UiT The Arctic University of Norway

Faculty of Health Sciences
Department of Pharmacy

# The Regulation of Steroid Receptor Co-activator-3 Activity by p38MAPK-MK2 Signaling Pathway

**Anup Shrestha**

A dissertation for the degree of Philosophiae Doctor-April 2022

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Tromsø, April 2022

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**Summary**

Steroid receptor co-activator-3 (SRC-3) is a co-activator that plays important roles in normal physiology and different diseases including cancer. Phosphorylation at serine 857 (S857) is the most frequently reported post translational modification regulating the activity of SRC-3. The aim of this thesis was to identify kinases phosphorylating SRC-3 at S857 and study the biological significance of this phosphorylation in lung and breast cancer cells.

In this study, the p38MAPK-MK2 signaling axis was identified to be involved in the phosphorylation of SRC-3 at S857 in several different cancer cell lines. This event was shown to be required for TNF-α mediated nuclear translocation of SRC-3, efficient transactivation of NF-κB and induction of IL-6 mRNA expression in A549 cells. In order to investigate the role of this phosphorylation site in triple negative breast cancer (TNBC) cells, MDA-MB-231 cells depleted for endogenous SRC-3 expression by CRISPR-Cas9 or shRNA were generated, and lentiviral re-expression of wild-type SRC-3 or a S857A mutant was used for rescue. Treatment with the chemotherapeutic drug doxorubicin induced activation of the p38MAPK-MK2-SRC-3 signaling axis. Interestingly, pretreatment with a MK2 specific inhibitor or depletion of SRC-3 resulted in increased sensitivity to doxorubicin, and rescue experiments indicated that phosphorylation at S857 was critical for the sensitivity towards doxorubicin. The TNBC cell models were further used to identify genes whose regulation were dependent on a functional SRC-3 S857 phosphosite and the p38MAPK-MK2-SRC-3 axis. For this purpose, sequencing of mRNA isolated from unstimulated cells, and cells stimulated with TNF-α and MK2 inhibitor alone or in combination was performed. Differentially expressed SRC-3 S857 dependent genes were identified for both unstimulated and TNF-α stimulated cells but showed very little overlap, indicating a major switch in the transcriptional complexes and promoters SRC-3 associates with and regulates upon TNF-α stimulation. Further, genes regulated specifically by the p38MAPK-MK2-SRC-3 axis were identified from samples pretreated with the MK2 inhibitor, and gene ontology analyses showed these genes to be associated with cell migration.

The major finding of this thesis is the identification of the p38MAPK-MK2 signaling pathway as responsible for the oncogenic SRC-3 S857 phosphorylation. In addition, efforts have been made to unravel the biological consequences of this phosphorylation, and use of a MK2 inhibitor indicate promising effects by causing changes in specific SRC-3 functions (e.g., sensitivity to a chemotherapeutic drug and target gene expression). More research needs to be done to investigate whether use of MK2 inhibitors will be applicable for future targeted therapies in cancers where SRC-3 is shown to have oncogenic activity.

**List of papers**

**Paper I**

Anup Shrestha, Henrike Bruckmueller, Hanne Kildalsen, Gurjit Kaur, Matthias Gaestel, Hilde Ljones Wetting, Ingvild Mikkola, and Ole-Morten Seternes. (2020). **Phosphorylation of steroid receptor coactivator-3 (SRC-3) at serine 857 is regulated by the p38MAPK-MK2 axis and affects NF-κB-mediated transcription.** Scientific Reports 10 (1): 11388.

**Paper II**

Anup Shrestha, Henrike Bruckmueller, Hanne Kildalsen, Rune Hogseth, Ingvild Mikkola, and Ole-Morten Seternes (2022). **A role of p38MAPK-MK2-SRC-3 signaling axis in the sensitivity to doxorubicin in triple negative breast cancer cells.**

Manuscript.

**Paper III**

Anup Shrestha, Henrike Bruckmueller, Julien Bruckmueller, Hanne Kildalsen, Ole-Morten Seternes, and Ingvild Mikkola (2022). **A Pilot study using RNA sequencing to identify genes regulated by phosphorylated SRC-3 S857 and the p38MAPK-MK2-SRC-3 signaling pathway in triple negative breast cancer cells.**

Manuscript.

**Abbreviations**

aa: amino acid

AR: androgen receptor

ARE: adenylate-uridylate (AU)-rich elements

ATP: adenosine tri-phosphate

bHLH/PAS: basic helix-loop-helix/Per-Arnt-Sim

CARM1: co-activator-associated arginine methyltransferase 1

Cas9: CRISPR associated protein

CBP: CREB-binding protein

CREB: cAMP response element-binding protein

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EMT: epithelia mesenchymal transition

ER: estrogen receptor

GFP: green fluorescent protein

GR: glucocorticoid receptor

GSK3: glycogen synthase kinase 3

HAT: histone acetyl transferase

HDAC: histone deacetylase

HER2: human epidermal growth factor receptor 2

hnRNPA0: heterogeneous nuclear Ribonucleoprotein A0

IKK: IκB kinase

IL-6: Interleukin-6

IκB: Inhibitor of κB

JNK: c-Jun-N-terminal kinase

KD: knockdown

KO: knockout

LPS: lipopolysaccharide

MAP2K: MAP kinase kinase

MAP3K: MAP kinase kinase kinase

MEF: mouse embryonic fibroblast

MSK: mitogen- and stress-activated kinases

MAPK: mitogen-activated protein kinases

MK: MAPK- activated protein kinases

NF-κB: Nuclear factor-κB

NR: nuclear receptor

NES: nuclear export signal

NLS: nuclear localization signal

p/CAF: p300/CBP-associated factor

PR: progesterone receptor

PRAK: p38-regulated and-activated protein kinases

RSK: p90 ribosomal S6 kinases

PKC: protein kinase C

Pol II: RNA polymerase II

PTM: post translational modification

RTK: receptor tyrosine kinase

shRNA: short hairpin RNA

siRNA: small interfering RNA

SMI: small molecule inhibitors

SR: steroid hormone receptor

SRC-3: steroid receptor co-activator-3

TNBC: triple negative breast cancer

TNF-α: tumor necrosis factor-α

TTP: tristetraprolin

UTR: untranslated region

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## 1. Introduction

Genomic alterations in cells resulting into changes in proteins that are vital for regulation of growth and proliferation might cause uncontrolled cell proliferation and ultimately invasive behavior. Such cells are called cancerous. The cause of cancers was found to be related to viruses [1], ionizing radiation [2], chemicals, stress and inflammation [3] and hereditary [4], while some cancers arose without any apparent external cause but due to mutation by chance [5, 6]. Cancer is a multistep process, and it may take several years from the initial mutation till the cells become malignant. Cancer progression requires modulation in one or several vital genes such as oncogenes, tumour suppressor genes and genes involved in DNA repair resulting in genetic instability. Besides the critical genes, it also requires modulation of several supportive genes. Different sets of genes need to be active in different stages of cancer [7]. Owing to this diversity, different cancer types respond to diverse therapies like chemotherapy, immunotherapy, hormone therapy, stem cell transplant, surgery and targeted therapy.

Hanahan and Weinberg have defined following features as hallmarks of cancer: evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis, resisting cell death, sustaining proliferative signaling, deregulation of cellular energetics and avoiding immune destruction [8]. These features are results of distortions in the cell signaling pathways that in normal cells control cell proliferation, survival and motility. The signaling network ameliorated in cancer is complex as there is redundancy of pathways, crosstalk between pathways, and feedback inhibition mechanisms that cause pathway reactivation. Deciphering how they are altered in cancer cells represents a major challenge [9, 10]. Understanding how these complex signaling networks function has major implications in our understanding of tumor cell behavior and in our ability to use this knowledge for cancer therapy. Transcription factors are integral members of cell signaling pathways. Transcription factors, cofactors, and chromatin regulators control the gene expression programs. Kinases are signaling molecules that play vital role in signaling pathways including the regulation of different transcription factors and coregulators. Misregulations in gene expression programs significantly contribute in acquisition of these tumor-related properties [11]. Many of the proteins that are components of these pathways can be possible targets for cancer therapy. Among them, kinases are of particular interest because they are druggable [12]. The steroid receptor co-activator-3 (SRC-3) is a transcriptional co-activator, which is widely reported to be involved in the development and progression of cancer. In this thesis, a kinase

that regulates the activity of this versatile co-activator is identified and the associated biological outcomes are explored.

### 1.1. Transcription

Transcription is the process by which the information from a DNA strand is transferred into a complementary RNA molecule by the enzyme RNA polymerase. It is a complex process that involves cis- and trans-regulatory factors and requires access to the DNA which is densely packed with histone proteins.

Histones are positively charged proteins that tightly bind to negatively charged DNA. Two copies of the four histone proteins H2A, H2B, H3, and H4 forms a protein core around which 146 base pairs of the double stranded DNA winds to form the most basic structure of chromatin called nucleosome [13]. Such nucleosome and linker histone H1 further coil and fold up with other additional proteins to produce densely packed chromatin. In this way, the DNA might be tightly packed in the nucleus and not easily accessible for the basal transcription machinery. However, the chromatin structure is dynamic. Post translational modifications (PTM) of histone proteins such as acetylation, methylation, ubiquitination, crotonylation and phosphorylation of certain amino acids within the histone proteins can alter the strong electrostatic interactions between the oppositely charged nucleosomal DNA and the histone proteins and thus regulate the accessibility of the DNA [14, 15]. The dynamic regulation of chromatin also involves specialized ATP-dependent chromatin-remodeling complexes. Such complexes ensure the proper density and spacing of nucleosomes, cooperate with sequence-specific transcription factors and histone modification enzymes to move or to eject histones to enable the binding of transcription factors at gene promoters or enhancers [16]. There are also regions, which are depleted of nucleosomes. Such regions are mostly associated with regulatory elements such as promoters and enhancers [17].

In eukaryotic cells, the RNA polymerase II (Pol II) core promoters are DNA sequences at transcription start sites (TSS) that support the assembly of the general transcription factors and Pol II. This is the minimum machinery necessary to allow transcription of the gene. The core promoter generally spans between -40 to +40 nucleotide relative to the TSS [18]. In a simple transcription unit, the core promoter acts in conjunction with an enhancer, a proximal promoter region and their associated regulatory proteins [19]. The proximal promoter is the regulatory DNA sequence that resides in the immediate vicinity of the core promoter (around 200 base pairs upstream of the TSS). This region comprises binding sites for sequence specific transcription factors whose binding enhances or represses transcription. Most genes are

controlled by multiple promoter-proximal elements [20]. Distal promoters include regulatory DNA motifs such as enhancer sequences, which are located tens of kilobases (kb) away from their target gene [21]. By means of DNA looping, enhancers accumulate the transcription factors close to the promoter and regulate the initiation, elongation and termination of transcription [22].

The transcriptional activation processes begin with the removal of repressor complexes from the promoter. At the next step, a DNA-binding transcriptional activator that recognizes specific DNA sequences binds the promoter and recruits co-activator complexes. The transcriptional activator interacts with the promoter to stimulate the subsequent recruitment of chromatin remodeling complexes that clear nucleosomes from the promoter sequence. General transcription factors are recruited at the next step of transcriptional activation, which results in Pol II recruitment and preinitiation complex (PIC) formation [23]. Phosphorylation of serine (S) 5 in the C-tail domain of Pol II then leads to initiation of transcription. This is followed by active transcription elongation [24].

### 1.1.1. Transcription factor

Transcription factors are proteins capable of binding to DNA in a sequence-specific manner and regulating transcription. General transcription factors bind to core promoters while site specific transcription factors bind to promoter-proximal elements and enhancers. The minimum requirement for being a transcription factor is having at least one DNA binding domain (DBD) which interacts with specific DNA sequences and a transcriptional activation or repression domain, which interacts with other proteins to regulate transcription from a nearby promoter. The three most common DBD of transcription factors are zinc fingers, homeodomains and helix–loop–helix domains [25]. Transcription factors can function as either activators or repressors while some can function as both depending on the local sequence context and the availability of cofactors and dimerization [26]. For example, MAX dimerizes with MYC to activate transcription but MAX homodimers or heterodimers with MNT or MAX dimerization protein 1 (MXD1) to inhibit transcription [27]. Transcription factors can slide, hop or induce local DNA bending to reach promoters [28]. Some transcription factors such as TFIIIC90 have intrinsic histone acetyl transferase (HAT) [29]. However, most eukaryotic transcription factors are thought to act by recruiting cofactors [30]. PTM such as phosphorylation and sumoylation can regulate the subcellular localization, protein-protein interactions, sequence-specific DNA binding, transcriptional regulatory activity, and protein stability of transcription factors [31].

Furthermore, their activity may be activated through ligand binding (e.g., nuclear receptors) and interaction with other transcription factors (e.g., NF-κB) [32] or coregulatory proteins [33].

### 1.2. Transcriptional co-regulator

Nuclear receptors (NRs) are a family of ligand-regulated transcription factors that are activated by steroid hormones, such as adrenal steroids (glucocorticoids and mineralocorticoids), sex steroids (progestins, estrogens, and androgens) and various other lipid-soluble signals such as vitamin D3, thyroid and retinoid (9-cis and all-trans) hormones [34]. In the early 1970s, it was noted that the extent of binding the NR complex to DNA appeared to be correlated with the levels of non-histone proteins [35]. By the late 1980s, it was found that besides NRs and general transcription factors, an additional factor was required for efficient hormone-stimulated transcriptional activity of NRs [36]. Finally, in 1994, the first nuclear receptor co-activator (SRC-1) was identified and shown to interact with NRs in a ligand-dependent manner [37]. Transcriptional co-regulators occupy an important place in regulation of gene expression by directly interacting with and modulating the activity of essentially all NRs and transcription factors [38].

Transcriptional co-regulators interact with transcription factors to either activate (co-activator) or inhibit (co-repressor) the transcription of specific genes. Co-activators, such as the CREB-binding protein (CBP) and p300 interact with transcription factors of all major families and regulate gene expression [39]. Nuclear receptor co-repressor 1 (NCoR1) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) are among the best-characterized co-repressors [40].

When bound to a transcription factor, the co-activator can play a role as a scaffold protein and facilitate recruitment of RNA Pol II and other basal transcription machinery. Some co-activators also have intrinsic enzyme activity such as histone acetyl transferase (HAT) or deacetylase (HDAC) activities that can play a role in covalent chromatin modification and nucleosome remodeling [33, 41, 42]. These activities of co-regulators are regulated in part by post translational modifications and small signaling molecules. Understanding the detailed mechanism of action of co-regulators has helped to define the physiological functions of co-regulators and their role in human disease states including cancer. This understanding of co-regulator biology can be used to develop drugs that target co-regulator dysfunctions [43, 44].

### 1.2.1. Steroid receptor co-activator

Steroid receptor co-activators (SRCs) belong to the p160 co-activator family and comprises three homologous members, SRC-1, SRC-2 and SRC-3. SRCs function as transcriptional co-activators of NRs and several other transcription factors [38] (Figure 1). SRCs play a role in transcription initiation, elongation, RNA splicing, recruitment of other co-regulators as well as in receptor and co-regulator turnover [45]. Hence, SRCs play important roles in the regulation of a variety of developmental events and physiological functions particularly in reproduction [46] and energy metabolism [47]. SRCs are abundantly expressed and amplified in breast, endometrial, ovarian, prostate, and other cancers and are reported to be involved in cancer progression and metastasis [43, 48] and inflammation [47]. The members of p160 family co-activators have sequence similarity of 50–55%. Some of their functions are redundant while others are unique to each member of the family [49].

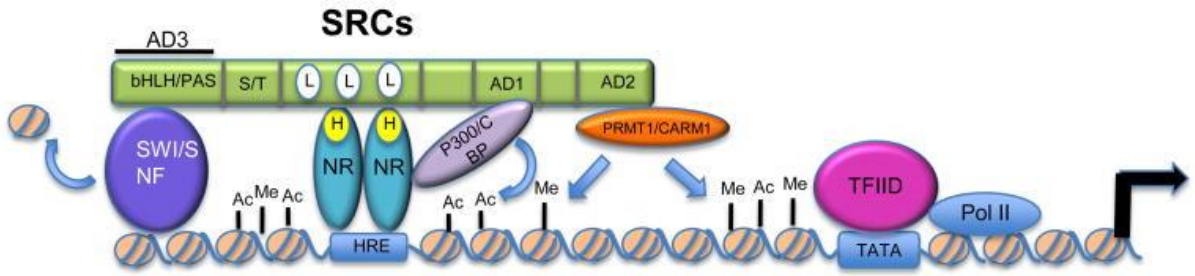


**Figure 1. SRC-mediated co-activation of NRs**. SRC proteins are recruited to hormone bound nuclear receptors (NR) and bind through their LXXLL motifs. SRCs then recruit different secondary co-activators such as histone acetyltransferase, p300/CBP; histone methyltransferases, PRMT1 and CARM1; and the chromatin remodeling complex, SWI/SNF that bind to their activation domains (ADs). SRCs: steroid receptor co-activators, bHLH/PAS : basic helix-loop-helix/Per-Arnt-Sim, S/T: serine/threonine–rich region, Ac: acetylation, Me: methylation, HRE: hormone response element, L: LXXLL motif, TFIID: transcription factor IID, Pol II: RNA polymerase II. Figure adopted from [50] with permission.

#### 1.2.1.1. SRC-1

SRC-1 (NCOA-1) is the first member identified of the p160 family of co-regulators [37, 51]. SRC-1 plays an important role in brain development and function [49]. SRC-1 has weak intrinsic histone acetyltransferase activity [52]. It can co-activate several NRs such as estrogen receptor (ER), progesterone receptor (PR), glucocorticoid receptor (GR), thyroid receptor, retinoid X receptor and other transcription factors such as Activator Protein-1 (AP-1), serum response factor, Nuclear factor-κB (NF-κB), ETS2, Polyomavirus Enhancer Activator 3 Homolog (PEA3) [53, 54]. SRC-1 knockout mice are viable and fertile and the response to steroid hormones are only partially impaired [55].

#### 1.2.1.2. SRC-2

SRC-2 (TIF2/GRIP1/NCOA-2) was identified in 1996 [56]. It plays a critical role in reproductive behavior and functions. SRC-2 knockout male mice display defective spermatogenesis and testicular degeneration while the female mice showed placental hypoplasia [57]. SRC-2 plays an important role in lipid metabolism and energy balance. SRC-2 knockout mice showed higher lipolysis in white fat and higher energy expenditure in brown fat tissue, thus they are able to protect themselves from high fat diet induced obesity [58]. SRC-2 positively regulates the circadian rhythm [59]. Besides this, it is involved in endometrial, pleural, pancreatic and prostate tumors [60].

#### 1.2.1.3. SRC-3

SRC-3 (ACTR/pCIP/RAC3/TRAM-1/AIB1/NCOA-3) is encoded by the gene *nuclear receptor co-activator 3 (NCOA3)* and is located on chromosome 20 (q12) in humans. When first identified, it was known as a nuclear receptor co-activator [61, 62] but now it is known to interact with several other transcription factors as listed in table 1. It is implicated in the regulation of reproductive health [63], cell proliferation, and inflammation [64] and in the progression of both hormone related and hormone independent cancers [65].

##### 1.2.1.3.1. Structure of SRC-3

SRC-3 is a protein of 165 kDa and has several fundamental and structurally conserved signature domains as shown in figure 2. At the N-terminus, the basic helix-loop-helix- Period [Per], Aryl hydrocarbon receptor [AhR], single-minded [Sim] (bHLH-PAS) domain is required for nuclear localization and serves as a protein interaction surface for different transcription factors. It interacts with the BAF57 subunit of the SWI/SNF (switch/sucrose nonfermentable) ATP dependent chromatin remodeling complex. The receptor interaction domain (RID) region contains three alpha-helical LXXLL (L: leucine, X: any amino acid) motifs. One of the three LXXLL motifs is essential for NR interaction and activation [66, 67].

SRC-3 has extensive binding sites for the recruitment of other co-regulators and histone-modifying enzymes giving them the opportunity to modulate gene expression in a context-specific manner. At the C-terminus, the activation domain (AD) 1 binds to histone acetylases; the cAMP response element-binding protein–binding protein (CBP) 1/p300 and the p300/CBP-associated factor (pCAF). AD2 interacts with protein methylases; co-activator-associated arginine methyltransferase 1 (CARM1) and protein arginine N-methyltransferase 1 (PRMT1) which promotes histone methylation and subsequent chromatin remodeling [68]. The C-terminus also has intrinsic mild histone acetyltransferase activity [52]. In this way, SRC-3 plays

a role in chromatin remodeling through both direct and indirect recruitment of other potent co-activators.

**Table 1. Transcription factors interacting with SRC-3**

| Transcription factor | Attributed function | Reference |
|---|---|---|
| Estrogen receptor | recruits p300/CBP and increases HAT activity | [69] |
| Progesterone receptor | gonadotropin-releasing hormone–induced progesterone receptor trans-activation of target genes | [70] |
| Androgen receptor | development and growth of prostate cancer | [66] |
| Activator Protein-1 (AP-1) | activation of IGF/AKT to promote cell proliferation and survival of prostate and breast cancer cells | [71] |
| TEA domain family member (TEAD) | co-activation of SRC-3-YAP target genes in mediating progression of early stage breast cancer | [72] |
| E2F Transcription Factor 1 (E2F1) | transcription cell cycle regulatory genes mediating breast cancer cell proliferation and tamoxifen resistance | [73] |
| Polyomavirus Enhancer Activator 3 Homolog (PEA3) | promotes upregulation of MMP gene expression and pro-invasive activity in lung cancer cells | [74] |
| NF-κB | reduced expression of interferon regulatory factor 1 and induction of IL6 | [75, 76] |
| Cyclic AMP-dependent transcription factor (ATF-4) | expression of adenosine monophosphate deaminase-1 (AMPD1) and xanthine dehydrogenase (XDH) that enhance purine synthesis, which promotes growth and metastasis of breast cancer | [77] |
| Hypoxia-inducible factor (HIF-1) | Induction of migration inhibitory factor (MIF) which is involved in suppression of autophagy thereby decreasing chemosensitivity and enhancing tumorigenicity. | [78] |
| SP1 | Promotes hepatocellular carcinoma growth and tumor progression through upregulation of the telomerase reverse transcriptase (TERT) signaling | [79] [80] |
| Liver receptor homolog 1 (LRH1) | SRC-3 acts synergistically with LRH1 to promote mediator of DNA damage checkpoint 1 (MDC1) expression and chemoresistance | [81] |

### 1.2.1.3.2. Post translational modification of SRC-3

The PTM of SRC-3 determines the interaction with NR and other proteins, the subcellular localization, the stability, the enzymatic activities and the conformational changes of the co-activators. SRC-3 is reported to contain more than 50 authentic PTMs of various types (Figure

3). These PTMs include phosphorylation, methylation, acetylation, sumoylation and ubiquitination of SRC-3 [82].

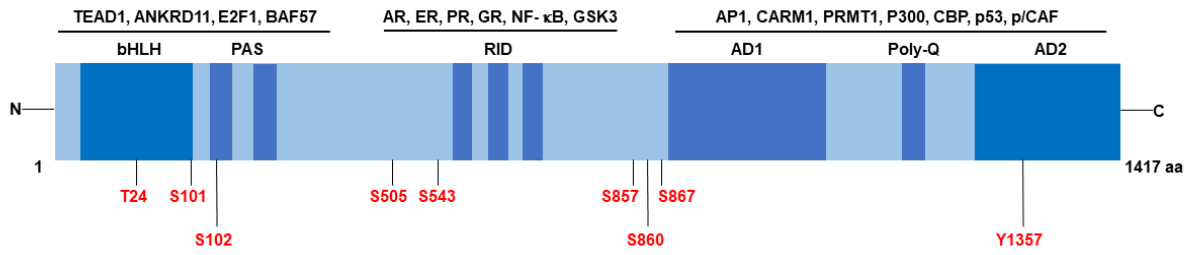


**Figure 2. Structural domains and phosphorylation sites of SRC-3.** SRC-3 co-activates nuclear receptors, as well as numerous other transcription factors. Some of SRC-3's interacting proteins are listed in the upper panel. In the lower panel, some of the frequent phosphorylation sites of SRC-3 are listed. bHLH-PAS: basic helix-loop-helix-Per/ARNT/Sim, RID: receptor-interacting domain, AD1: activation domain 1, Poly-Q: polyglutamate repeat tract, AD2: activation domain 2, T: threonine, S: serine, Y: tyrosine, TEAD1: TEA domain family member 1, ANKRD11: Ankyrin repeat domain-containing protein 11, AR: androgen receptor, ER: estrogen receptor, PR: progesterone receptor, GR: Glucocorticoid Receptor, AP1: Activator Protein 1, CARM1: co-activator-associated arginine methyltransferase 1, PRMT1: protein arginine methyltransferase 1, CBP: cAMP response element-binding protein (CREB)-binding protein, p/CAF: p300/CBP-associated factor, N: N-terminal, C: C-terminal, aa: amino acid. Figure modified from [50, 83] with permission.

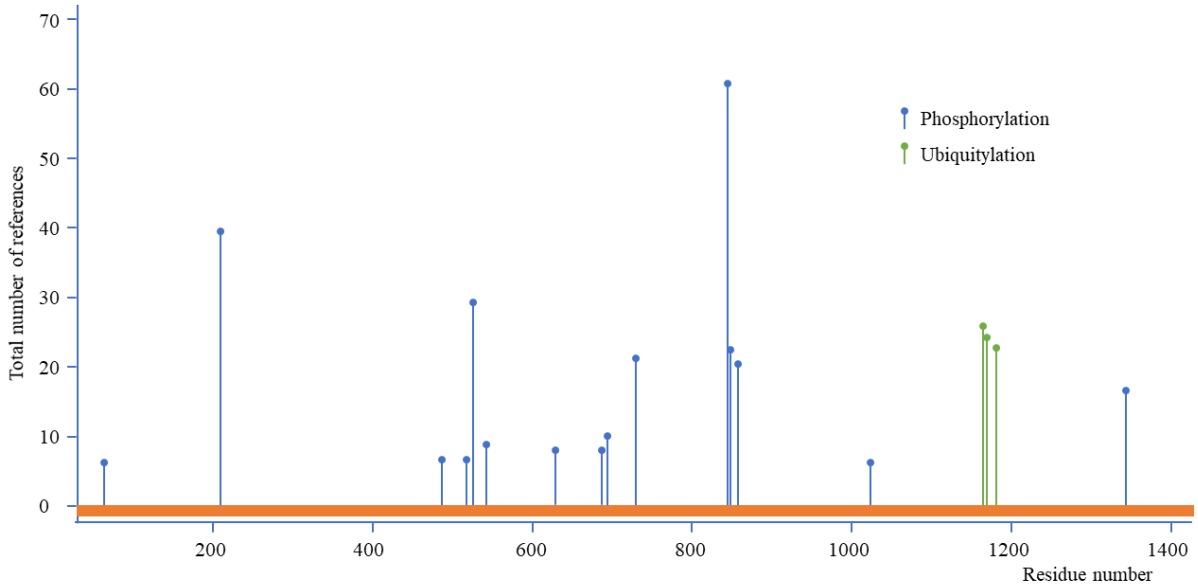


**Figure 3. Post translational modification of SRC-3.** The reported frequency of phosphorylation and ubiquitylation at different sites of SRC-3 are presented. Only the sites that are reported more than five times are mentioned in this figure. Image obtained from [84].

Different phosphorylation sites of SRC-3 and the corresponding kinases phosphorylating those sites along with the attributed functions are listed in Table 2. Serine 857 is the most intensively investigated phosphorylation site among all the SRC-3 phosphorylation sites [84].

Stimuli such as steroid hormones and TNF-α regulating SRC-3 activate different kinases upstream of SRC-3 [76]. Each kinase phosphorylates SRC-3 at specific residues and result into a phosphorylation code. Such unique phosphorylation code resulted into distinct outcomes. For example, estrogen and androgen induced phosphorylation of six phosphorylation sites (T24, S505, S543, S857, S860, and S867) were shown to be required for estrogen and androgen receptors interaction with CBP, while TNF-α induced phosphorylation of all six sites except S860 was enough for NF-κB activity [76]. In another study, S860 was the only site phosphorylated by the retinoic acid-induced p38MAPK pathway and it led to the degradation of SRC-3 [85]. Yet in another study, the four phosphorylation sites in mouse SRC-3 (S498, S536, S847 and S850, the mouse S847 corresponds to human S857) were found to regulate the peripheral insulin sensitivity by increasing IGF1 signaling, due to elevated IGFBP3 levels [86]. One of the outcomes of the phosphorylation of SRC-3 is the determination of its subcellular localization. Amazit et al. found that EGF-induced phosphorylation of SRC-3 at S857 and S860 via ERK1/2 in HeLa cells were important for the nuclear translocation of SRC-3 [87]. Phosphorylation of different phosphosites of SRC-3 was followed by ubiquitination which led to the proteasomal degradation of SRC-3 [88]. This has been further discussed in the section 1.2.1.3.3. Regulation of expression and stability of SRC-3, below. Sumoylation of SRC-3 at lysine (K)-723, 786 and 1194 was reported to attenuate the transactivation activity of SRC-3. Estradiol-induced activation of ERK1/2 phosphorylated SRC-3 which led to concomitant loss of the sumoylation indicating phosphorylation and sumoylation appear to inversely regulate the transcriptional activity of SRC-3 [89]. CBP/p300 was found to acetylate SRC-3 at K629 and K630. Such acetylation of SRC-3 neutralized the positive charges at the residues, disassociated the coactivator-receptor complexes, and consequently repressed the transcriptional activity [90]. SRC-3 has been demonstrated to act as substrate for CARM1. Like sumoylation and acetylation, methylation of SRC-3 also resulted in decreased transcriptional activity. Estrogen induced methylation of SRC-3 by CARM1 at arginine (R) 1171 which impaired the CBP-SRC-3 and CARM1-SRC-3 association thereby decreased the transcriptional activity of SRC-3 [91].

#### 1.2.1.3.3. Regulation of expression and stability of SRC-3

SRC-3 autoregulates its own expression and is recruited to its promoter in complex with the E2F Transcription Factor 1 (E2F1) to modulate SRC-3 expression [92]. The stability of the SRC-3 mRNA is regulated by two microRNAs, miR-17-5p and miR-20b [93, 94]. At protein level, its stability is regulated by proteasome dependent and independent mechanism [95]. GSK3 phosphorylates SRC-3 at S505 which is required for the ubiquitination of SRC-3 at lysine (K) residues at 723 and 786 positions. Multi-mono-ubiquitination, which is observed more frequently, enhances the transcriptional activity of SRC-3 because it increases the interaction with ER when stimulated with estradiol. Whereas the poly-ubiquitination at those sites causes degradation by proteasome. They suggest that phosphorylation-dependent ubiquitination of SRC-3 confers a transcription activation function before promoting SRC-3 degradation [88]. The nucleus is the principal site of SRC-3 proteasomal degradation [96]. Phosphorylation of SRC-3 at S101 and S102 signals for its proteasome-dependent turnover. Prevention of phosphorylation at these sites by Protein Phosphatase 1 (PP1) protects SRC-3 from the proteasome mediated turnover [97]. Retinoic acid receptor (RAR-α) mediated activation of p38MAPK induces the phosphorylation of SRC-3 at S860. This phosphorylation leads to increased proteasomal degradation of SRC-3 [85]. Two amino acid residues (lysine 17 and arginine 18) in the bHLH domain of SRC-3 are important for its nuclear localization and proteasome-dependent degradation [98]. The E3 ubiquitin ligase adaptor speckle-type poxvirus and zinc finger (POZ) domain protein (SPOP) interacts directly with SRC-3 and promotes ubiquitination and proteolysis. Mutations in SPOP in prostate cancer prevent the degradation of SRC-3 protein and suppression of androgen receptor transcriptional activity [99]. Besides, 20S proteosome regulator REGγ is involved in the degradation of SRC-3 in an ubiqutin- and ATP-independent manner in MCF-7 cells [100].

