1-2 hours with rotation. This was followed by overnight 65°C incubation to reverse the DNA to protein cross-links followed by RNase A (30 minutes at 37°C) and Proteinase K (1 hour at 55°C) treatments the following day. The DNA for each time point was purified using the PCR purification kit (Qiagen) and analyzed on an agarose gel or the bioanalyzer (Fig. 15). It is important to choose a specific MNase digestion time point that yields the highest amount of mononucleosomes compared to polynucleosomes. The use of a bioanalyzer provides fragment sizing, quantitation and purity assessment of the DNA by capillary electrophoresis.

A.



B.

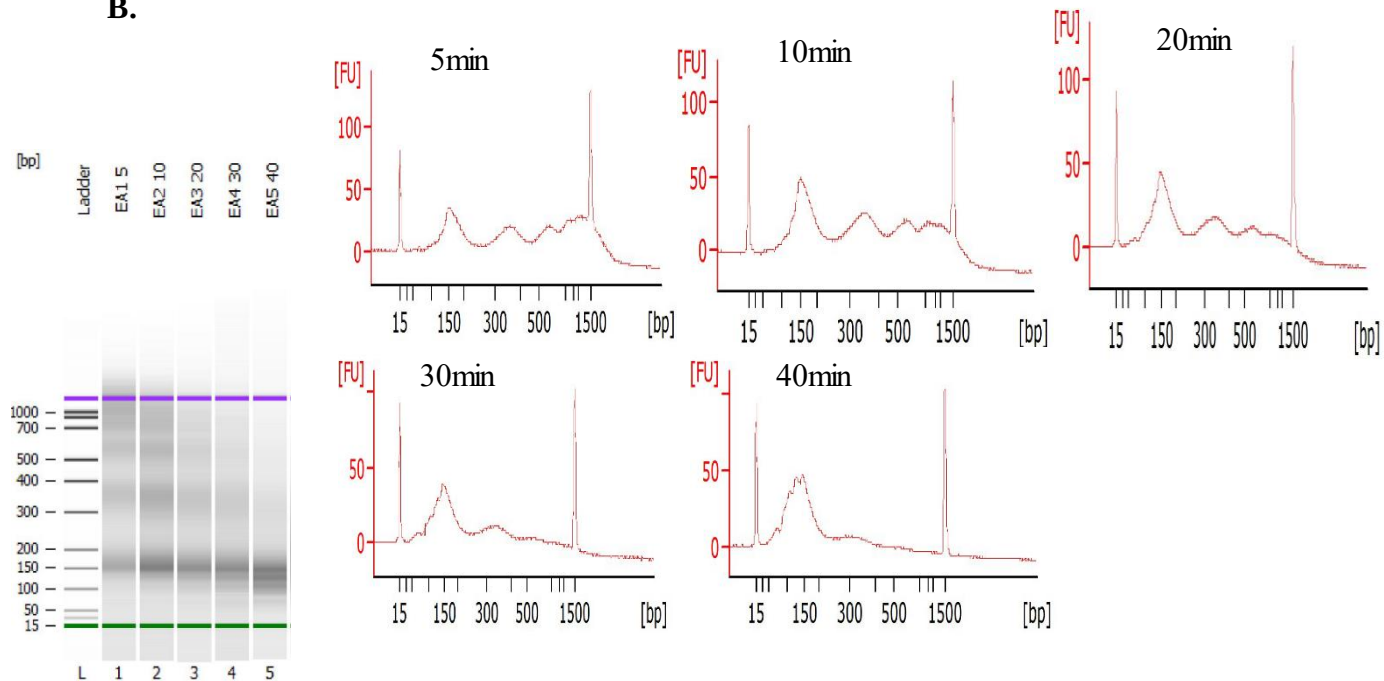


Figure 14. MNase optimisation

A. DNA of MNase digested MDA-MB-231 cells run on an agarose gel. **B.** Bioanalysis of DNA of MNase digested MDA-MB-231 on a bioanalyzer, showing the amount of mononucleosome, dinucleosome and trinucleosome fragments.

3.6.4.3.1 Agarose gels

One % agarose gel was prepared by dissolving 0.5 g of agarose (Invitrogen) in 50 mL of 1X TBE buffer (100 mM Tris base, 100 mM boric acid and 2 mM EDTA pH 8.0) and heated in the microwave for 1-2 minutes. The heated agarose was left to cool and 1xGelStar nucleic acid stain (Lonza) was added to the liquid agarose. The agarose gel was cast and left to solidify. The 1xTBE buffer was used as a running buffer for the agarose gel electrophoresis. A DNA loading buffer was added to each sample before loading them on the gel. The gel was run at 150V. Once the samples had reached three quarters of the GEL, the voltage was turned off and the gel was viewed using the FluorChem HD2 software (Alpha-Innotech).

CHAPTER FOUR

4.0 Results

4.1 Synchronization of MDA-MB-231 cells to G₀/G₁ cell cycle phase

Cell synchronization is the process of bringing cells at different stages of the cell cycle in a culture medium to the same phase. As previously mentioned in section 1.6, H3S10ph and H3S28ph occur minimally in interphasic cells, while majority of these phosphorylated PTMs are in mitotic cells. This study is interested in interphasic H3 serine phosphorylation responsible for immediate early gene expression (Barratt *et al.*, 1994). It is therefore crucial to ensure that cells are arrested at the G₀/G₁ phase of the cell cycle to eliminate any misreads of results.

MDA-MB-231 cells were grown to 90-100% confluency in a cell culture flask and a flow cytometric analysis was performed. Various serum starvation conditions were assayed for (Fig. 15A); however, a five-day serum starvation was the optimal condition as 80% of cells were at G₁ phase of the cell cycle (Fig. 15B).

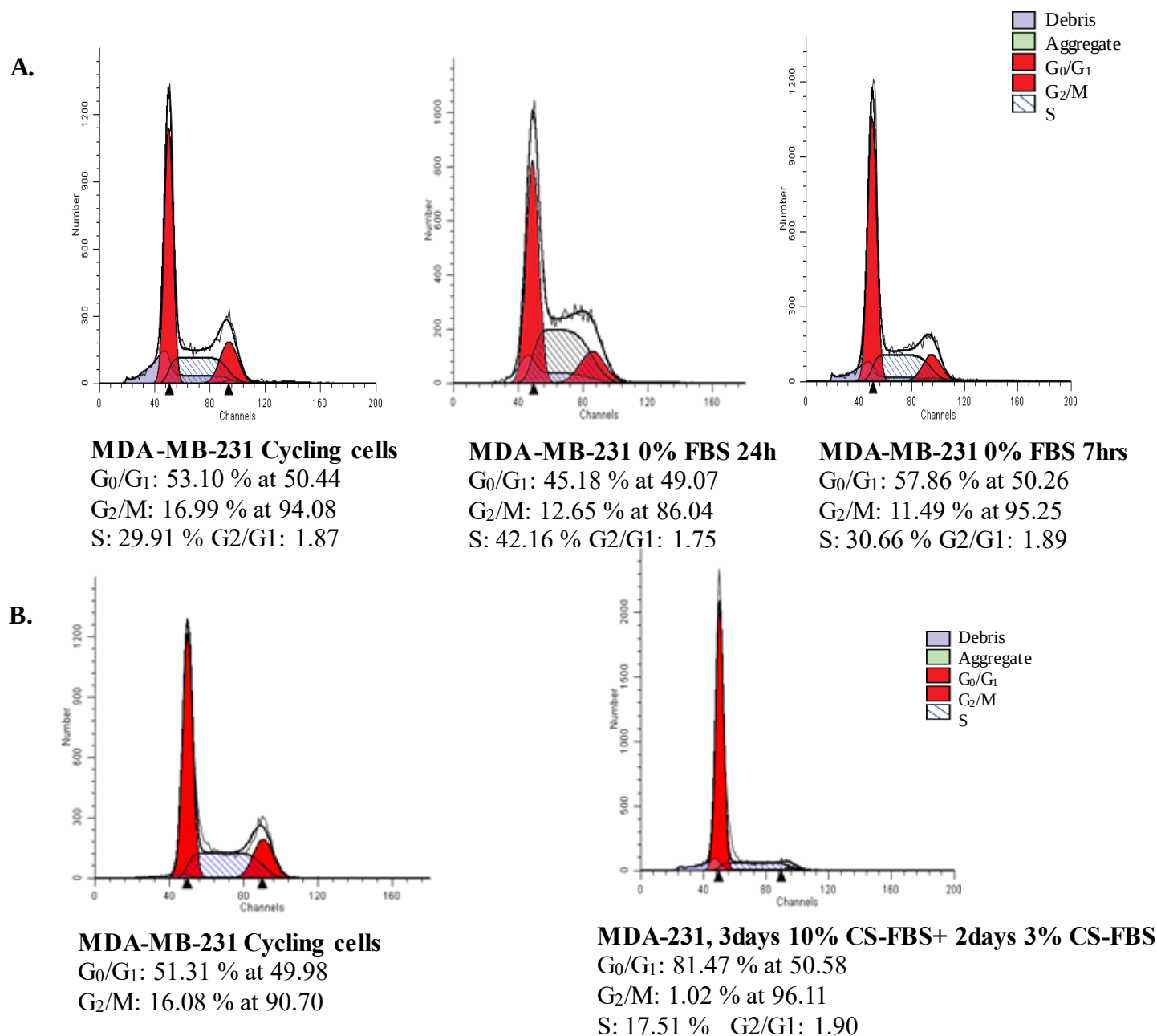


Figure 15. Flow cytometric analysis of serum starved MDA-MB-231 cells

A. (Left to right) Flow cytometric analyses were carried out on MDA-MB-231 cycling cells, 24 hours on cultured cells in L-15 medium without FBS and 72 hours on cultured cells in L-15 medium without FBS. **B.** (Left to right) Flow cytometric analyses were carried out on MDA-MB-231 cycling cells, and 5 days after serum starving cells in 10% charcoal stripped FBS for 2days and 3% charcoal stripped FBS for 3days. Flow cytometric analyses were carried out by Monroe Chan of the ReGen Flow Laboratory.

4.2 TNF α induced the nucleosomal response via the ERK1/2 and p38 MAPK pathways in MDA-MB-231 cells

To show that the nucleosomal response is activated, serum starved MDA-MB-231 cells were stimulated with TNF α for various lengths of time. Histone extracts were then prepared. The histones were resolved on a 15% SDS-PAGE, followed by western blot analysis with anti-H3S10ph and anti-H3S28ph antibodies, using anti-H3 as control. Results demonstrate that H3S10ph and H3S28ph are rapidly and transiently phosphorylated, although it appears that H3S10ph levels are greater than H3S28ph (Fig. 16).

A rapid increase in the phosphorylation of H3S10 would show activation of the nucleosome response pathway. To demonstrate that TNF α induction activates the ERK1/2 and/or p38 MAPK pathways, total cell lysates from serum starved MDA-MB-231 cells stimulated with TNF α for various lengths of time were prepared. The lysates were resolved by 10% SDS-PAGE, followed by western blot analysis with anti-pERK1/2 and anti-p38 antibodies, with anti-ERK1/2 and anti-p38 as controls. Results demonstrate that ERK1/2 and p38 pathways are activated following TNF α stimulation (Fig. 16).

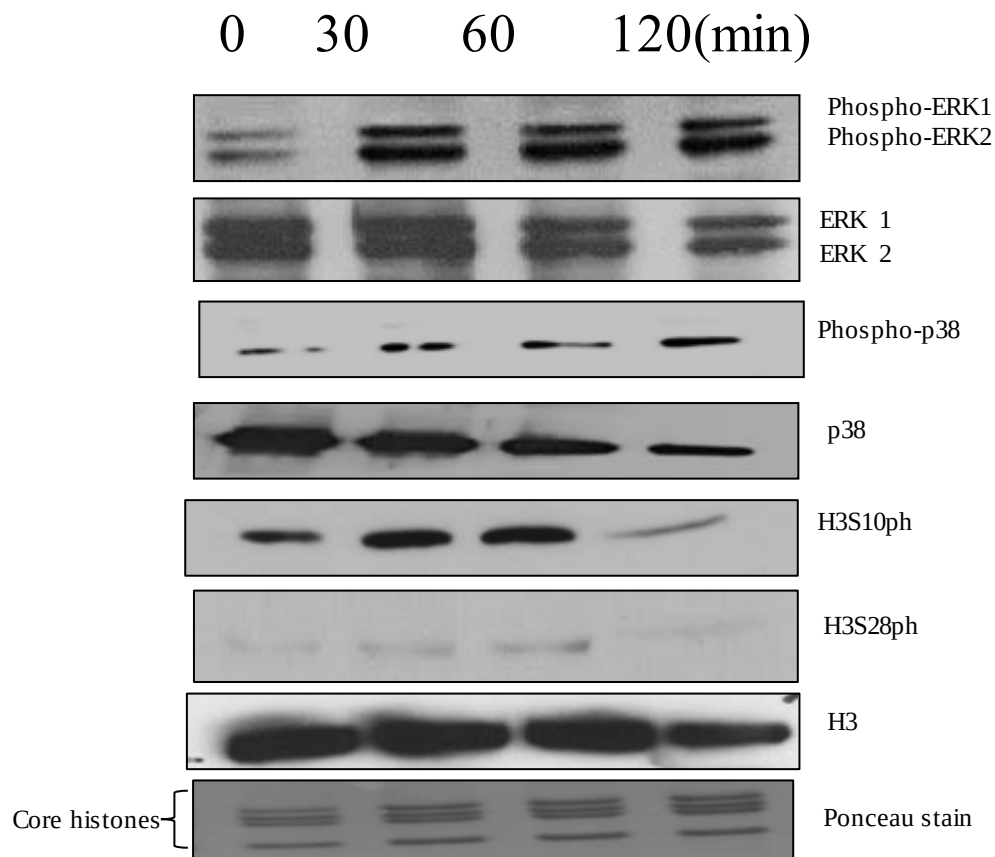


Figure 16. TNF α stimulation correlates with the induction of the nucleosomal response

Serum starved MDA-MB-231 cells were stimulated with TNF α for the indicated lengths of time. Top four panels: Western blots of total cellular proteins showing the relative protein levels of phosphorylated MAP kinases (ERK1/2 and p38). Bottom three panels: Western blots of histone extracts showing the relative protein levels of phosphorylated H3. Last panel: core histones of the same samples stained by Ponceau.

4.3 TNF α induced transcriptional response in MDA-MB-231 cells

4.3.1 Immediate early genes are amongst the strongest induced genes following TNF inductions

To our knowledge, the transcriptional program of TNBC induced by TNF α has not been investigated. Thus, serum starved MDA-MB-231 cells were treated with 20 ng/ml TNF α at 0, 30, 60, 120 minutes, and total RNA from these samples were isolated using the RNeasy Plus Mini Kit (Qiagen) as per the manufacturer's instructions. Ribosomal RNA was then removed from the isolated RNA using the RiboMinus™ Eukaryote Kit v2. By using this protocol, we capture nuclear and cytoplasmic, coding and non-coding RNA. The resulting RNA was then processed for RNA-Seq with the Illumina TruSeq Stranded mRNA Library Prep Kit. During the cDNA library construction, the Ambion ERCC Spike-In control mix 1 was added to standardize the RNA preparations. Library preparation was done by Aleksander Ilic of the Next Generation Sequencing Platform and bioinformatics analyses were done by Dr. Wayne Xu.

TNF α induced 905 genes out of the 28,375 genes found in the human genome and the top induced genes were genes involved in innate immunity and inflammation, followed by immediate early genes (Table. 6).

Table 6. Top genes induced by TNF α with a fold change greater than 2.0 at 30 minutes.