#### 1.2.1.3.4. SRC-3 in health and disease

Being closely associated to NR and several other transcription factors, SRC-3 plays important roles in multiple physiological processes including cell proliferation, cell survival, somatic cell growth, mammary gland development, female reproductive function and energy metabolism. In cancer, SRC-3 modulates various processes such as proliferation, development of metastasis [101] and resistances to anti-cancer drugs [102]. SRC-3 has been implicated in both hormone-related cancers, such as endometrial [103], epithelial ovarian [104], prostate [99] and breast cancer [83] and hormone-independent cancers such as esophageal, squamous cell, colorectal, hepatocellular, pancreatic and non-small cell lung cancer [65].

**Table 2. Post translational modifications of SRC-3 by phosphorylation and associated functions.**

| Phosphor-ylation site | Kinase phosphorylating the site | Attributed function | Reference |
|---|---|---|---|
| T56<br>S659<br>S676 | PAK1 | SRC-3Δ4 (SRC-3 without NLS) acting as a signaling adaptor at the cell membrane in the EGF signal transduction promoting cancer cell migration | [105] |
| S505<br>S509 | GSK3 | Influence ubiquitination dependent SRC-3 degradation and activation | [88] |
| S505, S543, S860, S867 | p38MAPK and JNK | Increase interaction with CBP and NRs | [76] |
| S860 | p38MAPK | Degradation of SRC-3 | [85] |
| S601 | CK1delta | Stablilizes SRC-3, influence the transcriptional activity of ER-α | [106] |
| S728<br>S867 | CDK1 | Redistribution of SRC-3 from a chromatin-associated state in interphase to a more peripheral localization during mitosis | [107] |
| S857 | IKK, PKA, | Essential for NF-κB activity and IL-6 expression | [76] |
| | PFKFB4 | ATF4 activity and purine synthesis | [77] |
| | ERK3 | Essential for interaction of SRC-3 with PEA3, which promotes upregulation of MMP gene expression and pro-invasive activity | [74] |
| | NLK | ER transcriptional activity | [108] |
| | IKK | Recruitment of CBP | [78] |
| S857, S860 | ERK1/2 | Enhancement of SRC-3 nuclear translocation | [87] |
| S1033 | Atypical PKC | Stabilizes SRC-3 protein in a ER-dependent manner | [109] |
| Y1357 | c-Abl (v-Abl Abelson murine leukemia viral oncogene homolog 1) | Facilitates the cross talk between steroid hormone and growth factor thereby regulating the cell growth and focus formation | [110] |

The SRC-3 gene is amplified in about 10%, while the protein is overexpressed in about 60% of breast cancer patients [111]. SRC-3 overexpression and over activation occurs in numerous other human cancers including prostate cancer [112], gastric cancer [113] and pancreatic [114] and is associated with poor clinical outcomes . Therefore, SRC-3 is considered a true oncogene involved in tumor promotion. It is exciting to see that SRC-3 could be a candidate drug target for Coronavirus 2 (CoV-2). SRC-3 was highly expressed in the human respiratory system after CoV2 infection. It possibly interacted with proteins of SARS-CoV-2 and regulated the viral pathogenesis through replication and development [115]. In the following sections, role of

SRC-3 in different activities that are crucial for normal health and tumor development and progression are discussed.

#### 1.2.1.3.4.1. SRC-3´s role in development and cell proliferation

SRC-3 is involved in normal somatic growth [116]. SRC-3 knockout mice displayed marked growth retardation and reduced adult body size. SRC-3 is required for estrogen and progesterone-induced mammary gland alveolar development and glandular differentiation in female mice. The estrogen levels and ovulation capacity were significantly lower in SRC-3 knockout mice [63].

As a master regulator of cellular growth and organism development, SRC–3 coordinates many intracellular signaling pathways that are critical for cancer cell proliferation. Epidermal growth factor receptor (EGFR/HER1) and human epidermal growth factor receptor 2 (HER2) are receptor tyrosine kinases (RTK). They mediate several functions including cell proliferation, cell survival, and development [117]. In a study by Lahusen et al., knockdown of SRC-3 resulted in a loss of the EGF-induced proliferation of lung, breast, and pancreatic cancer cell lines. This was due to reduced tyrosine phosphorylation of EGFR at multiple residues both at autophosphorylation and Src kinase phosphorylation sites caused by the SRC-3 depletion [118]. In SRC-3 knockout cultured non-tumorigenic mammary epithelial cells and tumor cells, phosphorylation of HER2, cyclin D1, and cyclin E and activity of AKT and JNK were markedly decreased, and proliferation was reduced. Furthermore, the homozygous deletion of SRC-3 in a mouse model completely inhibited HER2-induced mammary tumor development [119].

SRC-3 also plays role in cell proliferation by regulating genes involved in cell cycle. SRC-3 interacted with E2F family members to promote the transcription of genes important for initiation of DNA replication (G1 progression), which include cell division cycle 25A (CDC25A), cell division cycle 6 (CDC6), minichromosome maintenance complex component 7 (MCM7), cyclin E and cyclin dependent kinase 2 (CDK2). Depletion of SRC-3 prevented cells from entering S-phase and undergoing mitosis [120, 121].

In contrast to several studies reporting the proliferative role of SRC-3, depletion of the SRC-3 gene promoted proliferation of lymphocytes which often evolved into lymphoma upon aging in mice [122]. These findings suggest that SRC-3 as transcriptional co-activators can either promote or inhibit cell proliferation in cell and tissue context specific manner.

#### 1.2.1.3.4.2. SRC-3 and metastasis

Metastasis is responsible for 90% of deaths in patients with solid tumors [123]. Therefore, identifying genes involved in the development of metastasis, and factors that regulate their activity are important to improve the favorable prognosis of the patients.

Cancer cell metastasis is a complex phenomenon involving a number of sequential events. It involves steps like degradation of the local environment, epithelial mesenchymal transition (EMT), migration or chemotaxis and invasion [124]. SRC-3 has been reported to play a role in several of these steps [101]. Matrix metalloproteinases (MMPs) are enzymes involved in the degradation of extracellular matrix and hence play a role in invasion and migration of cancer cells [125]. SRC-3 was reported to be involved in increased expression of several MMPs. SRC-3 played an important part in prostate cancer cell invasion and metastasis by regulation of MMP-2 and MMP-13 expression mediated by AP-1 [126]. In hepatocellular carcinoma, SRC-3 regulated the expression of MMP-9 expression via NFκB and AP-1 [127]. In another study by Long et al., ERK3-mediated phosphorylation of SRC-3 at S857 was found to be important for interaction of SRC-3 with PEA3, which upregulated gene expression of MMP-2 and MMP-10 in lung cancer cells [74].

EMT involves the loss of epithelial cell junction proteins, including E-cadherin, and an increased expression of mesenchymal markers, such as N-cadherin and vimentin resulting in the conversion of apical-basal cell polarity into a spindle-shaped morphology [128]. According to a study by Wang et al., estrogen receptor and SRC-3 formed a complex that induced the transcription of SNAI1 which led to decrease of E-cadherin expression in T47D and MCF7 cells lines [129]. Similarly, studies have shown that overexpression of SRC-3 reduced the expression of E-cadherin in human pancreatic adenocarcinoma [130] and A549 cells [131]. Rohira et. al. found that mRNA and protein expression of the mesenchymal markers SNAI1, Slug, Vimentin and N-cadherin increased when SRC-3 was overexpressed and the expression of SNAI1 and Slug decreased when SRC-3 was knocked down using shRNA against SRC-3 [131].

Otaiby et al. showed that SRC-3 is important for migration in mouse endothelial cells as observed in *in vitro* scratch assays and wound healing assays in mouse models [132]. C-X-C motif chemokine receptor 4 (CXCR4) is a chemokine receptor and was positively co-related with metastasis [133]. The knockdown of SRC-3 substantially reduced both mRNA and protein expression of CXCR4, whereas the knockdown of CXCR4 in cells with SRC-3 ectopic overexpression diminished SRC-3-induced migration and invasion *in vitro* and tumor metastasis *in vivo* [134]. TEAD is an important member of the Hippo pathway, which is

involved in tissue regeneration [135]. All members of p160 family of co-activators are able to interact with transcription factors of TEAD family via their bHLH-PAS domain and potentiate the transcription [136]. SRC-3 and YES associated protein (YAP) converge at TEAD4. SRC-3 and YAP can together enhance the expression of genes that are important for the progression of invasion of breast cancer cells [72].

#### 1.2.1.3.4.3. SRC-3 and metabolism

SRCs was reported to be involved in a diverse array of metabolic functions [45, 59]. In particular, SRC-3 was reported to have significant role in brain and skeletal muscle metabolism [137]. SRC-3 was found to be necessary for proper transport of long chain fatty acids into mitochondria in skeletal muscle and its metabolism, likely through the regulation of carnitine acyl-carnitine translocase (CACT) gene expression. CACT is involved in the transport of fatty acids into the mitochondria. Thus, SRC-3 is a key regulator of β-oxidation [138]. Mouse model harboring serine to alanine mutations at four conserved phosphorylation sites S498, S536, S847 and S850 of SRC-3 displayed a phenotype with increased body weight and reduced peripheral insulin sensitivity. The underlying mechanism was linked to elevated IGFBP3 levels which enhanced IGF1 signaling [86]. However, other studies showed negative role of SRC-3 in lipid metabolism. PPARG co-activator-1 Alpha (PPARGC1A) plays a critical role in the maintenance of energy homeostasis [139]. SRC-3 facilitated the acetylation and the consecutive inactivation of PPARGC1A, through its effect on the expression of GCN5, the prime PPARGC1A acetyltransferase. Thereby, the genetic ablation of SRC-3 protected against obesity and improved insulin sensitivity by reducing the acetylation of PPARGC1A [140]. In line with this, Ma et. al. found that depletion of SRC-3 enhanced lipolysis by upregulating Peroxisome Proliferator Activated Receptor Alpha (PPARA) expression. In the study, SRC-3 coactivated retinoic acid receptor (RAR)-α to increase the expression of COUP transcription factor II (COUP-TFII), a transcription repressor. COUP-TFII decreased the expression of PPARA which is involved in lipolysis [141]. SRC-3 is reported to regulate metabolic pathways that supports tumorigenesis. Hypoxia inducible factor 1α (HIF1α) is a key transcription factor required for glycolysis [142]. SRC-3 promoted glycolysis in bladder cancer cells through HIF1α to facilitate tumorigenesis of urinary bladder cancer cell [143]. Similarly, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-4 (PFKFB4)-driven SRC-3 activation regulated glucose flux towards the pentose phosphate pathway that promoted the progress of breast cancer [77].

#### 1.2.1.3.4.4. SRC-3 and inflammation

Inflammation is a natural defense mechanism of the body where leucocytes travel to the damaged tissues to destroy the inflammatory trigger. Although acute inflammation mediates host defense against infections, chronic inflammation can predispose to various illnesses, including cancer [144]. Inflammation is a key component of the tumor microenvironment and it can promote or inhibit cancer. Inflammation can supply the tumor microenvironment with growth factors, survival factors, proangiogenic factors, and extracellular matrix-modifying enzymes that facilitate tumor initiation and progression [8]. The main players involved in cancer-related inflammation include infiltrating leukocytes, transcription factors such as NF-κB and STAT3, primary inflammatory cytokines, such as IL-1β, IL-6 and TNF-α, chemokines such as CCL2 and CXCL8 [145]. The nuclear factor NF-κB pathway is a pro-inflammatory signaling pathway, involved in the expression of pro-inflammatory genes such as cytokines, chemokines, and adhesion molecules [146]. IκB kinases (IKK) mediate the degradation of IκB, the inhibitor of NF-κB, in response to tumor necrosis factor (TNF)-α stimulation. IKK was reported to phosphorylate SRC-3, increasing its nuclear localization and then act on NF-κB resulting in the reduced expression of interferon regulatory factor-1 [75] or increased expression of Interleukin (IL)-6 [76]. IL-6 is an important modulator for the transition from acute phase to chronic phase of inflammation and plays role in JAK/STAT3, RAS/MAPK and PI3K–PKB/AKT pathways that are involved in regulation of many gene products that cause cell proliferation, differentiation, apoptosis, angiogenesis and metastasis [147]. SRC-3 can activate the NF-κB signaling pathway to promote C-X-C Motif Chemokine Ligand 2 (CXCL2) expression at the transcriptional level and contribute to host defense against enteric bacteria [148].

SRC-3 was also reported to suppress inflammatory response in mouse macrophages. SRC-3 cooperated with translational repressors such as T-cell intracellular antigen 1 (TIA-1) and TIA-1 related protein (TIAR) to regulate cytokine mRNA translation. In SRC-3 knockout macrophages, lipopolysaccharide (LPS) induced significant amount of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β compared to SRC-3 WT macrophages although both expressed similar amounts of the cytokine mRNA [149]. Peritoneal macrophages of SRC-3 deficient mice showed a decrease in bacterial phagocytosis and an increase in apoptosis of the macrophages. SRC-3 deficient mice produced more pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in plasma than wild-type mice during E. coli-induced peritonitis. Such SRC-3 deficient mice were more susceptible to peritonitis-induced lethality

caused by excessive local and systemic inflammatory responses [150]. In further study by the same group, SRC-3 was found to be involved in the activation of NF-κB signaling pathway in colon epithelial cells to enhance the secretion of CXCL2 which is required for the recruitment of neutrophils to ward off the intestinal bacteria. This suggests an important protective role of SRC-3 in the host defense against bacterial infection [148].

**1.2.1.3.4.5. Role of SRC-3 in hormone therapy and chemotherapy**

The choice of breast cancer treatment depends on the presence of ER, PR and HER2 [151]. For hormone receptor positive breast cancers, selective estrogen receptor modulators (for example tamoxifen) and aromatase inhibitors, which inhibit estrogen synthesis, are the drugs of choice [152] while for triple negative breast cancer cell (TNBC) cytotoxic drugs are the main treatment option [153].

SRC-3 was reported to play a role in hormone treatment in breast cancer cells and was used as a prognostic factor and predictive factor for hormone therapy. High SRC-3 level was a predictive marker for improved response to tamoxifen treatment in both pre and postmenopausal women [154, 155]. However, other studies have shown contradictory results. In several hormone receptor positive breast cancer cells treated with tamoxifen, high SRC-3 expression was associated with worse prognosis signifying the role of SRC-3 in tamoxifen resistance [102, 156-158]. Recently it was identified that Proline, glutamic acid, leucine-rich protein 1 (PELP1)/SRC-3 complexes enhance breast cancer cells activity and therapy resistance by promoting metabolic plasticity in ER positive breast cancer. Inhibiting this complexes in combination with endocrine therapies may be an effective strategy to inhibit breast cancer progression [159]. SRC-3 was significantly associated with disease recurrence and reduced disease-free survival in aromatase inhibitor resistant breast cancer. Estrogen-independent activation of ERα-SRC-3 led to marked increase in ER target genes involved in tumor cell proliferation [160]. There are only few studies regarding the role of SRC-3 in the treatment of TNBC. Treatment of TNBC cell lines with chemotherapeutic reagents resulted in survival of a set of cells that expressed SRC-3 at low levels. Though such cells had reduced tube forming and metastatic capacity, the prognosis was very poor. Therefore, the gene expression pattern of such SRC-3 low expressing cells may represent a signature indicative for poor response to chemotherapy in TNBC patients [161]. The role of SRC-3 in chemotherapy resistance is not limited to breast cancers. SRC-3 was found to be overexpressed in the chemo-radio therapy resistant group when compared to the chemo-radio therapy effective group in esophageal squamous cell carcinoma patients, suggesting that overexpression of SRC-3 is a useful predictor

of chemo-radio therapy resistance and an independent molecular marker of poor prognosis [162]. Down-regulation of SRC-3 expression enhanced the sensitivity of cholangiocarcinoma cells to chemotherapeutic drugs such as tamoxifen and cisplatin. This was achieved by the role of SRC-3 in promoting drug efflux, activation of AKT pathway and enhancing the expression of antioxidant genes [163]. In a colon cancer cell line, depletion of SRC-3 resulted in increased sensitivity to 5-Fluorouracil and oxaliplatin treatment compared to the wild type cells. High expression of SRC-3 led to over activation of NF-κB activity which was partially responsible for the chemoresistance [164]. SRC-3 prevented pemetrexed-induced cytotoxicity by inhibiting the chemotherapy-induced apoptosis via decreasing reactive oxygen species (ROS) level and regulating Nrf2 and AKT signaling pathway in lung adenocarcinoma [165]. Thus, SRC-3 seems to enhance the resistance to chemotherapeutic agents in different types of cancer.

#### 1.2.1.3.4.6. Involvement of SRC-3 S857 phosphosite in different functions

SRC-3 S857 is the most frequently reported phosphorylation site among the several phosphorylation sites of SRC-3 [84]. Phosphorylation of SRC-3 at S857 is important for the interaction of SRC-3 with ER, AR and CBP. It was demonstrated that mutation of SRC-3 at serine 857 to nonphosphorylatable alanine (SRC-3 S857A) diminished the interaction between SRC-3 and ER, AR or CBP and thereby greatly attenuated the ability of SRC-3 to activate the transcriptional activity of ER, AR and NF-κB [76]. Similarly, mutation of serine at 857 and 860 to alanine attenuated the nuclear translocation of SRC-3 thereby suggesting that phosphorylation at S857 is necessary for intracellular translocation of SRC-3 [87]. A Transwell Matrigel cell invasion assay demonstrated that phosphorylation at SRC-3 S857 is necessary for the lung cancer cell invasion. Phosphorylation of SRC-3 at S857 enhanced the binding of SRC-3 with the transcription factor PEA3 and upregulated the expression and activity of PEA3 target gene, MMP2 which is involved in cell invasion [74]. Phosphorylation of SRC-3 at S857 by IKKα enhanced the interaction of SRC-3 with HIF1α and CBP. Such interaction between HIF-1α, SRC-3 and CBP at the migration inhibitory factor (MIF) promoter increased the expression of MIF, which inhibits autophagic cell death thereby enhancing chemoresistance and tumorigenesis in xenograft mouse model [78]. In actively glycolytic breast cancers, phosphorylation of SRC-3 at S857 by PFKFB4 increased its interaction with the transcription factor ATF4 by stabilizing the recruitment of SRC-3 and ATF4 to target gene promoters. This enhanced the transcription of key metabolic enzymes which drive glucose flux into pentose phosphatase pathway thereby causing breast cancer cell proliferation and metastasis. This was demonstrated by the inability of the mutant SRC-3 S857A to rescue the growth of SRC-3-

depleted MCF7 cells compared to wild-type SRC-3 when stimulated with glucose and estradiol. Furthermore, in xenograft mouse model, mutant SRC-3 S857A suppressed breast tumour growth in mice and prevented metastasis to the lung from an orthotopic settings [77]. A knock-in mouse model containing serine to alanine mutations at four conserved phosphorylation sites of SRC-3 displayed a phenotype with increased body weight and adiposity coupled with reduced peripheral insulin sensitivity. Among the four SRC-3 phosphosites mutated in the mouse model, serine 847 corresponds to the human SRC-3 S857. In this light it is possible that human SRC-3 S857 could be important for insulin sensitivity and glucose homeostasis as suggested by York B. et al. [86]. However, it would be interesting to see whether all the four phosphorylation sites or only SRC-3 S857 is enough to display the described phenotype. Taken together, this single phosphorylation site modification contributes to proliferation, metastasis and metabolic changes that are involved in tumor progression. Therefore, prevention of phosphorylation of this site is important because it can prevent the action of pathways involved in tumorigenesis and tumor progression through SRC-3 S857.

### 1.3. Signal transduction

In an adult human being, about 37 trillion cells are trying to remain integrated as a single entity by communicating with each other, which occurs by means of exchanging signals like hormones, cytokines and neurotransmitters [166]. In addition, they also receive signaling cues from their environment. If soluble in lipid membrane such signal can cross the plasma membrane and bind to NRs [167], otherwise the signal is received by transmembrane receptors like heptahelical receptor, serine/threonine kinase-couple receptor, tyrosine kinase-coupled receptor, protein phosphatase coupled receptors or ion-channels. The exogenous signal is transmitted inside the cell by various intracellular signaling pathways, for example by signal transducing proteins such as G-proteins, second messengers and protein kinases [168]. This leads into a cellular response, which may involve progression through the cell cycle, changes in cellular gene expression, cytoskeletal architecture, protein trafficking, protein synthesis and stability, adhesion, migration, secretion, contraction and programmed cell death. The irregularities in signal transduction pathways results into diseases like cancers, diabetes, and disorders of the immune and cardiovascular systems. Hence, the knowledge of signaling transduction can be used in disease management that involves design and development of drugs that interferes with cell signaling.

As early as 1855, Claude Bernard has discovered that ductless glands like spleen released `internal secretions` to effect distant cells. This can be regarded as the earliest notion of cellular

signaling [169]. Regarding the intracellular signal transduction, the first pathway that was discovered was the course of the catalytic conversion of glycogen to glucose-I-phosphate catalyzed by the enzyme phosphorylase [170]. The discovery that phosphorylation can reversibly alter the activity of an enzyme through the combined action of a protein kinase and a protein phosphatase is another epoch-making discovery in cellular signaling [171]. Today many distinct cellular signaling pathways such as PI3K/AKT, Wnt/β-catenin, Delta/Notch, JAK-STAT and MAPK are well defined and understood.

### 1.4. Protein kinases

Phosphorylation is the single most important post translational modification of proteins regulating the cell signaling. Phosphorylation is addition of a phosphate group to a substrate. It is carried out by proteins called kinases, which catalyzes the transfer of the gamma-phosphate group from ATP to the substrate molecule. Phosphorylation is a reversible phenomenon. The removal of phosphate group from a substrate is called dephosphorylation and it is carried out by a protein phosphatase.

The characteristic feature of a protein kinase is the protein kinase domain [172, 173]. The kinase domain contains 12 conserved subdomains that fold into a common catalytic core structure [174]. The activation loop is one of them. Phosphorylation of the activation loop is essential for the activation of a kinase. Eukaryotic protein kinases catalyze the phosphorylation of other proteins though many of them also undergoing autophosphorylation of the activation loop within the catalytic center [175]. Protein kinases usually utilize two types of interactions to recognize their physiological substrates in cells. First is the recognition of the consensus phosphorylation sequence in the protein substrate by the active site of the protein kinase and the other are interactions between the kinase and the substrate mediated by binding of docking motifs [176-178].

There are 518 protein kinases encoded by the human genome. 478 of them are categorized as eukaryotic protein kinases (ePKs) and the remaining 40 are grouped as atypical protein kinases (aPKs) [179]. The atypical protein kinases lack the highly conserved eukaryotic kinase motifs but possess biochemical kinase activity and share the same characteristic eukaryotic protein kinase fold [180]. Eukaryotic protein kinases are further divided into seven groups based on the function and the sequence similarity of their catalytic domain [179]. On the basis of the amino acid phosphorylated by protein kinases, they can be classified as tyrosine kinases, serine/threonine kinases or dual-specificity kinases which phosphorylate tyrosine, serine/threonine or both amino acid residues on a substrate, respectively.

Protein kinases are the workhorses of signal transduction and are involved in a wide range of biological activities including proliferation, survival, apoptosis, metabolism, transcription, differentiation, and several other cellular processes. Dysregulation of protein kinases therefore plays a role in numerous diseases such as cancer [181], inflammatory diseases [182], central nervous system disorders [183], cardiovascular diseases [184] and complications of diabetes. Hence, these enzymes are important therapeutic targets for drug development.

### 1.4.1. Mitogen Activated Protein Kinases

Mitogen-activated protein kinases (MAPKs) are protein serine/threonine kinases that transmit extracellular stimuli into a variety of cellular activities including cell survival, apoptosis, cell differentiation, migration, motility and metabolism [185]. This group of kinases along with cyclin-dependent kinases (CDKs), glycogen synthase kinase 3 (GSK3), and CDK-like kinases (CLKs), constitutes a larger family referred to as the CMGC group kinases [179].

A MAPK has a strict three-tire activation module. MAP kinase kinase kinase (MAP3K) activates a MAP kinase kinase (MAP2K), which then activates a MAPK [186]. Further, down the signaling line, MAPK activates or inactivates other kinases and transcription factors. The MAP3K is a serine/threonine kinase activated by interactions with a small GTPase and/or phosphorylation by protein kinases downstream of cell surface receptors. As many as 20 MAP3Ks selectively activate seven different MAP2Ks [187]. MAP2Ks are dual specificity protein kinases that phosphorylate threonine (T) and tyrosine (Y) residue in conserved tripeptide T-X-Y motifs in the activation loop of MAPKs [188]. In order to be active, a MAP2K needs to be phosphorylated at its two serine or threonine residues in its activation loop [189].

MAPKs are proline-directed serine/threonine protein kinases. In mammals, MAPKs comprise 14 members, which can be broadly divided into conventional- and atypical-MAPKs [185]. Conventional MAPKs differ from atypical MAPKs by having a signature T-X-Y motif in the activation loop and following characteristic three-tire activation module. The group of the conventional MAPKs consist of the extracellular-signal regulated kinases (ERK 1/2 and ERK5), the c-*jun* N-terminal kinase (JNK 1/2/3), and the p38 MAPKs α/β/ϒ/*δ*, while atypical MAPK comprises ERK3, ERK4, ERK7/8 and nemo-like kinase (NLK) [190, 191]. Each MAPK subtype organizes a module of its own as shown in Figure 4.

### 1.4.1.1. ERK1/2

The ERK1/2 pathway is activated by mitogenic factors (for example epidermal growth factor (EGF), platelet derived growth factor (PDGF)), differentiation stimuli (for example nerve growth factor, vascular endothelial growth factor) and cytokines (for example TNF-α). In

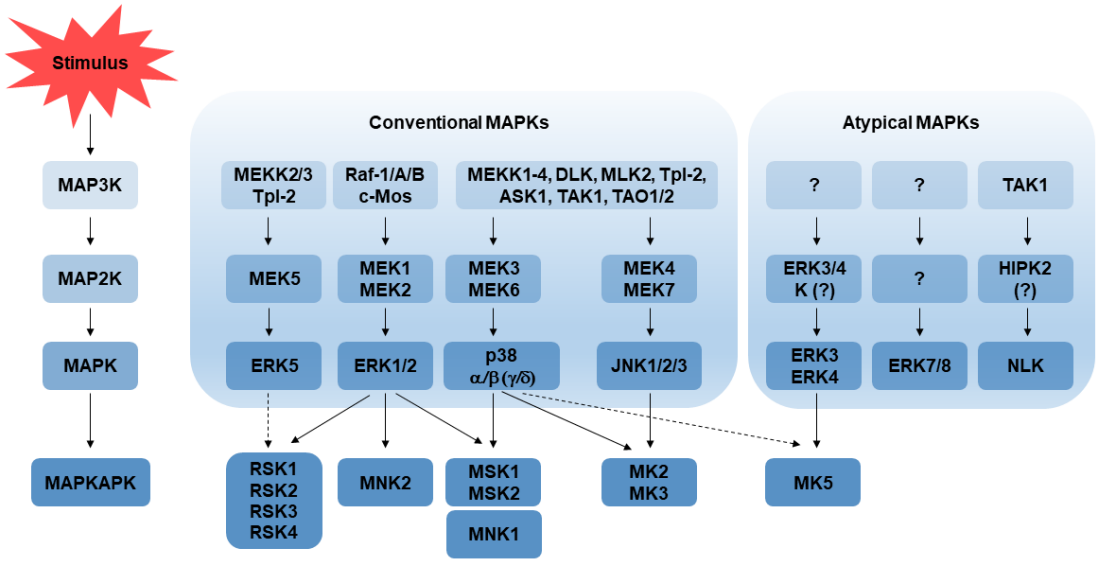


**Figure 4. MAPK signaling cascades.** Mitogens, cytokines, and cellular stress promote the activation of different MAPK pathways, which in turn phosphorylate and activate the five subgroups of MAPKAPKs, including RSK, MSK, MNK, MK2/3, and MK5. Dotted lines indicate that, although reported, substrate regulation by the respective kinase remains to be thoroughly demonstrated. The γ and δ isoforms of p38 are in parentheses to indicate that they have not been shown to promote MAPKAPK activation. Figure adapted from [185] with permission.

particular, when a growth hormone is bound to the extracellular domain of a receptor tyrosine kinase (RTK), the receptor dimerizes resulting in its autophosphorylation, which subsequently activates RAS, a small G-protein anchored to the cell membrane [192]. Activated RAS binds to RAF and makes RAF accessible for activation by autophosphorylation, PKC or SRC kinases (MAP4K). Activated RAF (MAP3K) phosphorylates MEK1/2 (MAP2K1/2). Activated MEK1/2 phosphorylates threonine and tyrosine residues of the T-E-Y motif in the activation loop of ERK1/2 [193]. ERK has two isoforms, ERK1 and ERK2, often just referred to as ERK1/2 because there is no significant difference in their activation mechanisms and substrate recognition and are hence functionally redundant. ERK2 is expressed at higher levels than ERK1 in most mammalian tissues [194].

Activated ERK1/2 phosphorylates numerous cytoplasmic and nuclear targets, including transcription factors, kinases, phosphatases, and cytoskeletal and scaffold proteins [195]. ERKs can phosphorylate transcription factors such as Elk 1, c-Fos, $PLA_2$, Ets-1, c-Jun, c-Myc etc. and

several members of MAPK activated protein kinases. With this, the ERK1/2 cascade will regulate different effector proteins that regulate a wide variety of cellular processes, including cell growth, proliferation, cell cycle, differentiation, and survival [196]. Mutations in RAF for instance are linked to 66% of melanomas [197].

#### 1.4.1.2. ERK5

ERK5 is composed of two distinct functional domains. One is a catalytic N-terminal region with a T-E-Y motif whose sequence is 50% identical to ERK1/2 and is responsible for its protein kinase activity. The other one is a C-terminal extension, which is unique to this MAPK and regulates its subcellular distribution and transcriptional co-activator function [198].

ERK5-mediated signaling is prompted by a variety of extracellular stimuli, including mitogens, oxidative and osmotic stress, hypoxic conditions as well as pro-inflammatory cytokines [199]. On stimulation, MEKK2 and MEKK3 activate MEK5, which then phosphorylates ERK5 at its regulatory T-E-Y motif, followed by auto-phosphorylation at multiple residues within its C-terminal tail. Other upstream activators of MEK5-ERK5 are TPL2/COT, RAS and AKT [200]. The activated ERK5 phosphorylates downstream substrates such as myocyte enhancer factor 2 (MEF2) [201], serum response factor accessory protein 1 (SAP1) [202] or the p90 ribosomal S6 kinase (RSK) [203]. Non-canonical pathways are involved in the MEK5-independent phosphorylation of the C-terminal residues. This includes phosphorylation by either cyclin-dependent kinase 1 (CDK1) [204], ERK1/2 [205] or CDK5 [206] resulting in nuclear shuttling and transcriptional transactivation irrespective of ERK5 T-E-Y phosphorylation status.

ERK5 plays a well-established role in cell proliferation and is essential for cardiovascular development and neural differentiation [207]. Recently, MEK5-ERK5 signaling is demonstrated to be involved in sustaining malignant cell proliferation and tumor growth, invasion–metastasis cascade, anti-cancer drug resistance and sustaining inflammatory tumor milieu [208].

#### 1.4.1.3. p38MAPK and JNK

p38MAPK and JNK are the pathways involved in regulation of stress activated by a diverse array of intra- and extracellular stimuli. They show a certain degree of redundancy in their actions, however, the extent of crosstalk between them is cell and tissue specific [209]. p38MAPK and JNK are activated by environmental stress factors like UV radiation, DNA damage, heat shock, hypoxia, osmotic pressure, cytotoxic chemicals, oxidative stress and inflammatory stimuli such as LPS, TNF-α, IL-1 and mitogenic stimuli [210, 211].

Plasma membrane receptors for example serine/threonine receptor kinase-coupled receptors receive the signals and transmit them to MAP3Ks such as Apoptosis signal-regulating kinase 1 (ASK1), MEK Kinase (MEKK), mixed-lineage kinase (MLK), transforming growth factor-β-activated kinase 1 (TAK1), and tumor progression locus-2 (TPL-2). In many cases, the MAP3Ks are recruited to the inner side of the plasma membrane by small G-proteins of the Rho family such as RAC and CDC42 [212]. The MAP3Ks are then activated by putative MAP4K as germinal-center kinases (GCK), while in other cases the MAP3Ks are activated by interaction with the RIPK1-TRAF2 complex or the IRAK1-TRAF6 complex. The MAP3Ks activate different MAP2Ks. The MAP2K named MKK4/7 phosphorylate the T-P-Y motif of JNK, while MKK3/6 phosphorylate the T-G-Y motif of p38MAPK in the activation segment (reviewed in [213]). There are also non-canonical p38MAPK activations. In T-cells, upon antigen presentation the antigen T cell receptor (TCR) is involved in phosphorylation of Y323, which promotes an auto-phosphorylation of activation loop [214]. In addition, p38 can be activated by the presence of other stimuli such as intracellular infection, myocardial ischemia or dendritic cells maturation signals. In these cases, TAK1-binding protein 1 (TAB1) associates with p38MAPK and promotes its auto-phosphorylation [215].

p38MAPK comprises four isoforms p38α, p38β, p38γ and p38δ. The four isoforms differ in sequence homology, substrate specificity and sensitivity to inhibitors. The p38α and p38β are both ubiquitously expressed. While p38α is expressed at high level, p38β is expressed only at lower level. On the other hand, p38δ and p38γ have a more restricted expression pattern [216]. They are expressed at significantly different levels in each tissue and hence the isoforms have distinct biological functions [217].

As many as hundred proteins that are located in either the nucleus or cytoplasm are reported to be directly phosphorylated by p38MAPK (Figure 5). Furthermore, p38MAPK can phosphorylate kinases such as MK2, MSK1 and MSK2 which in turn can phosphorylate additional proteins. p38MAPK phosphorylated MK2/MK3 regulates mRNA stability and cytoskeleton organization. MSK1 and MSK2 can regulate gene expression by phosphorylation of transcription factors. p38MAPK can regulate protein stability by either phosphorylation of substrate proteins leading to subsequent degradation or activation of enzymes involved in proteasomal degradation (reviewed in [218, 219]) [220].

The p38MAPK is also involved in stress-induced apoptosis, cell differentiation, cell cycle regulation, migration, senescence and aging [221]. A strong and sustained p38MAPK activation has been linked to apoptosis, senescence, and terminal cell differentiation, whereas low

p38MAPK activation has a cell survival effect [209]. p38MAPK is implicated in cell apoptosis mediated by different chemotherapeutic reagents in breast and colon cancer cell lines. Furthermore, inhibition of p38MAPK has been associated with resistance to gemcitabine and cytarabine (reviewed in [222]). As the overwhelming inflammatory response in COVID-19 infection may be due to hyperactivation of p38MAPK, Grimes et. al. has proposed that therapeutic inhibition of p38MAPK could attenuate COVID-19 infection [223].

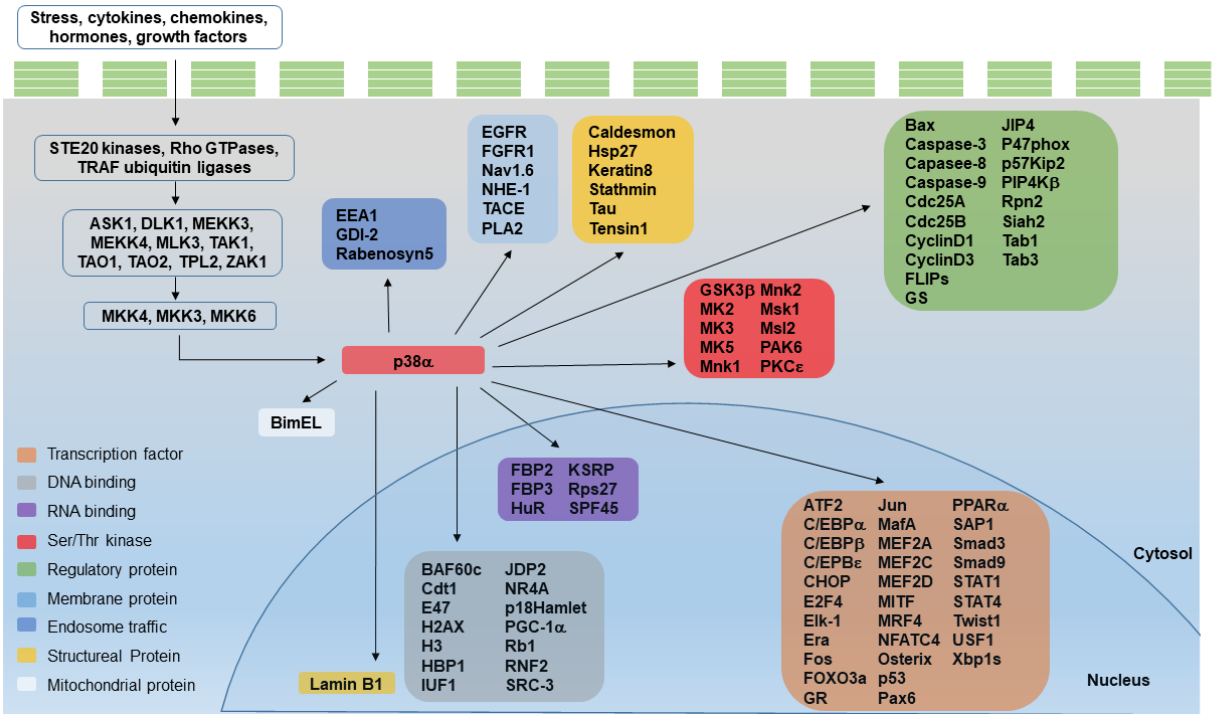


**Figure 5. The p38MAPK signaling pathway.** Different stimuli such as growth factors, inflammatory cytokines and a wide variety of environmental stress factors can activate p38MAPK. Around hundred downstream targets including transcription factors, DNA and RNA binding protein, membrane protein, structural protein, kinases, mitochondrial protein and regulatory proteins are reported. Figure adapted from [219] with permission.

The p38MAPK and NF-κB pathways are important determinants involved in the regulation of stress. Although p38MAPK and NF-κB are both stimulated by stress, only a few stress stimuli activate both pathways at the same time. Besides, p38MAPK itself does not phosphorylate NF-κB subunits. This indicates that primary events of both pathways are not, or are only partially, overlapping [224, 225]. However, p38MAPK regulates transcriptional activity of p65 indirectly by regulating its acetylation via phosphorylation-coupled acetyltransferase activity of p300 [226].

### 1.4.1.4. ERK3 and ERK4

ERK3 and ERK4 are atypical members of MAPK family. The two proteins have high sequence similarity. Although they were among the first MAPKs to be identified they are yet not well

characterized [190, 227]. The upstream kinases and extracellular stimuli activating ERK3 and ERK4 are still not well known. Unlike conventional MAPK, phosphorylation of ERK4 is detected in resting cells and is not modulated by common mitogenic or stress stimuli [228]. However, recent data have suggested that ERK3 and ERK4 may be regulated by group 1 p21-activated kinase (PAK) downstream of RAC/Rho/CDC42 [229]. ERK3 is a highly unstable kinase and is subject to rapid degradation by the ubiquitin-proteasome system [230]. ERK3 and ERK4 possess highest homology with ERK1/2 but unlike ERK1/2, they have a S-E-G motif in their activation loop. Activated ERK3 binds, translocates and phosphorylates MK5 [231, 232]. The interaction between ERK4 and MK5 prevents ERK4 dephosphorylation [228]. Unlike the classical MAPKs, interaction between the docking (D) domains of ERK3/ERK4 and their substrate MK5 is not important for phosphorylation. Rather, it is the FRIEDE motif within MK5 that is necessary for the interaction with ERK3/ERK4 [233]. Recently, DUSP2 a known phosphatase of ERK1/2 and p38MAPK is reported to dephosphorylate ERK3 and ERK4 and prevent the activation of their downstream target MK5 [234].

Genetic ablation of the ERK3 gene has revealed that ERK3 plays an important role in fetal growth and lung maturation [235], however recent studies using ERK3 conditional knockout mice has revealed that deletion of ERK3 is not essential for viability, pulmonary function or T-cell development [236, 237]. ERK3 plays a role in invasive breast cancer morphology and increases the cell migration speed [238]. Furthermore, ERK3 regulates IL8-mediated chemotaxis of human neutrophils and monocytes [239]. Depending on the cell type, ERK3 can either promote or suppress cell invasiveness. ERK3 is reported to promote migration and invasion of lung cancer cells [74, 240] but inhibits the migration, proliferation and colony formation of melanoma cells [241]. New substrates of ERK3 have been uncovered recently. ERK3 has been reported to phosphorylate SRC-3 at S857 in lung cancer cells, and this promotes lung cancer cell invasiveness [74] and the ERK3-SRC-3 axis is also reported to be involved in regulation of endothelial cell functions [79]. ERK3 is reported to phosphorylate Tyrosyl DNA phosphodiesterase 2 (TDP2) at S60, and this regulates TDP2's phosphodiesterase activity, thereby cooperatively protecting lung cancer cells against Topoisomerase 2 inhibitors-induced DNA damage and growth inhibition [242]. A role of ERK3 has also been established in adipose tissue. In a recent study, the ERK3-MK5 signaling axis was found to target the transcription factor FOXO1 to promote lipolysis in mouse adipocytes [243].

#### 1.4.1.5. ERK7/8

ERK7/8 is an atypical MAPK, which contains a T–E–Y motif in its activation loop. ERK7/8 is the most recently identified and comparatively the least studied atypical MAPK [244]. Unlike the conventional MAPK, it is activated by auto-phosphorylation or by kinases like RET/PTC3 [245, 246]. ERK7/8 is involved in autophagy [247], ciliogenesis [248], protein trafficking/secretion [249], cell proliferation [250] and genome integrity [251]. Interestingly, ERK7/8 is also found to be involved in formation of cilium-like structure in apicomplexan parasite *Toxoplasma gondi* [252].

### 1.4.2. MAPK-Activated Protein Kinase

The MAPK-activated protein kinases (MAPKAPK) are a group of kinases activated by MAPKs [253]. They consist of 11 members divided into five groups: four p90 ribosomal S6 kinases (RSK1-4), two mitogen- and stress-activated kinases (MSK1-2), two MAPK-interacting kinases (MNK1-2) and three MAPK- activated protein kinases (MKs) (where MK2, MK3 makes one subgroup and MK5 is by itself in another subgroup). The RSKs are direct downstream targets of ERK1/2, while the MSKs and MNKs are targets of both ERK1/2 and the p38MAPKα and β. MK2 and MK3 are downstream targets of p38MAPK, while MK5 is a direct downstream target of the atypical MAPKs, ERK3 and ERK4 [185]. These MAPKAPKs belong to the calcium/calmodulin-dependent protein kinase (CaMK) group of protein kinases [254] and mediate a wide range of biological functions in response to mitogens and stress stimuli. RSKs are involved in cell growth, proliferation, survival and migration [255] while MSKs [256], MNKs [257], and MKs [258] are involved in inflammation, neuronal development, and mRNA stability.

#### 1.4.2.1. MK2

MK2 is phosphorylated by p38MAPK under stress [259]. It is activated when phosphorylated at any two of the three residues T222, S272 and T334. The activation of MK2 results in nuclear export of MK2. Cytoplasmic, active MK2 then phosphorylates downstream targets. The N-terminus of MK2 contains a proline-rich region which is able to bind to SH3 domain containing proteins, while the C-terminus contains different signals for regulation of subcellular localization [260, 261]. MK2 exists in two alternatively spliced variants, one of which contains a nuclear localisation signal (NLS) and nuclear export signal (NES) close to its carboxyl terminus [262, 263]. Recently, a third isoform has been described which arises from alternative translation initiation sites [264].

MK2 is a serine/threonine kinase that phosphorylates serine in the peptide sequence, Hyd-X-R-X-X-S, where Hyd is a large hydrophobic residue, X can be any amino acids except cysteine, serine, threonine and tyrosine. MK2 displays a strong selection for the hydrophobic residues leucine (L), phenylalanine (F), isoleucine (I) and valine (V) at the S-5 position and S+1 position in its substrate in a peptide library screening [265]. An overview of different MK2 substrates, the consensus phosphorylation sequence (where known) and attributed functions of these target proteins are listed in table 3. The motif determined for MK2 using the peptide library screening [265] is in good agreement with the sequence of MK2 phosphorylation sites on known substrates listed in table 3 which primarily contains L, I, or F in the S−5 position and arginine (R) in the S−3 position.

**Table 3. List of MK2 substrate proteins**

| Substrate | Phosphorylation site/sequence | Attributed functions | Reference |
|---|---|---|---|
| Arachidonate 5-Lipoxygenase (5-LO) | 271: LERQLS*LEQ | Enzyme activation for leucotriene biosynthesis | [266] |
| bHLH transcription factor E47 | ? | E47 activity and E47-dependent gene expression | [267] |
| Bcl-2-associated athanogene (BAG2) | 20: FCRSSS*MAD | regulate Hsp70-mediated molecular chaperone activities and apoptosis | [268] |
| Beclin 1 | 90: PARMMS*TE | Increases starvation-induced autophagy | [269] |
| B-Related Factor 2 (BRF2) | 54: FPRRHS*VT<br>92: RDRSFS*EG<br>203: LQHSFS*FA | Interfere mRNA decay promotion of activity of BRF2 | [270] |
| Centrosomal Protein 131 (CEP131) | 47: IVRSVS*VV<br>78: LRRSNS*TT | Centriolar satellites remodeling | [271] |
| Ubiquitin Conjugating Enzyme E2 J1 (UBE2J1) | 184: LARQIS*FK | Translational control of TNF-α synthesis | [272] |
| ETS transcription factor 1 (ER81) | 191: FRRQLS*EPC<br>216: YQRQMS*EPN | Suppress Transcription | [273] |
| F-actin capping protein Z-interacting protein (CAPZIP) | 179: FRRSQS*DCG | Remodel actin filament assembly | [274] |
| Glycogen Synthase | 7: KKPLNRTLS*VASLPGL (*in vitro*) | - | [275] |
| gp130-IL6R-β | 782: FSRSES*TQ | Internalization and degradation of gp130-166R | [276] |
| Heat shock factor 1 (HSF1) | 121: KRKVTS*VST | Inhibition of binding to heat shock element | [277] |

| Substrate | Phosphorylation site/sequence | Attributed functions | Reference |
|---|---|---|---|
| Heterogeneous nuclear ribonucleoprotein A0 (HnRNP A0) | 84: LKRAVS*RED | ARE-dependent stabilization and translation of mRNA | [278] |
| Inhibitor of neuronal regeneration ( NOGO-B) | 107: PERQPS*WDP | Axonal regeneration | [279] |
| Keratin 18 | 52: VSRSTS*F | Regulation of mucin secretion | [280] |
| Keratin 20 | 13: FHRSLS*S | Regulation of mucin secretion | [280] |
| LIM domain kinase 1 (LIMK1) | 323: LGRSES*L | Regulates VEGFA-induced actin reorganization and cell migration | [281] |
| Lymphocyte-specific protein (LSP)1 | 204: LARQAS*IEK<br>252: LSRQPS*IEL | F-actin binding and actin remodeling stabilization of F-actin polarization during neutrophil chemotaxis | [282] |
| p16 subunit of the seven-member actin related protein-2/3 complex (p16-Arc) | 77: KDRAGS*IVL | Remodeling of actin cytoskeleton | [283] |
| poly A binding protein 1 (PABP1) | ? | Stabilization and translation of mRNA | [284] |
| Poly (A)-specific ribonuclease (PARN) | 557: YRNNS*F | Control G2/M checkpoint | [285] |
| Phosphoserine/threonine binding protein 14-3-3 zeta | 58: GARRSS*WRV | Regulation of dimerisation and target binding | [286] |
| Protein phosphatase Cdc25B1,2 | 323: LFRSPS*MPC | Cell cycle checkpoint control | [265] |
| Protein phosphatase Cdc25C | 216: LYRSPS*MPE | Cell cycle checkpoint control | [265] |
| RNA-binding protein 7 (RBM7) | 136: IQRSFS*SP | Increases the stability of noncoding RNA | [287] |
| Serum response factor (SRF) | 103: LKRSLS*EME | Transcription | [288] |
| Small heat shock protein (HSP27) | 15: LLRGPS*WDP<br>82: YSRALS*RQL<br>86: LSRQLS*SGV | Actin remodelling, regulation of chaperone properties | [275] |
| Small Heat shock protein (Hsp25) | 86: LNRQLS*SGV | regulation of the renal stress response and in the development of glomerulonephritis | [289] |
| TGF-β Activated Kinase 1 Binding Protein 3 (TAB3) | 506: YQRSSS*SG | - | [290] |
| Tristetraprolin (TTP) | 52: PGRSTS*LVE<br>178: LRQSIS*FSG | ARE-dependent mRNA stabilization and translation | [291]<br>[292] |
| Tuberin (TSC2) | 1254: LYKSLS*VPA | Cell size regulation | [293] |
| Vimentin | ? | Intermediate filament assembly | [294] |

| Substrate | Phosphorylation site/sequence | Attributed functions | Reference |
|---|---|---|---|
| Tyrosine hydroxylase | 19: FRRAVS*EQD | Enzyme activation for catecholamine biosynthesis | [295] |
| Ubiquitin ligase HDM2 | 157: LVSRPS*TSS<br>166: RRRAIS*ETE | Regulation of p53 | [296] |
| alphaB-crystallin | 59: FLRAPS*WFD | Regulation of chaperone properties or cytoskeleton rearrangement in cell division | [297] |
| Receptor Interacting Serine/Threonine Kinase 1 (RIPK1) | 320: VKRMQS*LQ | Inhibits apoptosis | [298] |
| Ataxia-telangiectasia group D-associated gene (ATDC) | 550: PSLMRS*QS | Resistance to ionizing radiation | [299] |
| RNA-binding protein deleted in azoospermia-like (DAZL) | 65: FARYGS*VK | Regulation of spermatogenesis | [300] |
| Jun activation domain-binding protein 1 (JAB1) | 177: PTRTIS*AG | promotes tumorigenesis by sustaining AP1 activity | [301] |

#### 1.4.2.1.1. MK2 and inflammation

MK2 plays a significant role in stress and immune response [258]. MK2 knockout mice are resistant to endotoxic shock [302], collagen induced arthritis [303] and show increased susceptibility to infection [304]. A possible contribution of MK2 in inflammatory processes in osteoarthritis [305], inflammatory bowel disease [306], cerulein-induced pancreatitis [307], skin inflammation [308], angiotensin II-induced vascular inflammation [309], inflammatory bone turnover [310], acute proliferative glomerulonephritis [289] and dextran sodium sulfate-induced mouse colitis [311] has been observed.

The pro-inflammatory property of MK2 is attributed to its ability to interfere with the stability of mRNAs containing Adenylate-Uridylate (AU)-rich elements (ARE). MK2 is a master regulator of RNA binding proteins [312]. MK2 phosphorylates RNA binding proteins such as tristetraprolin (TTP), Heterogeneous Nuclear Ribonucleoprotein A0 (hnRNPA0), poly (A) binding protein 1 (PABP1). TTP can regulate the stability of several genes involved in inflammation, including TNF-α, IL-2, IFN-gamma [313], IL-6 [314], IL-10 [315] and TTP itself. TTP competes with human antigen R (HuR) in binding to the ARE-containing mRNA. When bound to an ARE-containing mRNA, TTP degrades the mRNA while human antigen R (HuR) stabilizes it. Phosphorylation of TTP by MK2 decreases its affinity for the ARE-containing mRNA and results in its sequesteration by binding to 14-3-3. This allows HuR to bind to the ARE-containing mRNA and promote its translation [316].

MK2 can also stimulate the transcription of pro-inflammatory genes by modifying the action of transcriptional activators or repressors. Heat-shock factor 1 (HSF1) represses the transcription of pro-inflammatory IL-1β cytokine [317]. MK2 phosphorylates HSF1 and prevents the binding of HSF1 to cognate elements in promoters [277]. Thus, MK2 can increase the production of IL-1 by phosphorylation and inhibition of HSF1.

When comparing TNF-α treated wild type and MK2 deficient HUVEC cells, it was found that MK2 is essential for sustained nuclear localization of the p65 subunit of NF-κB by reducing expression of its exporter IκB-α [318]. MK2 phosphorylates the common gp130 subunit of the IL-6 receptor. This results in increased internalization and degradation of the receptor and subsequently declined STAT3 activation and reduced stimulation of STAT3-mediated gene transcription [276].

Receptor Interacting Serine/Threonine Kinase 1 (RIPK1) is a master regulator of cell fate, which may signal inflammation and cell survival via TAK1/p38MAPK and the IKK complex or promote apoptosis through FADD and caspase-8 (CASP8) under diverse cellular stress situations. RIPK1 activity is controlled by complex post translational modifications [319]. Recently, RIPK1 was identified as a direct substrate of MK2. MK2-mediated RIPK1 phosphorylation promotes RIPK1 pro-survival functions in TNF-α-treated fibroblasts and LPS-stimulated macrophages. Therefore, inhibition of MK2 in anti-inflammatory therapy could both inhibit cytokine biosynthesis at the cellular level and reduce the number of cytokine-producing cells, leading to resolution of inflammation [320] (reviewed in [321]).

#### 1.4.2.1.2. MK2 and actin remodeling

MK2 is reported to play a role in actin remodeling. This could be achieved by regulating F-actin capping protein Z-interacting protein (CAPZIP) [274], Lymphocyte-specific protein 1 (LSP1) [282], p16-Arc [283], heat shock protein 27 (Hsp27) and LIM domain kinase 1 (LIMK1) [281], which are proteins shown to participate in actin filament reorganization.

#### 1.4.2.1.3. MK2 and cell cycle

Another important physiological role of MK2 includes cell cycle checkpoint control following DNA damage [322]. MK2 appears to control the cell cycle checkpoint response, at least in part, through phosphorylation of the RNA binding proteins heterogeneous nuclear ribonucleoprotein A0 (HnRNPA0) and Poly (A) specific ribonuclease (PRAN) to stabilize growth arrest and DNA damage inducible alpha (Gadd45-α) transcript. Gadd45-α blocks the premature activation and nuclear translocation of Cdc25B and C in the presence of ongoing DNA damage [322].

Cdc25B/C protein phosphatases are positive regulators of Cyclin/Cdk complexes. In addition, MK2 phosphorylates and inactivates these Cdc25B/C protein phosphatases [323]. In this way, MK2 prevents the cell from entering into cell cycle upon DNA damage. In p53 mutant cells, MK2 can act as a checkpoint kinase involved in DNA damage response. MK2 activates HnRNPA0, which is a RNA binding protein and increases the stability of its target mRNAs such as p27 (Kip1) and Gadd45α mRNAs. This pathway is involved in cisplatin resistance in lung cancer, highlighting the role of MK2 in cell cycle modulation [324].

#### 1.4.2.2. MK3

MK2 and MK3 are closely related isoenzymes. They show 75% overall sequence identity on the amino acid level with the highest similarity within the catalytic domain [254]. Under stress, p38MAPK phosphorylates and activates both, MK2 and MK3. They are indistinguishable concerning substrate specificity [325]. Between MK2 and MK3, MK2 has higher expression and activity level than MK3 [326]. They have both common and unique functions.

They are demonstrated to cooperate in the regulation of certain LPS-induced inflammatory gene expression such as IL-10, IL-19, CXCL2 and IL-4 receptor (IL-4R) α subunit [327]. Moreover, ectopically expressed MK3 can rescue MK2 deficiency similar to MK2 [328]. On the other hand, in a study based on the differential regulation of gene expression by MK2 and MK3, in MK2 free genetic background, MK3 negatively regulates Interferon (IFN)-β, Cr1A, Nucleotide Binding Oligomerization Domain Containing 1(NOD1) and Serpin Family A Member 3 (Serpina3f) and delays the nuclear translocation of NF-κB by delaying the ubiquitination and subsequent degradation of IκB-β [327].

#### 1.4.2.3. MK5

MK5/p38-regulated and -activated protein kinases (PRAK) is a serine/threonine-protein kinase. There is 42% sequence similarity between the full-length human MK2 and MK5 and 48% sequence similarity between their catalytic domains [254]. However, there is a significant difference between their upstream regulators and biological functions between MK2 and MK5. Though p38MAPK is known to phosphorylate MK5 in *in vitro* experiments and overexpression systems [329], it is not observed in *in vivo* experiments [330]. The atypical MAPKs, ERK3 and ERK4 interact with MK5 and forms a complex. In the complex, ERK3 or ERK4 mediates phosphorylation and activation of MK5. Furthermore, this complex formation also results in the relocalization of MK5 from nucleus to cytoplasm [231]. So far, no stimulant is reported that is able to induce the activation of MK5 in an endogenous setting [331].

Unlike MK2 knockout mice, MK5 knockout mice did not show increased resistance to endotoxic shock or decreased LPS-induced cytokine production [330]. MK5 activates p53 by direct phosphorylation and mediates RAS-induced senescence and thereby suppresses the tumor progression. This was demonstrated using a MK5 knockout mouse model obtained by deletion of exon 8 of MK5 [332]. However, another study using MK5 mouse model obtained by deleting either exon 6 or 8 of MK5 contrasts the earlier finding. Here, the authors showed that deletion of exon 6 of MK5 has no effect on tumor progression while deletion of exon 8 of MK5 actually does not completely remove the expression of MK5. Rather the truncated MK5 still behaves as a wild type MK5 [333]. MK5 phosphorylates FoxO3a and promotes miR-34b/c expression which downregulates the MYC expression and arrests proliferation [334]. MK5 physically interacts with the transcriptional co-activator YAP and prevents its proteasomal degradation. Moreover, MK5 upregulation results in high levels of YAP expression and poor prognosis in clinical tumor samples. However, ERK3 and ERK4 which are the major regulators of MK5 were not involved in regulation of YAP [335]. In this way, MK5 can act as either tumor suppressor or tumor promoter depending on cell type (reviewed in [331]).

Recently MK5 is reported to play role in heart functions. MK5 haplodeficiency decreased the progression of hypertrophy, reduced collagen type 1 mRNA, and protected diastolic function in response to chronic pressure overload [336] and also reduced infarct size, scar area, and scar collagen content post-myocardial infarction [337].

### 1.5. NF-κB

NF-κB is a transcription factor consisting of hetero- or homo-dimers of the Rel transcription factor family members p50, p52, Rel A (p65), Rel B, and c-Rel [338]. Among them only Rel A, Rel B and c-Rel contain the transcriptional transactivation domain (TAD). The TAD-containing heterodimers are transcriptional activators whereas p50 or p52 homodimers are repressors unless bound to secondary proteins [32]. NF-κB binds to κB sites found in the enhancers or promoters of hundreds of genes (www.bu.edu/nf-kb/gene-resources/target-genes/) [339].

In unstimulated cells, the NF-κB proteins are sequestered in the cytoplasm by IκBs (Inhibitor of κB). NF-κB activation involves post translational modification of IκB. This occurs by either a canonical or an alternative pathway. In the canonical pathway, a kinase complex called IκB kinase (IKK) specifically phosphorylates IκB proteins leading to their degradation. This causes NF-κB dimers, which actively shuttle between the nucleus and cytosol, to stay nuclear and induce gene expression. The cytosolic IKK holoenzyme is composed of a regulatory subunit,

NF-κB essential modifier (NEMO, also called IKKγ), and two kinase subunits, IKKα and IKKβ. NEMO is a non-catalytic subunit that binds IKKα and IKKβ into a regulatory holoenzyme. Upstream signaling causes the ubiquitination of NEMO leading to its proteasomal degradation and thereby release of IKK [338].

The NF-κB-induced genes include chemokines, cytokines, adhesion molecules, inflammatory mediators, and apoptosis inhibitors, giving NF-κB a pivotal role in immune, inflammatory and stress responses. It is also involved in cell growth, maturation and survival. More recently, constitutive expression of NF-κB has been associated with several types of cancer [32, 340].

### 1.6. p38MAPK-MK2 signaling pathway

Downstream signaling of p38MAPK is extremely diverse, resulting in the phosphorylation of more than 100 different substrate proteins including MK2 [218]. p38MAPK is distributed both in the cytoplasm and nucleus in resting cells, while MK2 is located predominantly in the nucleus [263]. Cytoplasmic p38MAPK translocates into the nucleus upon stimulation [341]. A docking domain in p38MAPK (called common docking domain), serves as a site for binding to MK2 [342]. Similarly, MK2 also contains a p38MAPK specific docking domain in the C-terminal tail that confer specificity for the upstream activator [343]. Activated p38MAPK forms a high affinity complex with MK2 [344]. p38MAPK binds and subsequently phosphorylates MK2. Such phosphorylation results in exposure of nuclear export signal (NES) of MK2 leading to its translocation from nucleus to cytosol along with p38MAPK. Nuclear export of p38MAPK and MK2 may permit them to phosphorylate substrates in the cytoplasm [260, 263].

Interestingly, beside nuclear translocation, direct interaction between p38MAPK and MK2 is also important for stabilization of each other. The amount of p38MAPK is significantly reduced in cells and tissues lacking MK2 [328, 345] and MK2 expression is reduced in p38α knockout MEF cell lines [346]. The p38MAPK-MK2 signaling pathway has been studied for many years for its involvement in inflammation, cell migration and cell cycle regulation [258, 347]. Inhibition of the p38MAPK-MK2 pathway in stromal cells reduces breast cancer metastases and chemotherapy-induced bone loss [348]. p38MAPK-MK2 pathway is reported to be involved in redox stress, cell death and ischemia injury [349]. Senescent cells express pro-tumorigenic factors termed the senescence-associated secretory phenotype (SASP). The p38MAPK-MK2-HSP27 pathway regulates mRNA stability of SASP. Furthermore, the tumor-promoting abilities of senescent stromal cells were lost upon inhibition of MK2 [350].

### 1.7. Targeted therapy

A major objective of signal transduction research is the development of signaling pathway-targeted therapy. The fundamental research in cell signal transduction has led to the success of targeted therapies, such as imatinib for the treatment of chronic myelogenous leukemia, and trastuzumab for the treatment of HER2- positive breast cancer [351].