Inflammation and Innate immunity	Immediate early genes
<i>EDN2</i>	<i>FOSB</i>
<i>SLP1</i>	<i>FOS</i>
<i>TNFAIP3</i>	<i>JUNB</i>
<i>PTGS2</i>	<i>JUN</i>
<i>TNFRSF11B</i>	<i>EGR1</i>
<i>IL6</i>	<i>EGR2</i>
<i>CXCL3</i>	<i>EGR3</i>
<i>CXCL8</i>	<i>EGR4</i>
<i>CXCL1</i>	<i>IER1</i>
<i>CSF2</i>	<i>IER2</i>
<i>NFKIB</i>	<i>IER3</i>

4.3.2 Validation of immediate early genes identified from RNA-Seq data

From RNA-Seq data, TNF α induced genes that had the greatest level of induction at 30 and 60 minutes, with roles in tumorigenesis and metastasis were prioritized. The genes selected were: *FOS*, *JUN*, immediate early response 3 (*IER3*) and *IL6* (Fig. 17). Subsequently, these genes were validated by real time RT-qPCR in TNF α -induced MDA-MB-231 cells. As expected, the mRNA expression for these genes followed the same trend as those observed by RNA-Seq track maps (Fig. 18).

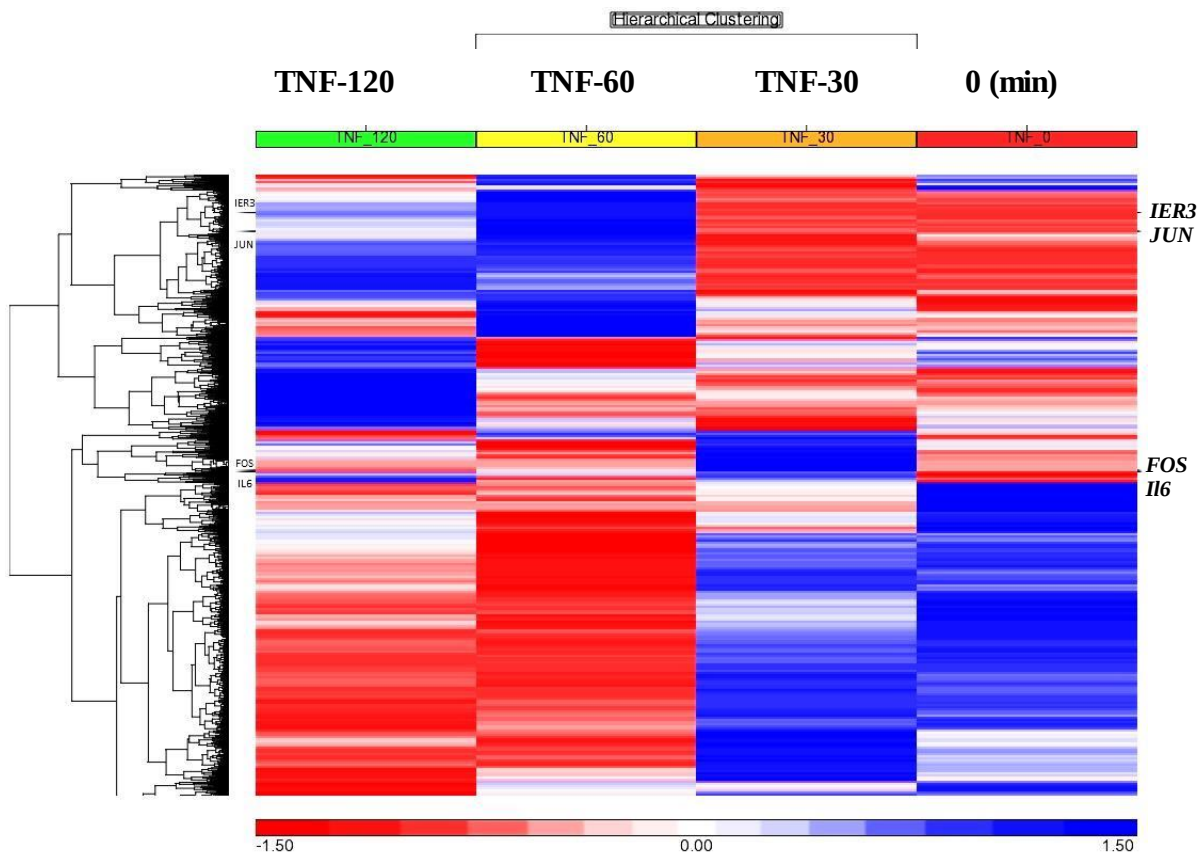
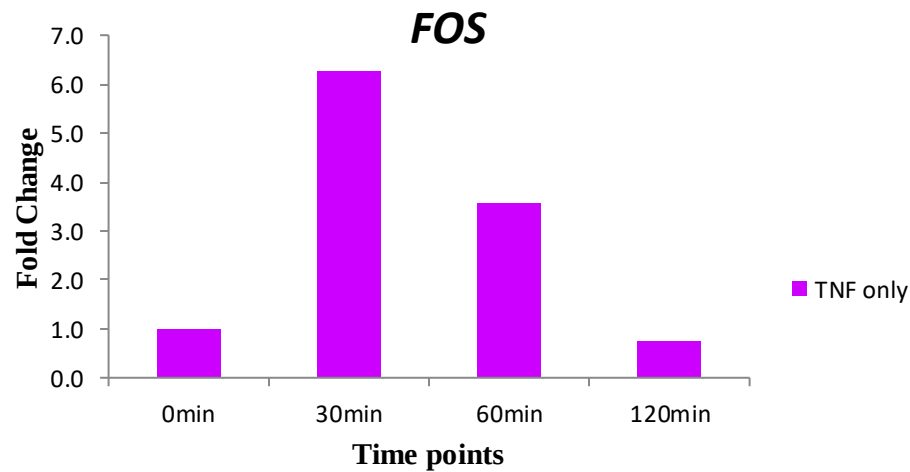


Figure 17. Heat map of TNF α induced MDA-MB-231.

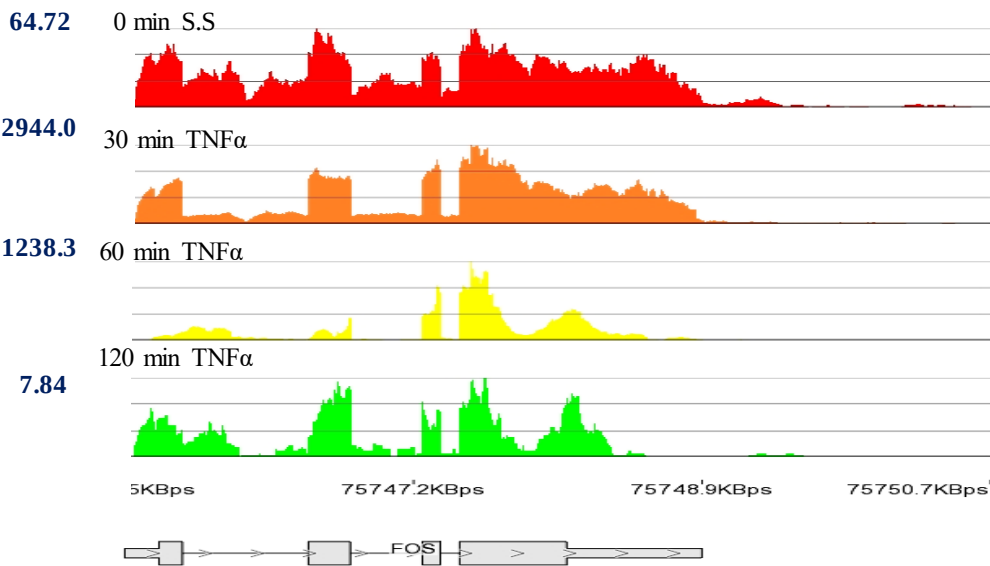
MDA-MB-231 cells were rendered quiescent by serum starvation for five days and then stimulated with TNF α for 30, 60 and 120 minutes. Results from the RNA-seq analyses are presented as a heat

map diagram illustrating average log₂ ratios for genes that were induced greater than 1.5-fold by TNF α . Indicated on the heat map is the location of *FOS*, *JUN*, *IER3* and *IL6*. This figure was provided by Dr. Wayne Xu.

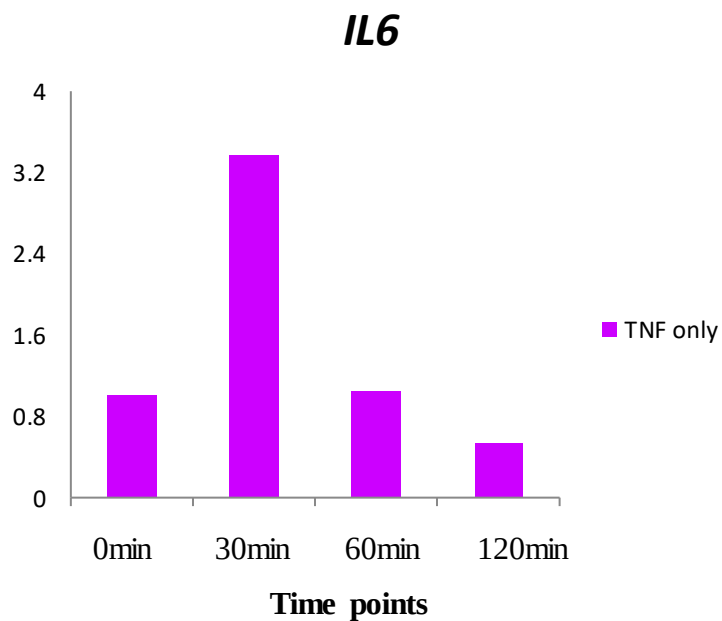
A1.



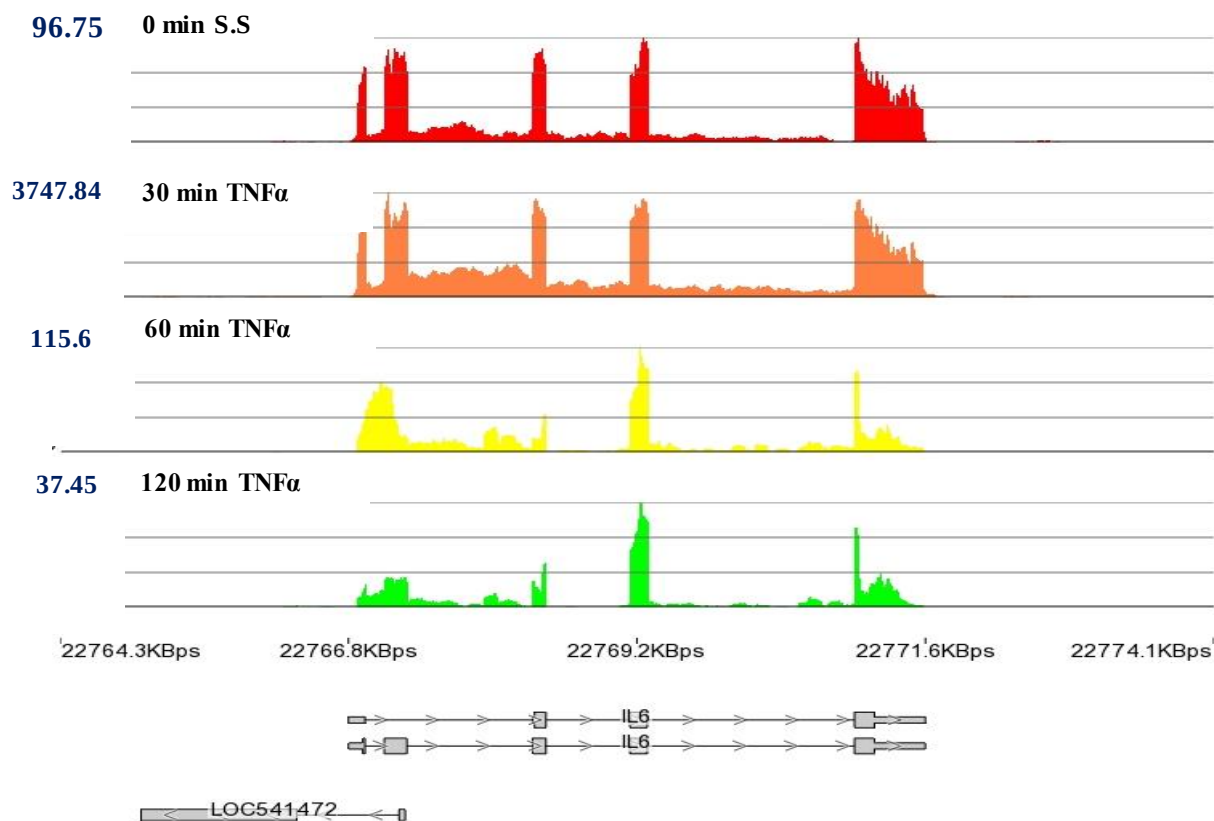
A2.



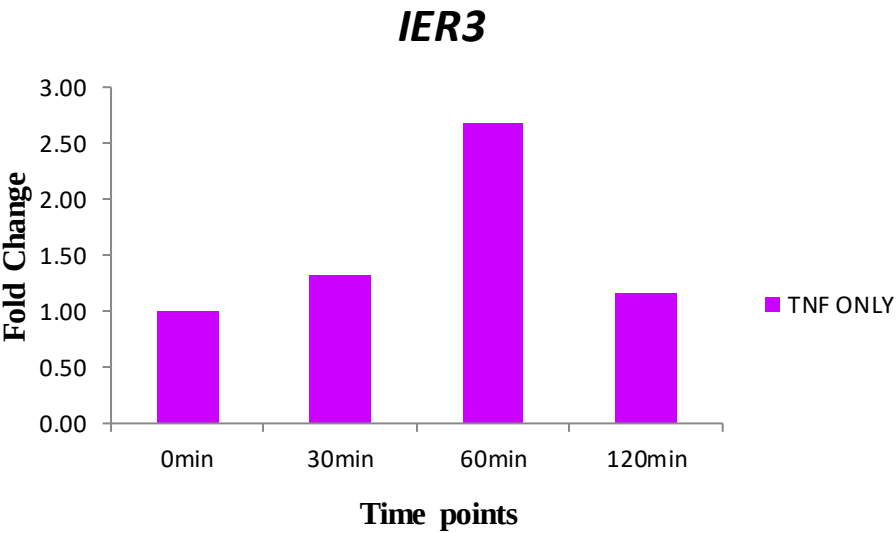
B1.



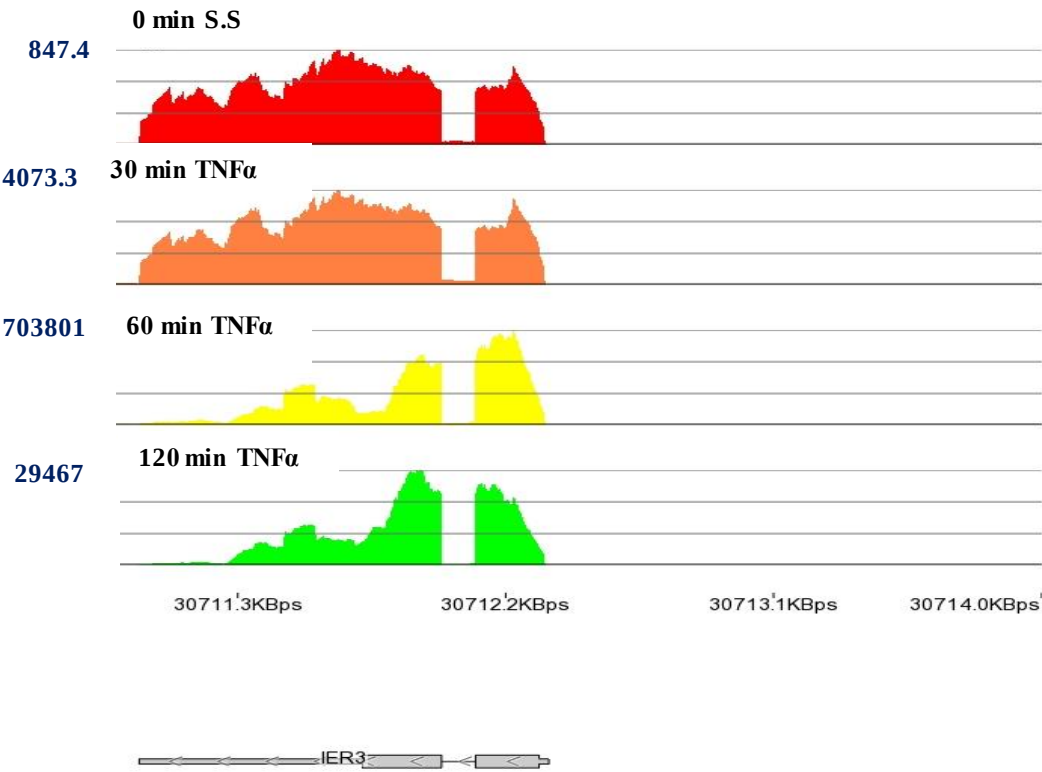
B2.