Targeted therapy aims to develop drugs targeting particular genes or proteins that are the cause of the disease. In cancer, targeted therapy can basically be of two types. The first type is immunotherapies based on monoclonal antibodies. Antibody-conjugated nanoparticles are also being used for targeted delivery of chemotherapeutics where an antibody is used to target cell-surface markers of disease that are frequently upregulated or are specifically expressed in tumor cells. The second type are small-molecule drugs (molecular weight <1,000 Da), which target specific proteins that are important for disease progression [352].

Targeted therapy is of growing importance because of its ability to specifically target the cancer cells. Unlike the traditional chemotherapy that targets all growing cells, in targeted therapy drugs target only selected cells with particular mutation and hence the unwanted effects are minimal. Targeted cancer therapies are more favorable for cancers like lung, colorectal, breast, lymphoma and leukemia as they focus on particular molecular changes unique to a specific cancer [353].

#### 1.7.1. Kinases in targeted therapy

Kinases play a central role in the growth, survival, and therapy resistance of tumor cells. They are the cornerstone of targeted cancer therapy because they are druggable, and can be targeted selectively using small-molecule inhibitors [354]. Kinase inhibitors are very efficacious drugs for the treatment of individuals with cancer that carry specific genetic alterations, whereas treatment is generally not efficacious for individuals with cancer that have a set of different mutations. Fasudil (HA-1077) was one of the first protein kinase inhibitors used in clinic. It was approved in Japan in 1995 for cerebral vasospasm [355] and inhibited myosin light chain protein kinase and protein kinase C [356]. Imatinib, approved in 2001, is the first tyrosine kinase inhibitor for the treatment of Philadelphia chromosome positive chronic myeloid leukemia targeting Bcr-Abl tyrosine kinase [357]. Imatinib increase overall survival by a decade or more [358]. The success of these small molecule kinase inhibitors sharply raised the interest in protein kinases as drug targets. Since then several kinase inhibitors have been approved for therapeutic uses. As of January 2022, 70 small molecule protein kinase inhibitors are approved by FDA (www.brimr.org/PKI/PKIs.htm). A monthly-updated database of approved or currently

in clinical trials protein kinase inhibitors compiles 218 inhibitors [359]. Among the FDA-approved small molecule protein kinase inhibitors, more than 46 are prescribed for the treatment of neoplasms and few are indicated for inflammatory disease [12]. So far, only a small fraction of the kinome is being targeted. Owing to the wide role of protein kinases, we can expect the approval of new drugs inhibiting other protein kinases in the treatment of illnesses such as hypertension, Parkinson's disease, and autoimmune diseases [360].

However, development of resistance poses a serious challenge in the use of these kinase inhibitors [361]. The conserved nature of kinases, particularly in the catalytic domains may result in limitation in regards of the specificity of kinase inhibitors. This promiscuity of the kinase inhibitors may contribute to severe unexpected side effects [362]. Therefore, only few drugs targeting kinases have been completely successful in the clinic. Thus, peptides that interfere with protein-protein interaction involving kinases and scaffold proteins or other binding proteins are also being developed as an alternative approach (discussed in [363]).

#### 1.7.1.1. p38MAPK inhibitors in different diseases

The p38MAPK pathway is a target for anti-inflammation and cancer therapy [364]. Several small molecule inhibitors of p38MAPK are proposed for different inflammatory conditions such as in rheumatoid arthritis, [365] and neural disease [366]. Targeting the p38MAPK kinase has been less successful in the clinic, with most trials failing owing to toxicity or a lack of efficacy [367]. Accumulating evidence suggests a dual role of p38MAPK signaling in various types of cancers, wherein the p38MAPK pathway can both suppress and promote tumor growth, metastasis and chemoresistance. This is due to the pleiotropic effects on the immune system stemming from p38MAPK inhibition. p38MAPK is involved in a wide range of physiological activities regulated by multiple downstream signaling pathways [218]. It exists in multiple isoforms that differ in terms of activation, substrate specificity and tissue specificity. Thus, p38MAPK forms a complex signaling network and hence targeting p38MAPK in therapeutics results in severe unwanted effects (reviewed in [368]). There are also other problems in using p38MAPK as a drug target. Dual Specificity Phosphatase (DUSP) 1 is a phosphatase involved in the dephosphorylation of p38MAPK and JNK [369]. p38MAPK-MK2 pathway is involved in induction of DUSP1 in innate immune responses [370]. p38MAPK is reported to suppress the activity of TAK1 by regulating TAB1. TAK1 is involved in activation of JNK and IKK along with p38MAPK itself [371]. Thus, inhibition of p38MAPK interferes the delicate negative feedback mechanism thereby resulting in the over activation of JNK and NF-κB

pathway. Furthermore, inhibition of p38MAPK drastically destabilize anti-inflammatory cytokine, IL-10 [372]. In this way, inhibition of p38MAPK can have deleterious effect.

#### 1.7.1.2. MK2 inhibitors in different diseases

The p38MAPK-MK2 pathway is involved in a series of pathological conditions including inflammation, metastasis and in the resistance mechanism to antitumor agents. None of the p38MAPK inhibitors have entered advanced clinical trials because of severe side effects. For this reason, MK2 was identified as an alternative target to block the pathway but avoiding the side effects of p38MAPK inhibition. There are ATP-competitive and non-ATP competitive MK2 inhibitors showing different levels of solubility, cell permeability and selectivity [373]. CDD-450 inhibited inflammation in mice with cryopyrinopathy, and attenuated arthritis in rats and cytokine expression by cells from patients with cryopyrinopathy and rheumatoid arthritis [374]. MMI-0100 attenuated Dextran Sulfate Sodium (DSS)-induced body weight loss, colon length shortening, and colonic pathological injury, inflammatory cell infiltration in mouse models [375]. A combination of MK2 inhibitor IV and the key multiple myeloma therapeutic agents prevented human multiple myeloma cell proliferation, while multiple myeloma mouse model treated with MK2 inhibitor IV survived longer [376]. Few specific inhibitors of MK2 are at different stages in clinical trials as listed in table 4.

**Table 4. MK2 inhibitors in clinical trial** (as of February 2022)

| Drug | Developer | Phase | Disease condition | Identifier |
|---|---|---|---|---|
| ATI-450 | Aclaris Therapeutics | II | Rheumatoid arthritis | NCT04247815 |
| ATI-450 | Aclaris Therapeutics | II | COVID-19 | NCT04481685 |
| MMI-0100 | Moerae Matrix, Inc | I | Acute Inflammatory Response to Non-antigenic Stimulus | NCT02515396 |
| ATI-450 | Aclaris Therapeutics | II | Hidradenitis Suppurativa | NCT05216224 |
| ATI-450 | Aclaris Therapeutics | II | Cryopyrin-Associated Periodic Syndrome | NCT04524858 |
| CC-99677 | Celgene | II | Active Ankylosing Spondylitis | NCT04947579 |

### 1.7.2. SRC-3 inhibitors in different diseases

Most cancers are highly adaptable and often escape the growth inhibiting actions of drugs targeting only a single pathway. SRC-3 is at the nexus of many intracellular signaling pathways

critical for cancer progression. Therefore, small molecule inhibitors (SMIs) that inhibit co-activator functions like that of SRCs could be very useful for cancer treatment. Besides, new approaches that combine existing targeted therapy with co-regulator targeting drugs might be more effective in treatment of cancers [38]. However, developing an effective SRC-3 inhibitor faces critical challenges as it lacks a high affinity and high specificity ligand binding sites, enzymatic activation surface and has a large and flexible structure. But with increased understanding of SRC-3 biology and with technological advances, targeting them is becoming possible. For example, a novel series of biphenyl proteomimetic compounds, which are SMIs that interfere with the binding of co-regulators to NRs (e.g. the ER-α co-activator binding site) can be promising [377]. Even better might be compounds targeting the SRC-3 protein directly thereby resulting in the modulation of the co-activator. Examples for this kind of compounds are gossypol [378], bufalin [379] and SI-2 [380]. Gossypol binds directly to SRC-3 in its receptor interacting domain and reduces the cellular protein concentrations of SRC-3 [378]. Bufalin promotes the degradation of SRC-3 in a proteasome-dependent manner and efficiently blocks cancer cell growth *in vitro* and *in vivo* [379]. SI-2 reduces the transcriptional activities and the protein concentrations of SRC-3 and significantly inhibits primary tumor growth in a breast cancer mouse model. Unlike gossypol and bufalin, SI-2 is a drug-like molecule, effective at comparatively lower concentration [380]. MCB-613, an SRC small-molecule stimulator, inhibits tumor growth in a different way. MCB-613 hyperstimulates SRC-3 that causes cell stress and massive production of reactive oxygen species (ROS) resulting in selective killing of cancer cells [381]. Drugs like bufalin are in very early stages of clinical trial and hence reports about resistance to SMI are not yet reported [382]. However, provided the fact that the biological activity of SRC-3 is controlled by its phosphorylation at several sites, a possible way to modify SRC-3 activity in cancer could be to target the kinases responsible for the disease promoting phosphorylation with specific kinase inhibitors.

## 2. Aims of present study

SRC-3 is involved in several biological activities. Its role is also well established in different types of cancer including breast and lung cancer. The activity of SRC-3 is regulated by phosphorylation at several sites and phosphorylation of SRC-3 at S857 is most frequently reported. The aim of this thesis was to identify the kinase(s) that phosphorylates SRC-3 at S857 and explore the biological function of the signaling pathway involved therein.

In particular, the aim was to

a. Validate the newly identified ERK3-SRC-3 signaling axis.

b. Identify kinase(s) phosphorylating SRC-3 at position S857.

c. Determine a biological function of the identified p38MAPK-MK2-SRC-3 signaling axis.

d. Identify genes regulated by p38MAPK-MK2-SRC-3 signaling axis.

## 3. Methodological considerations

### 3.1. Generation of SRC-3 depleted cell lines using CRISPR-Cas9 and shRNA targeting SRC-3

In order to study the role of SRC-3 in different biological activities, we needed a cell line that lacked the expression of SRC-3. There are several methods to deplete the expression of a gene. One approach is editing the target genome. With the help of this approach, complete depletion of targeted gene expression can be obtained. CRISPR-Cas9 stands out among other gene editing technologies as it is efficient, specific, fast, cost-effective and easy to use [383].

The type of Cas9 used in CRISPR-Cas9 gene editing technique can influence the on-target effect. Compared to the wild-type Cas9, modified Cas9 1.1 in which three positively charged amino acids has been mutated to neutral amino acids is reported to reduce off-target cleavage [384]. Therefore, we used this modified Cas9 1.1 in this study. For the determination of oligos for guide RNAs of SRC-3, we used chopchop.cbu.uib.no database [385]. The guide RNAs we selected were among the ones rated as highly efficient.

As a tool for determining the function of SRC-3, we tried to generate several SRC-3 KO cell lines using the CRISPR-Cas9 technique. We tried to generate two SRC-3 KO lung cancer cell lines (H1299 and A549) and two SRC-3 KO breast cancer cell lines (MDA-MB-231 and MCF7). First of all, we attempted to generate SRC-3 KO H1299 cells using guide RNA that targets the fourth exon. In the resulting cell clones, the use of this guide RNA either gave no loss of nucleotides, or loss of nucleotides in the multiple of three. Such deletions did not result into frame shift, and hence the SRC-3 protein (only missing one or a few amino acids) could still be detected. That no other combination of deleted nucleotides was detected indicates that SRC-3 is vital for the survival of H1299 cell line, and hence any successful knockout of SRC-3 would not be able to generate viable cells. Then we used a different guide that targets the eighth exon to edit SRC-3 gene in A549, MDA-MB-231 and MCF7 cells. With the help of this guide RNA, we successfully generated SRC-3 KO A549 and MDA MB 231 cell lines. We were not, however, able to generate SRC-3 KO MCF7 cells. We obtained a few clones of SRC-3 KO A549 and several clones of SRC-3 KO MDA-MB-231. However, we did not get any clones of SRC-3 KO MCF7 cell lines. The GFP marker in the CRISPR-cas9 was well expressed in all of the transfected cell lines, so we ruled out low transfection efficiency as cause of this observed discrepancy in the gene editing success. One reason might be that it is more difficult for the MCF7 cells to grow from a single cell into colonies. In addition, a recent study has shown that Cas9 is less active in p53 wild-type than in p53 mutant cell lines [386]. MCF7 and A549 cells

expresses wild-type p53 [387], MDA-MB-231 harbors a missense mutation in p53 [388] and H1299 cell lines lack expression of p53 protein. This discrepancy in p53 expression in these cell lines might partly explain our observed differences in the generation of SRC-3 KO cells using CRISPR-Cas9 technology. Another possibility is that since SRC-3 gene is amplified in MCF7 cells [111], SRC-3 gene needs to be targeted at several sites and hence complete depletion of SRC-3 might be challenging.

We confirmed the knockout of SRC-3 protein by Western-blotting. Furthermore, in order to identify indels, we performed DNA sequencing. DNA sequencing of the target sequence revealed insertion of an extra nucleotide at the target site resulting in a frame shift which generated a stop codon further down. We also examined the SRC-3 mRNA expression in the SRC-3 KO MDA-MB-231 cells and found that it was significantly lower than the wild-type cells. The presence of a premature termination codon in an mRNA can trigger non-sense mediated mRNA decay (NMD) resulting in the degradation of the transcript [389]. NMD might have played role in the degradation of SRC-3 mRNA in SRC-3 KO MDA-MB-231.

The success of generation of a cell line by CRISPR-Cas9 depends on generation of cell line from a single cell. So, it is possible that we can observe a clonal effect. Another possible bias in using KO cell lines is that in KO cells the functions lost due to degradation of a protein can be compensated by activity of another protein with overlapping functions and expression pattern (reviewed in [390]). In order to overcome these possible biases, we used a lentiviral system to stably introduce shRNA targeting SRC-3 (shSRC-3) into the genome to generate SRC-3 knockdown (SRC-3 KD) cell lines to decipher the function of SRC-3. Use of SRC-3 KD cell lines gave us an opportunity to further confirm the findings obtained from SRC-3 KO cell lines as the drawbacks of the CRISPR-Cas9 method would not exist in this technique. It is a fact that shSRC-3 cannot completely knockout the target gene expression. We managed however to achieve cells with only a very low expression of SRC-3 in our SRC-3 KD cell line. Even such low amount of SRC-3 can be enough to regulate some functions of SRC-3, therefore we have utilized comparative study between SRC-3 KD and SRC-3 KO cell lines to establish a role of SRC-3. Drawing a conclusion utilizing more than one SRC-3 depletion techniques increased the confidence of our findings.

### 3.2. Generation of SRC-3 KO and KD cell lines re-expressing either wild type or mutant SRC-3

Rescue cell lines obtained by reintroducing a gene into knockout or knockdown cells is a powerful tool to study the function of a gene. Comparison of phenotype among wild type, SRC-

3 KO and KD cell lines and SRC-3 KO or KD cells re-expressing wild-type or S857A mutant SRC-3 provided evidence of a phenotype regulated by SRC-3. After developing SRC-3 KO and KD cell lines, we reintroduced wild-type or mutant SRC-3 into SRC-3 KO and SRC-3 KD cell lines using the lentiviral system and generated SRC-3 rescue cell lines. We first tried to rescue SRC-3 depleted cells using constitutive and tetracycline-inducible expression vector. However, with this approach we did not succeed in generating any cell lines expressing near endogenous level of re-expressed SRC-3. There is batch to batch variation in the lentiviral particles production. It is possible that the amount of virus particles in some batches was too small for successful transduction. The other reason could be the PGK promoter which we used in the vector system because PGK promoters are comparatively a weak promoter [391]. Next, we used a vector harboring a promoter from the elongation factor 1 (EF1) and a selection marker expressed from an internal ribosomal entry site (IRES) from the same transcript as SRC-3. Use of IRES from the same transcript as the gene of interest ensures that the cells resistant to the selection marker also expresses the gene of interest. With this approach, we were able to generate SRC-3 depleted MDA-MB-231 cells re-expressing wild-type SRC-3 tagged to mClover2 or SRC-3 mutated at S857 tagged to mScarlet. The expression of SRC-3 as fusion protein with fluorescent protein would have given the possibility to visualize the expression of SRC-3 proteins in living cells. Although we could detect expression of the fusion proteins in Western-blotting we were not able to detect the expressed fusion proteins in the fluorescent microscope using live cells. This could be because the amount of the fusion protein expressed is too low for detection with the fluorescent microscope.

### 3.3. Identification of a kinase phosphorylating SRC-3 at S857 using *in vitro* kinase assay, siRNA against specific kinases, kinase inhibitors, and rescue cell lines

In this study, one of the objectives was to identify a specific kinase responsible for phosphorylation of SRC-3 at S857. We utilized different techniques to identify the kinase required for phosphorylating SRC-3 at S857. First of all, we used an *in vitro* kinase assay as they are useful in screenings for substrates of a kinase. A kinase assay is a technique generally performed *in vitro* (in this study by *in vitro* we mean experiments performed with purified proteins in Eppendorf tubes) to examine if the kinase is able to phosphorylate the substrate [392]. This is a quick but preliminary method. In an *in vitro* kinase assay, the concentration of the kinase tends to be higher than in the *in vivo* situation. Similarly, there is also no competition between the kinase and other putative candidate kinases. In such conditions, it is possible that a kinase can phosphorylate a substrate which it would not phosphorylate in living cells.

Therefore, we also confirmed our findings from *in vitro* experiments *in vivo* (in this study by *in vivo*, we mean experiments performed in living cells). The source of the kinase used in kinase assay can also interfere the findings. For example, if an immunoprecipitated kinase is used to verify the substrate it is always a possibility that it is a co-immunoprecipitated active kinase that is doing the job. In order to prevent this, in case of ERK3 we used purified recombinant active kinase expressed in insect Sf9 cells in our kinase assay.

For *in vivo* kinase assay, we have used siRNA against specific kinase to determine if the particular kinase is responsible for the phosphorylation of the target substrate. Here the use of validated phospho-specific antibodies is mandatory for confirming that the kinase phosphorylates its substrate at specific site and hence we have generated phospho-S857 SRC-3 antibody. We have also used radioactive phosphate to determine the phosphorylation of SRC-3 by autoradiography. In case of MK2, we have used MK2 inhibitor instead of siRNA against MK2. This is because MK2 is important for the stability of p38MAPK [345]. Therefore, depletion of MK2 would also deplete p38MAPK and hence it would not be possible to determine if the observed phenotype is due to inhibition of MK2 or p38MAPK. Substrates of a kinase can also be determined by using inhibitors like we did in our study. However, one has to be careful in use of kinase inhibitors to determine substrate specificity since many kinase inhibitors are promiscuous and often inhibit multiple kinases in key signaling pathways [393]. For example, the MK2 inhibitor PF-3644022 is reported to inhibit MK3 and MK5 besides MK2 activity [394]. Hence, it can be difficult to conclude if the observed phenotype is actually due to inhibition of the target kinase or due to inhibition of other kinases. To overcome this, we have used cell lines depleted of MK2 and MK3 and rescued with kinase activity dead MK2 compared to those rescued with wild-type MK2. Therefore, several approaches were utilized to verify that the correct kinase was identified.

### 3.4. Determination of cell proliferation by live cell imaging and ATP detection assay

We used IncuCyte for comparing cell proliferation among different variants of MDA-MB-231 cells. The IncuCyte has an incubator with attached live cell imaging microscope. Cells are seeded in desired plates and placed inside IncuCyte then the live cell imaging microscope is set to take pictures at regular intervals. Phase contrast images of cells are captured for over a period of time. A mask, which indicates area that overlaps the cells, is defined and confluency of the cells is determined with Cell-by-Cell Analysis Software Module of the IncuCyte. This is an automated method and data can be obtained without removing cells from the incubator. Furthermore, it monitors the cell proliferation in real time. However, defining a mask for

determining the area occupied by the proliferating cells can be challenging if reagents added to the culture media precipitate. We found that the MK2 inhibitor we used in our cytotoxicity experiments precipitated making it difficult to use the IncuCyte for determining the cell proliferation. Therefore, we used CellTiter-Glo® 2.0 assay kit to measure cell proliferation in those experiments. This is an indirect method of measuring the viable cells where the amount of ATP released by cells is used as surrogate for living cells. ATP detection assay is by far the most sensitive and rapid method to measure the cell viability.

### 3.5. Generation and characterization of phospho-S857 SRC-3 antibody

We needed an antibody to assess the phosphorylation status of SRC-3 at S857 but the antibody was not available commercially. Therefore, we produced our own phospho-S857 specific SRC-3 antibody. Many experiments in this study utilized antibodies, including Western-blotting, immunoprecipitation, immunostaining and kinase assay. A well characterized and validated antibody is very important for the trustworthiness and reproducibility of results from these experiments. The reproducibility of research findings has been a growing concern. One of the reasons is lack of reagent validation, including antibodies [395, 396]. In order to assure the validity of antibodies, International Working Group for Antibody Validation (IWGAV) suggested five different conceptual pillars for validation of antibodies. These include genetic and orthogonal approaches, use of two or more independent antibodies binding to different regions of the protein, expression of tagged proteins, and immunocapture followed by mass spectrometry. They recommended that at least one of these criteria should be fulfilled for claiming that a particular antibody has been adequately validated for a specific application [397]. Therefore, we validated the phospho-S857 specific SRC-3 antibody rigorously.

For the initial antibody validation, we used a recombinant GST fusion protein encoding the CBP-interacting domain (CID) of SRC-3 (SRC-3 aa 840–1,080) wild-type (WT) or mutant version where serine 857 was replaced with alanine (S857A). In an *in vitro* kinase assay, GST-CID-SRC-3 WT and GST-CID-SRC-3 S857A were incubated with and without active MK5. In western-blotting of the proteins from this *in vitro* reaction we could observe that the antibody could recognize the GST-CID-SRC-3 protein only when it was incubated with active MK5. The antibody was unable to recognize GST-CID-SRC-3 S857A protein even if it was incubated with active MK5. The antibody specifically recognized the phosphorylation of GST-CID-SRC-3 WT at S857, while no signal was detected when incubated with the mutated GST-CID-SRC-3 S857A protein. This indicated that our antibody could discriminate between phosphorylated and non-phosphorylated wild-type GST-CID-SRC-3 and that this ability depends on S857. We

could also show that the antibody could detect the MK5 phosphorylated GST-SRC-3 in a dose dependent manner. In order to test the antibody ability to detect SRC-3 phosphorylated at S857 in the full-length protein and in mammalian cells we transfected H1299 cells with expression vector encoding wild-type SRC-3 or a mutant form of SRC-3 where the serine 857 is replaced by an alanine. Western-blotting of the extracts from these transfections confirmed that the antibody could discriminate between a protein with an intact serine at 857 and a protein where this residue is replaced by a alanine. The data from the *in vitro* kinase reactions indicate that SRC-3 has to be phosphorylated in order for the antibody to detect it. Moreover, an intact serine at residue 857 is required for the antibody to detect SRC-3. The requirement for serine at position 857 was also required for the ability of the antibody to detect full-length SRC-3 ectopically expressed in mammalian cells. In order to further show that SRC-3 has to be phosphorylated in order for the antibody to detect it also in endogenous setting we immunoprecipitated endogenous SRC-3 from mammalian cells. After immunoprecipitation, the precipitate was split into two fractions and one fraction was dephosphorylated by lambda phosphatase and the other fraction was left untreated. Western-blotting of the fractions demonstrate that our antibody could recognize phosphorylated SRC-3 but not SRC-3 dephosphorylated by the phosphatase. Thus, our data show that serine at residue 857 is required for the antibody to detect SRC-3 in western-blotting. Phosphorylation by a kinase like MK5 or MK2 increase the signal detected by the antibody in a dose-dependent manner and dephosphorylation of the SRC-3 decrease the signal detected by the antibody. Altogether, this indicates that our antibody is able to detect SRC-3 when it is phosphorylated at S857.

In our experience using this antibody in detecting endogenous SRC-3, we found it to give better resolution if SRC-3 is immunoprecipitated from the extract before the western-blotting. This is because the antibody also gives rise to a lot of unspecific signals when detecting endogenous P-S857 SRC-3 in direct western-extracts. The reason for this is probably the level of phosphorylated SRC-3 expressed in cells are quite low and the blots have to be scanned at high sensitivity in order to detect the phosphorylated form of SRC-3. We did not observe these extra bands when SRC-3 were detected from extracts where SRC-3 was expressed ectopically at high level or from immunoprecipitate. In this way, we validated the antibody by several methods.

### 3.6. Visualization of SRC-3 using confocal microscopy

In order to determine how SRC-3 is distributed in nucleus and cytoplasm of a cell, we performed subcellular fractionation and confocal microscopy. In subcellular fractionation, we separated the proteins into different fractions and used control proteins to show that the lysates were

separated into a particular fraction. In this approach, protein from one compartment contaminated the protein from other compartment and our attempt to separate the cell extracts into nuclear and cytosolic fractions did not succeed. It is reported that considerable amount of nuclear proteins are lost from the nucleus within 10 minutes of cell disruption [398]. This might be the reason for the inability to completely separate the proteins into their respective fractions. Then we utilized confocal imaging technique to determine the localization of SRC-3. Confocal imaging gives visual impression of the location of the target protein. In confocal microscopy, the nucleus can be stained with DAPI and hence the distribution of SRC-3 when located by an antibody tagged with a distinct color, can be easily determined. Demonstrating the location of a protein visually is more convincing than by the fractionation experiments. However, a specific antibody is very crucial in the successful staining of the target protein [399]. The SRC-3 antibody we used could detect SRC-3 in wild-type cell lines but did not give any signal in SRC-3 KO cell lines. Furthermore, when SRC-3 was overexpressed in SRC-3 KO cell line, the antibody could detect SRC-3.

## 4. Summary of main results

### 4.1. Paper I

SRC-3 is a versatile co-activator whose function is modulated by its phosphorylation at different sites. S857 is the most studied phospho-acceptor site, and its modification has been reported to be important for SRC-3-dependent tumor progression. In this study, we examined if ERK3 phosphorylated SRC-3 as reported by others, explored other kinases phosphorylating SRC-3 at S857 and the biological function incurred therein.

A phospho-specific S857 SRC-3 antibody was not commercially available, but we were able to successfully generate this, and its specificity was verified in different ways. Based on the literature review, we then used this antibody to examine some of the kinases reported to phosphorylate this site. We were unable to verify that the atypical MAPK ERK3 phosphorylates SRC-3 at S857, neither *in vivo* nor *in vitro,* in our experimental conditions. Similarly, we did not find that the individual inhibition of IKK-β activity or IKK-α expression influenced the TNF-α-induced phosphorylation of SRC-3 at S857. However, with the mutual inhibition of IKK-β activity and IKK-α expression a slight decrease in the phosphorylation was observed. Instead, we found that even though MK2 and MK5 both phosphorylate SRC-3 at S857 *in vitro,* only MK2 phosphorylates the site *in vivo*. Then we assessed the role of the MK2 upstream kinase, p38MAPK, in the phosphorylation of SRC-3 at S857. Stimulation of the human lung cancer cell lines H1299 and A549, the human embryonic kidney cell line HEK293, the human cervical carcinoma cell line HeLa, and the human breast cancer cell line MDA MB 231 with well-known p38MAPK activators (TNF-α, anisomycin and sodium arsenite) all resulted in the phosphorylation of SRC-3 at S857. Inhibition of p38MAPK activation with a specific p38MAPK inhibitor prevented this. Furthermore, using a MK2 KO mouse cell line and a specific MK2 inhibitor, we showed that MK2 is the kinase responsible for phosphorylation of SRC-3 S857. This was shown to be true both for the mouse cell lines and the various human cancer cell lines. We also showed that stimulation with TNF-α induced phosphorylation at SRC-3 S857 in a time and dose dependent manner. In the lung cancer cell line A459, the SRC-3 protein was located both in the nucleus and cytosol under unstimulated condition. When cells were stimulated with TNF-α, it translocated into the nucleus. Phosphorylation of SRC-3 at S857 played an important role in this translocation as prevention of phosphorylation of this site with a p38MAPK inhibitor or a MK2 inhibitor significantly reduced the nuclear translocation of SRC-3. Moreover, there was no significant translocation of SRC-3 into the nucleus when serine 857 was mutated to an alanine (SRC-3 S857A). This was observed when SRC-3 KO A549 cells

transfected with SRC-3 S857A were stimulated with TNF-α in contrast to the same cells transfected with wild-type SRC-3. Inside the nucleus, SRC-3 played a significant role for the transcriptional activity of NF-κB and was involved in transcription of IL-6. When we further examined some of the NF-κB target genes whose expression was enhanced by stimulation with TNF-α, we found that only the IL-6 mRNA expression was significantly reduced when p38MAPK and MK2 activities were inhibited.

We identified the p38MAPK-MK2 signaling axis as a key regulator of the SRC-3 phosphorylation and activity. The p38MAPK-MK2-SRC-3 signaling axis can be a novel therapeutic target to control inflammation.

### 4.2. Paper II

Triple negative breast cancer accounts for nearly 15% of all invasive breast cancers and have the highest rate of metastatic disease and poorest overall survival among breast cancer subtypes. SRC-3 is reported to play role in breast cancer progression and sensitivity to chemotherapeutic regimens. Anthracyclines like doxorubicin is one of the important chemotherapy regimen in breast cancer treatment and acts by inducing the p38MAPK pathway. We have identified that the p38MAPK-MK2 pathway phosphorylated SRC-3 at S857, therefore we wanted to investigate the role of p38MAPK-MK2-SRC-3 signaling pathway in doxorubicin-induced cytotoxicity.

Doxorubicin treatment of MDA-MB-231 cells induced phosphorylation of p38MAPK and SRC-3 S857, suggesting activation of the p38MAPK-MK2-SRC-3 signaling axis which we have identified recently. In this study, we aimed to explore the role of SRC-3 S857 in doxorubicin-induced cytotoxicity. For this, we generated SRC-3 depleted MDA-MB-231 cells using shRNA directed against SRC-3 in addition to CRISPR-Cas9 mediated gene editing technology. shRNA efficiently knocked down the expression of SRC-3, leaving some expression of residual endogenous SRC-3 protein. While CRISPR-Cas9 mediated gene-editing completely knocked out the expression of SRC-3. The generated SRC-3 knockdown (KD) and SRC-3 knockout (KO) MDA-MB-231 cells were then rescued with lentiviral vectors expressing either wild-type SRC-3 or mutant SRC-3 S857A, where serine 857 was mutated to an alanine. We successfully re-expressed SRC-3 to near endogenous protein level in the SRC-3 KD and SRC-3 KO MDA-MB-231 cells using the lentiviral rescue technique. Then we studied the role of SRC-3, and in particular the role of the S857 phosphorylation, with regard to doxorubicin sensitivity using the obtained cell lines. Depletion of SRC-3 increased MDA-MB-231 cells sensitivity towards doxorubicin both in the SRC-3 KD and SRC-3 KO MDA-MB-231 cells. However, the SRC-3 KD cells seemed to be more sensitive to doxorubicin than SRC-3 KO cells when looking at the IC50 values. Furthermore, the re-expression of wild-type SRC-3 or mutant SRC-3 S857A had different outcomes in the SRC-3 KD and SRC-3 KO cells. In addition, we observed that inhibition of MK2 activity with MK2 inhibitor also increased the sensitivity to doxorubicin. This suggests that phosphorylation of SRC-3 S857 by the p38MAPK-MK2 signaling pathway contributes to enhanced resistance to doxorubicin in the MDA-MB-231 cells. Phosphorylation of SRC3 at S857 has been shown to play a role in both increased proliferation and migration of cancer cells. However, in our study we found no significant difference between wild type MDA-MB-231 cells and SRC-3 depleted (SRC-3 KD and SRC-

3 KO) MDA-MB-231 cells neither in cell proliferation nor colony formation ability, indicating that SRC-3 does not play role in these events in MDA-MB-231 cells.

In this study, we have indications that this phosphorylation is involved in sensitivity to doxorubicin in MDA-MB-231 cells. The rescue of SRC-3 KO cells with mutant SRC-3 S857A makes the cells more sensitive to the drug, as does the use of an MK2 inhibitor. We have shown that doxorubicin induces the phosphorylation of SRC-3 at S857 via p38MAPK-MK2 signaling pathway. Therefore, we conclude that use of a MK2 inhibitor in combination with doxorubicin may be a way to increase the sensitivity of the TNBC cells to the drug and improve the therapeutic output. Further research is needed to verify this.

### 4.3. Paper III

SRC-3 is an oncogene whose activity is regulated by PTM. Phosphorylation of SRC-3 at S857 is frequently reported and was found to regulate lung and breast cancer progression. Recently, phosphorylation at this residue was shown to be mediated by TNF-α-induced activation of the p38MAPK-MK2 signaling pathway.

In an attempt to identify the genes regulated by functional SRC-3 S857 phosphosite and the newly identified p38MAPK-MK2-SRC-3 pathway, we performed RNA-seq of wild-type and CRISPR-Cas9-mediated SRC-3 KO MDA-MB-231 cells. The SRC-3 KO cell line was rescued by lentiviral transduction of either wild-type SRC-3 or mutant SRC-3 S857A, and differential gene expression between these two cell lines were used for identification of SRC-3 S857 dependent genes. The p38MAPK-MK2-SRC-3 signaling pathway was activated by TNF-α stimulation, and a MK2 inhibitor was used either alone or in combination with TNF-α. We have identified 340 genes dependent on SRC-3 S857 phosphosite. Gene ontology analysis showed that such genes were particularly associated with regulation of transcription, cell adhesion and different types of cancer. Stimulation with TNF-α identified 101 genes dependent on SRC-3 S857. Such genes were associated with regulation of transcription, adherens junction organization and different metabolic processes. Interestingly, comparison of genes dependent on a functional SRC-3 S857 phosphosite showed that only 12 genes were common between the unstimulated (340) and TNF-α stimulated (101) SRC-3 S857 dependent genes indicating a major change in the transcriptional complexes and promoters that SRC-3 associates with upon TNF-α stimulation. Thirty-seven of the 101 SRC-3 S857 dependent genes regulated by TNF-α were identified to be dependent on p38MAPK-MK2-SRC-3 pathway, as identified by use of the MK2 inhibitor. Such genes were associated with biological properties related to cell migration which included genes such as SET Binding Protein 1 (SETBP1), praoxonase 3 (PON3), periplakin (PPL) etc. Further, we found that MK2 inhibitor alone caused differential expression of 1,200 genes in unstimulated condition. Among them 131 genes were dependent on SRC-3 S857 phosphosite. Several of these genes were associated with cell adhesion.

In this study, we have found that genes dependent on SRC-3 S857 phosphosite seems to be determined by the signaling pathways that are active in the stimulated and unstimulated condition. Many genes dependent on this phosphosite and the p38MAPK-MK2-SRC-3 signaling axis are associated with tumorigenesis. Hence, further research should be performed to see if and how the knowledge about the p38MAPK-MK2-SRC3 axis can be used in a clinical setting.

## 5. Discussion

This PhD work aimed at identifying the protein kinase/s responsible for phosphorylation of SRC-3 at S857, and physiological outcomes of this phosphorylation. SRC-3 promotes tumorigenesis through multiple mechanisms. SRC-3 amplification and overexpression have been associated with tumor aggressiveness and poor prognosis [400]. The activity, stability and cellular localization of SRC-3 is highly regulated by phosphorylation. Phosphorylation of SRC-3 at S857 is of particular interest because it is involved in lung cancer cell invasion [74], tumor growth and metastasis of breast cancer cells [77] and enhancement of chemoresistance and tumorigenesis in xenograft mouse model [78]. Therefore, identification of the protein kinase that phosphorylates SRC-3 at S857 in cancer cells could be of importance with regard to future development of targeted therapies. In paper I, we explored possible protein kinases phosphorylating SRC-3 at S857 and identified that the p38MAPK-MK2 signaling axis phosphorylates SRC-3 at S857. Then we went on to explore the role of this signaling axis in regulation of NF-κB activity in lung cancer cell lines (Paper I) and doxorubicin induced-cytotoxicity in TNBC (Paper II). Further, by employing SRC-3 depleted cells re-expressing either WT SRC-3 or mutant SRC-3 S857A treated or untreated with an activator and/or specific inhibitor of MK2, we aimed to identify the genes dependent on SRC-3 S857 and those regulated by the p38MAPK-MK2-SRC-3 signaling pathway. Here, I will discuss the obtained results in connection with kinase/s that phosphorylates SRC-3 at S857, the role of p38MAPK-MK2-SRC-3 pathway in SRC-3's subcellular localization, regulation of NF-κB activity and expression of IL6. Further, I will discuss the possible mechanism underlying p38MAPK-MK2-SRC-3 pathway regulated doxorubicin sensitivity in TNBC and the potential clinical application of MK2 inhibitor.

### 5.1. Kinase/s phosphorylating SRC-3 at S857

SRC-3 is well known to promote tumorigenesis by regulating proliferation, metastasis and chemoresistance [400]. In order to control SRC-3 mediated tumorigenesis, identifying a kinase phosphorylating SRC-3 can be crucial because small molecules modulating kinase activity have been successfully used in cancer treatment [401]. Protein kinase substrate identification is a significant challenge in cell signaling research. Though over 100,000 phosphosites in the human proteome have been registered in public databases [84] using various computational tools, a lot of them needs to be further validated experimentally. In paper I, we examined the ability of several protein kinases that were reported to phosphorylate SRC-3 at S857 to actually induce

phosphorylation of S857 in both biochemical and cell-based assays. We showed that the protein kinase ERK3 did not phosphorylate SRC-3 in intact cells or *in vitro* settings while MK5 phosphorylated SRC-3 at S857 in an *in vitro* setting. In contrast to this, Long et. al. [74], Alsaran et. al. [240], Elkhadragy et. al. [402] found that ERK3 phosphorylated SRC-3 at S857 in *in vitro* experiments. We propose that this discrepancy is due to the source of ERK3 protein used in the experiment. Phosphorylation of ERK3 at S189 leads to the formation of a stable active complex of ERK3 and MK5, and ERK3 in this complex phosphorylates MK5 at T182 resulting in activation of MK5 [231]. The ERK3 protein used for the *in vitro* kinase assay in Long et. al. [74], Alsaran et. al. [240] and Elkhadragy et. al. [402] was obtained by immunoprecipitation of ERK3 expressed in HEK293T mammalian cells. Therefore, the phosphorylation of SRC-3 by ERK3 as described by these researchers could be due to active MK5 which co-immunoprecipitated along with the immunoprecipitated ERK3 used in the experiment. In our experimental setting, we used ERK3 expressed and purified from insect cells and was not able to verify it as a kinase phosphorylating SRC-3. In future, use of a specific ERK3 inhibitor can also be used to validate the finding. Though, ERK3 kinase inhibitors with both reversible and irreversible modes of action are identified, the development of a potent and selective inhibitors is still at infancy [403].

Further support of the fact that it is MK2 and not ERK3 which is the kinase phosphorylating SRC-3 S857 was obtained by looking at the amino acid sequence surrounding the phosphorylation site. The amino acid sequence surrounding SRC-3 S857 is Y-N-R-A-V-S (857)-L. The putative MAPKAPK consensus sequence is R-X-X-S-X [185, 404]. In agreement with this, the MK2 substrates listed in table 3 in the introduction section of this thesis reveal that MK2 mostly phosphorylates serine of Hyd-X-R-X-X-S-X consensus sequence where Hyd is a bulky hydrophobic residue. MAPK, on the other hand, is a proline guided serine/threonine kinase [176]. Therefore, it is more likely that a MAPKAPK rather than a MAPK, such as ERK1/2 and ERK3, would phosphorylate SRC-3 at S857. MEK1/2-ERK1/2 and MEK3/6-p38MAPK are the major pathways regulating the MAPKAPK activities. We therefore used the MEK1/2 specific inhibitor PD-184352 and the p38MAPKα/β specific inhibitor SB-202190 to determine which MAPKAPK is involved in the phosphorylation of SRC-3. Inhibition of MEK1/2 activity thereby inhibiting ERK1/2 activity did not change the phosphorylation status of SRC-3 at S857. However, inhibition of the p38MAPK activity and thereby inhibiting MK2/3 drastically decreased the phosphorylation of SRC-3 suggesting that the p38MAPK-MK2 pathway is involved in the phosphorylation of SRC-3. In further support of this we showed that reagents stimulating the MK2 activity increased phosphorylation of SRC-3 while the MK2

specific inhibitor PF-3644022 decreased phosphorylation of SRC-3. However, a kinase inhibitor can be promiscuous and may inhibit many other kinases [405]. Therefore, we also utilized MK2/MK3 double KO cell lines to validate our finding. Cells depleted of both MK2 and MK3 are required in this case because MK2 and MK3 have many overlapping functions [326, 328]. Since MK2/MK3 double KO cell line was used, we cannot conclude whether MK2 or MK3 was the main kinase involved in the phosphorylation of SRC-3 S857. Furthermore, owing to the fact that the MK2 inhibitor used in our study is reported to inhibit MK3 to some extent [373] the role of MK3 in the phosphorylation of SRC-3 cannot be excluded. However, the *in vivo* activity and expression level of MK2 is always markedly higher than that of MK3 [326] and hence MK2 can be assumed to be the major kinase phosphorylating SRC-3. MK2 is required for the stability of p38MAPK [345]. Therefore, use of MK2 depletion methods such as siRNA or shRNA would not confirm if the change in the phosphorylation of SRC-3 was due to loss of MK2 or destabilization of p38MAPK. We addressed this issue using MK2/MK3 double KO cell lines re-expressing kinase activity dead MK2 and MK2 specific inhibitor because in both cases, scaffolding property of MK2 is intact and hence p38MAPK is available. We found that in MK2/MK3 double KO mouse cell lines re-expressing kinase activity dead MK2, the stimulation with reagents activating MK2 activity could not induce phosphorylation of SRC-3 at S857. The conclusion is that MK2/MK3 and not p38MAPK directly phosphorylates SRC-3 at S857.

Wu et al. found that inhibition of IKK-α and IKK-β significantly prevented the phosphorylation of SRC-3 at S857 [76]. In our study, we found that inhibition of IKK-α and IKK-β resulted in only slight decline of phosphorylation of SRC-3 at S857. The difference in these observations could be due to different experimental settings and cell lines used. For example, Wu et al. [76] performed their experiment in the breast cancer cell line MCF7 while we studied it in the lung cancer cell line A549. Similarly, they performed the experiments either *in vitro* or in an overexpressed system while we performed our experiments *in vivo* by knocking out endogenous gene expression. In an overexpressed system, stoichiometric imbalance of the expressed protein can result in promiscuous interactions of the protein. Hence, results obtained at endogenous protein level are more reliable than one obtained from an overexpression system [406]. However, even if we feel confident that IKK-α and IKK-β does not play a significant part in the phosphorylation of S857 in the A549 cells, it cannot be ruled out that they might contribute to a greater extent in other cell lines and tissues.

Furthermore, our finding in Paper III, hint the possibility of kinase/s other than MK2 that could phosphorylate SRC-3 at S857. In Paper III, we found that only 37 of the 101 TNF-α stimulated SRC-3 S857 dependent genes were affected by the use of a MK2 inhibitor. The discrepancy between the number of S857 dependent genes identified and the number of these genes being affected by the MK2 inhibitor suggests that there could be other kinases phosphorylating SRC-3 at S857 and regulating the expression of genes dependent on SRC-3. For example, we showed that IKK-α and IKK-β in combination, but not independently, may slightly upregulate phosphorylation of SRC-3 at S857 in certain cell line. Kinases that are activated by TNF-α stimulation and involved in the regulation of the remaining SRC-3 S857 dependent genes could be interesting to examine if they are involved in the phosphorylation of SRC-3 at S857. However, there are alternative reasons for the inability of the MK2 inhibitor to affect the expression of S857 dependent SRC-3 target genes. Firstly, use of a MK2 inhibitor will not completely inhibit the phosphorylation of S857 at all SRC-3 molecules in the cell, so there might exist a "background level" of phosphorylated S857, while in the rescue mutant S857A cell line the possibility of phosphorylation at this site is completely removed. Secondly, the S857A mutation in SRC-3 could in theory cause small structural changes in the CBP interaction domain (CID) of SRC-3 (S857 lies within the CID domain) which could have influenced the role of SRC-3 in gene expression. Thirdly, the cells are treated with MK2 inhibitor only for a fixed time (2 hours) whereas the S857A mutation was permanently present in the cells. It is therefore a possibility that the continuous expression of SRC-3 S857A could lead to regulation of a set of genes which can further lead to another secondary set of gene in due course of time. This could result in permanent changes in the transcriptome that are indirectly caused by the inhibition of the SRC-3 S857 phosphorylation, and therefore would not be mimicked by the brief use of a MK2 inhibitor.

### 5.2. Role of SRC-3 S857 phosphosite regarding its nuclear translocation, NF-κB activity and doxorubicin sensitivity

We found that phosphorylation of SRC-3 at S857 is involved in the nuclear translocation of SRC-3 in A549 cells. Prevention of SRC-3 phosphorylation using MK2 inhibitor retained a fraction of SRC-3 in the cytoplasm (Paper I). That phosphorylation regulates the nuclear translocation of SRC-3 is supported by other studies. In a study by Wang et. al., okadaic acid was found to inhibit the insulin-induced nuclear translocation of SRC-3 in rat cells suggesting that the mechanism of SRC-3 nuclear import may be phosphorylation dependent [116]. Amazit et al. found that EGF induced phosphorylation of SRC-3 at S857 and S860 via ERK1/2 in HeLa

cells. Interestingly, when these two sites were mutated in combination, but not independently, nuclear translocation of SRC-3 decreased in HEK293 cells suggesting that phosphorylation at S857 (in combination with S860) is important for nuclear translocation of SRC-3 [87]. SRC-3 harbors a NLS within the first 34 aa and a NES within its CID domain [407, 408]. As a general rule, the NLS and NES motifs present in a protein bind the importins and exportins which imports the protein into the nucleus or exports it out of the nucleus respectively [409]. However, phosphorylation adds another level of regulation to this already tightly controlled trafficking of proteins between the cytoplasm and nucleus. It is not clear how phosphorylation of SRC-3 at S857 regulates its nuclear translocation. As NES and the phosphosite S857 are both located in the CID domain of SRC-3, it is possible that phosphorylation of SRC-3 S857 results into masking of the NES or attenuation of binding between SRC-3 and exportin. However, it cannot be denied that such phosphorylation could result in un-masking of the NLS or enhancement of the binding between SRC-3 and importin. It is also possible that the phosphorylated cytoplasmic SRC-3 binds to other proteins which would piggyback SRC-3 into the nucleus. Piggybacking of SRC-3Δ4 (SRC-3 without NLS) by p300 into nucleus has been previously reported [410].

The intracellular localization of a protein plays a significant role in determining its function. Several studies have reported that the nucleus is the major site of SRC-3 proteasomal degradation [96, 98]. In our study, we showed that TNF-α-induced phosphorylation of SRC-3 at S857 is important for nuclear translocation and after 120 minutes of TNF-α treatment, SRC-3 started to degrade in both MDA-MB-231 and A549 cells (Paper I). As a transcriptional coactivator, SRC-3 exerts its major function in the nucleus. The observed decrease in SRC-3 after TNF-α stimulation (Paper I) indicates that SRC-3 is degraded after it has executed its function in the nucleus. However, high level of nuclear SRC-3 was found exclusively in about 10% of breast tumor tissues [411]. Recently, in xenograft mouse model, immunostaining of the primary tumors with a phospo-SRC-3 S857 antibody detected increased nuclear SRC-3 in the tumors that progressed to aggressive metastatic disease [77]. Therefore, identification of mechanisms regulating SRC-3 nuclear translocation is critical because such mechanism can be targeted to modulate the stability of SRC-3 and hence its oncogenic potential. Based on the observations presented in our study, use of MK2 inhibitor can limit the availability of SRC-3 in the nucleus and thus its function as a transcriptional co-activator. Interestingly, several studies reveal that SRC-3 can also have functions outside the nucleus. For example, in a study by Long et. al., EGF-induced phosphorylation of SRC-3Δ4 (SRC-3 without NLS) promotes the localization of SRC-3Δ4 to the plasma membrane and mediates interactions with EGFR and

focal adhesion kinase (FAK) to promote cell migration [105]. Similarly, Yu et. al. showed that SRC-3 may cooperate with translational repressors such as TIA-1 and TIAR in the cytoplasm to repress the translation of several cytokines [149]. The cytoplasmic functions of SRC-3 would be abolished when SRC-3 is translocated into the nucleus due to SRC-3 S857 phosphorylation. In this way, it can be expected that inhibition of nuclear translocation of SRC-3 using MK2 inhibitor can prevent its oncogenic activities related to its genomic transcriptional functions, but its oncogenic properties related to non-genomic cytoplasmic functions will remain the same or may even be enhanced.

Next, we investigated the physiological role of p38MAPK-MK2-SRC-3 signaling pathway regarding regulation of inflammation. Knockdown of SRC-3 downregulated, while re-expression of SRC-3 in SRC-3 KO A549 cells upregulated, NF-κB activity when stimulated with TNF-α. Further, we found that SRC-3 S857 phosphorylation is a key PTM event involved in promoting transcriptional activity of NF-κB in A549 lung cancer cell (Paper I). In line with our study, SRC-3 was found to upregulate NF-κB activity in colon epithelial cells [148] and HeLa cells [76, 412]. However, findings in different cell type and species contrast our finding. E.g., in mouse macrophages, SRC-3 deficiency did not affect LPS-induced activation of NF-κB [149]. This discrepancy might be due to cell and species-specific role of SRC-3 in regulation of NF-κB. Though we did not explore the underlying mechanism of how SRC-3 would regulate NF-κB activity there are studies investigating this. Wu et al. found that phosphorylation at SRC-3 S857 is not necessary for interaction between SRC-3 and NF-κB but is required for the binding of SRC-3 with the secondary coactivator CBP [76]. Likewise, Saha and colleagues have shown that p38MAPK phosphorylates and activates p300 which then binds to NF-κB and enhances the transcription of NF-κB [226]. Hence, p38MAPK would modulate NF-κB's transcriptional activity by regulating the coactivators associated with NF-κB. However, it was a different scenario in mouse B-lymphocytes where IKK bound to SRC-3 was found to prevent IκB phosphorylation and hence stabilized IκB. This inhibited NF-κB nuclear accumulation and transcriptional activity [122].

IL-6 is a downstream target of NF-κB. We showed that in A549 cells SRC-3 upregulates IL-6 expression, and that inhibition of MK2 activity, thereby prevention of phosphorylation of SRC-3 at S857, inhibited IL-6 transcription. In accordance, Wu et al. also showed that SRC-3 S857 is important for expression of IL-6 mRNA utilizing SRC-3 KO MEF cell lines re-expressing mutant SRC-3 S857A [76]. However, it has been previously known that p38MAPK-MK2 signaling itself regulates IL-6 mRNA stability. RNA binding protein TTP binds to IL-6 mRNA

and leads to the degradation of IL-6. MK2 phosphorylation of TTP prevents the binding between TTP and IL-6 mRNA and inhibits the degradation of IL-6 mRNA [312]. Here, we have reported a novel p38MAPK-MK2-mediated pathway for the regulation of IL-6 expression via SRC-3. In contrast to our finding, Yu et. al. reported that in mouse macrophages no significant differences in IL-6 mRNA expression was observed between the wild-type and SRC-3 KO macrophages. Rather, SRC-3 was found to repress the translation of IL-6 by cooperating with the translational repressor TIA-1 and TIAR [149]. This further strengthens that the role of SRC-3 in regulation of NF-κB activity is cell and species dependent. p38MAPK was reported to regulate TNF-α-induced IL-6 expression via NF-κB activity without influencing its binding to DNA and without inducing the phosphorylation of its subunits. This suggested that the TNF-α-induced IL-6 transcription is regulated through another factor involved in the transactivation by NF-κB [413, 414]. In agreement with these studies, we showed that p38MAPK-MK2 signaling induced phosphorylation of SRC-3 is involved in the regulation of the transcriptional activity of NF-κB and thereby transcription of IL-6 (Paper I). Our study might have identified the missing link between p38MAPK and NF-κB signaling that is involved in upregulation of IL-6 transcription.

Among the several downstream targets of NF-κB including IL-6, IL-8, MMP9, TRAF1 and ICAM-1 examined, we found only IL-6 to be regulated by SRC-3 in A549 cells (Paper I). We did not find IL-6 to be regulated by SRC-3 in MDA-MB-231 cells in the RNA-seq analysis (Paper III). This might be due to the cell specificity of SRC-3 regulation. There could be different explanations for inability of SRC-3 to regulate the other reported NF-κB target genes. SRC-3 recruits other co-activators such as CARM1 [415], CBP [76] and HDAC1 [416] which are involved in modifying chromatin structure and thus enhance or prevent the transcriptional accessibility. It is possible that the co-activator complex assembled by NF-κB act differently at different promoters resulting in the discrepancy in the expression of NF-κB target genes. Furthermore, in SRC-3 KO cells, the role of SRC-3 might be compensated by other members of SRC family. For example, in colon epithelial cells obtained from SRC-3 KO mouse, SRC-1 was found to cooperate with NF-κB to enhance CXCL2 expression [148]. As IL-6 is involved in the pathophysiology of different diseases, it an important target for treatment of those diseases [417, 418]. Thus, targeting p38MAPK-MK2-SRC-3 signaling pathway could also regulate the expression of IL-6 for certain cancer cells.

Though SRC-3 has been previously reported to be involved in increased resistance to different chemotherapeutic drugs in several cancer types such as lung [165], esophageal [162], hepatic

[163], colon [164] and ER/PR positive breast cancers [419], its role in chemotherapeutic drug resistance in TNBC is not well known. In Paper II, we found that depletion of SRC-3 by either CRISPR-cas9 or shRNA enhanced the sensitivity to doxorubicin in TNBC. Furthermore, we found that doxorubicin-induced phosphorylation of SRC-3 at S857 might be involved in the enhancement of resistance to doxorubicin. However, the underlying mechanism by which SRC-3 depletion leads to doxorubicin sensitization remains to be elucidated in our study. It is known that in MCF7 breast cancer cells, depletion of SRC-3 increased cell sensitivity towards doxorubicin [419]. In this case, SRC-3 cooperated with AP1 to enhance the expression of TRAF4 which was found to compete with p53 for access to a deubiquitinating enzyme, HAUSP. This binding competition resulted in decreased p53 protein levels and subsequently reduced stress-induced cell apoptosis [419]. Since SRC-3 is able to abrogate p53 function, SRC-3 overexpression may be especially important in regulation of doxorubicin sensitivity in tumors where p53 is not mutated. However, since MDA-MB-231 harbors mutated p53 [388], this is less likely to be the mechanism in our study. SRC-3 is known to mediate upregulation of genes that are involved in efflux of anticancer drugs. The three key transporters involved in the transport of anticancer drugs such as doxorubicin are P-glycoprotein (P-gp/ABCB1), multidrug-resistance protein-1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP/MXR/ABCG2) [420]. SRC-3 is a coactivator of Nuclear factor erythroid 2-related factor (Nrf2) [421] which regulates the expression of ABCC1, ABCG2 [422]. Doxorubicin is reported to upregulate the expression and activity of Nrf2 in MDA-MB-231 [423] so it is possible that SRC-3 could contribute to doxorubicin resistance by decreasing the intracellular accumulation of doxorubicin by regulating Nrf2 mediated upregulation of ABCC1, ABCG2. Another possible mechanism is upregulation of genes involved in DNA repair by SRC-3. Since DNA damage has been proposed as one of the potential modes of actions for doxorubicin [424], upregulation of genes involved in DNA repair by SRC-3 may result in reduced sensitivity to doxorubicin leading to drug resistance. Wang et. al. found that SRC-3 coactivated Liver receptor homology 1 (LRH1) and enhanced the expression of its downstream target, Mediator of DNA damage checkpoint protein 1 (MDC1). Upregulation of MDC1 improved the ability of cell DNA damage repair resulting into resistance to doxorubicin in MDA-MB-231 cells [81]. In our study we were not able to identify ABCC1, ABCG2 or MDC1 as SRC-3 S857 dependent genes. But since our analyses only identified the S857 dependent genes, it is still possible that these are SRC-3 target genes and the S857 phosphosite is not necessary for their regulation.

The sensitivity to doxorubicin was lower in SRC-3 depleted cells re-expressing WT SRC-3 than in SRC-3 depleted cells re-expressing mutant SRC-3 S857A, suggesting the role of SRC-3 S857

phosphorylation in the sensitivity to doxorubicin. This implicates that prevention of S857 phosphorylation would sensitize the cells to doxorubicin treatment. This is in line with our observation that MK2 is involved in the phosphorylation of SRC-3 S857 and prevention of this phosphorylation with MK2 inhibitor enhanced the sensitivity to doxorubicin (Paper II). In other words, MK2-SRC-3 pathway decreases doxorubicin sensitivity in TNBC cells. MK2 inhibitor was also found to enhance doxorubicin and bortezomib sensitivity in multiple myeloma (MM) [376]. While a recent study showed that inhibition of SRC-3 improved bortezomib sensitivity in MM [425]. As suggested by our study, it would be interesting to investigate if MK2-SRC-3 pathway is involved in the downregulation of doxorubicin sensitivity in MM. In a study, phosphorylation of SRC-3 at S857 was shown to be required for the coactivation of migration inhibitory factor (MIF) promoter by SRC-3, CBP and HIF-1α. Downregulation of MIF enhanced autophagic cell death thereby improving sensitivity to doxorubicin and suppressing tumorigenicity of MCF-7 cells in a tumor xenograft mouse model [78]. Though, in this study, we provide a direct evidence of the involvement of the phosphorylation of SRC-3 at S857 in sensitivity to a chemotherapeutic reagent in TNBC cell line we did not find MIF to be dependent on SRC-3 S857 phosphosite. We were therefore excited by the fact that genes involved in doxorubicin and daunorubicin metabolic processes were enriched for among the biological properties identified for SRC-3 S857 dependent target genes in paper III. Three genes of the aldo-keto reductase family 1, namely AKR1C1, AKR1C3 and AKR1C4 were associated with this activity. However, while these genes are known to contribute to doxorubicin resistance by increasing the metabolism of this drug [426], we found that these genes were downregulated by SRC-3 in MDA-MB-231 cell and thus unlikely to contribute to the decreased sensitivity to doxorubicin caused by SRC-3. In paper III, we found that MK2-SRC-3 pathway regulates the expression of 37 genes. One of them is praoxonase 3 (PON3) which prevents oxidative stress [427]. PON3 is reported to enhance sensitivity to cisplatin in esophageal cancer cells by repressing the NF-κB and PI3K/AKT pathway [428]. Among several molecular mechanism of action proposed for cisplatin [429] and doxorubicin [424], some such as DNA damage are common to both. Therefore, it is possible that PON3 might be involved in SRC-3 S857 mediated doxorubicin sensitivity in MDA-MB-231 cells. However, further study is needed to confirm this.

### 5.3. Possible reason for little overlap between SRC-3 S857 dependent genes identified in unstimulated and TNF-α stimulated conditions

In paper III, it was interesting that only 12 out of the 101 genes identified as TNF-α induced SRC-3 S857 dependent genes were also identified as SRC-3 S857 dependent genes in the unstimulated cells. TNF-α stimulation thus seem to mediate a switch in target genes regulated by SRC-3. This might be explained by induction of multifaceted signaling pathways by TNF-α stimulation and their possible crosstalk with SRC-3 (Figure 6). Briefly, TNF-α binds to its receptors, mainly Tumor necrosis factor receptor superfamily member 1 (TNFR1) and TNFR2 and triggers the activation and expression of various transcription factors e.g., NF-κB, AP-1 [430] and several kinases including MAPKs, protein kinase B (AKT) [430, 431]. The status of these transcription factors can affect the SRC-3 mediated gene expression. Let's look into c-Fos, a subunit of AP-1 transcription complex which is co-activated by SRC-3 [71]. In paper III, we observed that TNF-α stimulation markedly enhanced the expression of c-Fos. Such abundance of c-Fos could result into the expression of new set of SRC-3 target genes that were unaffected in unstimulated condition. Though the duration of TNF-α stimulation was only 2 hours, such short duration could still be enough for expression of primary set of genes by c-Fos. TNF-α induced activation of kinases including p38MAPK [430], JNK [432], IKKα/β [433], ERK1/2 [434] could also influence the SRC-3 mediated gene expression. p38MAPK and JNK phosphorylate multiple sites on SRC-3 including S505, S543, S860 and S867, IKKα/β is reported to phosphorylate S857 [76] and ERK1/2 is reported to phosphorylate S857 and S860 [87]. TNF-α induced phosphorylation of SRC-3 enhanced association between SRC-3 and cofactor CBP. Such co-operation between the two cofactors increases the transcriptional activity of NF-κB [76]. TNF-α stimulation also activates MK2. MK2 is a master regulator of RNA binding proteins thereby interfering the stability of mRNAs containing Adenylate-Uridylate (AU)-rich elements [312]. In paper III, we identified that TNF-α decreased the expression of histone deacetylases (HDAC4, 9, 11) which are involved in the modification of chromatin remodeling thereby modulating transcription [435]. It is possible that the decrease in the expression of HDACs contributed to expression of set of genes in TNF-α stimulated cells that were otherwise not expressed in unstimulated condition. Taken together, stimulation with TNF-α results into different set of SRC-3 S857 dependent genes that are unique to the stimulated condition.