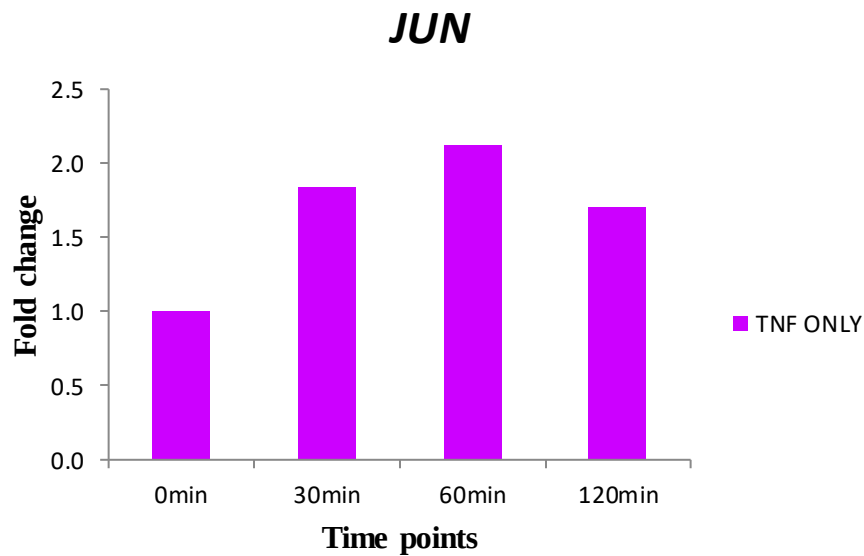
C1.



C2.



D1.



D2.

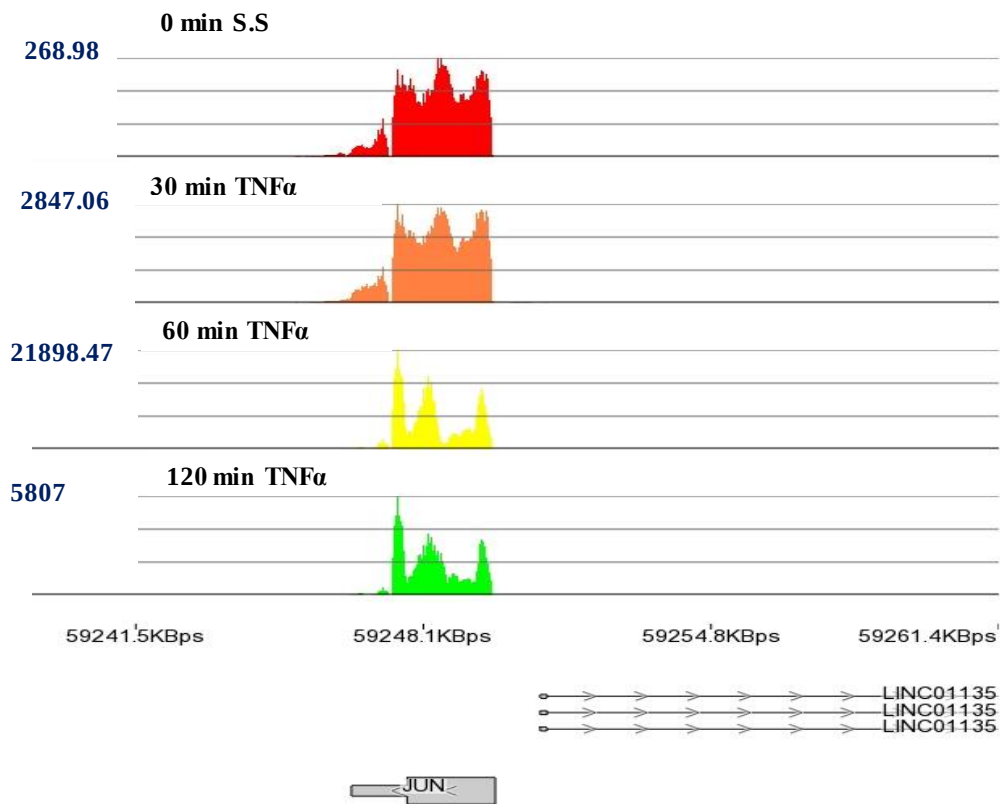


Figure 18. Track maps for selected immediate early genes and RNA-Seq validation.

A1, B1, C1 and D1. Quantitative real-time RT-PCR analyses of serum starved MDA-MB-231 cells stimulated with TNF α , showing the mRNA expression levels of *FOS*, *IL6*, *IER3* and *JUN* at

various lengths of time. Fold change values, normalized to 36B4 levels in untreated and treated samples and to time 0 values. **A2, B2, C2 and D2.** *FOS*, *IL6*, *IER3* and *JUN* track maps generated from RNA-Seq of serum starved MDA-MB-231 cells stimulated with TNF α , showing the temporal expression of transcripts at various lengths of time. The track maps were provided by Dr. Wayne Xu. The peak reads were normalized manually and calculated as follows:

$$\frac{\text{specific read}}{\text{total reads}} = \text{Factor}$$

$$\text{Normalized reads} = \text{Factor} \times \text{specific read}$$

4.4 MSK mediated TNF induction of immediate early genes

4.4.1 MSK inhibitor H89 attenuated TNF induced genes

In mouse fibroblast cells, treatment of cells with MSK inhibitor H89 prevented the EGF- or TPA-induced recruitment of MSK 1 and 2, 14-3-3 proteins and SWI/SNF to the upstream promoter region and enhancer of immediate early genes, preventing their initiation (Drobic *et al.*, 2010; Pérez-Cadahía *et al.*, 2011). Thus, the role of MSK in the TNF α -induced gene program was examined using the MSK inhibitor H89. Cells were serum starved and treated with or without H89 30 minutes prior to TNF α treatment. Quantitative real-time RT-PCR analyses of these cells were then performed and bar charts of the mRNA expression levels of *FOS*, *JUN*, *IL6* and *IER3* were plotted. Results demonstrated that H89 attenuated mRNA levels of *FOS*, *JUN* and *IL6* but not *IER3* (Fig. 19).

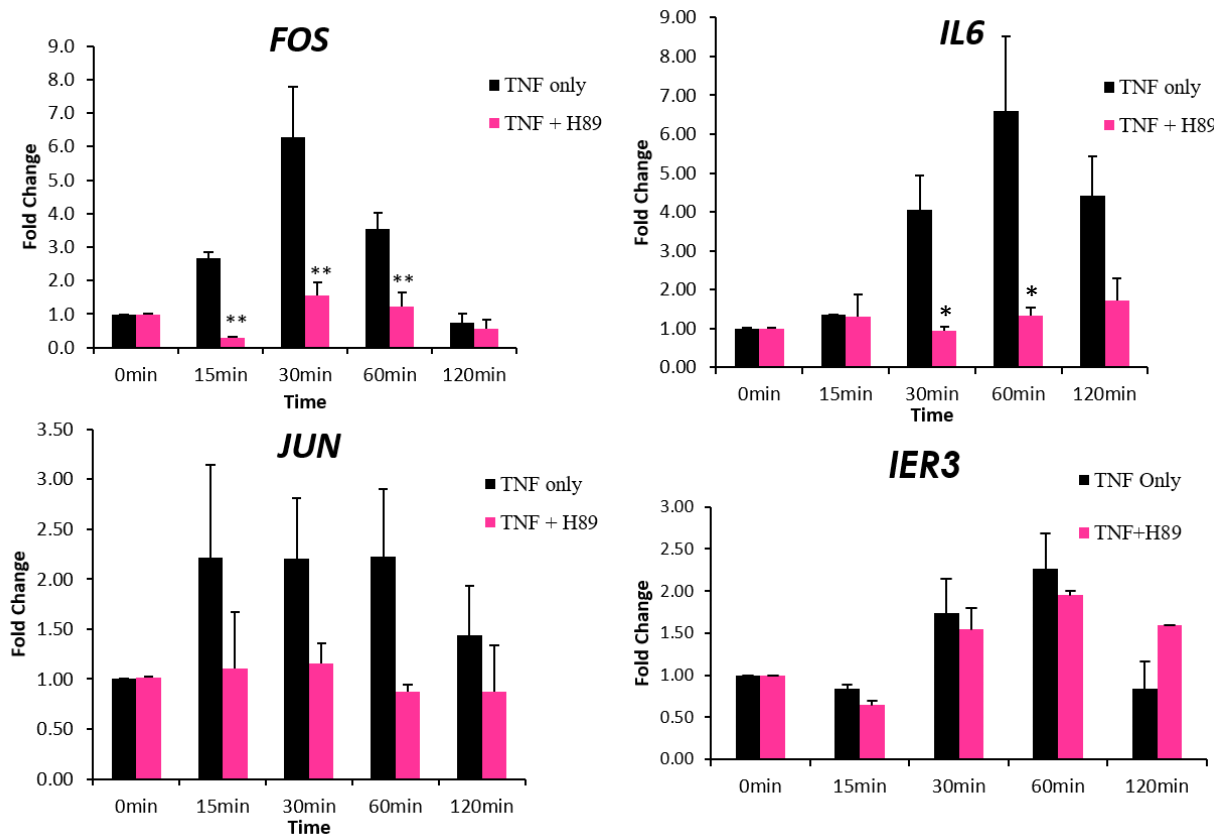


Figure 19. mRNA expression of FOS, JUN, IL6 and IER3 genes with or without H89

Serum-starved MDA-MB-231 cells were pre-treated or not with H89 prior to TNF α stimulation for 0, 15, 30, 60 or 120min. Total RNA was isolated and quantified by real time RT-PCR. Fold change values, normalized to *36B4* levels in untreated and treated samples and to time 0 values are the mean of three independent experiments, and the error bars represent the standard error of mean. ** = $P < 0.01$, * = $P < 0.05$.

4.4.2 PKA inhibitor Rp-cAMPs does not attenuate TNF induced genes

Although H89 is a potent MSK inhibitor, it was originally marketed as a PKA inhibitor (Lochner and Moolman, 2006). Thus, to control for PKA, the PKA inhibitor Rp-cAMPs, which does not inhibit MSK, was used (Thomson *et al.*, 1999). Cells were treated or not treated with 100 nM of Rp-cAMPs 1 hour prior to TNF α treatment as described by (Drobic *et al.*, 2010), and quantitative real-time RT-PCR analyses of these cells were performed. Subsequently bar charts of the mRNA expression levels of *JUN* and *IL6* were plotted. Results demonstrated that Rp-cAMPs does not attenuate *IL6* and *JUN*, except at 120 minutes.

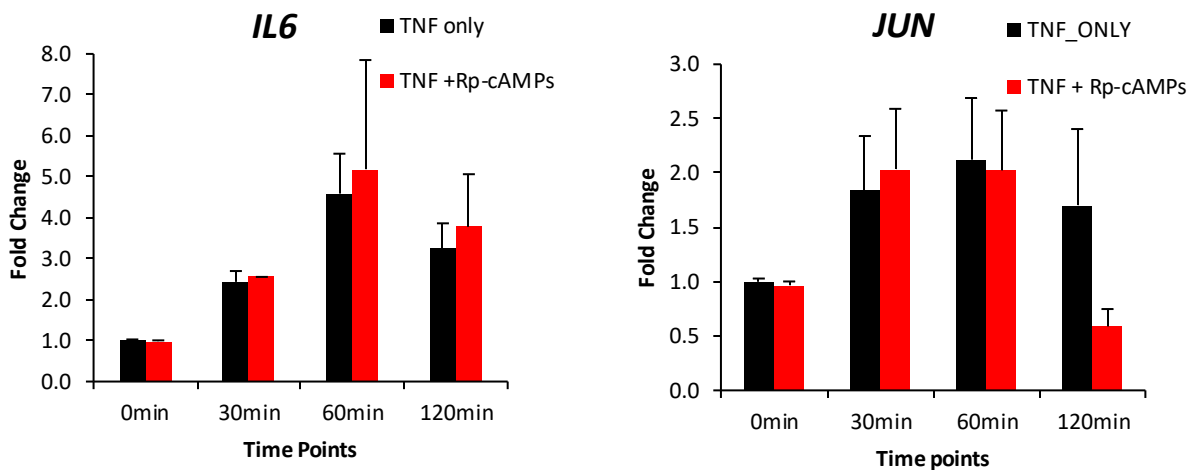


Figure 20. mRNA expression of *IL6* and *JUN* genes with or without PKA inhibitor Rp-cAMPs

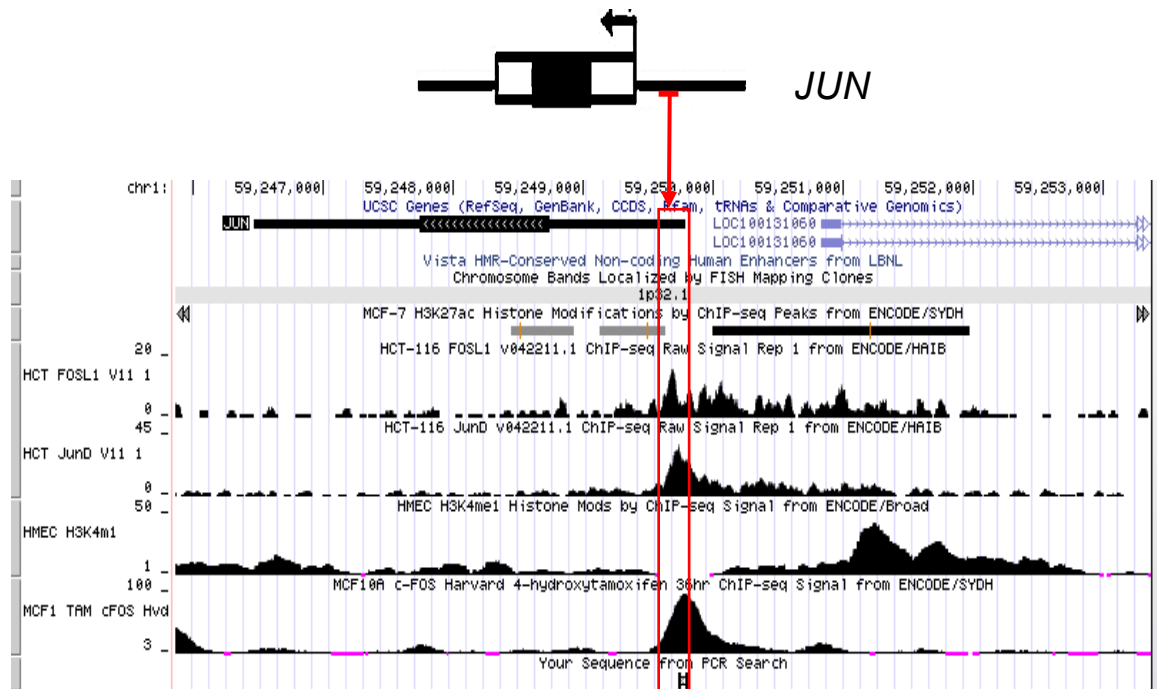
Serum-starved MDA-MB-231 cells were pre-treated or not with Rp-cAMPs prior to TNF α stimulation for 0, 15, 30, 60 or 120 min. Total RNA was isolated and quantified by real time RT-PCR. Fold change values, normalized to *36B4* levels in untreated and treated samples and to time

0 values are the mean of two independent experiments and the error bars represent the standard deviation.