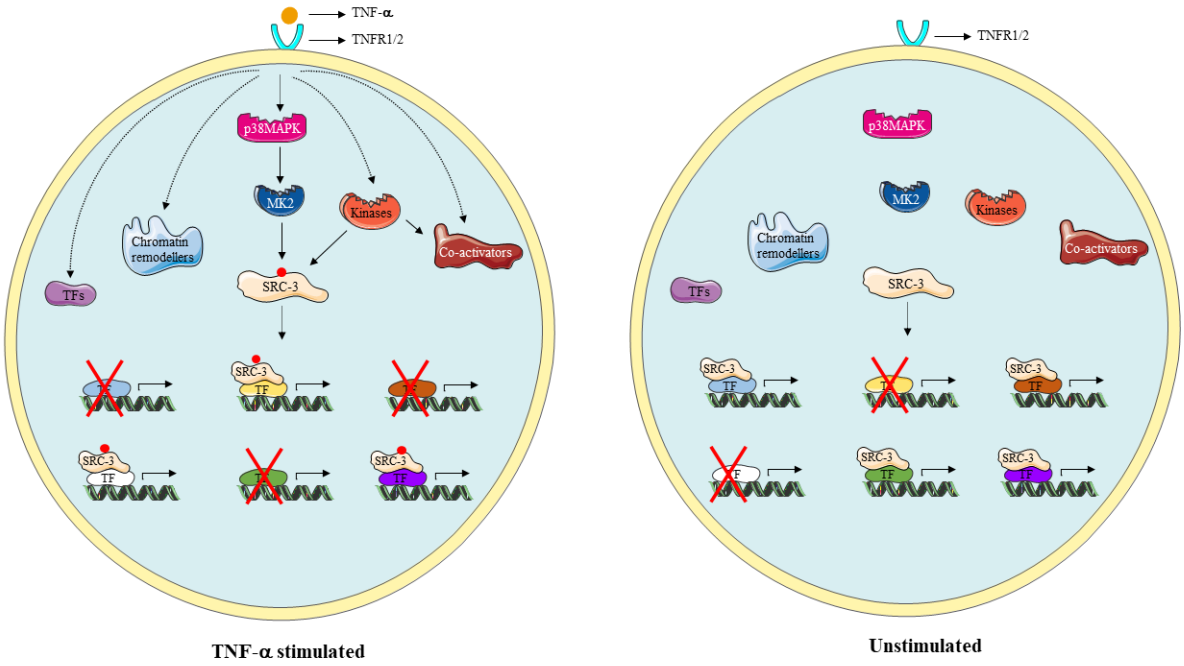


**Figure 6. Simplified figure illustrating different events unique to TNF-α stimulated and unstimulated conditions that could result into different sets of SRC-3 target genes in the two conditions.** On the figure to the left, pathways activated upon TNF-α stimulation are shown. For simplicity only a few representative events such as modification of different transcription factors (TF), chromatin remodelers, kinases and co-activators including SRC-3 are illustrated. Such modifications would influence the association of SRC-3 with transcription factors and thereby the expression of genes regulated by them. While on the figure to the right, status of TFs, chromatin remodelers, kinases and co-activators in unstimulated condition are illustrated. In unstimulated condition, SRC-3 would co-operate with TFs different than the ones in TNF-α stimulated condition resulting into expression of different set of genes.

In paper III, we used SRC-3 KO cells re-expressing wild-type SRC-3 or mutant SRC-3 S857A to identify genes dependent on SRC-3 S857 phosphorylation site. With this, we identified 340 genes dependent on S857 phosphorylation site. In order to be able to include all conditions and controls (and keep the costs as low as possible) we chose to pool the RNA from three biological replicates before sequencing. However, we kept the RNA samples of the individual replicates so that they could be used for verification of target genes by RT-qPCR after the RNA-seq analysis. RNA sample pooling before sequencing reduced the costs, and others have in fact shown that it works good because average level of expression across all samples is obtained [436]. However, as pointed out by Rajkumar and colleagues [437], in practice such pooling has a risk to influence an experiment as an outlier among the pooled sample can skew the average value. Another drawback of pooling samples was that we only had a single biological replicate instead of three and hence we could not perform statistics (calculate p-values) to identify

statistically significant target genes [438]. This could have led to some false positive results in our study. In hindsight, performing this study with individual sequences of 3-4 biological replicates for each sample would have produced more reliable results. However, as a pilot study this has been useful for setting up the analyses to find the SRC-3 S857 dependent genes in the future study. It will be exciting to see how much of these results could be reproduced when the experiment is repeated.

### 5.4. Clinical implications of MK2 inhibitor

In this study, we identified that the p38MAPK-MK2-SRC-3 pathway is involved in expression of pro-inflammatory cytokine IL-6 in lung cancer cells (paper I), doxorubicin sensitivity (paper II) and expression of genes involved in the regulation of cancer in TNBC (paper III). Targeting members of this pathway could therefore be an option to regulate inflammation and cancer progression. Several p38MAPK inhibitors have been identified but such inhibitors have not been successful in clinical trials due to their severe side effects and unspecific targeting [439]. SRC-3 is also a possible drug target, and several SRC-3 specific inhibitors such as gossypol [378], bufalin [379] and SI-2 [380] has been developed. However, none of the SRC-3 inhibitors have reached clinical trials till date. Due to the lack of high affinity and high specificity ligand binding sites and the large and flexible structure of SRC-3, developing effective SRC-3 inhibitors faces critical challenges. MK2 is an attractive target to treat inflammation and related diseases and to increase tumor sensitivity to chemotherapeutics [373]. In our study, we have shown that inhibition of MK2 activity with a specific inhibitor reduced the transcription of pro-inflammatory gene IL-6 in A549 cells (Paper I) and enhanced the doxorubicin sensitivity in MDA-MB-231 cells (Paper II). Further, pretreatment with MK2 inhibitor affected the expression of several genes reported to be involved in tumorigenesis (Paper III). Since the MK2 inhibitors are already in clinical trials (ClinicalTrials.gov Identifier: NCT04247815, NCT04481685, NCT05216224 and NCT04947579) it makes sense that using MK2 inhibitor is a better approach than using SRC-3 inhibitors for the treatment of diseases where the S857 phosphorylation of SRC-3 is proven to have an effect. This particularly saves valuable time and resource for development of a novel drug. However, inhibition of MK2 activity does not prevent only the SRC-3 regulated activities. As enlisted in the introduction section of this thesis (Table 3), MK2 has several targets and therefore inhibition of MK2 activity can regulate the associated biological activities. Synthetic lethality is defined as the setting in which loss of function of two genes together results in cell death however, inactivation of only one of the two genes has little effect on cell viability [440]. MK2 and p53 have synthetic lethal relationship. MK2 can work

as a cell cycle checkpoint kinase in response to DNA damage [322]. As p53 also possesses cell cycle checkpoint function, in cells devoid of p53 if MK2 activity is inhibited then there is drastic reduction in DNA damaging chemotherapeutic agent-induced cell cycle arrest. This leads to mitotic catastrophe and ultimately cell death thereby enhancing the sensitivity to the chemotherapeutic agent [285]. In our study, we found that MK2 inhibitor enhanced doxorubicin sensitivity in MDA-MB-231 cells which possess p53 R280K mutation (Arg 280 is mutated to a Lys). However, it is not known whether such mutation results into loss of the cell cycle checkpoint control function of p53. Nevertheless, it was reported that such mutation in p53 decreases its DNA binding ability [441]. Further study comparing the effect of doxorubicin and MK2 inhibitor between breast cancer cells that has wild-type and p53 R280K mutation is needed to confirm this. Such an increased sensitivity to chemotherapeutic reagent between the use of a MK2 inhibitor and chemotherapeutic drugs have also been observed for doxorubicin in multiple myeloma [376] and temozolomide in glioblastoma cells [442]. The other factor determining sensitivity to a chemotherapeutic agent when combined with MK2 inhibitor seems to be mechanism of action of the chemotherapeutic reagent used. For example, MK2 inhibition sensitized pancreatic cancer cells against cisplatin but protected against gemcitabine [443] while we have found that MK2 inhibitor enhanced doxorubicin sensitivity. Both cisplatin [429] and doxorubicin [424] are known to act by damaging DNA, indicating that the MK2 inhibitor enhances the sensitivity of chemotherapeutic drugs that act by damaging DNA. On the other hand, gemcitabine is chemotherapeutic nucleoside analogue which incorporates into the nascent DNA strands during replication interfering DNA synthesis [444]. However, in the presence of MK2 inhibitor, DNA replication continues despite the presence of the gemcitabine resulting into resistance to gemcitabine. Identification of MK2 as a promising actionable target in TNBC in our study is of special interest because TNBC lacks a drug target, and it is among some of the most chemotherapy-resistant cancers [445]. Interestingly, it seems that the p38MAPK and MK2 activation is more prominent in TNBC (basal like) cancer compared to ER positive (luminal type) cancer [301], and thus the MK2 inhibition could be of particular importance in the treatment of TNBC. Furthermore, we found that treatment with MK2 inhibitor alone influenced a huge number of genes (Paper III). Identification of the functions of these target genes in cancer development will therefore be important to predict and evaluate the effect a MK2 inhibitor will have in cancer treatment. Further study needs to be performed to firmly establish the potential of MK2 inhibitors in targeted therapy.

## 6. Conclusion

SRC-3 is a co-activator involved in lung and breast cancer. The activity of SRC-3 is regulated by phosphorylation and S857 is the most frequently reported phosphosite. In order to regulate the activity of SRC-3, identifying a kinase phosphorylating SRC-3 at S857 can be crucial because kinases are an ideal drug target. In this study, we have identified that p38MAPK-MK2 signaling axis is involved in phosphorylation of SRC-3 at S857 in a wide range of cell lines. This phosphorylation leads to its nuclear translocation. Inside the nucleus, the phosphorylation of SRC-3 at S857 enhances the transcriptional activity of NF-κB and upregulates the transcription of pro-inflammatory cytokine, IL-6 in A549 lung cancer cells. In addition, the p38MAPK-MK2-SRC-3 signaling axis is also involved in the enhancement of resistance to doxorubicin in TNBC cell line. Furthermore, this study shows that several genes involved in cancer progression might also be regulated by the p38MAPK-MK2-SRC-3 signaling axis in the TNBC cell line. Thus, targeting this signaling pathway to modulate the role of SRC-3 in cancer is warranted. MK2 inhibitor is already in clinical trials for treatment of inflammatory disease. Repurposing MK2 inhibitor to curb the role of SRC-3 S857 phosphosite in cancer will save valuable time in development of a novel cancer therapy.

## 7. Future perspective

In the present study, we have identified SRC-3 as a substrate of MK2 in a wide range of cell lines. Furthermore, we established the role of p38MAPK-MK2-SRC-3 pathway in the expression of pro-inflammatory cytokine IL-6 in lung cancer cells, and sensitivity towards doxorubicin in breast cancer cells. We have identified SRC-3 S857 dependent genes and genes regulated by p38MAPK-MK2-SRC-3 pathway in TNBC cells. The involvement of this pathway in other biological activities can be explored.

We have generated SRC-3 KO MDA-MB-231 cells re-expressing wild-type SRC-3 and mutant SRC-3 S857A, and used RNA-seq of stimulated and unstimulated cells in a pilot study to try to identify specific target genes regulated by SRC-3 phosphorylated at S857. This was a useful exercise and generated a lot of data, but preliminary RT-qPCR data indicate that several biological replicates should be included for each cell line and condition when repeating the experiment. The clinical relevance of our findings can be studied by immunohistochemical staining of patient samples to analyze the correlation between MK2 activity, phosphorylation of SRC-3 at S857 and expression levels of selected target genes. The goal should be to identify those target genes that can be used as biomarkers for identification of patients for treatment with the MK2 inhibitor, and for monitoring the outcome of this treatment. Further, the role of the MK2-SRC-3 signaling axis in tumor development in xenograft mouse models can be determined by use of the cell models we have generated. In such pre-clinical study, the efficacy of MK2 inhibitor can also be validated. In Paper II, we have explored the importance of phosphorylation of SRC-3 at S857 in sensitivity to doxorubicin. In this study, we need to perform more experiments to further strengthen the finding that doxorubicin induced the stimulation of p38MAPK-MK2-SRC-3 signaling axis. Doxorubicin-induced phosphorylation of SRC-3 at S857 should be verified using MK2 inhibitor and the phosphorylation of SRC-3 at S857 should also be studied at endogenous level. The study should be performed in additional TNBC cell lines. Experiments showing that MK2 is involved in doxorubicin-induced cytotoxicity should be performed using different concentrations of MK2 inhibitor. The doxorubicin-induced cytotoxicity should be assessed by more than one method. Eventually, the natural progression is studying the combined effect of doxorubicin and MK2 inhibitor in pre-clinical mouse models. It can also be worth exploring the role of SRC-3 S857 in the sensitivity to other members of anthracycline drugs such as epirubicin. The downstream target of SRC-3 that is involved in regulation of the cytotoxicity can be explored.

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## 9. List of papers

**Paper I**

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# Phosphorylation of steroid receptor coactivator-3 (SRC-3) at serine 857 is regulated by the p38$^{MAPK}$-MK2 axis and affects NF-κB-mediated transcription

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**Steroid receptor coactivator-3 (SRC-3) regulates the activity of both nuclear hormone receptors and a number of key transcription factors. It is implicated in the regulation of cell proliferation, inflammation and in the progression of several common cancers including breast, colorectal and lung tumors. Phosphorylation is an important regulatory event controlling the activities of SRC-3. Serine 857 is the most studied phospho-acceptor site, and its modification has been reported to be important for SRC-3-dependent tumor progression. In this study, we show that the stress-responsive p38$^{MAPK}$-MK2 signaling pathway controls the phosphorylation of SRC-3 at S857 in a wide range of human cancer cells. Activation of the p38$^{MAPK}$-MK2 pathway results in the nuclear translocation of SRC-3, where it contributes to the transactivation of NF-kB and thus regulation of IL-6 transcription. The identification of the p38$^{MAPK}$-MK2 signaling axis as a key regulator of SRC-3 phosphorylation and activity opens up new possibilities for the development and testing of novel therapeutic strategies to control both proliferative and metastatic tumor growth.**

The steroid receptor coactivator 3 (SRC-3) is a transcriptional coactivator of the p160 family encoded by the gene *nuclear receptor coactivator 3* (*NCOA3*). It was originally identified as a coactivator for nuclear receptors[1], but is now recognized as a coactivator of several other transcription factors including E2F transcription factor 1 (E2F1)[2], polyomavirus enhancer activator 3 (PEA3)[3], activator protein-1 (AP-1)[4,5], and nuclear factor-κB (NF-κB)[6,7]. Based on this broad spectrum of transcriptional activities, SRC-3 has been shown to play important roles in a wide range of physiological processes, such as cell proliferation, cell survival, mammary gland development[8] and metabolism[9]. Since 1997, when SRC-3 was found to be amplified in breast cancer[10] its role in cancer progression has been broadly investigated. It has been shown to be implicated in hormone-related cancers, such as endometrial[11], ovarian[12], prostate[13] and breast cancer[14], but also in in hormone-independent cancer types such as esophageal, squamous cell, colorectal, hepatocellular, pancreatic and non-small cell lung cancer[15]. SRC-3 modulates various processes, for example cell proliferation[16] , development of metastasis[17], and resistances to anti-cancer drugs[18,19].

The function of the SRC-3 protein is highly regulated by post-transcriptional modifications through phosphorylation. SRC-3 is phosphorylated at multiple residues mediated by distinct protein kinases, suggesting that SRC-3 might be controlled by several different signaling pathways in health and disease[20,21]. Among the different phosphorylation sites, the most frequently reported modification of SRC-3 is the phosphorylation at serine 857 (S857)[22]. This phosphorylation has been shown to be important for regulation of estrogen receptor, androgen receptor and NF-κB-mediated transcription[20]. In addition, more recent data indicate that phosphorylation at S857 is also essential for the ability of SRC-3 to promote lung and breast cancer progression and metastasis[23].

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With regards to these observations, the protein kinase (or kinases) responsible for this specific phosphorylation of SRC-3 might be attractive therapeutic targets for treatment of lung and breast cancer. However, to date the identity of the protein kinases able to phosphorylate SRC-3 at S857 remains unclear. Suggested candidates include protein kinase A (PKA)[20], I kappa B kinase (IKK)[7] and the metabolic enzyme 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 4 (PFKFB4)[23].

Recently, SRC-3 was also reported to be a novel target for the extracellular regulated kinase 3 (ERK3)[3,24]. ERK3 is an atypical member of the Mitogen-Activated Protein Kinases (MAPKs) family of protein kinases. So far, little is known about the biological function of these atypical kinases. Our limited knowledge can partly be attributed to a lack of identified physiological substrates. The first physiological substrate identified for ERK3 was the MAPK-Activiated Protein Kinase (MAPKAPK) MK5[25]. However, besides being a regulator and downstream substrate of ERK3 and ERK4, the biological function of MK5 is unknown. Thus, the data indicating that SRC-3 is a substrate for ERK3, could be an important step towards our understanding of the biological role of ERK3[3,24].

In the present study, we aimed to confirm that S857 of SRC-3 is a *bona fide* substrate for ERK3 using the purified recombinant kinase. Unexpectedly, we found that ERK3 was not able to phosphorylate SRC-3 at S857 efficiently in vitro. Instead, we observed that SRC-3 was efficiently phosphorylated at S857 by the MAPKAP kinases MK2 and MK5 in vitro. However, only MK2, a downstream effector of the activated $p38^{MAPK}$ pathway, could mediate this specific phosphorylation in living cells. The phosphorylation of SRC-3 at S857 was efficiently inhibited by specific inhibitors of MK2 and MK3 in unstimulated cells and in cells with active $p38^{MAPK}$ signaling. Moreover, our data demonstrate that SRC-3 is an important regulator of the inducible expression of the pro-inflammatory cytokine IL-6 in response to activation of the $p38^{MAPK}$-MK2 signaling pathway by TNF-α.

## Results

**SRC-3 is not a substrate of ERK3 in vitro.** As SRC-3 was described as substrate for ERK3 in lung cancer cells[3], we aimed to confirm this finding in an in vitro approach. First, we tested whether recombinant active ERK3 could phosphorylate a recombinant GST fusion protein encoding the CBP-interacting domain (CID) of SRC-3 (SRC-3 aa 840–1,080). As shown in Fig. 1A, recombinant active ERK3 was unable to phosphorylate the GST-CID-SRC-3 WT (wild type) fusion protein. In contrast, when MK5, a *bona fide* ERK3 substrate, was added to the reaction efficient phosphorylation of GST-CID-SRC-3-WT was readily observed and was also seen after incubation with activated MK5 alone (Fig. 1A). Importantly, no phosphorylation was observed when a mutant version of the protein (GST-CID-SRC-3 S857A), in which serine 857 was replaced with alanine was used as substrate (Fig. 1A). These findings indicate that SRC-3 is phosphorylated at S857 by the ERK3 downstream effector MK5 rather than by ERK3 itself.

Next, we aimed to determine if MK5 is also responsible for the phosphorylation of SRC-3 at S857 in vivo. We first generated a S857 phospho-specific SRC-3 antibody. The specificity of the antibody generated (P-S857-SCR-3 antibody) was then tested in an in vitro kinase assay by incubating GST-CID-SRC-3 WT and GST-CID-SRC-3 S857A with and without active MK5. The anti-P-S857-SRC-3 antibody specifically recognized the phosphorylation of GST-CID-SRC-3 WT at S857, while no signal was detected when incubating the mutated GST-CID-SRC-3 S857A protein (Fig. 1B). The sensitivity of the anti-P-S857-SRC-3 antibody was then determined by Western-blot analysis of a serial dilution of MK5-phosphorylated GST-CID-SRC-3 WT fusion protein revealing that the signal detected with this antibody was linear over a wide range of concentrations of phosphorylated SRC-3 (Fig. 1C). Next, we determined whether the anti-P-S857-SRC-3 antibody was able to discriminate between unphosphorylated SRC-3 and SRC-3 phosphorylated at S857 in vivo in mammalian cells. The human lung cancer cell line H1299 was transfected with expression vectors encoding either SRC-3 WT or SRC-3 S857A. Western-blot analysis confirmed the specificity of the anti-P-S857-SRC-3 antibody for the phosphorylation of S857, as a clear signal was only detected for SRC-3 WT but not for SRC-3 S857A (Fig. 1D). In a final step, we confirmed that the anit-P-S857-SRC-3 antibody could also discriminate between endogenous SRC-3 phosphorylated or unphosphorylated at S857. After immunoprecipitation of endogenous SRC-3 from extracts of H1299 cells, the precipitated SRC-3 was split into two fractions and one fraction was treated with lambda phosphatase. Western-blot analysis showed that the anti-P-S857-SRC-3 antibody detected a signal only in the untreated fraction, and not in the fraction treated with lambda phosphatase (Fig. 1E). Taken together these results clearly demonstrate both the sensitivity and the specificity of the newly generated anti-P-S857-SRC-3 antibody.

**SRC-3 is phosphorylated at S857 by the MAPKAP kinases MK2 and MK5 in vitro.** In the next experiments, we used the anti-P-S857-SRC-3 antibody to identify the kinase(s) that mediate the phosphorylation of SRC-3 at S857. As we could not detect any efficient phosphorylation of SRC-3 by ERK3, but only by MK5 (Fig. 1A,B), we examined the sequence surrounding serine 857 in SRC-3. This sequence (Y-N-R-A-V-S-L) is more closely related to the optimal phosphorylation site sequences for either a MAPKAPK or protein kinase A (X-R-X-X-S-L), than to recognition sequence for a proline-directed MAPK such as ERK3 (T/S-P) (Fig. 2A)[26]. To investigate and compare the preference of MAPKAPKs and MAPKs for the S857 phosphorylation site in SRC-3 in vitro, we set up a kinase assay using the GST-CID-SRC-3 WT fusion protein as substrate. The amount of each kinase used in the assay (MAPKAPKs MK2 and MK5, and the MAPKs ERK2, ERK3 and p38α) was adjusted to give equal input of kinase activity of 0.1 units. Under these conditions, both active MK2 and MK5 phosphorylated SRC-3 at S857 efficiently, while active ERK2, ERK3 and p38α were unable to phosphorylate this residue (Fig. 2B). To further explore the dynamics of S857 phosphorylation by MK2 and MK5, in vitro kinase assays were performed in dose- (Fig. 2C) and time-dependent (Fig. 2D) manners. The results showed that phosphorylation of SRC-3 increased gradually both with increasing amounts of active MK2 and MK5 (Fig. 2C) and with increasing incubation time (Fig. 2D). Taken together, these experiments demonstrated that SRC-3-S857 is an in vitro substrate for both of MK2 and MK5.

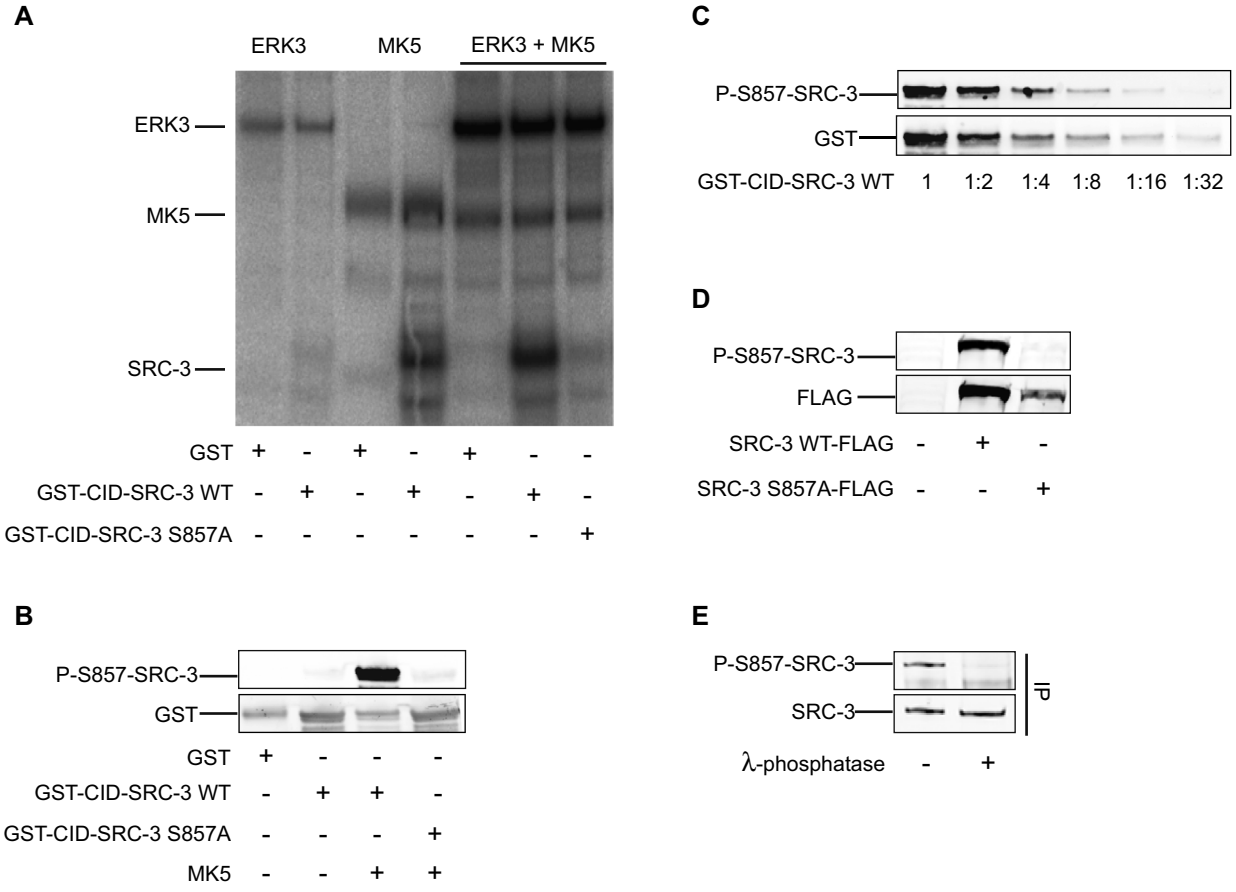



**Figure 1.** ERK3 does not phosphorylate SRC-3. (**A**) MK5, but not ERK3, phosphorylates SRC-3-S857 in vitro. For in vitro kinase assay, either 300 ng of active recombinant ERK3 protein (83.5 kDa) or 50 ng active recombinant MK5 (54 kDa) or both was incubated with 2 µg GST or GST-CID-SRC-3 WT or GST-CID-SRC-3 S857A in kinase buffer and 1 µCi [$\Upsilon^{32}$P]-ATP. The reaction was carried out at 30 °C for 15 min. Proteins were resolved by SDS-PAGE gel and visualized by autoradiography. (**B**) In vitro kinase assay was performed by incubating 2 µg GST or wild type (WT) or mutant (S857A) GST-CID-SRC-3 fusion proteins with and without 50 ng active MK5 in the kinase buffer for 15 min. Serine 857 phosphorylation and total amount of GST-CID-SRC-3 WT and GST-CID-SRC-3 S857A fusion proteins were detected by Western-blotting using anti-P-S857-SRC-3 and anti-GST antibodies, respectively. The full-length blots are presented in supplementary figure S4. (**C**) MK5 phosphorylated GST-CID-SRC-3 fusion protein (2 µg) was diluted 2, 4, 8, 16 and 32 times before separation on SDS-PAGE followed by Western-blotting. The membrane was then probed with anti-GST and anti-P-S857-SRC-3 antibodies. The full-length blots are presented in supplementary Figure S5. (**D**) H1299 wild type cells were seeded in 6-well plates and left overnight followed by transfection with 1 µg vector encoding either SRC-3 wild type-FLAG (SRC-3 WT-FLAG) or SRC-3 S857A-FLAG (SRC-3 S857A-FLAG). After 48 h of transfection, the cells were lysed. FLAG-tagged SRC-3 and level of serine 857 phosphorylation of SRC-3 in the lysate was detected by Western-blotting with anti-FLAG and anti-P-S857-SRC-3 antibodies, respectively. The full-length blots are presented in supplementary figure S6. (**E**) Endogenous SRC-3 protein was immunoprecipitated from H1299 cells. After the last wash step, half of the precipitate was treated for 30 min with 400U lambda phosphatase. Western-blot was performed with anti-SRC-3 and anti-P-S857-SRC-3 antibodies. The full-length blots are presented in supplementary Figure S7.

**The p38$^{MAPK}$ signaling pathway controls the phosphorylation of SRC-3 at S857 in vivo.** MK2 and MK5 are controlled by two different signaling pathways; while MK5 is located downstream of the atypical MAPKs ERK3 and ERK4, MK2 lies downstream of the p38$^{MAPK}$. First, we aimed to determine whether ERK3 and MK5 are required in vivo for phosphorylation of SRC-3 at S857. Therefore, we analyzed the phosphorylation status of endogenous SRC-3 at S857 in H1299 lung cancer cells, which were transfected with siRNA against MK5 or ERK3. Loss of either MK5 or ERK3 did not affect the phosphorylation state of SRC-3 at S857 in these cell extracts (Fig. 2E). These results suggest that SRC-3 is not a physiological substrate of neither ERK3 nor MK5.