4.5 TNF α induced H3S10ph at the upstream promoter regulatory of TNF α induced genes

High-resolution ChIP assay was used to determine the levels of H3S10ph at the upstream promoter region of selected TNF α induced genes. Following stimulation of serum starved MDA-MB-231 cells with TNF α for 0 and 30 minutes, nuclei from formaldehyde crosslinked cells were isolated, and the chromatin was processed to mononucleosome length with micrococcal nuclease. Antibodies against H3S10ph were used to immunoprecipitate the crosslinked mononucleosomes, ChIP DNA was then isolated and purified after a series of washes, and ChIP qPCR was performed (see section 3.6). Amplicons were selected on ENCODE based on regions enriched for transcription factors such as AP-1 (*FOS* and *JUN* families), which are known to recruit MSK (Fig. 21). H3S10ph is present at the upstream promoter region of the *JUN* and *IL6* genes, and are enriched only in TNF α stimulated cells (Fig. 21 and 22).

A.



B.

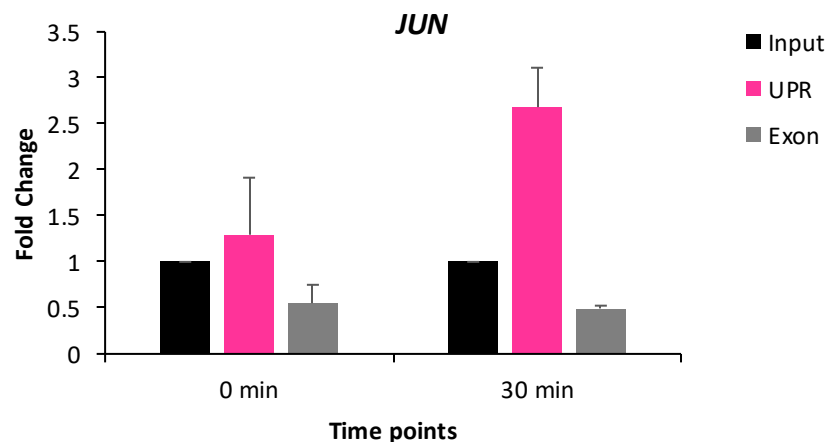


Figure 21. Levels of H3S10ph at the upstream promoter region of JUN.

A. Amplicon location, showing regions enriched for FOSL1 and JUND. The red box shows the exact location of the amplicon in ENCODE. **B.** H3S10ph ChIP assays in uninduced and induced MDA-MB-231 cells at the upstream promoter region (UPR) of *JUN* gene. Serum-starved MDA-MB-231 cells were treated or not treated with $\text{TNF}\alpha$ for 30 min. Formaldehyde crosslinked mononucleosomes were prepared and used in ChIP assays with antibodies against H3S10ph. Equal

amounts of input and immunoprecipitated DNAs were quantified by real-time quantitative PCR. Fold change values, normalized to input level, and then two to the power of the calculated value was used (2ⁿ). Exon represents the negative control region where H3S10ph is not enriched. Bar charts are the mean of two independent experiments with the values 2.07 and 2.68 respectively. The error bars represent the standard deviation.

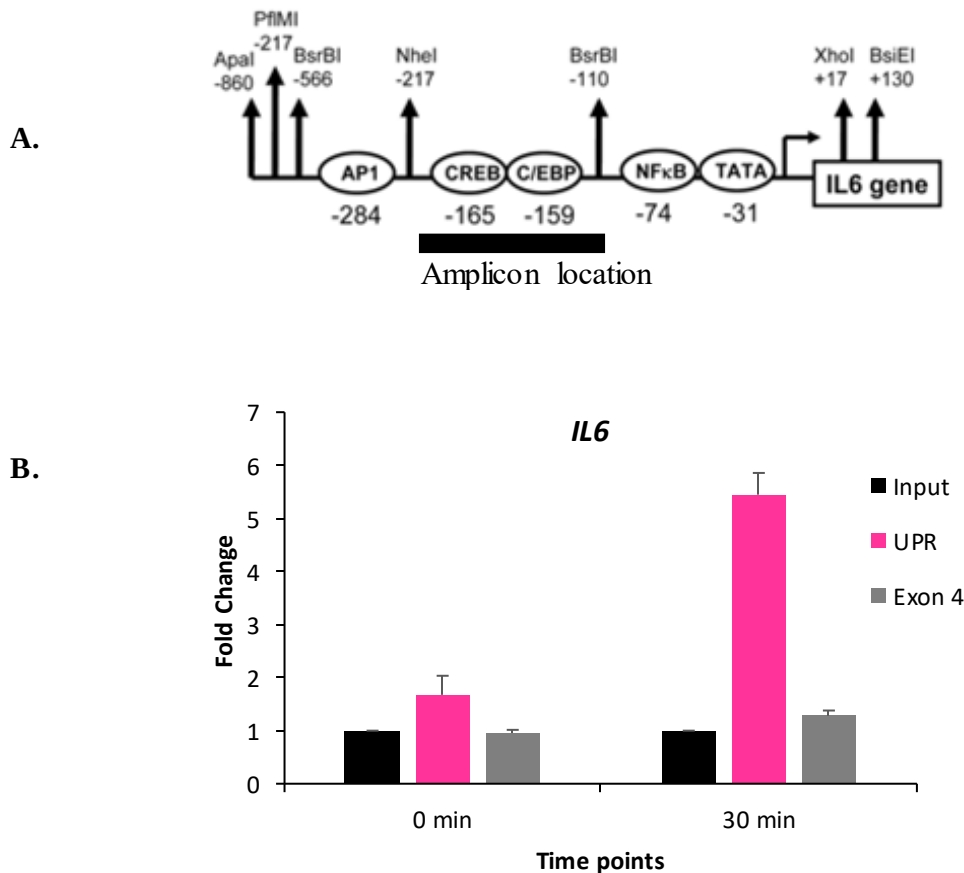


Figure 22. Levels of H3S10ph at the upstream promoter region of *IL6*

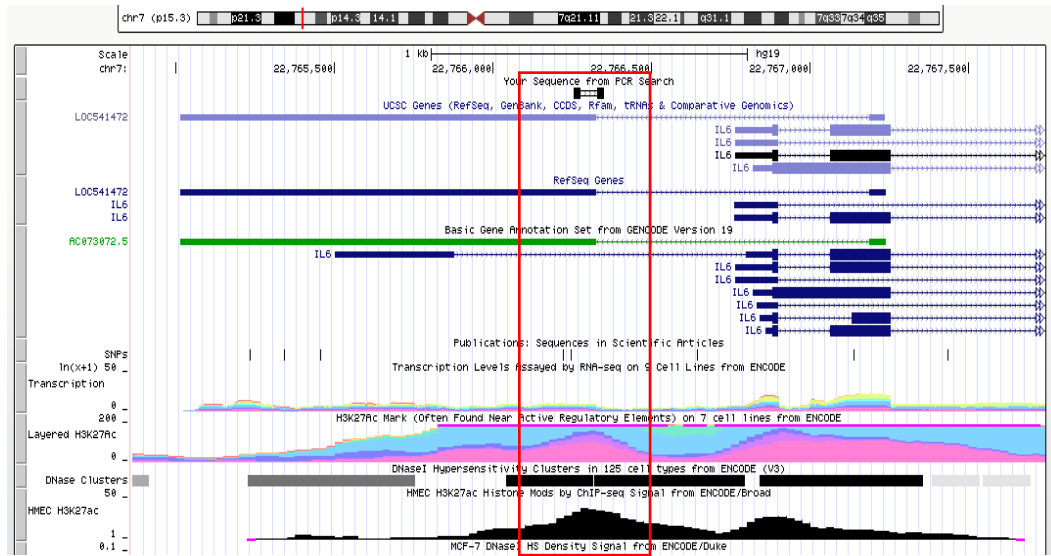
A: Schematic representation of the highly conserved IL-6 promoter region with indication of the various embedded transcription factor and restriction enzyme motifs reproduced with permission from (Ndlovu *et al.*, 2009). **B:** H3S10ph ChIP assays in uninduced and induced MDA-MB-231 cells at the upstream promoter region (UPR) of *IL6* gene. Serum-starved MDA-MB-231 cells were

treated or not treated with TNF α for 30 min. Formaldehyde crosslinked mononucleosomes were prepared and used in ChIP assays with antibodies against H3S10ph. Equal amounts of input and immunoprecipitated DNAs were quantified by real-time quantitative PCR. Fold change values, normalized to input level, and then two to the power of the calculated value was used (2^n). Exon 4 represents the negative control region where H3S10ph is not enriched. Bar charts are the mean of two independent experiments with the values 5.4 and 4.82 respectively. The error bars represent the standard deviation.

4.6 TNF α induced H3K27ac at the upstream promoter and enhancer region of *IL6*

As previously stated, H3K27ac is present at active upstream promoter regions and at active enhancers (Heintzman *et al.*, 2009). Thus, to determine the levels of H3K27ac at the upstream promoter region and putative enhancers of immediate early genes, a candidate approach, using high-resolution ChIP assays was performed. *IL6*, a known pro-tumorigenic cytokine involved in metastasis of a variety of cancers including TNBCs (Ndlovu *et al.*, 2009; Erez *et al.*, 2013), was selected as a candidate gene. Following stimulation of serum starved MDA-MB-231 cells with TNF α for 0 and 30 minutes, nuclei from formaldehyde crosslinked cells were isolated, and the chromatin was processed to mononucleosome length with micrococcal nuclease. Antibodies against H3K27ac were used to immunoprecipitate the crosslinked mononucleosomes, ChIP DNA was then isolated and purified after series of washes and a ChIP qPCR was performed (see section 3.6). Amplicon was selected based on H3K27ac ChIP-Seq data from GEO on MBA-MB-231 cells (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM1204474>). Interestingly H3K27ac was not enriched at the upstream promoter region of *IL6* but was enriched at a putative enhancer region of the gene (Fig 23 and 24).

A.



B.

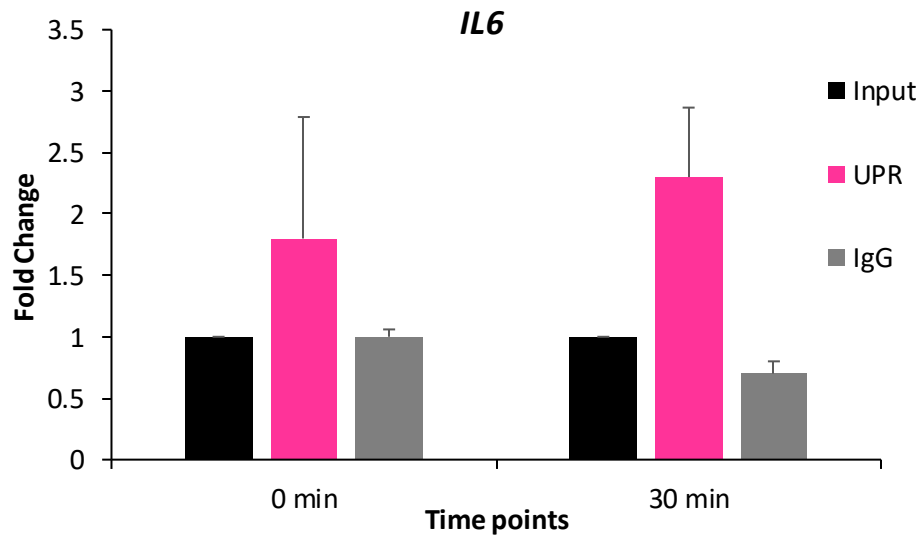
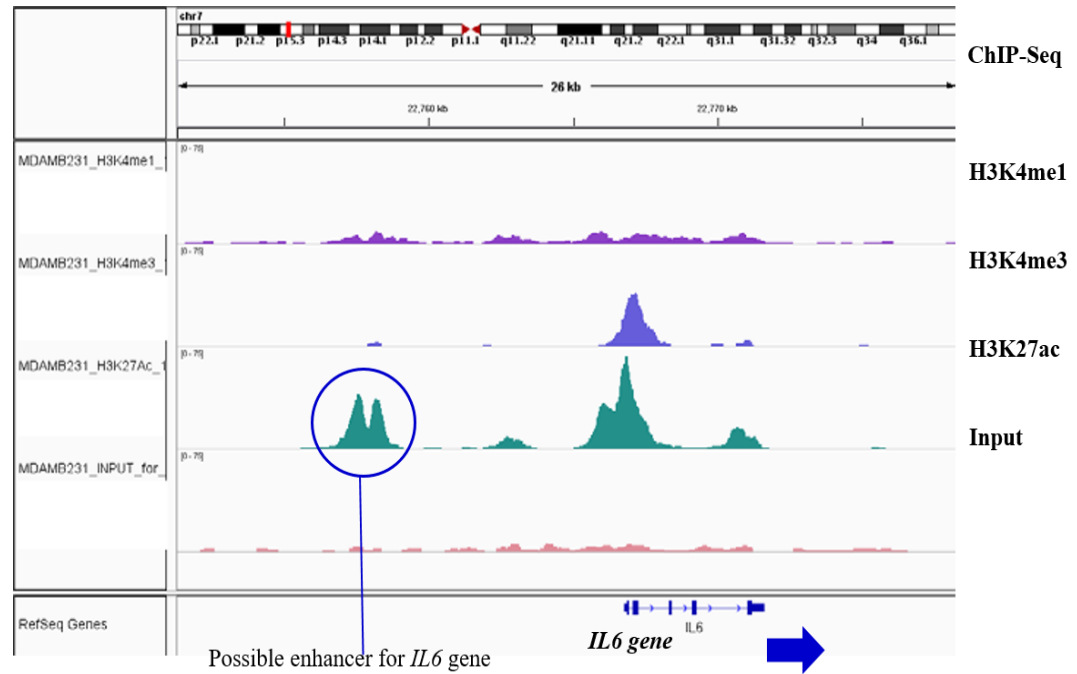


Figure 23. Levels of H3K27ac at the upstream promoter region of *IL6*

A: Amplicon location, showing regions enriched for H3K27ac on ENCODE. The red box shows the exact location of the amplicon. **B:** H3K27ac ChIP assays in uninduced and induced MDA-MB-231 cells at the upstream promoter region (UPR) of *IL6* gene. Serum-starved MDA-MB-231 cells were treated or not treated with $\text{TNF}\alpha$ for 30 min. Formaldehyde crosslinked mononucleosomes

were prepared and used in ChIP assays with antibodies against H3K27ac. Equal amounts of input and immunoprecipitated DNAs were quantified by real-time quantitative PCR. Fold change values, normalized to input level, and then two to the power of the calculated value was used (2^n). IgG represents the negative control pull down using normal rabbit IgG and not H3S10ph antibody. Bar charts are the mean of two independent experiments and the error bars represent the standard deviation.

A.



B.

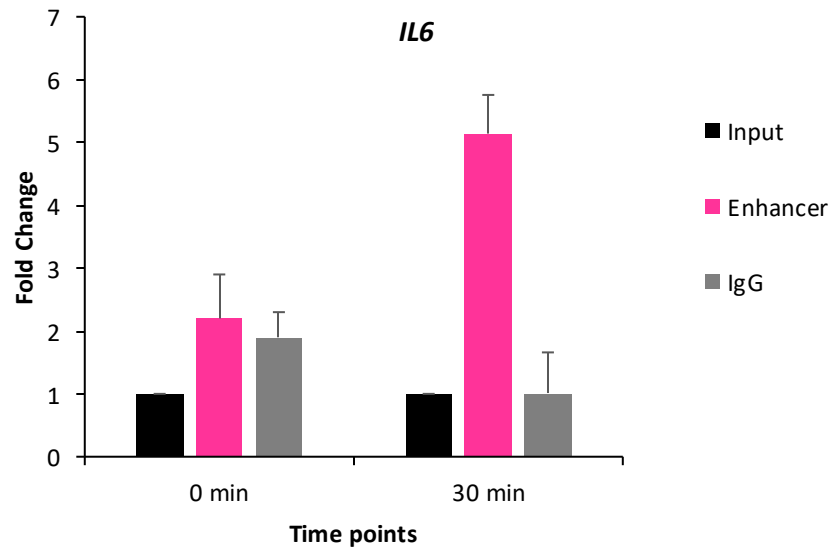


Figure 24. Levels of H3K27ac at a putative enhancer region of *IL6* gene

A: Amplicon location, showing regions enriched with H3K27ac mark on ENCODE. **B:** H3K27ac ChIP assays in uninduced and induced MDA-MB-231 cells at *IL6* gene. Serum-starved MDA-MB-231 cells were treated or not treated with $\text{TNF}\alpha$ for 30 min. Formaldehyde crosslinked

mononucleosomes were prepared and used in ChIP assays with antibodies against H3K27ac. Equal amounts of input and immunoprecipitated DNAs were quantified by real-time quantitative PCR. Fold change values, normalized to input level, and then two to the power of the calculated value (2^n), are the mean of two independent experiments (5.84 and 4.89). The error bars represent the standard deviation.

4.7 Dynamic levels of H3K27ac at a putative enhancer region of *IL6*

Khan et al. 2017 concluded that H3K27ac directs MSK1/2 to phosphorylate H3S28. Thus, to determine if the $\text{TNF}\alpha$ induced H3K27ac is MSK sensitive in MDA-MB-231 cells, a ChIP assay on serum starved cells treated with $\text{TNF}\alpha$, with or without the MSK inhibitor H89 at various lengths of time, at the putative enhancer region of *IL6* was performed. Figure 27 demonstrated that the levels of H3K27ac varied at different time points with the greatest induction observed at 60-minute time point. Furthermore, H89 attenuated the induction of H3K27ac.

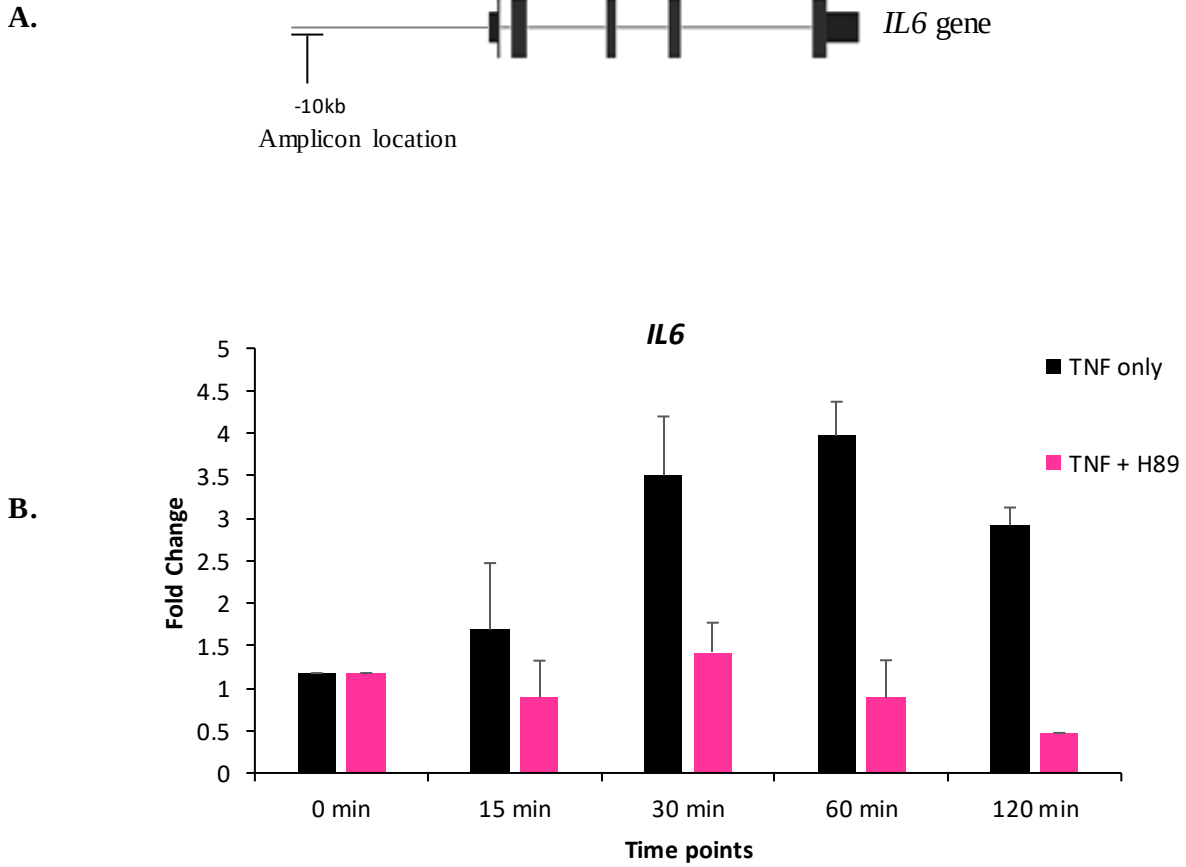


Figure 25. *TNF α* -induced H3K27ac at a putative enhancer region of *IL6*

A. Schematic representation of *IL6* gene showing the region amplified in the ChIP assay. **B.** ChIP experiments were performed using antibodies against H3K27ac on formaldehyde-crosslinked mononucleosomes prepared from serum starved MDA-MB-231 cells treated or not with H89 prior to induction with *TNF α* for 0, 15, 30, 60 and 120 minutes. Equal amounts of input and immunoprecipitated DNA were quantified by real-time quantitative PCR. The bar chart represents the mean of two independent experiments and the error bar represents standard deviation.

CHAPTER FIVE

DISCUSSION

The TNF α induced transcriptional program in MDA-MB-231 cells resulted in the induction of several genes involved in innate immunity and inflammation, as well as several immediate early genes with known roles in tumorigenesis and metastasis. Subsequently, the induction of these genes was attenuated by the MSK inhibitor H89. Furthermore, the regulatory regions of selected immediate early genes showed an increase in the levels of H3S10ph and H3K27ac following TNF α stimulation of cells. Together, these results suggest that the MSK-mediated nucleosomal response pathway, plays a role in the transcriptional program induced by TNF α in MDA-MB-231 cells.

5.1 TNF α induced transcriptional response in MDA-MB-231 cells

The RNA-seq data revealed that of the 28,375 genes in the human genome, 905 genes were induced by TNF α with a fold change greater than 2.0 at 30 minutes in MDA-MB-231 cells. The strongest induced genes involved genes with roles in epithelial to mesenchymal transition, such as: *IGFB4*, *SOX10*, *MMP15*, *IL6* and *SNAI1* and immediate early genes such as, *FOSB*, *FOS*, *JUNB*, *JUN*, *IER1-3*, *EGR1-4* and. This TNF α induced transcriptional response, suggests that patients with mesenchymal stem like subtype of TNBC may benefit from agents that preferentially target immediate early genes, as well as pathways involved in epithelial to mesenchymal transition. However, further validations will need to be done to determine if this signature is ‘prognostic’ or ‘predictive’.

A gene signature should be specific in terms of diagnosis, prognosis or prediction and this specificity should be validated in independent groups of tumors. Subsequently, the signature must be clinically validated to test for correlation with event free survival of patients (Chibon, 2013)

(Fig. 26). Studies with TNBC patient tumors (clinical samples) as well as other TNBC cell lines would be conclusive in demonstrating whether this signature is ‘prognostic’ or ‘predictive’ for patients.

The TNF-induced genes *FOS*, *JUN*, *IER3* and *IL6* were prioritized from RNA-Seq data. Furthermore, validation studies by RT-qPCR of these selected genes correlated with RNA-Seq data, as the same pattern of induction of genes was observed in both data (Fig. 18). *FOS* is not functional as an independent transcription factor and must heterodimerize with members of the *JUN* family to form the transcription factor AP-1. *FOS* promotes tumor initiation through the down-regulation of tumor suppressor genes through the induction of DNA methylation and it directly regulates several genes involved in tumor invasion such as *MMP1*, *3* and *9* (Healy, Khan and Davie, 2012). In SKBR3 (HER2 positive breast cancer cell line) cells, *JUN* was shown to upregulate vascular endothelial growth factor and *CD44* involved in metastasis (Zhang *et al.*, 2007). *IL6* has been shown to promote repression of E-Cadherin and overexpression of vimentin, N-cadherin and Snail, which are phenotypes of epithelial to mesenchymal transition (Dethlefsen, Højfeldt and Hojman, 2013). *IER3* promoted colony formation of NIH 3T3 cells in soft agar assays and it was shown to have higher expression levels in invasive ductal carcinoma compared with normal samples (Yang, 2006).

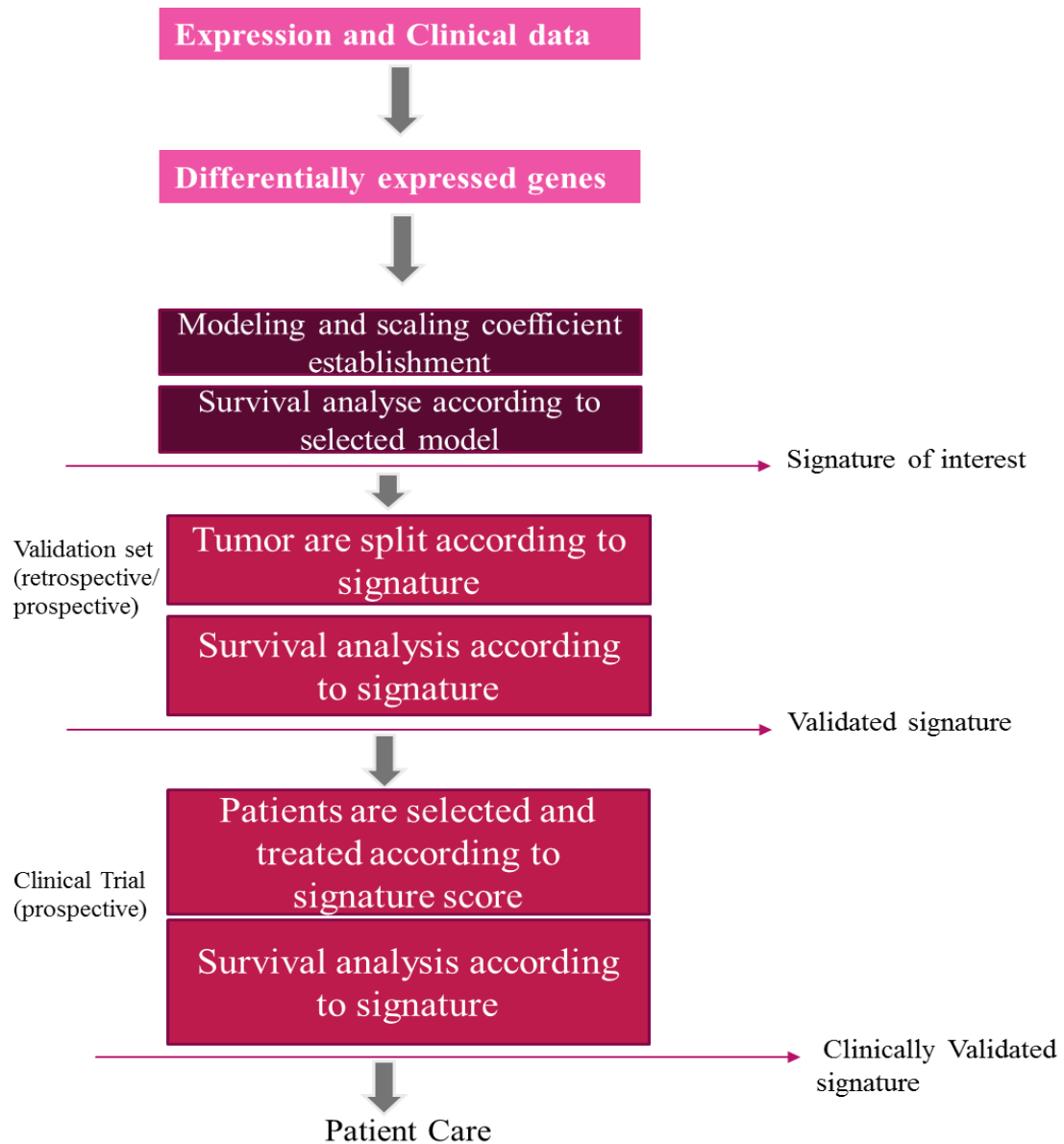


Figure 26. Approaches leading to clinical validation.

The workflow to clinical validation demonstrating the three steps required. These include: signature identification (training set), signature validation (validation set) and finally the clinical validation. Based on (Chibon, 2013).

5.2 TNF α induced nucleosomal response and MSK-mediated induction of immediate early genes in MDA-MB-231 cells.

TNF α stimulation induced a rapid yet transient increase in the levels of H3S10ph and H3S28ph, although the levels of H3S28ph appeared lower than H3S10ph. Espino *et al.* and others have shown that the mitogenic stimulation of human cells (MCF 7, Hela and pancreatic cells) resulted in the induction of H3S10ph and not H3S28ph (Espino *et al.*, 2006, 2009). Western blot analyses suggest that the predominant H3 phosphorylation present in MDA-MB-231 cells is H3S10ph. The H3S28ph antibody was tested for epitope specificity under dot blot conditions, and it recognized the H3S28ph peptide at a concentration of 0.4 μ g, suggesting that the antibody had no issues binding its target epitope. However, different antibodies have different binding affinities for their epitope. Therefore, it could be that the H3S10ph antibody has a greater binding affinity than the H3S28ph antibody. Mass spectrometric analysis could be used to quantify the levels of phosphorylated H3 in MDA-MB-231 cells. Nevertheless, the in-source fragmentations that occur with phosphorylation make phosphorylation very difficult to detect using this technique.

This TNF α induced nucleosomal response may be mediated by the ERK1/2 and p38 MAPK pathways, as TNF stimulation resulted in the activation of phospho-ERK and -p38. Interestingly, H89 does not affect the levels of activated ERK (Appendix, Fig 1). This suggests that H89 may act directly on activated MSK by blocking its ability to phosphorylate histone H3. These data support the results observed in mouse 10T1/2 fibroblast cells published by Thomson *et al.*, 1999. At 10 μ M the H89 inhibits MSK, PKA, S6K1, ROCK II and PKB α with differing IC₅₀ (Davies *et al.*, 2000). However, ligand binding of TNF α to its receptor leads to activation of NF- κ B, JNK, p38 MAPK, ERK and PI3K pathways (Balkwill, 2006) and studies using several kinase

inhibitors have shown that the nucleosomal response is via the ERK and p38 MAPK pathways and not JNK or PI3K (Hazzalin *et al.*, 1996; Deak *et al.*, 1998; Thomson *et al.*, 1999). Furthermore, MSK has been shown to directly activate transcription factors such as NF- κ B (Thomson *et al.*, 1999; Soloaga *et al.*, 2003; Ndlovu *et al.*, 2009; Healy *et al.*, 2012). Altogether, the use of H89 as a selective MSK inhibitor in a TNF-induced system is justifiable.