As the consensus sequence around S857 in SRC-3 resembles a MAPKAPK phosphorylation motif, it is possible that other MAPKAP kinases lying downstream of the MAPK's ERK1/2 or p38$^{MAPK}$ could be responsible for the phosphorylation of this site in vivo. To investigate this, we treated the lung cancer cell line H1299 with specific kinase inhibitors before analyzing the phosphorylation state of SRC-3 at S857. Since all of the MAPKAPKs except MK5 are activated by either MAP Kinase Kinase (MKK) 1/2-ERK1/2 or MKK3/6-p38$^{MAPK}$ α/β, we treated the

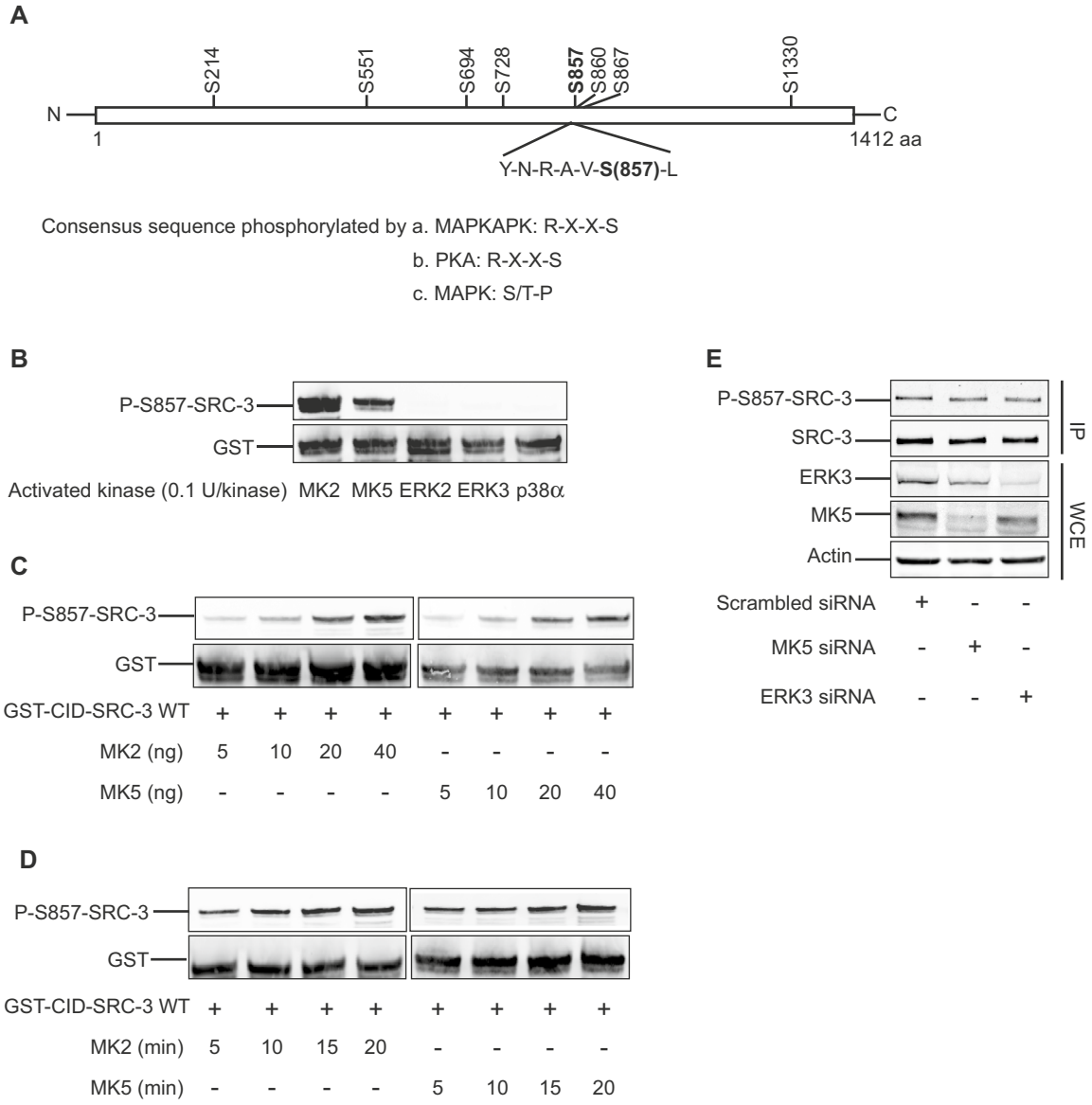



**Figure 2.** MK2 and MK5 phosphorylate SRC-3-S857 in vitro. (**A**) Schematic diagram of SRC-3 amino acid sequence (aa). Common phosphorylation sites are indicated above the sequence and the consensus sequence of the S857 phosphorylation site is indicated below. Preferred consensus sequences for phosphorylation by MAPKKAPK (i), PKA (ii) and MAPK (iii) are shown in the lower part of the figure; Hyd indicates hydrophobic aa, X indicates any amino acid[57]. (**B**) MK2 and MK5 phosphorylates SRC-3 at S857 in vitro. The in vitro kinase assay was performed by incubating GST-CID-SRC-3 WT (2 µg) with 0.1U of different active kinases at 30 °C for 15 min. Phosphorylation of SRC-3-S857 in GST-CID-SRC-3 WT and the total amount of GST-CID-SRC-3 WT was detected using anti-P-S857-SRC-3 and anti-GST antibodies, respectively. The full-length blots are presented in supplementary figure S8. (**C**,**D**) MK2 and MK5 phosphorylate SRC-3-S857 in a dose and time dependent manner in vitro. (**C**) An in vitro kinase assay was performed by incubating increasing amounts (5, 10, 20 and 40 ng) of active MK2 or active MK5 together with 2 µg GST-CID-SRC-3 WT at 30 °C for 15 min. (**D**) An in vitro kinase assay was performed by incubating 100 ng of active MK2 or MK5 together with 2 µg GST-CID-SRC-3 WT at 30 °C for different periods of time (5, 10, 15, 20 min). For both (**C**) and (**D**), anti-P-S857-SRC-3 antibody was used to detect SRC-3-S857 phosphorylation and an anti-GST antibody visualize the input of GST-CID-SRC-3 WT fusion protein for each reaction. The full-length blots are presented in supplementary figures S9,S10. (**E**) Neither MK5 nor ERK3 phosphorylate SRC-3-S857 in vivo. H1299 cells were transfected with either 20 nM scrambled siRNA, siRNA against MK5 or against ERK3. After 48 h, the cells were lysed and SRC-3 was immunoprecipitated with anti-SRC-3 antibody. The immunoprecipitate (IP) and whole cell extracts (WCE) were analyzed by Western-blotting. The full-length blots are presented in supplementary figure S11.

cells with the MKK1/2 specific inhibitor PD-184352 or with the $p38^{MAPK}\alpha/\beta$ specific inhibitor SB-202190. As shown in Fig. 3A, inhibition of MKK1/2 activity using PD-184352 (thereby inhibiting RSKs, MSKs and MNKs activity via ERK1/2) did not affect the phosphorylation of SRC-3 at S857. However, inhibition of the $p38^{MAPK}$ activity using SB-202190, and thereby inhibition of MK2/3 (and MSK1/2 and MNK1/2), profoundly inhibited the phosphorylation of SRC-3.

Based on these findings, we aimed to further validate the role of the $p38^{MAPK}$ signaling pathway in the regulation of SRC-3 phosphorylation at S857. Therefore, we stimulated various cell lines with well-known $p38^{MAPK}$ activators (TNF-α, anisomycin and sodium arsenite (SA)). All cell lines analyzed, namely the lung cancer cell lines H1299 (Fig. 3B) and A549 (Fig. 3C), the human embryonic kidney cell line HEK293 (Fig. 3D), the human cervical carcinoma cell line HeLa (Fig. 3E) and the human breast cancer cell line MDA MB 231 (Fig. 3F) showed an enhanced phosphorylation of SRC-3 at S857 upon exposure to the three $p38^{MAPK}$ activators. In addition to this, when $p38^{MAPK}$ activity was inhibited using SB-202190, the TNF-α and anisomycin-induced (Fig. 3G) and SA-induced (Fig. 3H) SRC-3 phosphorylation at S857 was not observed. Since the results were obtained by activation of the $p38^{MAPK}$ signaling pathway using different types of $p38^{MAPK}$ activators in several different cell lines, it indicates that the phosphorylation of SRC-3 at S857 by activation of $p38^{MAPK}$ signaling is a general phenomenon.

**Phosphorylation of SRC-3 at S857 is dependent on MK2.** The results from in vitro kinase assays and cell culture experiments with specific inhibitors suggest MK2 as a *bona fide* downstream target of $p38^{MAPK}$, rather than the MAPKAPKs downstream of ERK1/2, is the kinase responsible for phosphorylation of SRC-3 at S857. To investigate this hypothesis further, we utilized mouse embryonic fibroblast (MEF) cells and bone marrow derived cells (BMDC) isolated from double knockout (DKO) mice lacking both MK2 and MK3. As the expression of both of these downstream kinases is required for $p38^{MAPK}$ stability[27] and thus to ensure that our results reflect the loss of MK2/MK3 and not the resulting depletion of $p38^{MAPK}$, we re-expressed either wild type MK2 ($MK2^{WT}$) or a kinase dead mutant of MK2 ($MK2^{K72A}$) into the DKO cell lines. The three different cell lines ($MK2/MK3^{-/-}$; $MK2/MK3^{-/-} + MK2^{WT}$; $MK2/MK3^{-/-} + MK2^{K72A}$) were then treated with the $p38^{MAPK}$ pathway activators (TNF-α, lipopolysaccharide (LPS) or sodium arsenite (SA)), and the phosphorylation status of SRC-3 at S857 was analyzed. In the $MK2/MK3^{-/-}$ cells and the $MK2/MK3^{-/-}$ cells expressing the kinase-dead MK2 ($MK2^{K72A}$), none of the $p38^{MAPK}$ pathway stimulants resulted in induction of S857 phosphorylation. However, SA and LPS induced S857 phosphorylation in the $MK2/MK3^{-/-}$ cells rescued with $MK2^{WT}$ (MEF cell, Fig. 4A; BMDC cells, Fig. 4B). Overall, these results indicate that MK2 activity is required for phosphorylation of SRC-3 at S857 in murine cells.

Next, we studied the role of MK2 (and MK3) for phosphorylation of SRC-3 at S857 in different human cancer cell lines. For these experiments, we used the specific MK2 kinase inhibitor PF-3644022. A549 cells pretreated with different doses of PF-3644022 were stimulated with TNF-α (Fig. 4C) and anisomycin (Fig. 4D). Increasing doses of PF-3644022 markedly inhibited TNF-α and anisomycin-induced MK2 activity, as shown by the decrease in phosphorylation of HSP27, a known substrate of MK2[28]. Moreover, PF-3644022 effectively prevented the phosphorylation of SRC-3 at S857 (Fig. 4C,D). Then, we used A549 cells where we knocked down endogenous SRC-3 expression with a specific siRNA, and at the same time co-transfected the cells with vectors encoding siRNA resistant FLAG-tagged SRC-3 WT or the phosphorylation site mutant SRC-3-S857A. Analysis of ectopically expressed SRC-3 revealed that activation of the $p38^{MAPK}$ pathway with TNF-α or anisomycin resulted in increased phosphorylation of SRC-3 at S857, which was prevented by pre-incubation with the MK2-inhibitor PF-3644022 (Fig. 4E) (Supplementary Fig. S1A). No phosphorylation of SRC-3 was observed in the cells transfected with SRC-3-S857A. The effect of PF-3644022 on anisomycin-stimulated S857 phosphorylation was also observed for endogenous SRC-3 protein in five human cell lines: HeLa (Fig. 4F), A549 (Fig. 4G), H1299 (Fig. 4H), HEK 293 (Fig. 4I) and the breast cancer cell line MDA MB 231 (Fig. 4J). These results indicate that MK2 is responsible for phosphorylation of SRC-3 at S857 in response to activation of the $p38^{MAPK}$ signaling pathway.

In the next step, we examined the dose and time dependent effect of TNF-α on MK2 activation (as indicated by phosphorylation of MK2 at T334), and the phosphorylation of SRC-3 at S857 in the breast cancer cell line MDA MB 231 and the lung cancer cell line A549. We observed that the phosphorylation of SRC-3 at S857 follows similar pattern as the activation of MK2, both in relations to dose in MDA-MB 231 cells (Fig. 5A), and time after stimulation in both MDA-MB 231 and A549 cells (Fig. 5B,C).

**IKK is not involved in TNF-α induced phosphorylation of SRC-3 at S857.** In unstimulated cells, the NF-κB proteins are sequestered in the cytoplasm by IκBs (Inhibitor of κB). When stimulated, IκB kinase (IKK) becomes activated and phosphorylates IκB proteins. This results in degradation of IκB and activation of NF-κB. IKK is composed of heterodimer of the catalytic IKK-α and IKK-β subunits and a regulatory subunit IKK-γ[29]. IKK-α and IKK-β are reported to phosphorylate SRC-3 at S857[20]. In order to investigate whether IKK is involved in $p38^{MAPK}$-MK2 signaling pathway in phosphorylation of SRC-3, we studied the role of IKK-β and IKK-α on TNF-α-induced phosphorylation of SRC-3 at S857 in A549 cells using IKK-β inhibitor, BI-605906[30] (Fig. 6A) and siRNA against IKK-α (Fig. 6B) respectively. We found that neither exclusive inhibition of IKK-β activity (Fig. 6A) nor the exclusive inhibition of IKK-α expression (Fig. 6B) influenced the TNF-α-induced phosphorylation of SRC-3 at S857. However, at higher concentration of BI-605906 (Fig. 6A, last lane) and mutual inhibition of IKK-β activity and IKK-α expression together (Fig. 6B, last lane), a slight decrease in the phosphorylation of SRC-3 was observed. Moreover, no effect on the TNF-α-induced phosphorylation of HSP27 was observed when IKK-β activity (Fig. 6A) and IKK-α expression (Fig. 6B) were inhibited, thereby suggesting that IKK does not influence the $p38^{MAPK}$-MK2 pathway. Based on these findings, we conclude that MK2 is the major kinase phosphorylating SRC-3 at S857 in A549 cells.

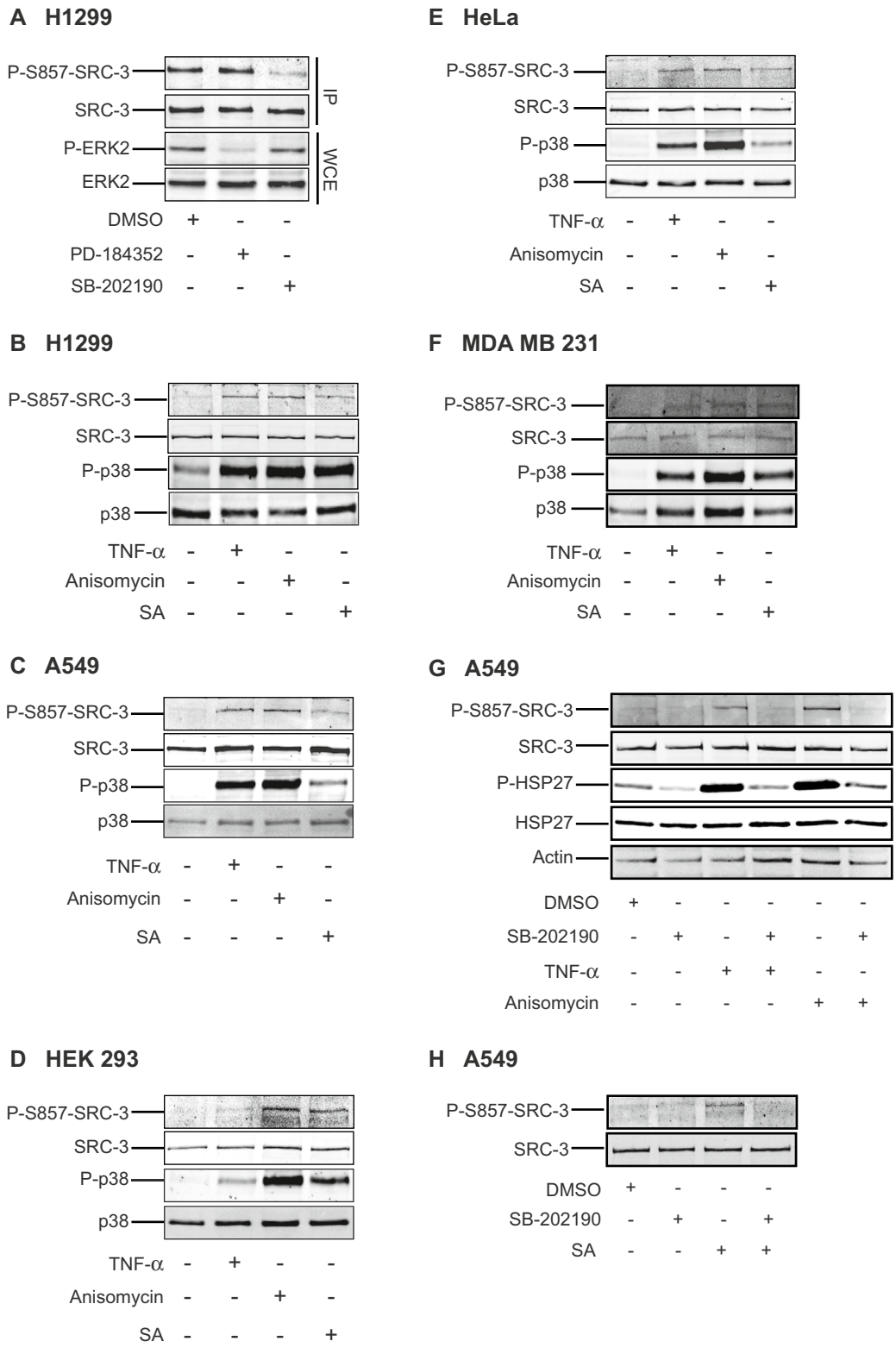
**Figure 3.** Activation of $p38^{MAPK}$ results in phosphorylation of SRC-3 at S857. (**A**) $p38^{MAPK}$ but not ERK1/2 is involved in phosphorylation of SRC-3 at S857. H1299 cells were incubated with either 10 μM MEK1/2 inhibitor (PD-184352) or 10 μM $p38^{MAPK}$ inhibitor (SB-202190) for 2 h before SRC-3 was immunoprecipitated (IP). The IP lysate and whole cell extract (WCE) were analyzed by Western-blotting using anti-P-S857-SRC-3, anti-SRC-3, anti-phospho ERK1/2 MAPK and anti-ERK2 antibodies. (**B**–**F**) $p38^{MAPK}$ activation phosphorylates SRC-3 at S857. The full-length blots are presented in supplementary figure S12. H1299 (**B**), A549 (**C**), HEK 293 (**D**), HeLa (**E**) and MDA MB 231 (**F**) cells were stimulated with either 10 ng/ml TNF-α (15 min), 10 μg/ml anisomycin or 250 μM sodium arsenite (SA) for 30 min. Unstimulated cells were used as control. The cells were lysed and the level of phosphorylation of SRC-3 at S857 and $p38^{MAPK}$ at T180/Y182 was analyzed by Western-blotting using anti-P-S857-SRC-3, anti-SRC-3, anti-phospho-$p38^{MAPK}$ and anti-$p38^{MAPK}$ antibodies. The full-length blots are presented in supplementary figures S13–S17. (**G**,**H**) Inhibition of $p38^{MAPK}$ activation prevents TNF-α and anisomycin-induced phosphorylation of SRC-3 at S857. A549 cells were seeded and left overnight. On the other day, the cells were pretreated either with DMSO or 10 μM SB-202190 for 30 min. Then they were stimulated with 10 ng/ml TNF-α (15 min) or 10 μg/ml anisomycin (**G**) or 500 μM sodium arsenite (SA) (**H**) for 30 min. Finally, the cells were lysed and level of phosphorylation of SRC-3 at S857, HSP27 at S82, total amount of SRC-3, HSP27 and actin were detected by Western-blotting using appropriate antibodies. The full-length blots are presented in supplementary figures S18,S19.

**$p38^{MAPK}$ and MK2 activity is important for the nuclear translocation of SRC-3.** SRC-3 contains both a nuclear localization signal (NLS) and a nuclear export signal (NES)[31], and has been demonstrated to shuttle between the nucleus and cytoplasm depending on both its phosphorylation state, and interaction with the estrogen receptor[32]. Having identified $p38^{MAPK}$ and MK2 as crucial mediators of SRC-3 phosphorylation, we determined whether $p38^{MAPK}$ and MK2 activity might also regulate subcellular distribution of SRC-3. As shown in Fig. 7A,B,E,F (left panels), immunostaining of SRC-3 in untreated A549 cells indicate abundant SRC-3 protein both in the cytosol outside the nucleus, as well as in the nucleus. Upon stimulation with TNF-α, SRC-3 translocates into the nucleus leading to almost complete nuclear localization of SRC-3. However, after pretreatment with the specific $p38^{MAPK}$ inhibitor SB-202190 (Fig. 7A,E (right panels)) and the specific MK2 kinase inhibitor PF-3644022 (Fig. 7B,F (right panels)), TNF-α stimulation did not lead to a significant translocation of SRC-3 into the nucleus.

Together with the observation that SB-202190 and PF-3644022 prevented phosphorylation of SRC-3 at S857, these findings strongly suggest that phosphorylation of SRC-3 at S857 by $p38^{MAPK}$ and MK2 may be important for efficient nuclear localization of SRC-3.

Since phosphorylation of SRC-3 at S857 seems to be important for TNF-α-induced nuclear translocation of SRC-3, we further wanted to study whether the mutated SRC-3 S857A is translocated into nucleus when stimulated with TNF-α. For this, we generated A549 cells where we knocked out endogenous SRC-3 using CRISPR-Cas9 mediated gene editing (Fig. 7C) (Supplementary Fig. S3). When we transfected the $SRC\text{-}3^{KO}$ A549 cells with an expression vector encoding wild type SRC-3 and mutated SRC-3 S857A and stimulated with TNF-α, we found that SRC-3 WT was more efficiently translocated into nucleus than SRC-3 S857A (Fig. 7D,G). This finding further strengthens our hypothesis that phosphorylation of SRC-3 at S857 significantly enhances its nuclear translocation.

**SRC-3 is required for MK2-mediated induction of IL-6 mRNA expression in response to TNF-α.** After we established that the $p38^{MAPK}$-MK2 pathway is involved in phosphorylation of SRC-3 at S857, we aimed to explore the biological function of this modification. SRC-3 has been reported to co-activate NF-κB-mediated gene expression, and this was shown to depend on the phosphorylation of S857[7]. Therefore, we aimed to determine the role of S857 in regulating NF-κB-mediated transcription in A549 cells. In order to investigate this, we transfected $SRC\text{-}3^{KO}$ A549 cells with a NF-κB-dependent luciferase reporter gene. A basal induction of luciferase activity in response to treatment with TNF-α was observed. Interestingly, we noticed a significant increase in TNF-α-induced luciferase activity when the $SRC\text{-}3^{KO}$ cells were co-transfected with an expression vector encoding wild type SRC-3 (Fig. 8A). Of note, this SRC-3 mediated increase in luciferase activity was not observed when the $SRC\text{-}3^{KO}$ cells were co-transfected with a vector encoding the mutated SRC-3 S857A (Fig. 8A). This result shows that phosphorylation of SRC-3 at S857 is required for the ability of SRC-3 to co-activate NF-κB in response to TNF-α in A549 cells. Furthermore, knockdown of SRC-3 expression by specific siRNA or inhibition of MK2 activity with the inhibitor PF-3644022 both resulted in significant decrease in NF-κB-driven luciferase activity in response to treatment with TNF-α (Fig. 8B) (Supplementary Fig. S1B). These findings strongly indicate that both SRC-3 and MK2 activity are required for the induction of NF-κB-dependent transcription in response to TNF-α. Interleukin-6 (IL-6) is a well-known downstream target of NF-κB[33]. Earlier studies have shown that the phosphorylation of SRC-3 at S857 is necessary for the TNF-α stimulated IL-6 mRNA expression[20]. When $SRC\text{-}3^{WT}$ and $SRC\text{-}3^{KO}$ A549 cells were stimulated with TNF-α, the TNF-α-induced IL-6 mRNA expression was significantly lower in the $SRC\text{-}3^{KO}$ compared to the $SRC\text{-}3^{WT}$ A549 cells (Fig. 8C). This indicates a role of SRC-3 in IL-6 mRNA expression. MMP9 is another known downstream target of NF-κB[34]. When $SRC\text{-}3^{WT}$ and $SRC\text{-}3^{KO}$ A549 cells were stimulated with TNF-α, MMP9 mRNA expression increased substantially in both cell lines. There was however, no significant difference in the increase of MMP9 mRNA expression between $SRC\text{-}3^{WT}$ and $SRC\text{-}3^{KO}$ cell lines stimulated with TNF-α (Fig. 8D). These results indicate that although the expression of both IL-6 and MMP9 mRNA are induced by TNF-α only IL-6 expression is specifically dependent on SRC-3.


A H1299
P-S857-SRC-3
SRC-3
P-ERK2
ERK2
IP
WCE
DMSO + - -
PD-184352 - + -
SB-202190 - - +
B H1299
P-S857-SRC-3
SRC-3
P-p38
p38
TNF-α - + - -
Anisomycin - - + -
SA - - - +
C A549
P-S857-SRC-3
SRC-3
P-p38
p38
TNF-α - + - -
Anisomycin - - + -
SA - - - +
D HEK 293
P-S857-SRC-3
SRC-3
P-p38
p38
TNF-α - + - -
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E HeLa
P-S857-SRC-3
SRC-3
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SRC-3
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DMSO + - - - - -
SB-202190 - + - + - +
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P-S857-SRC-3
SRC-3
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**Figure 4.** MK2 phosphorylates SRC-3 at S857. (**A**,**B**) MK2 phosphorylates SRC-3 at S857 in mouse cell lines. Mouse embryonic fibroblast (MEF) cells (**A**) or bone marrow derived dendritic cells (BMDC) cells (**B**) derived from mice knocked out for MK2 and MK3 expression (MK2/MK3$^{-/-}$), rescued with retroviral transduced GFP-MK2 wild type (MK2/MK3$^{-/-}$ + MK2$^{WT}$), or rescued with retroviral transduced kinase dead MK2 (MK2/MK3$^{-/-}$ + MK2$^{K72A}$) were seeded and left overnight. Then the cells were treated with either 10 ng/ml TNF-α, 250 μM SA or 5 ng/ml Lipopolysaccharide (LPS) as indicated for 30 min. Cell lysates were analyzed by Western-blotting using anit-P-S857-SRC-3, anti-SRC-3, anti-MK2 and anti-actin antibodies. *indicates the phosphorylated band. The full-length blots are presented in supplementary figures S20,S21. (**C**,**D**) PF-3644022 prevents TNF-α and anisomycin-induced phosphorylation of SRC-3 at S857. A549 cells were seeded and left overnight. On the other day, the cells were pretreated with DMSO or 1, 2.5, 5, 10 μM of PF-3644022 for 30 min followed by stimulation with either 10 ng/ml TNF-α (**C**) or 10 μg/ml anisomycin (**D**) for 30 min. Then the cells were lysed and level of phosphorylation of SRC-3 at S857, HSP27 at S82, total SRC-3 and total HSP27 were analyzed by Western-blotting using anti-PS857-SRC-3, anti-PS82-HSP27, anti-SRC-3 and anti-HSP27 antibodies respectively. The full-length blots are presented in supplementary figures S22,S23. (**E**) MK2 phosphorylates SRC-3 at S857 in the human lung adenocarcinoma cancer cell line A549. A549 cells were seeded in a 6-well plate and left overnight then co-transfected with 20 nM siRNA against SRC-3 and 1 μg vector expressing either siRNA resistant SRC-3 wild type-FLAG (SRC-3 WT-FLAG) or siRNA resistant SRC-3 S857A-FLAG (SRC-3 S857A-FLAG). After 48 h, cells were treated with 10 ng/ml TNF-α (15 min) or 10 μM anisomycin for 30 min in absence or presence of 10 μM PF-3644022, which was added 30 min before the treatment. The cells were lysed and phosphorylation of SRC-3 at S857 was investigated by Western-blotting using anit-P-S857-SRC-3, anti-FLAG and anti-actin antibodies. The full-length blots are presented in supplementary figure S24. (**F**–**J**) MK2 phosphorylates endogenous SRC-3 at S857 in human cell lines. HeLa (**F**), A594 (**G**), H1299 (**H**), HEK 293 (**I**) and MDA MB 231 (**J**) cells were seeded and left overnight. Then the cells were treated with 10 μM PF-3644022 for 30 min followed by stimulation with 10 μg/ml anisomycin for 30 min. Cells were lysed and level of phosphorylation of SRC-3-S857 and p38$^{MAPK}$ were detected, before the total amount of SRC-3, p38$^{MAPK}$ and actin were detected by Western-blotting using appropriate antibodies. Presented here is a representative image of three independent experiments that showed similar result. The full-length blots are presented in supplementary figure S25–S29.

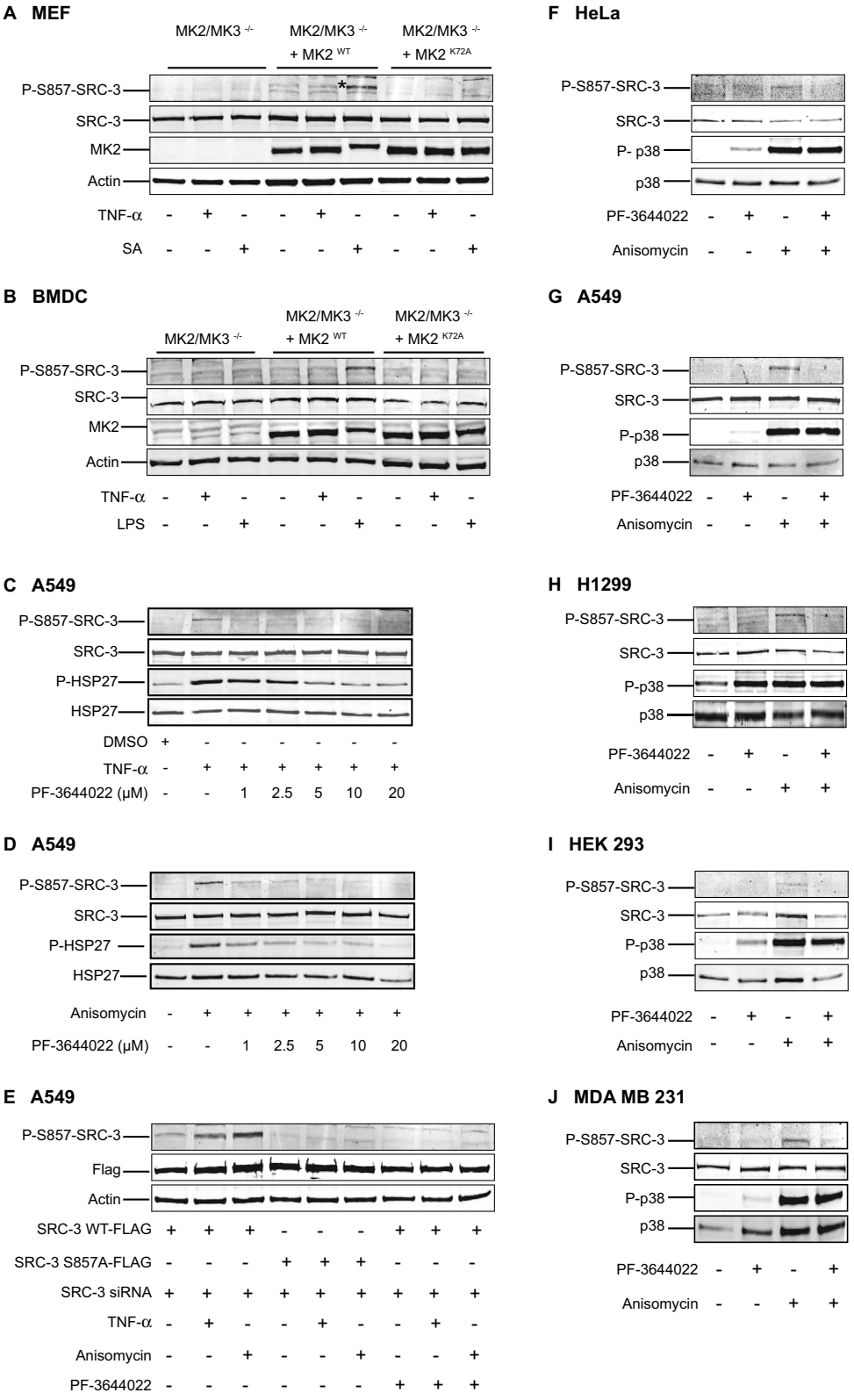
As MK2 was shown to be involved in NF-κB-dependent transcription (Fig. 8B), we performed experiments to investigate the role of MK2 in TNF-α stimulated IL-6 transcription in lung cancer cells. We transfected A549 cells with an IL-6 promoter-dependent luciferase vector. The cells were then stimulated with TNF-α after pretreatment with either the MK2 inhibitor PF-3644022, or DMSO as control. TNF-α-induced luciferase activity decreased significantly when MK2 activity was inhibited, suggesting involvement of MK2 activity in IL-6 transcription (Fig. 8E). Furthermore, we studied the role of MK2 in transcription of other NF-κB target genes namely, TNF receptor-associated factor 1 (TRAF1)[35], IL-8[36] and Intercellular Adhesion Molecule 1 (ICAM1)[37]. A549 cells pretreated with DMSO or PF-3644022 for 30 min were stimulated with TNF-α for 5 h or left untreated. We found that mRNA expression of TRAF1 (Fig. 8F), IL-8 (Fig. 8G) and ICAM1 (Fig. 8H) increased significantly when stimulated with TNF-α compared to control. However, when MK2 activity was inhibited there was a significant decrease in mRNA expression of TRAF1 and IL-8 but not ICAM1. This indicates that MK2 is involved in regulation of selective NF-κB target genes.

As p38$^{MAPK}$ is an upstream activator of MK2 and SRC-3, we examined the role of p38$^{MAPK}$ in NF-κB mediated transcription. First, we determined the role of p38$^{MAPK}$ in NF-κB-dependent luciferase activity. The significant increase in TNF-α-induced NF-κB luciferase activity in A549-NF-κB-Luc cell decreased significantly when inhibited with SB-202190 (Fig. 8I). This suggests that p38$^{MAPK}$ is involved in transcriptional activity of NF-κB. Next, we analyzed the involvement of p38$^{MAPK}$ in TNF-α-stimulated IL-6 transcription in A549 cells. The TNF-α-induced increase in IL-6 transcription activity was significantly decreased when p38$^{MAPK}$ activity was inhibited (Fig. 8J). Furthermore, TNF-α-induced upregulation of mRNA expression of NF-κB target genes IL-6 (Fig. 8K), IL-8 (Fig. 8L) and MMP9 (Fig. 8M) were significantly inhibited when the p38$^{MAPK}$ activity was inhibited in A549 cells. This confirmed the role of p38$^{MAPK}$ in regulation of NF-κB target genes that are upregulated by TNF-α.