The effector kinase mediating the nucleosomal response was controversial. MSK 1/2 and RSK1/2 were the two kinases found to be activated by the MAPK pathways, although RSK1/2 are only activated by ERK and not p38 pathways (Deak *et al.*, 1998). However, using MSK 1/2 knockout strategy, it was demonstrated that MSK and not RSK was responsible for phosphorylating H3, as RSK1/2 knockouts failed to block the induction of H3S10ph (Soloaga *et al.*, 2003). Furthermore, the use of H89 as a MSK inhibitor, was debatable. This is because H89 is also a PKA inhibitor. Moreover, PKA has been shown to phosphorylate H3S10 (Lochner and Moolman, 2006). Therefore, its inhibitory effects cannot be ascribed to MSK alone. Mahadevan and colleagues demonstrated with the use of the PKA inhibitor Rp-cAMPs that the inhibitory effects of H89 on H3 phosphorylation is not due to the inhibition of cAMP-dependent kinase by H89, as Rp-cAMPs failed to inhibit the nucleosomal response even at concentrations as high as 1mM (Thomson, Louis and Clayton, 1999). Furthermore, I have demonstrated that Rp-cAMPs did not attenuate the TNF α induced immediate early genes in MDA-MB-231 cells whereas H89 did. This observation suggests that MSK and not PKA is involved in immediate early gene induction. Nonetheless, MSK can directly activate transcription factors that induce these genes without resulting in the nucleosomal response. Thus, to show the involvement of the nucleosomal response, ChIP assays for H3S10ph at the upstream promoter regions of select genes were performed and results demonstrated the presence and induction of H3S10ph at the upstream promoter region of

immediate early genes following TNF α stimulation in MDA-MB-231 cells, further providing evidence of MSK involvement. These results corroborate those reported by Ndlovu et al. 2009, which showed that the proximal *IL6* promoter in MDA-MB-231 cells were enriched with H3S10ph and MSK 1. By ChIP assays in MDA-MB231 cells, they found strongly elevated levels of H3S10K14 phosphoacetylation concomitantly with the recruitment of the histone lysine acetyltransferases CBP and p300, as well as MSK 1, and the chromatin-remodeling factor Brg1 at the *IL6* promoter region during *IL6* gene transcription. In contrast, in MCF7 cells, the levels of MSK1 and H3S10K14 phosphoacetylation were lower.

5.3 TNF α induced H3K27ac at the upstream promoter region and enhancer region of *IL6*

The Davie group had previously characterised MSK as a multiprotein complex containing p300 and PCAF, which is recruited to the regulatory regions of immediate early genes. Furthermore, performing inhibition and co-immunoprecipitation assays, they showed the acetylation state of H3K27 dictates the serine residue MSK phosphorylates (Drobic *et al.*, 2010; Khan *et al.*, 2017). That is, if H3K27 is acetylated, MSK phosphorylates H3S28 whereas if H3K27 is not acetylated, MSK phosphorylates H3S10.

H3K27ac is indicative of an active enhancer and promoter and to date most studies have focused on the MSK-mediated nucleosomal response at upstream promoter region, but not enhancers of genes induced by serum or other agents. Nonetheless, it has been shown that immediate early gene activation involves enhancers that regulates their gene expression (Bahrami and Drabløs 2016, and Khan and Davie 2013). Hence, ChIP assays for H3K27ac at the regulatory regions of *IL6* were performed. *IL6* acts as a growth factor for many tumor cells and it has been

associated with poor prognosis in breast cancer patients (van't Veer *et al.*, 2002). Additionally TNF α induced *IL6* expression requires activation of MSK 1 to elicit selective chromatin relaxation at the *IL6* promoter, upon H3S10ph (Vermeulen *et al.*, 2003). I have shown that TNF stimulation of MDA-MB-231 cells did not result in an increase in H3K27ac at the upstream promoter region of *IL6*. In contrast, there was an increase in H3K27ac at the putative enhancer region, consistent with studies that demonstrate immediate early gene activation involves enhancers and I have shown for the first time in a triple-negative breast cancer inducible system that this H3k27ac dynamics (change in levels of H3K27ac from serum starvation to when induction happens) was H89 responsive, suggesting MSK sensitivity.

One possible mechanism to why H3S10ph appears to be more than H3S28ph in TNF α stimulated MDA-MB-231 cells, could be the levels of CBP/p300 or PCAF associated with the MSK complex (Fig. 26). As previously mentioned (see section 1.6.2), CBP/p300 acetylates H3K27 while PCAF (GCN5/p300/CBP-associated factor) acetylates H3K9 and co-immunoprecipitation studies in human fibroblast cells have demonstrated that H3K9ac goes together with H3S10ph while H3K27ac goes with H3S28ph (Khan *et al.*, 2017). Furthermore, it was shown that H3K27ac is required for MSK to phosphorylate H3S28. The lack of increase in H3K27ac at the upstream promoter region of induced *IL6* gene suggest that the levels of PCAF in the MSK complex might be higher than the levels of CBP/p300 within the complex. Nevertheless, ChIP assays with CBP/p300 and PCAF with their inhibitors would provide more insight and be conclusive.

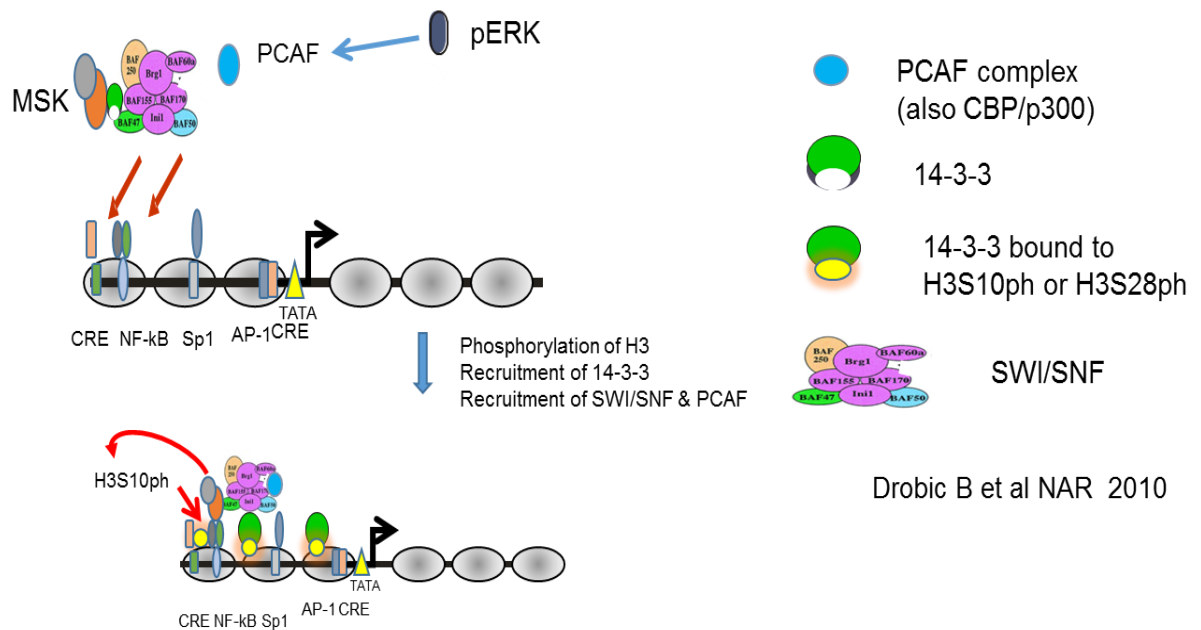


Figure 27. Mechanism of MSK-mediated nucleosomal response and induction of immediate early genes.

MSK (shown as orange oval) is in a multiprotein complex with PCAF or CBP/p300, 14-3-3 proteins and the chromatin remodeler SWI/SNF. The grey ovals along the DNA represent nucleosomes, the NF- κ B transcription factor (p65/p50 dimer) is shown as a purple/green pair of ovals binding to the NF- κ B binding site (blue oval). In response to the activation of the ERK and p38 MAPK pathways, the MSK1 multiprotein complex is recruited to the regulatory regions of immediate early genes by transcription factors such as Elk1, NF- κ B or C/EBP β . MSK1 phosphorylates either H3 S10 or H3 S28, with H3S10ph being shown (yellow circles) depending on lysine acetyltransferase in the complex. H3S10ph and H3S28ph recruit 14-3-3 proteins, which mediate the recruitment of the chromatin remodeler SWI/SNF, with BRG1 being the ATPase subunit. The ensuing remodeling of promoter nucleosomes allows the access of transcription factors such as AP-1 to the promoter target sequences. The pre-initiation complex is recruited at the TATA box (yellow triangle), and initiation of transcription.

5.4 Significance

MSK enzymes play a role in the induction of several inflammatory and immediate early genes relevant to oncogenic processes. Thus, targeting MSK enzymes may lead to a reduction in the expression levels of these genes and thereby reducing tumor formation. Knowledge from this project explores the molecular mechanisms that induce the genes involved in cancer proliferation and metastasis. This study also suggests that MSK plays a role in the TNF-induced transcriptional program in TNBC, suggesting that MSK could be pursued as a therapeutic target in TNBC.

5.5 Future Directions

To further investigate the MSK-mediated nucleosomal response in the transcriptional program induced by TNF α in TNBC, several experiments are proposed. Firstly, RNA-Seq of MSK knockdown MDA-MB-231 cells treated with TNF α , together with RNA-Seq with/without MSK inhibitor H89, will provide a better understanding on the role of MSK. Secondly, it is vital to assay for the levels of MSK in different TNBC, as studies show that MSK 1 is overexpressed in more than 40% of colorectal cancer patients and is associated with poor prognosis (Xinhui *et al.*, 2017). Thirdly, there is a need to investigate by co-immunoprecipitation if H3K27ac goes together with H3S28ph or H3S10ph in MDA-MB-231 cells, as previous studies were done in fibroblast cells. A ChIP-Seq will also provide a global distribution of H3K27ac, as the lack of increase in H3K27ac at the upstream promoter region of *IL6* may be as a result of the amplicon location.

Moreover, there is a need to further investigate clinical samples and other triple-negative breast cancer cell lines such as, MDA-MB-468, HCC70, HCC187, BT-549, and MDA-MB-453 which represent all subtypes of triple-negative breast cancers, as this will give a complete understanding of the role of MSK in these tumors.

REFERENCES

- Aggarwal, B. B. (2003) 'Signalling pathways of the TNF superfamily: a double-edged sword.', *Nature reviews. Immunology*, 3(9), pp. 745–56.
- Ananieva, O., Darragh, J., Johansen, C., Carr, J. M., McIlrath, J., Park, J. M., Wingate, A., Monk, C. E., Toth, R., Santos, S. G., Iversen, L. and Arthur, J. S. C. (2008) 'The kinases MSK1 and MSK2 act as negative regulators of Toll-like receptor signaling.', *Nature immunology*, 9(9), pp. 1028–36.
- Arranz, E. E., Vara, J. Á. F., Gámez-Pozo, A. and Zamora, P. (2012) 'Gene signatures in breast cancer: current and future uses.', *Translational oncology*. Neoplasia Press, 5(6), pp. 398–403.
- Arthur, J. S. (2004) 'Mitogen- and stress-activated protein kinase 1 mediates cAMP response element-binding protein phosphorylation and activation by neurotrophins', *J. Neurosci.*, 24(18), pp. 4324–4332.
- Arthur, J. S. C. and Cohen, P. (2000) 'MSK1 is required for CREB phosphorylation in response to mitogens in mouse embryonic stem cells', *FEBS Letters*, 482(1–2), pp. 44–48.
- Bahrami, S. and Drabløs, F. (2016) 'Gene regulation in the immediate-early response process', *Advances in Biological Regulation*, 62, pp. 37–49.
- Bain, J., Plater, L., Elliott, M., Shpiro, N., Hastie, C. J., Mclauchlan, H., Klevernic, I., Arthur, J. S. C., Alessi, D. R. and Cohen, P. (2007) 'The selectivity of protein kinase inhibitors: a further update', *Biochemical Journal*, 408(3), pp. 297–315.
- Balkwill, F. (2006) 'TNF- α in promotion and progression of cancer', *Cancer and Metastasis Reviews*, 25(3), pp. 409–416.

- Bannister, A. J. and Kouzarides, T. (2011) 'Regulation of chromatin by histone modifications.', *Cell research*. Nature Publishing Group, 21(3), pp. 381–95.
- Barratt, M. J., Hazzalin, C. A., Cano, E. and Mahadevan, L. C. (1994) 'Mitogen-Stimulated Phosphorylation of Histone H3 is Targeted to a Small Hyperacetylation-Sensitive Fraction', *Source: Proceedings of the National Academy of Sciences of the United States of America Cell Biology*, 9117425038(91), pp. 4781–4785.
- Beck, I. M. E., Vanden Berghe, W., Vermeulen, L., Bougarne, N., Vander Cruyssen, B., Haegeman, G. and De Bosscher, K. (2008) 'Altered subcellular distribution of MSK1 induced by glucocorticoids contributes to NF-kappaB inhibition.', *Embo J*, 27(12), pp. 1682–93.
- Ben-Baruch, A. (2012) 'The Tumor-Promoting Flow of Cells Into, Within and Out of the Tumor Site: Regulation by the Inflammatory Axis of TNF α and Chemokines.', *Cancer microenvironment : official journal of the International Cancer Microenvironment Society*, 5(2), pp. 151–64.
- Berger, S. L. (2007) 'The complex language of chromatin regulation during transcription', *Nature*, 447(7143), pp. 407–412.
- Bianchini, G., Balko, J. M., Mayer, I. A., Sanders, M. E. and Gianni, L. (2016) 'Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease.', *Nature reviews. Clinical oncology*. Nature Publishing Group, 13(11), pp. 674–690.
- Bosch, A., Eroles, P., Zaragoza, R., Viña, J. R. and Lluch, A. (2010) 'Triple-negative breast cancer: Molecular features , pathogenesis , treatment and current lines of research', *Cancer Treatment Reviews*. Elsevier Ltd, 36(3), pp. 206–215.

Carey, L. A., Perou, C. M., Livasy, C. A., Dressler, L. G., Cowan, D., Conway, K., Karaca, G., Troester, M. A., Tse, C. K., Edmiston, S., Deming, S. L., Geradts, J., Cheang, M. C. U., Nielsen, T. O., Moorman, P. G., Earp, H. S. and Millikan, R. C. (2006) 'Race, Breast Cancer Subtypes, and Survival in the Carolina Breast Cancer Study', *JAMA. American Medical Association*, 295(21), p. 2492.

Chandramohan, Y., Droste, S. K., Arthur, J. S. C. and Reul, J. M. H. M. (2008) 'The forced swimming-induced behavioural immobility response involves histone H3 phospho-acetylation and c-Fos induction in dentate gyrus granule neurons via activation of the N-methyl-D-aspartate/extracellular signal-regulated kinase/mitogen- and stress-a', *European Journal of Neuroscience*, 27(10), pp. 2701–2713.

Chang, H. Y., Nuyten, D. S. A., Sneddon, J. B., Hastie, T., Tibshirani, R., Sørlie, T., Dai, H., He, Y. D., Van, L. J., Bartelink, H., Rijn, M. Van De and Brown, P. O. (2005) 'Robustness , scalability , and integration of a wound-response gene expression signature in predicting breast cancer survival', *PNAS*, 102(10), pp. 3738–3743.

Chang, H. Y., Sneddon, J. B., Alizadeh, A. A., Sood, R., West, R. B., Montgomery, K., Chi, J., Rijn, M. Van De, Botstein, D. and Brown, P. O. (2004) 'Gene Expression Signature of Fibroblast Serum Response Predicts Human Cancer Progression : Similarities between Tumors and Wounds', 2(2), pp. 206–214.

Chang, S., Iversen, L., Kragballe, K., Arthur, J. S. C. and Johansen, C. (2011) 'Mice lacking MSK1 and MSK2 show reduced skin tumor development in a two-stage chemical carcinogenesis model.', *Cancer investigation*, 29(3), pp. 240–5.

Chibon, F. (2013) 'Cancer gene expressio n signatures – The rise and fall?', *European Journal of*

Cancer, 49, pp. 2000–2009.

Clayton, A. L., Rose, S., Barratt, M. J. and Mahadevan, L. C. (2000) ‘Phosphoacetylation of histone H3 on c- *fos* - and c- *jun* -associated nucleosomes upon gene activation’, *The EMBO Journal*. John Wiley & Sons, Ltd, 19(14), pp. 3714–3726.

D’Anello, L., Sansone, P., Storci, G., Mitrugno, V., D’Uva, G., Chieco, P. and Bonafé, M. (2010) ‘Epigenetic control of the basal-like gene expression profile via Interleukin-6 in breast cancer cells.’, *Molecular cancer*. BioMed Central Ltd, 9(1), p. 300.

Davie, J. R. (2003) ‘MSK1 and MSK2 Mediate Mitogen- and Stress-Induced Phosphorylation of Histone H3: A Controversy Resolved’, *Science Signaling*, 2003(195), p. pe33-pe33.

Davie, J. R. and Spencer, V. a (1999) ‘Control of histone modifications.’, *Journal of cellular biochemistry*, Suppl 32-3, pp. 141–148.

Davie, J. R. and Spencer, V. A. (2001) ‘Signal transduction pathways and the modification of chromatin structure.’, in *Progress in nucleic acid research and molecular biology*, pp. 299–340.

Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) ‘Specificity and mechanism of action of some commonly used protein kinase inhibitors’, *Biochemical Journal*, 351(Pt 1), pp. 95–105.

Deak, M., Clifton, A. D., Lucocq, J. M. and Alessi, D. R. (1998) ‘Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB’, *EMBO Journal*, 17(15), pp. 4426–4441.

Dethlefsen, C., Højfeldt, G. and Hojman, P. (2013) ‘The role of intratumoral and systemic IL-6 in breast cancer’, *Breast Cancer Research and Treatment*, 138(3), pp. 657–664.

Drobic, B., Perez-Cadahia, B., Yu, J., Kung, S. K.-P. and Davie, J. R. (2010) ‘Promoter chromatin

remodeling of immediate-early genes is mediated through H3 phosphorylation at either serine 28 or 10 by the MSK1 multi-protein complex', *Nucleic Acids Research*, 38(10), pp. 3196–3208.

Duda, K. (2012) 'Katarzyna Duda and Morten Frödin* ' MSKs, edited by J. Simon C. Arthur. c2012 Landes Bioscience., 2, pp. 1–27.

Duncan, E. a, Anest, V., Cogswell, P. and Baldwin, A. S. (2006) 'The kinases MSK1 and MSK2 are required for epidermal growth factor-induced, but not tumor necrosis factor-induced, histone H3 Ser10 phosphorylation.', *The Journal of biological chemistry*, 281(18), pp. 12521–5.

Dunn, K. L. and Davie, J. R. (2005) 'Stimulation of the Ras-MAPK pathway leads to independent phosphorylation of histone H3 on serine 10 and 28', *Oncogene*, 24(21), pp. 3492–3502.

Dunn, K. L., Espino, P. S., Drohic, B., He, S. and Davie, J. R. (2005) 'The Ras-MAPK signal transduction pathway, cancer and chromatin remodeling', *Biochemistry and Cell Biology*. NRC Research Press Ottawa, Canada, 83(1), pp. 1–14.

Dyson, M. H., Thomson, S., Inagaki, M., Goto, H., Arthur, S. J., Nightingale, K., Iborra, F. J. and Mahadevan, L. C. (2005) 'MAP kinase-mediated phosphorylation of distinct pools of histone H3 at S10 or S28 via mitogen- and stress-activated kinase 1/2.', *Journal of cell science*, 118, pp. 2247–2259.

Erez, N., Glanz, S., Raz, Y., Avivi, C. and Barshack, I. (2013) 'Cancer associated fibroblasts express pro-inflammatory factors in human breast and ovarian tumors.', *Biochemical and biophysical research communications*. Elsevier Inc., 437(3), pp. 397–402.

Espino, P. S., Drohic, B., Dunn, K. L. and Davie, J. R. (2005) 'Histone modifications as a platform for cancer therapy', *Journal of Cellular Biochemistry*. Wiley Subscription Services, Inc., A Wiley

Company, 94(6), pp. 1088–1102.

Espino, P. S., Li, L., He, S., Yu, J. and Davie, J. R. (2006) ‘Chromatin Modification of the Trefoil Factor 1 Gene in Human Breast Cancer Cells by the Ras / Mitogen-Activated Protein Kinase Pathway Breast Cancer Cells by the Ras / Mitogen-Activated Protein Kinase Pathway’, (9), pp. 4610–4617.

Espino, P. S., Pritchard, S., Heng, H. H. Q. and Davie, J. R. (2009) ‘Genomic instability and histone H3 phosphorylation induction by the Ras-mitogen activated protein kinase pathway in pancreatic cancer cells’, *International Journal of Cancer*. Wiley Subscription Services, Inc., A Wiley Company, 124(3), pp. 562–567.

Flier, J. S., Underhill, L. H. and Dvorak, H. F. (1986) ‘Tumors: Wounds That Do Not Heal’, *New England Journal of Medicine*. Massachusetts Medical Society , 315(26), pp. 1650–1659.

Fry, C. J., Shogren-Knaak, M. A. and Peterson, C. L. (2004) ‘Histone H3 amino-terminal tail phosphorylation and acetylation: Synergistic or independent transcriptional regulatory marks?’, *Cold Spring Harbor Symposia on Quantitative Biology*, 69(February 2004), pp. 219–226. doi: 10.1101/sqb.2004.69.219.

Halegoua, S. and Patrick, J. (1980) ‘Nerve growth factor mediates phosphorylation of specific proteins’, *Cell*, 22(2), pp. 571–581. doi: 10.1016/0092-8674(80)90367-0.

Healy, S., Khan, P. and Davie, J. R. (2012) ‘Immediate early response genes and cell transformation’, *Pharmacology & Therapeutics*. Elsevier Inc., 137(1), pp. 64–77.

Healy, S., Khan, P., He, S. and Davie, J. R. (2012) ‘Histone H3 phosphorylation, immediate-early gene expression, and the nucleosomal response: a historical perspective’, *Biochem. Cell Biol.*, 90,

pp. 39–54.

Hehlgans, T. and Pfeffer, K. (2005) ‘The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games’, *Immunology*. Blackwell Science Ltd, 115(1), pp. 1–20.

Heintzman, N. D., Hon, G. C., Hawkins, R. D., Kheradpour, P., Stark, A., Harp, L. F., Ye, Z., Lee, L. K., Stuart, R. K., Ching, C. W., Ching, K. A., Antosiewicz-Bourget, J. E., Liu, H., Zhang, X., Green, R. D., Lobanenkov, V. V., Stewart, R., Thomson, J. A., Crawford, G. E., Kellis, M. and Ren, B. (2009) ‘Histone modifications at human enhancers reflect global cell-type-specific gene expression.’, *Nature*. NIH Public Access, 459(7243), pp. 108–12.

Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P. and Allis, C. D. (1997) ‘Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation’, *Chromosoma*. Springer-Verlag, 106(6), pp. 348–360.

Hoadley, K. A., Yau, C., Wolf, D. M., Cherniack, A. D., Tamborero, D., Ng, S., Leiserson, M. D. M., Niu, B., McLellan, M. D., Uzunangelov, V., Zhang, J., Kandoth, C., Akbani, R., Shen, H., Omberg, L., Chu, A., Margolin, A. A., van’t Veer, L. J., Lopez-Bigas, N., Laird, P. W., Raphael, B. J., Ding, L., Robertson, A. G., Byers, L. A., Mills, G. B., Weinstein, J. N., Van Waes, C., Chen, Z., Collisson, E. A., Benz, C. C., Perou, C. M. and Stuart, J. M. (2014) ‘Multiplatform Analysis of 12 Cancer Types Reveals Molecular Classification within and across Tissues of Origin’, *Cell*. Elsevier Inc., 158(4), pp. 929–944.

Hogh Annabeth Laursen and Nielsen Kare Lehmann (2008) *Serial Analysis of Gene Expression*

(SAGE). 1st edn. Edited by Kare Lehmann Nielsen. Totowa, New Jersey: Human Press.

Hwang-Verslues, W. W., Kuo, W.-H., Chang, P.-H., Pan, C.-C., Wang, H.-H., Tsai, S.-T., Jeng, Y.-M., Shew, J.-Y., Kung, J. T., Chen, C.-H., Lee, E. Y.-H. P., Chang, K.-J. and Lee, W.-H. (2009) 'Multiple Lineages of Human Breast Cancer Stem/Progenitor Cells Identified by Profiling with Stem Cell Markers', *PLoS ONE*. Edited by M. V. Blagosklonny. Public Library of Science, 4(12), p. e8377.

Italiano, A. (2011) 'Prognostic or predictive? It's time to get back to definitions!', *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. American Society of Clinical Oncology, 29(35), p. 4718; author reply 4718-9.

Iyer, V. R., Eisen, M. B., Ross, D. T., Schuler, G., Moore, T., F Lee, J. C., Trent, J. M., Staudt, L. M., Hudson Jr, J., Boguski, M. S., Lashkari, D., Shalon, D., Botstein, D., Iyer, V. R., Ross, D. T., Eisen, M. B., Botstein, D., Schuler, G., Boguski, M. S., Moore, T. and Hudson Jr, J. (1999) 'The Transcriptional Program in the Response of Human Fibroblasts to Serum', *Science J. Microsc. AIDS Res. Hum. Retroviruses Virology J. Biol. Chem. EMBO J. Proc. Natl. Acad. Sci. U.S.A. J. T. Finch et al*, 283(85), pp. 83–87.

Jahan, S. and Davie, J. R. (2015) 'Protein arginine methyltransferases (PRMTs): Role in chromatin organization', *Advances in Biological Regulation*, 57, pp. 173–184.

Jansen, M. P. H. M., Sieuwerts, A. M., Look, M. P., Ritsier, K., Meijer-van Gelder, M. E., van Staveren, I. L., Klijn, J. G. M., Foekens, J. A. and Berns, E. M. J. J. (2007) 'HOXB13-to-IL17BR expression ratio is related with tumor aggressiveness and response to tamoxifen of recurrent breast cancer: a retrospective study.', *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. American Society of Clinical Oncology, 25(6), pp. 662–8.

Jenuwein Thomas and Allis C. David (2001) 'Translating the Histone Code', *Science*, 293(5532), pp. 1074–1080.

Jin, Q., Yu, L.-R., Wang, L., Zhang, Z., Kasper, L. H., Lee, J.-E., Wang, C., Brindle, P. K., Dent, S. Y. R. and Ge, K. (2011) 'Distinct roles of GCN5/PCAF-mediated H3K9ac and CBP/p300-mediated H3K18/27ac in nuclear receptor transactivation', *The EMBO Journal*. John Wiley & Sons, Ltd, 30(2), pp. 249–262.

Josefowicz, S. Z., Shimada, M., Armache, A., Li, C. H., Miller, R. M., Lin, S., Yang, A., Dill, B. D., Molina, H., Park, H.-S., Garcia, B. A., Taunton, J., Roeder, R. G. and Allis, C. D. (2016) 'Chromatin Kinases Act on Transcription Factors and Histone Tails in Regulation of Inducible Transcription', *Molecular Cell*. Elsevier Inc., 64(2), pp. 347–361.

Josefowicz, S. Z., Shimada, M., Armache, A., Li, C. H., Miller, R. M., Lin, S., Yang, A., Dill, B. D., Molina, H., Park, H. S., Garcia, B. A., Taunton, J., Roeder, R. G. and Allis, C. D. (2016) 'Chromatin Kinases Act on Transcription Factors and Histone Tails in Regulation of Inducible Transcription', *Molecular Cell*, 64(2), pp. 347–361.

Karolin Luger, Armin W. Mañder, Robin K. Richmond, D. F. S. & T. J. R. (1997) 'Crystal structure of the nucleosome core particle at 2.8 Å resolution.: EBSCOhost', *Nature*, 389(6648), p. 251.

Khan, D. H. (2013) *Role of Histone Deacetylases in Gene Expression and RNA Splicing Doctor of Philosophy*. University of Manitoba.

Khan, D. H. and Davie, J. R. (2013) 'HDAC inhibitors prevent the induction of the immediate-early gene *FOSL1*, but do not alter the nucleosome response', *FEBS Letters*, 587(10), pp. 1510–1517.

Khan, D. H., Healy, S., He, S., Lichtensztein, D., Klewes, L., Sharma, K. L., Lau, V., Mai, S., Delcuve, G. P. and Davie, J. R. (2017) 'Mitogen-induced distinct epi-alleles are phosphorylated at either H3S10 or H3S28 depending on H3K27 acetylation.', *Molecular biology of the cell*, (January), p. mbc.E16-08-0618.

Khan, P., Drohic, B., Pérez-Cadahía, B., Healy, S., He, S. and Davie, J. R. (2013) 'Mitogen- and stress-activated protein kinases 1 and 2 are required for maximal trefoil factor 1 induction.', *PLoS one*, 8(5), p. e63189.

Khan, S. A., Amnekar, R., Khade, B., Barreto, S. G., Ramadwar, M., Shrikhande, S. V and Gupta, S. (2016) 'of histone H3 serine 10 phosphorylation defines distance-dependent prognostic value of negative resection margin in gastric cancer', *Clinical Epigenetics*. Clinical Epigenetics, pp. 1–16.

Kim, H.-G., Lee, K. W., Cho, Y.-Y., Kang, N. J., Oh, S.-M., Bode, A. M. and Dong, Z. (2008) 'Mitogen- and Stress-Activated Kinase 1–Mediated Histone H3 Phosphorylation Is Crucial for Cell Transformation', *Cancer Research*, 68(7).

Kittaneh, M. and Montero, A. J. (2013) 'Biomarkers in Cancer Molecular Profiling for Breast Cancer: A Comprehensive Review', pp. 61–70.

Klose, R. J. and Zhang, Y. (2007) 'Histone H3 Arg2 methylation provides alternative directions for COMPASS', *Nature Structural & Molecular Biology*, 14(11), pp. 1058–1060.

Kornberg, R. D. and Lorch, Y. (1999) 'Twenty-Five Years of the Nucleosome, Fundamental Particle of the Eukaryote Chromosome', *Cell*, 98(3), pp. 285–294. doi:

Lau, P. N. I. and Cheung, P. (2011) 'Histone code pathway involving H3 S28 phosphorylation and

K27 acetylation activates transcription and antagonizes polycomb silencing', *Proceedings of the National Academy of Sciences of the United States of America*, 108(7), pp. 2801–2806.

De Laurentiis, M., Cianniello, D., Caputo, R., Stanzione, B., Arpino, G., Cinieri, S., Lorusso, V. and De Placido, S. (2010) 'Treatment of triple negative breast cancer (TNBC): current options and future perspectives', *Cancer Treatment Reviews*, 36, pp. S80–S86.

Lee, D. Y., Teyssier, C., Strahl, B. D. and Stallcup, M. R. (2005) 'Role of Protein Methylation in Regulation of Transcription', *Endocrine Reviews*. Oxford University Press, 26(2), pp. 147–170.

Lee, J.-S., Smith, E. and Shilatifard, A. (2010) 'The Language of Histone Crosstalk', *Cell*, 142(5), pp. 682–685.

Lehmann, B. D. B., Bauer, J. a J., Chen, X., Sanders, M. E., Chakravarthy, a B., Shyr, Y. and Pietenpol, J. a (2011) 'Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies', *Journal of Clinical Investigation*, 121(7), pp. 2750–2767.

Lehmann, B. D. and Pietenpol, J. A. (2015) 'Clinical implications of molecular heterogeneity in triple negative breast cancer', *The Breast*. Elsevier Ltd, 24, pp. S36–S40.

Lehn, S., Tobin, N. P., Berglund, P., Nilsson, K., Sims, A. H., Jirström, K., Härkönen, P., Lamb, R. and Landberg, G. (2010) 'Down-regulation of the oncogene cyclin D1 increases migratory capacity in breast cancer and is linked to unfavorable prognostic features.', *The American journal of pathology*. American Society for Investigative Pathology, 177(6), pp. 2886–97.

Leibovich-Rivkin, T., Liubomirski, Y., Bernstein, B., Meshel, T. and Ben-Baruch, A. (2013) 'Inflammatory factors of the tumor microenvironment induce plasticity in nontransformed breast

epithelial cells: EMT, invasion, and collapse of normally organized breast textures.’, *Neoplasia* (New York, N.Y.), 15(12), pp. 1330–46.

Lejeune, F. J. (2002) ‘Clinical use of TNF revisited: improving penetration of anti-cancer agents by increasing vascular permeability’, *The Journal of Clinical Investigation J. Clin. Invest.*, 110(110), pp. 433–435.

Linn, S. C. and Van ’t Veer, L. J. (2009) ‘Clinical relevance of the triple-negative breast cancer concept: Genetic basis and clinical utility of the concept’, *European Journal of Cancer*. Elsevier Ltd, 45(SUPPL. 1), pp. 11–26.

Lochner, A. and Moolman, J. A. (2006) ‘The many faces of H89: A review’, *Cardiovascular Drug Reviews*, 24(3–4), pp. 261–274.

Locksley, R. M., Killeen, N. and Lenardo, M. J. (2001) ‘The TNF and TNF receptor superfamilies: Integrating mammalian biology’, *Cell*, 104(4), pp. 487–501.

Macdonald, N., Welburn, J. P. I., Noble, M. E. M., Nguyen, A., Yaffe, M. B., Clynes, D., Moggs, J. G., Orphanides, G., Thomson, S., Edmunds, J. W., Clayton, A. L., Endicott, J. a and Mahadevan, L. C. (2005) ‘Molecular basis for the recognition of phosphorylated and phosphoacetylated histone H3 by 14-3-3.’, *Mol. Cell*, 20, pp. 199–211.

Mahadevan, L. C., Willis, A. C. and Barratt, M. J. (1991) ‘Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors’, *Cell*, 65(5), pp. 775–783.

Mayer, I. A., Abramson, V. G., Lehmann, B. D. and Pietenpol, J. A. (2014) ‘New Strategies for Triple-Negative Breast Cancer--Deciphering the Heterogeneity’, *Clinical Cancer Research*, 20(4),

pp. 782–790.

McCoy, C. E., Campbell, D. G., Deak, M., Bloomberg, G. B. and Arthur, J. S. C. (2005) ‘MSK1 activity is controlled by multiple phosphorylation sites.’, *The Biochemical journal*, 387(Pt 2), pp. 507–517.

McGuire L. Williamd (1991) ‘Breast Cancer Prognostic Factors: Evaluation Guidelines’, *Journal of the National Cancer Institute*, 83(3), pp. 154–5.

Naqvi, S., Macdonald, A., McCoy, C. E., Darragh, J., Reith, A. D. and Arthur, J. S. C. (2012) ‘Characterization of the cellular action of the MSK inhibitor SB-747651A’, *Biochemical Journal*, 441(1), pp. 347–357.

Ndlovu, Matladi N, Van Lint, C., Van Wesemael, K., Callebert, P., Chalbos, D., Haegeman, G. and Vanden Berghe, W. (2009) ‘Hyperactivated NF- κ B and AP-1 transcription factors promote highly accessible chromatin and constitutive transcription across the interleukin-6 gene promoter in metastatic breast cancer cells.’, *Molecular and cellular biology*, 29(20), pp. 5488–504.

Oakman, C., Viale, G. and Di, A. (2010) ‘Management of triple negative breast cancer’, *The Breast*. Elsevier Ltd, 19(5), pp. 312–321.

Pavri, R., Zhu, B., Li, G., Trojer, P., Mandal, S., Shilatifard, A. and Reinberg, D. (2006) ‘Histone H2B Monoubiquitination Functions Cooperatively with FACT to Regulate Elongation by RNA Polymerase II’, *Cell*, 125(4), pp. 703–717.

Pérez-Cadahía, B., Drohic, B. and Davie, J. R. (2009) ‘H3 phosphorylation: dual role in mitosis and interphase’ This paper is one of a selection of papers published in this Special Issue entitled

30th Annual International Asilomar Chromatin and Chromosomes Conference and has undergone the Journal's usual peer review process.', *Biochemistry and Cell Biology*, 87(5), pp. 695–709.

Pérez-Cadahía, B., Drohic, B., Espino, P. S., He, S., Mandal, S., Healy, S. and Davie, J. R. (2011) 'Role of MSK1 in the malignant phenotype of Ras-transformed mouse fibroblasts.', *The Journal of biological chemistry*, 286(1), pp. 42–9.

Perou, C. M., Sùrlie, T., Eisen, M. B., Rijn, M. Van De, Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, I., Pergamenschikov, A., Williams, C., Zhu, S. X., Lunning, P. E., Brown, P. O., Botstein, D. and Grant, S. (2000) 'Molecular portraits of human breast tumours', 533(May), pp. 747–752.

Peterson, C. L. and Laniel, M.-A. (2004) 'Histones and histone modifications', *Current Biology*, pp. R546–R551.

Pierrat, B., Correia, J. d. S., Mary, J.-L., Tomas-Zuber, M. and Lesslauer, W. (1998) 'RSK-B, a Novel Ribosomal S6 Kinase Family Member, Is a CREB Kinase under Dominant Control of p38 Mitogen-activated Protein Kinase (p38 MAPK)', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 273(45), pp. 29661–29671.

Prigent, C. and Dimitrov, S. (2003) 'Phosphorylation of serine 10 in histone H3, what for?', *Journal of Cell Science*, 116(18).

Rakha, E. A. and Chan, S. (2011) 'Metastatic Triple-negative Breast Cancer Statement of Search Strategies Used and Sources of Information Epidemiology of Triple-negative Breast Cancer Diagnostic Features of Triple-negative Breast Cancer', *Clinical Oncology*. Elsevier Ltd, 23(9), pp. 587–600.

Reyskens, K. M. S. E. and Arthur, J. S. C. (2016) 'Emerging Roles of the Mitogen and Stress Activated Kinases MSK1 and MSK2', *Frontiers in Cell and Developmental Biology*. Frontiers, 4, p. 56.

Rossetto, D., Avvakumov, N. and Côté, J. (2012) 'Histone phosphorylation: A chromatin modification involved in diverse nuclear events', *Epigenetics*, 7(10), pp. 1098–1108.

Roze, E., Betuing, S., Deyts, C., Marcon, E., Brami-Cherrier, K., Pagès, C., Humbert, S., Mérienne, K. and Caboche, J. (2008) 'Mitogen- and stress-activated protein kinase-1 deficiency is involved in expanded-huntingtin-induced transcriptional dysregulation and striatal death.', *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. Federation of American Societies for Experimental Biology, 22(4), pp. 1083–93.

Sawicka, A. and Seiser, C. (2012) 'Histone H3 phosphorylation - A versatile chromatin modification for different occasions', *Biochimie*. Elsevier, 94(11), pp. 2193–2201.

Schena, M., Shalon, D. and Davis, R. W. (1995) 'Quantitative Monitoring of Gene Expression Patterns with a Complementary DNA', *Science, New Series*, 270(5235), pp. 467–470.

Schneider, J. and Shilatifard, A. (2006) 'Histone Demethylation by Hydroxylation: Chemistry in Action', *ACS Chemical Biology*. American Chemical Society, 1(2), pp. 75–81.

Simpson, E. R. and Brown, K. a (2013) 'Minireview: Obesity and breast cancer: a tale of inflammation and dysregulated metabolism.', *Molecular endocrinology (Baltimore, Md.)*, 27(5), pp. 715–25.

Soloaga, A., Thomson, S., Wiggin, G. R., Rampsaud, N., Dyson, M. H., Hazzalin, C. a., Mahadevan, L. C. and Arthur, J. S. C. (2003) 'MSK2 and MSK1 mediate the mitogen- and stress-

induced phosphorylation of histone H3 and HMG-14', *EMBO Journal*, 22(11), pp. 2788–2797.

Sørlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Lønning, P. E. and Børresen-Dale, A. L. (2001) 'Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 98(19), pp. 10869–74.

Strelkov, I. S. and Davie, J. R. (2002) 'Ser-10 Phosphorylation of Histone H3 and Immediate Early Gene Expression in Oncogene-transformed Mouse Fibroblasts Ser-10 Phosphorylation of Histone H3 and Immediate Early Gene Expression in', (11), pp. 75–78.

Taverna, S. D., Li, H., Ruthenburg, A. J., Allis, C. D. and Patel, D. J. (2007) 'How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers', 14(11), pp. 1025–1041.

Thompson, L. L., Guppy, B. J., Sawchuk, L., Davie, J. R. and McManus, K. J. (2013) 'Regulation of chromatin structure via histone post-translational modification and the link to carcinogenesis', *Cancer and Metastasis Reviews*. Springer US, 32(3–4), pp. 363–376.

Thomson, S., Clayton, A. L., Hazzalin, C. A., Rose, S., Barratt, M. J. and Mahadevan, L. C. (1999) 'The nucleosomal response associated with immediate-early gene induction is mediated via alternative MAP kinase cascades: MSK1 as a potential histone H3/HMG-14 kinase', *EMBO Journal*, 18(17), pp. 4779–4793.

Thomson, S., Louis, M. C. and Clayton, A. L. (1999) 'MAP kinase-mediated signalling to nucleosomes and immediate-early gene induction', *seminars in cell and developmental biology*, 10, pp. 205–214.

Tomás-Zuber, M., Mary, J. L., Lamourt, F., Bur, D. and Lesslauer, W. (2001) 'C-terminal Elements Control Location, Activation Threshold, and p38 Docking of Ribosomal S6 Kinase B (RSKB)', *Journal of Biological Chemistry*, 276(8), pp. 5892–5899.

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J. and Jemal, A. (2015) 'Global cancer statistics, 2012', *CA: A Cancer Journal for Clinicians*, 65(2), pp. 87–108.

Trojer, P. and Reinberg, D. (2007) 'Facultative Heterochromatin: Is There a Distinctive Molecular Signature?', *Molecular Cell*, 28(1), pp. 1–13.

van't Veer, L. J., Dai, H. Y., van de Vijver, M. J., He, Y. D. D., Hart, a a M., Mao, M., Peterse, H. L., van der Kooy, K., Marton, M. J., Witteveen, a T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Linsley, P. S., Bernards, R. and Friend, S. H. (2002) 'Gene expression profiling predicts clinical outcome of breast cancer', *Nature*, 415(345), pp. 530–536.

Veer, L. J. Van, Dai, H., Vijver, M. J. Van De, Kooy, K. Van Der, Marton, M. J., Witteveen, A. T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Bernards, Â., Friend, S. H. and Linsley, P. S. (2002) 'Gene expression profiling predicts clinical outcome of breast cancer', 415(345).

Vermeulen, L., Berghe, W. Vanden, Beck, I. M. E., De Bosscher, K. and Haegeman, G. (2009) 'The versatile role of MSKs in transcriptional regulation', *Trends in Biochemical Sciences*, 34(6), pp. 311–318.

Vermeulen, L., De Wilde, G., Van Damme, P., Vanden Berghe, W. and Haegeman, G. (2003) 'Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1).', *The EMBO journal*, 22(6), pp. 1313–24.

Viale, G. (2012) 'The current state of breast cancer classification', *Annals of Oncology*,

23(SUPPL. 10).

Viatour, P., Merville, M.-P., Bours, V. and Chariot, A. (2005) 'Phosphorylation of NF- κ B and I κ B proteins: implications in cancer and inflammation', *Trends in Biochemical Sciences*, 30(1), pp. 43–52.

Vicent, G. P., Ballaré, C., Nacht, A. S., Clausell, J., Subtil-Rodríguez, A., Quiles, I., Jordan, A., Beato, M., Reuter, G. and Auricchio, F. (2006) 'Induction of progesterone target genes requires activation of Erk and Msk kinases and phosphorylation of histone H3.', *Molecular cell*. Elsevier, 24(3), pp. 367–81.

Wang, Y., Zhang, T., Kwiatkowski, N., Young, R. A., Gray, N. S., Zhao, J. J., Wang, Y., Zhang, T., Kwiatkowski, N., Abraham, B. J., Lee, T. I. and Xie, S. (2015) 'CDK7-Dependent Transcriptional Addiction in Triple-Negative Breast Cancer Article CDK7-Dependent Transcriptional Addiction in Triple-Negative Breast Cancer', *Cell*. Elsevier Inc., 163(1), pp. 174–186.

Wiggin, G. R., Soloaga, A., Foster, J. M., Murray-Tait, V., Cohen, P. and Arthur, J. S. C. (2002) 'MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF1 in fibroblasts.', *Molecular and cellular biology*. American Society for Microbiology, 22(8), pp. 2871–81.

Winter, S., Simboeck, E., Fischle, W., Zupkovitz, G., Dohnal, I., Mechtler, K., Ammerer, G. and Seiser, C. (2008) '14-3-3 Proteins recognize a histone code at histone H3 and are required for transcriptional activation', *The EMBO Journal*. John Wiley & Sons, Ltd, 27(1), pp. 88–99.

Woon Ki Paik and Sangduk Kim (1971) 'Protein Methylation', *Science*, 174(4005), pp. 114–119.

Xiao-Jun Ma, Paula D. Ryan, Steven J. Isakoff, Anne Barnettler, A. F., Beth Muir, Gayatry Mohapatra, Ranelle Salunga, J. Todd Tuggle, Yen Tran, D. T., Ana Tassin, Paul Amon, Wilson Wang, Wei Wang, Edward Enright, K. S., Eden Estepa-Sabal, Barbara Smith, Jerry Younger, Ulysses Balis, J. M. and Atul Bhan, Karleen Habin, Thomas M. Baer, Joan Brugge, D. A. H. (2004) 'A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen', *Cancer Cell*, 5(6), pp. 607–616.

Xinhui Fu, Xinjuan Fanc, Jun Hu, Hongzhi Zou, Zhiting Chen, Q. L. and Beibei Ni, Xiaoli Tan, Qiao Su, Jingxuan Wang, Lei Wang, J. W. (2017) 'Overexpression of MSK1 is associated with tumor aggressiveness and poor prognosis in colorectal cancer', *Digestive and Liver Disease*. Editrice Gastroenterologica Italiana, pp. 1–9.

Yang, X., Sun, Y., Li, H., Shao, Y., Zhao, D., Yu, W. and Fu, J. (2017) 'C-terminal binding protein-2 promotes cell proliferation and migration in breast cancer via suppression of p16 INK4A', 8(16), pp. 26154–26168.

Zhang, Y. A. N., Pu, X., Shi, M., Chen, L., Qian, L. U., Song, Y., Yuan, G., Zhang, H. A. O., Yu, M., Hu, M., Shen, B. and Guo, N. (2007) 'c-Jun , a crucial molecule in metastasis of breast cancer and potential target for biotherapy', pp. 1207–1212.

APPENDIX

TNF α + H89

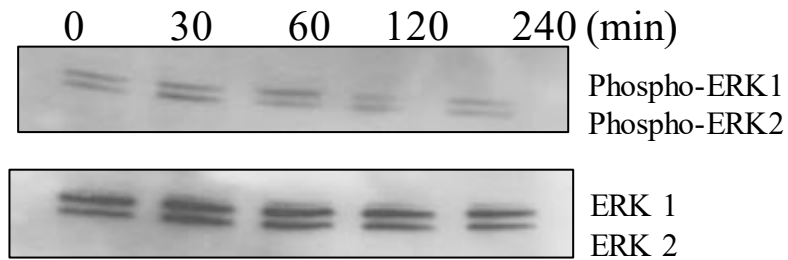


Figure 1. H89 does not affect ERK1/2. Total cell lysates from serum starved MDA-MB-231 cells stimulated with H89 30 minutes prior to TNF α for various lengths of time were prepared. The lysates were resolved on a 10% SDS-PAGE, followed by western blot analysis with anti-pERK1/2 (upper panel) using anti-ERK1/2 as control (lower panel).