## Discussion

Although SRC-3 was first described as coactivator for nuclear receptors, it turned out that it can also act as coactivator for several other transcription factors, including PEA3[3], AP-1[5], TEAD4[38] and NF-κB[6]. A concept has emerged which indicates that SRC-3 can act as an integrator able to connect the activation of protein kinase signaling cascades to the control of specific transcriptional programs[39]. This integration is controlled by specific kinases that phosphorylate SRC-3 at distinct sites. These phosphorylations may form a code that determine the ability of SRC-3 to be recruited to different transcription factors. The most frequent reported phosphorylation site of SRC-3 is S857[22]. In the present study, we identified MK2 as the kinase responsible for both the basal, and the stress-induced phosphorylation of SRC-3 at S857 in a wide variety of cell types. This suggests that SRC-3 could be crucial for the control of various transcriptional programs via the p38$^{MAPK}$-MK2-SRC-3-S857 signaling axis.

Earlier studies suggested that SRC-3-S857 is a substrate of the atypical MAPK ERK3[3,40]. Unexpectedly, we observe in our study that recombinant ERK3 was not able to efficiently phosphorylate this site of SRC-3 in in vitro kinase assays. The main difference between our in vitro kinase assays and the previous studies was the source of the ERK3 protein. In the present study, we have used recombinant ERK3 purified from insect cells, while the previous studies used immunoprecipitated HA-tagged ERK3 protein transiently expressed in HEK 293 cells. For ERK3 from mammalian cells, it is well known that it forms strong and stable complexes with the protein kinase


A MEF
MK2/MK3 -/-
MK2/MK3 -/- + MK2 WT
MK2/MK3 -/- + MK2 K72A
P-S857-SRC-3
SRC-3
MK2
Actin
TNF-α
SA
B BMDC
LPS
C A549
P-HSP27
HSP27
DMSO
PF-3644022 (μM)
D A549
Anisomycin
E A549
Flag
SRC-3 WT-FLAG
SRC-3 S857A-FLAG
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H H1299
I HEK 293
J MDA MB 231

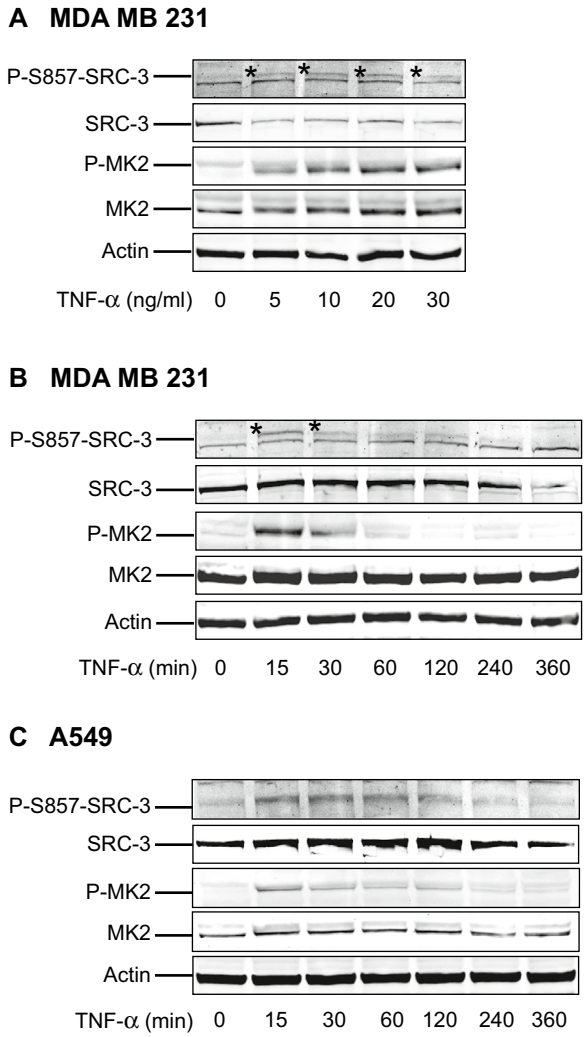



**Figure 5.** MK2-mediated phosphorylation of SRC-3 at S857 in response to TNF-α is dose and time dependent. (**A**) TNF-α stimulation phosphorylates SRC-3 and MK2 in a dose dependent manner. MDA MB 231 cells were seeded and left in incubator overnight. After that, the cells were treated with 0, 5, 10, 20 or 30 ng/ml of TNF-α as indicated in the figure for 15 min. Then the cells were lysed and the phosphorylation statuses of SRC-3 at S857 and MK2 at threonine (T) 334 as well as total protein amounts of these proteins and actin were examined by Western-blotting using anti-P-S857-SRC-3, anti-SRC-3, anti-phospho-MK2, anti-MK2 and anti-actin antibodies. *indicates the phosphorylated band. The full-length blots are presented in supplementary figure S30. (**B**, **C**) TNF-α stimulation causes phosphorylation of SRC-3 at S857 and MK2 in a time dependent manner. MDA MB 231 (**B**) or A549 (**C**) wild type cells were seeded and left overnight. Then the cells were treated with 10 ng/ml TNF-α for 0, 15, 30, 60, 120, 240 or 360 min as indicated in the figures. The cells were lysed and phosphorylation status was examined by Western-blotting as described in (**A**) above. *indicates the phosphorylated band. The full-length blots are presented in supplementary figures S31,S32.

MK5[25,41,42]. Furthermore, this complex formation between ERK3 and MK5 is dependent on ERK3 phosphorylation at Serine 189. In this complex, MK5 becomes activated by ERK3, and this activation is dependent on the kinase activity of ERK3[25,41,43]. In our study, we observed that S857 of SRC-3 is a *bona fide* substrate of MK5 in vitro, which may suggest that the kinase activity observed with HA-tagged ERK3 immunoprecipitated from HEK 293 could be due to co-precipitation of MK5. This hypothesis is further supported by the observation that MK5 is active in complex with ERK3, while MK5 is inactive in complex with kinase deficient ERK3[3,40]. Moreover, it was further supported by the observation that a functional S189 in ERK3 is required for phosphorylation of SRC-3 when ERK3 was immunoprecipitated from HEK 293 cells and used in an in vitro kinase assay. These findings strengthen the assumption that MK5 co-precipitating with ERK3 is the kinase responsible for SRC-3 phosphorylation observed in these studies[40].

When examining the sequence surrounding S857 in SRC-3 (YNRAV**S**L) it became clear that this sequence more closely resembled the consensus for efficient phosphorylation by MK2 which is HydXRXXSX (where Hyd is a bulky hydrophobic residue), than a phosphorylation site for a proline-directed MAPK[44,45]. In agreement with the MAPKAPK substrate consensus sequence, we found that S857 of SRC-3 was efficiently phosphorylated

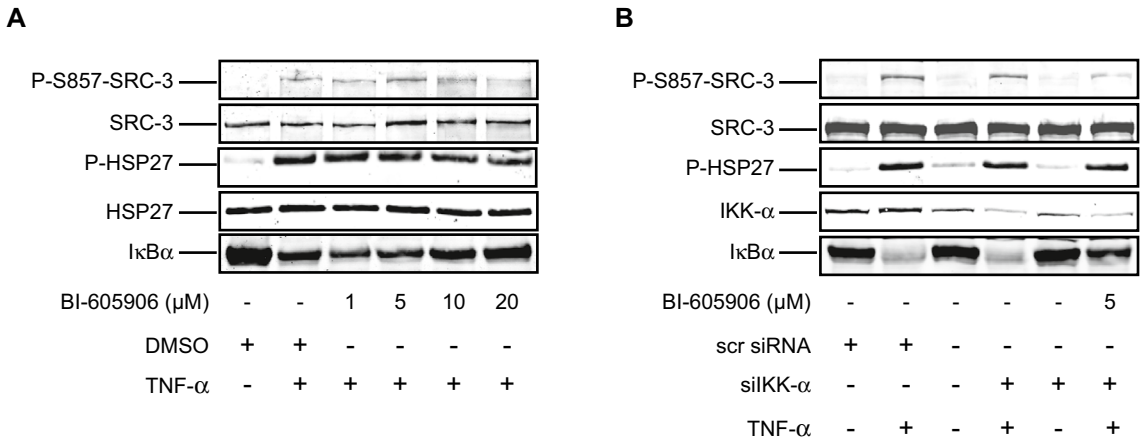



**Figure 6.** IKK-β and IKK-α are not involved in TNF-α induced phosphorylation of SRC-3 at S857. (**A**) IKK-β does not phosphorylate SRC-3 at S857. A549 cells pretreated with either DMSO or 1, 5, 10, 20 μM BI-605906 for 30 min were stimulated with 10 ng/ml TNF-α for 15 min. Then the cells were lysed and Western-blotting was carried out to examine the phosphorylation of SRC-3 at S857, HSP27 at S82 and expression of total SRC-3, HSP27, IκBα using anti-P-S857-SRC-3, anti-P-HSP27, anti-SRC-3, anti-HSP27 and anti-IκBα antibodies respectively. The full-length blots are presented in supplementary figure S33. (**B**) IKK-α does not phosphorylate SRC-3 at S857. A549 cells were transfected with either 10 nM scrambled siRNA, siRNA against IKK-α or left untransfected. After 48 h, the cells were either pretreated with 5 μM BI-605906 for 30 min or left untreated. Then the cells were either stimulated with 10 ng/ml TNF-α or left unstimulated for 15 min. Finally, the cells were lysed and Western-blotting was performed to examine the phosphorylation of SRC-3 at S857, HSP27 at S82 and expression of total SRC-3, HSP27, IKK-α and IκBα using anti-P-S857-SRC-3, anti-P-HSP27, anti-SRC-3, anti-HSP27, anti-IKK-α and anti-IκBα antibodies respectively. The full-length blots are presented in supplementary figure S34.

by both MAPKAPK, MK2 and MK5, but not by MAP kinases in vitro. Using a phospho-specific antibody in combination with specific siRNAs and protein kinase inhibitors together with cells derived from mice deficient for MK2 and MK3 expression, we found that S857 is phosphorylated by MK2 in response to activation of the $p38^{MAPK}$ signaling pathway in several human cancer cells, as well as in mouse embryonal fibroblast and bone marrow derived mouse cells. The MK2 inhibitor PF-3644022 used in this study is reported to inhibit MK2, MK3 and MK5 activities[46]. Besides, the mouse cell lines used for validation of the MK2 involvement in SRC-3 phosphorylation at S857, were knocked out for both MK2 and MK3. Therefore, we cannot completely exclude that MK3, which has a much lower expression, is also involved in the SRC-3 phosphorylation at S857 in addition to MK2.

In order to investigate the downstream targets of $p38^{MAPK}$-MK2 induced phosphorylation of SRC-3 at S857, we studied the role of SRC-3 on the transcriptional activation of NF-κB. The phosphorylation of SRC-3 at S857 was earlier described to be important for SRC-3 for complex formation with the transcription factor NF-κB, and the transcriptional activation of several cytokines including IL-6. This coactivation of NF-κB was linked to IKK-α-mediated phosphorylation of S857 in response to TNF-α stimulation[7]. We investigated the role of IKK-β and IKK-α in TNF-α-induced phosphorylation of SRC-3 at S857 in A549 cells and found that neither IKK-β nor IKK-α was involved in the phosphorylation. Besides, in all the cancer cell lines examined in the present study, we observed that pretreatment with the MK2-specific kinase inhibitor PF-3644022 efficiently blocked the phosphorylation at S857 in response to TNF-α. This finding suggests that MK2 rather than IKK-α is the major TNF-α induced kinase responsible for phosphorylation of SRC-3 at S857 in cancer cells. However, it cannot be excluded that IKK-α might be responsible for regulation of this phosphorylation in other cell types or tissues.

The $p38^{MAPK}$-MK2 signaling axis plays a prominent role in controlling cytokine expression in response to proinflammatory cytokines and cellular stress. MK2 is well known for its post-transcriptional regulation of genes harboring adenine/uridine-rich elements (AREs) in their 3′-untranslated region (3′-UTR), including proinflammatory genes such as IL-6, TNF-α, and IL-1β[47]. Our data indicated that MK2 may also contribute to transcriptional regulation of certain cytokines such as IL-6, TRAF1 and IL-8. However, due to the profound role of MK2 in regulation of cytokine expression at the post-transcriptional level, it is difficult to exactly assess its role for the transcriptional regulation. Nonetheless, we could show that deletion of SRC-3 expression by CRISPR-Cas9 mediated gene editing significantly decreased both, basic and TNF-α induced IL-6 mRNA expression in our A549 lung cancer cell system. To further evaluate the role of MK2 in direct transcriptional activation, we employed reporter gene assays. Using a NF-κB-driven reporter assay, we could show that both SRC-3 and MK2 activity are required for full activation of the NF-κB promoter in response to TNF-α. Moreover, we also demonstrated that a functional S857 in SRC-3 is required for its ability to transactivate NF-κB in response to TNF-α. $p38^{MAPK}$ was earlier described to be required for transcriptional activation of NF-κB and this activation was independent of IκB (Inhibitor of κB) phosphorylation or NF-κB translocation and DNA-binding[48]. Our data support this, and suggests that the requirement for a full NF-κB-mediated transcriptional activation might be the phosphorylation of SRC-3 at S857 via the activation of the $p38^{MAPK}$-SRC-3-MK2 axis. One specific role for MK2-mediated phosphorylation of SRC-3 could be to further facilitate the TNF-α-induced nuclear translocation of SRC-3. The

**Figure 7.** Activation of $p38^{MAPK}$ and MK2 is required for efficient nuclear translocation of SRC-3 in response to TNF-α. (**A**,**B**) $p38^{MAPK}$ and MK2 is involved in nuclear translocation of SRC-3. A549 WT cells were seeded on coverslip and left overnight. The next day, cells were treated with either DMSO, 10 ng/ml TNF-α or SB-202190 (**A**) or 10 μM PF-3644022 (**B**) separately, or in combination with SB-202190 (**A**) or PF-3644022 (**B**) for 30 min followed by TNF-α stimulation for 60 min. Representative images of the $SRC\text{-}3^{WT}$ A549 cells stained for SRC-3 (red) using anti-SRC-3 antibody and nucleus ( blue, DAPI). The specificity of the antibody for SRC-3 was verified using SRC-3 $^{KO}$ cells (Supplementary Fig. S2A,B). (**C**) Generation of $SRC\text{-}3^{KO}$ A549 cells. Expression of SRC-3 and actin in $SRC\text{-}3^{WT}$ and $SRC\text{-}3^{KO}$ A549 cells were analyzed by Western-blotting. (**D**) SRC-3 WT is more efficiently translocated into nucleus than SRC-3 S857A in response to TNF-α. $SRC\text{-}3^{KO}$ A549 cells were seeded in 24 well plate and left overnight. The next day, the cells were transfected with 200 ng of vector expressing either SRC-3 wild type (WT)-FLAG or SRC-3 S857A-FLAG. After 48 h, the cells were either stimulated with 10 ng/ml TNF-α for 60 min or left unstimulated. Representative images of the $SRC\text{-}3^{KO}$ A549 cells stained for SRC-3 (red, anti-SRC-3) and nucleus (blue, DAPI). (**E**–**G**) Quantitative presentation of the distribution of SRC-3 in conditions described in (**A**,**B**,**D**) respectively. The cellular localization of SRC-3 was determined as either cytoplasmic and nuclear or mainly nuclear. The SRC-3 overlapping nucleus (DAPI) is considered nuclear and the SRC-3 overlapping the nucleus and present around and outside the nucleus is considered cytoplasmic + nuclear. For quantification, minimum 100 cells were counted for each condition described in (**A**,**B**,**D**) and expressed in percentage. Data in (**E**,**F**,**G**) are presented as mean ± SD of three replicates. Unpaired t-test was used for analysis of significance between groups compared in the figure. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P \leq 0.001$. Now 345.

complete nuclear translocation of SRC-3 is efficiently blocked by pretreatment of cells with the MK2 inhibitor. The requirement for SRC-3 in co-activation of genes downstream of TNF-α could be both cell and gene specific. In our experiments, we found that SRC-3 is required for TNF-α-induced IL-6 expression while it is dispensable for TNF-α-induced MMP9 expression.

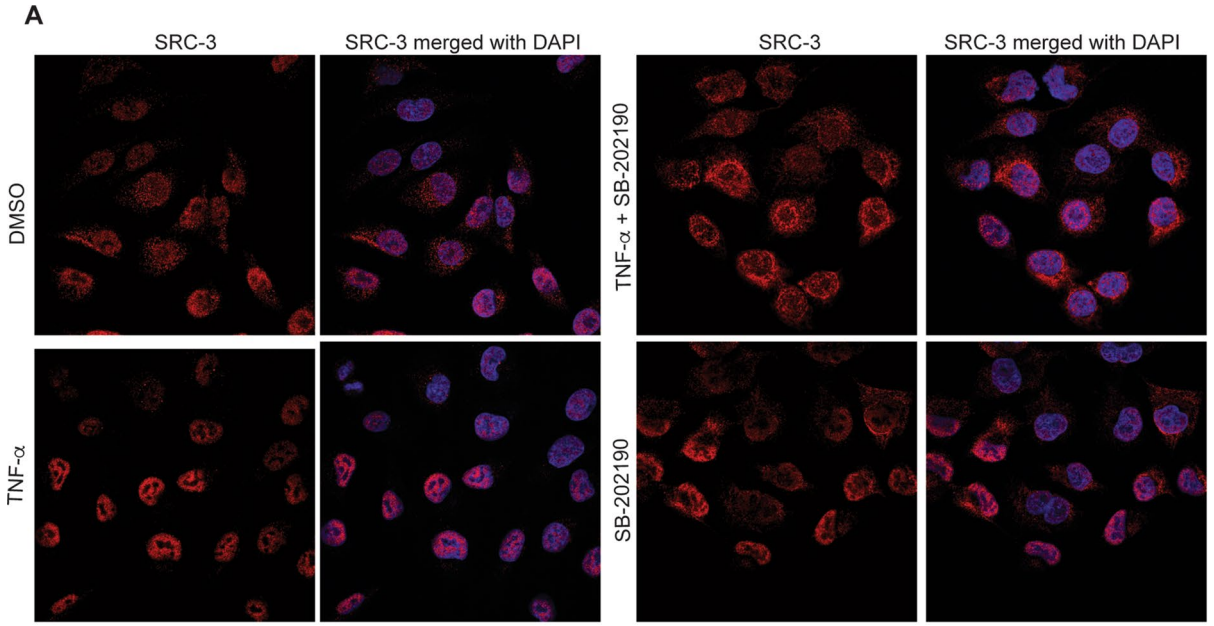
The gene encoding for SRC-3 is amplified in 5–10% of breast cancer patients, and is often found to be overexpressed on both mRNA and protein level[49,50]. Together with other members of the steroid receptor coactivators, such as SRC-1 and SRC-2, SRC-3 has been shown to be important for initiation and progression of estrogen receptor (ER) positive breast cancer[39]. Recently, Dasgupta et. al. showed that SRC-3 is important for the ability to promote tumorgenicity in both ER-dependent (MCF7 cells) and -triple negative (MDA MB 231 cells) breast cancer models[23]. Knock down of SRC-3 in these models inhibited either growth (MCF-7 cells) or both growth and metastasis (MDA MB 231 cells). As these phenotypes could not be rescued by expression of the phosphorylation-defective S857A mutant of SRC-3, these results suggest that the phosphorylation of SRC-3 at S857 might be crucial for breast cancer progression.

Since the present study demonstrates that MK2 is responsible for phosphorylation of SRC-3 at S857 in a wide variety of cell lines, including triple negative breast cancer cells (MDA MB 231 cells), the results indicate that the $p38^{MAPK}$-MK2-SRC-3 signaling axis could be a relevant therapeutic target in treatment of breast cancer.

## Methods

**Reagents.** Penicillin/streptomycin (#P0781), DMSO (#472301), anisomycin (# A5862), LPS, Sodium arsenite, PF-3644022 (#PZ0188) were purchased from Sigma-Aldrich, MO, USA. SB-202190 (#BML-EI294-001), PD-184352 (#ALX-270-471) were purchased from Alexis Biochemicals, CA, USA. Recombinant human TNF-α (#300-01A) was purchased from PeproTech, NJ, USA. Lambda phosphatase was purchased from New England Biolabs, MA, USA, BI-605906 was purchased from R&D systems, UK.

**Generation of vectors.** The mammalian expression vectors for expression of SRC-3 wild type (WT)-FLAG and SRC-3 S857A-FLAG as well as the vectors for expression of GST-CID-SRC-3 WT and GST-CID-SRC-3 S857A were kind gifts from Dr. Weiwen Long, Baylor College of Medicine, Texas, USA and are described in[3]. The expression vectors expressing siRNA resistant SRC-3 wild type (WT)-FLAG and SRC-3 S857A-FLAG were generated with quick-change mutagenesis (ThermoFisher Scientific, MA, USA) using the primers SRC-3siRF and SRC-3siRR (listed in Table 2) to introduce four silent mutations in the binding sequence for siRNA. The luciferase reporter vector κB-ConA-luc containing the binding site for NF-κB was kindly provided by Dr. Estelle Sontag, University of Texas South Western Medical Center, Texas, USA and is described in[51]. The plasmids PX458 (Addgene # 48138) and eSpCas9 (1.1) (Addgene # 71814) were a kind gift from Dr. Feng Zhang (MIT). To generate vector PX458 (1.1) for delivery of a Cas9 enzyme with less of target activity as described by Slaymaker et. al. the 2,281 base pair (bp) ApaI-BsmI fragment of the plasmid PX458[52] was exchanged with corresponding fragment derived from the plasmid eSpCas9 (1.1)[53]. The reporter gene vector pGL3-IL-6-promoter was generated by cloning of a 1,136 bp KpnI-Hind III fragment, containing the IL-6 promoter (1,186 bp) amplified from human genomic DNA by PCR using the primers IL-6-prom F and IL-6-prom R (listed in Table 2), into the vector pGL3-basic (Promega, WI, USA) linearized with the restriction sites KpnI and Hind III.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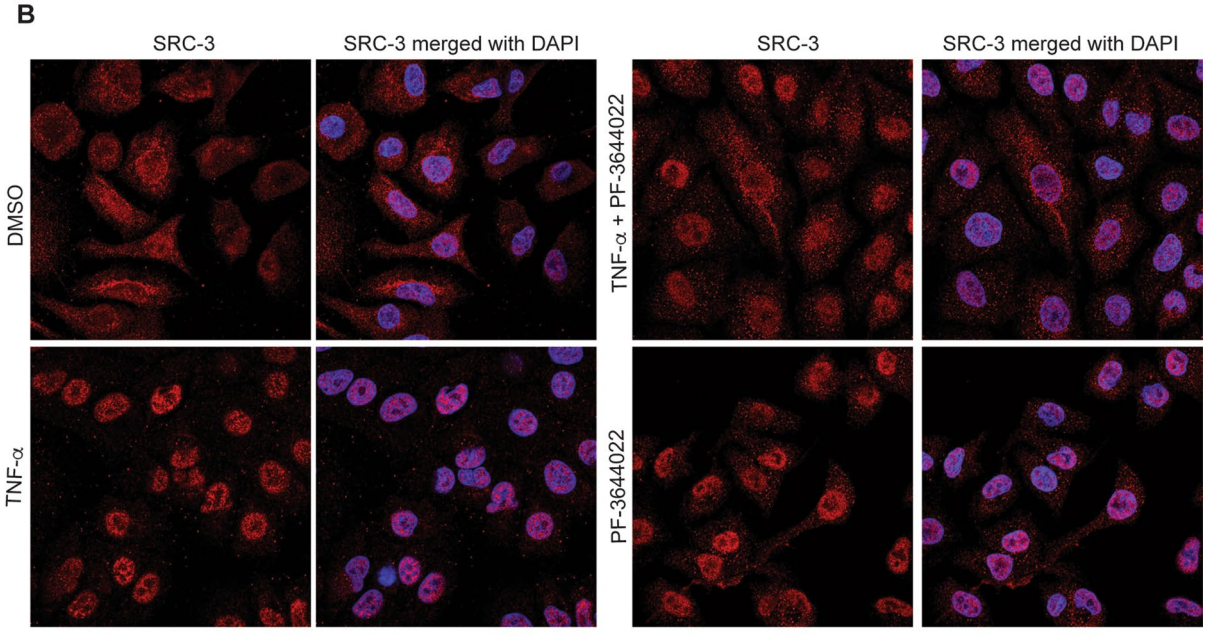
SRC-3 merged with DAPI
SRC-3
SRC-3 merged with DAPI
DMSO
TNF-α + SB-202190
TNF-α
SB-202190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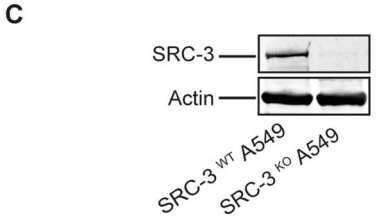
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Actin
SRC-3 WT A549
SRC-3 KO A549

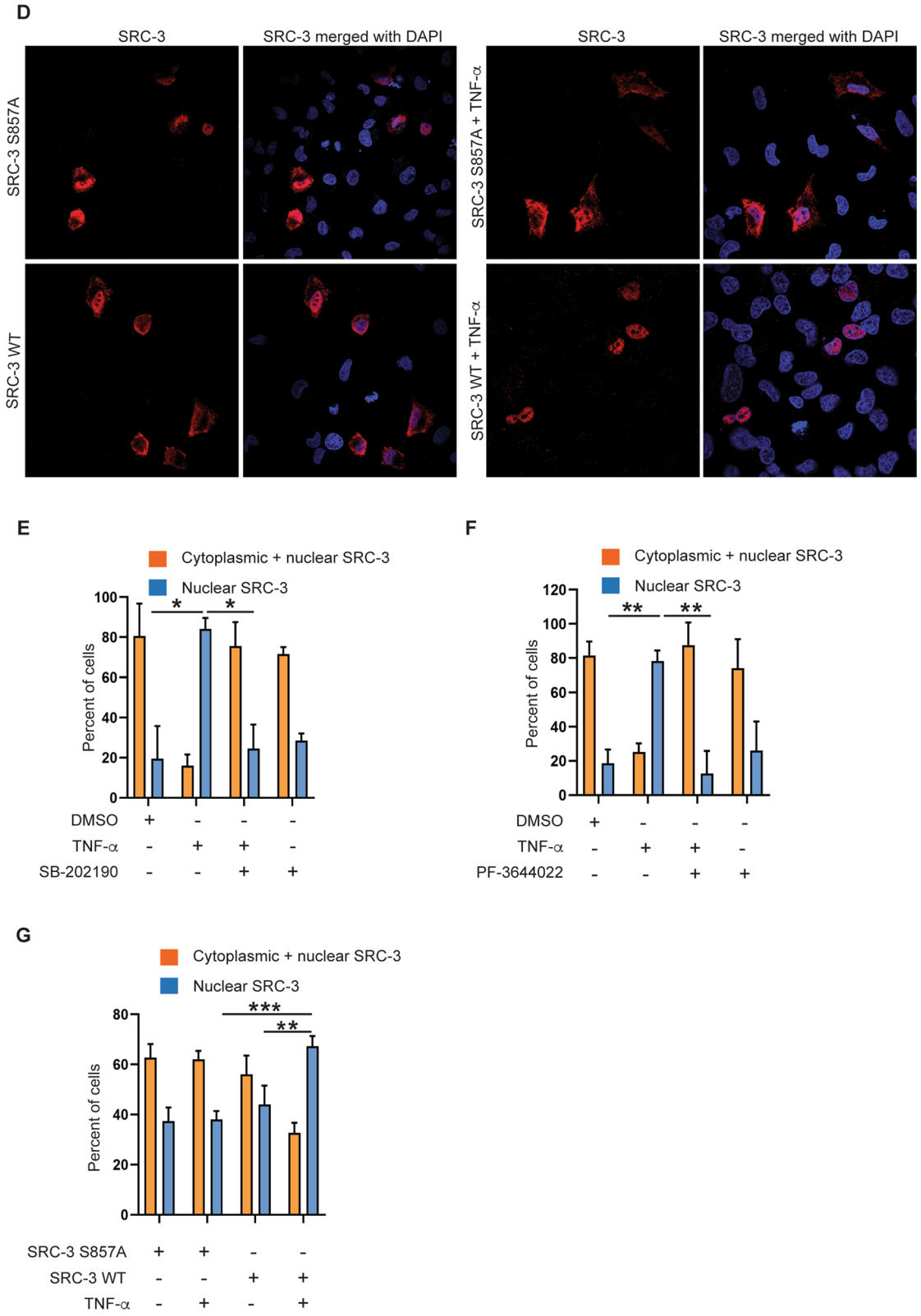



**Figure 7.** (continued)

**Cell lines, siRNA and transfection.** MDA MB 231 (American Type Culture Collection (ATCC) Virginia, USA, HTB-26), H1299 (ATCC CRL-580), A549 (ATCC CCL-185), HeLa (ATCC CCL-2), HEK 293 (ATCC CRL-11268), Mouse Embryonic Fibroblast (MEF) and Balb/c mouse bone marrow derived dendritic cells (BMDC) cells were maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich (D 5796)) supplemented with 10% fetal bovine serum (FBS) (Millipore, MA, USA, TMS-013-B), penicillin (100 units/ml) and streptomycin (100 mg/ml) in a humidified 5% $CO_2$ atmosphere at 37 °C. Cell lines were authenticated by comparing DNA profiles of the cell lines with the reference cell lines. Cell lines were routinely screened for mycoplasma and mycoplasma-free cells were used for all the experiments. Generation of MK2/3 KO and rescue MEF and BMDC cells are explained in[27]. The A549-NF-κB-Luc cells (RC0002) were obtained from Panomics San Diego, CA, USA. These cells are stably transfected with a luciferase reporter gene, which is under the transcriptional control of NF-κB.

siRNA against target genes were transfected into A549 and H1299 with Lipofectamine 2,000 or 3,000 (Invitrogen, CA, USA) prepared in OptiMEM (ThermoFisher Scientific) according to the manufacturer's instructions. Scrambled siRNA was used as control. The siRNA duplexes were purchased from ThermoFisher Scientific and are listed in Table 2. In each well of a 6-well plate, $3 \times 10^5$ cells were seeded and left overnight in incubator, then the cells were transfected with 20 ng/ml target siRNA or scrambled siRNA. The cells were lysed and lysates were harvested after 48 h. Successful knockdown was verified by Western-blotting analysis using antibodies listed in Table 1. Vectors were transfected into HeLa cells with Lipofectamine 2,000 or 3,000 (ThermoFisher Scientific), into A549 and H1299 with Lipofectamine LTX plus (ThermoFisher Scientific) and into HEK 293 cells with Trans IT-LT1 reagent (Mirus, WI, USA) according to the manufacturer's instructions.

**SRC-3 knock out by CRISPR-Cas9.** Oligos for guide RNAs targeting SRC-3 were determined using the chopchop.cbu.uib.no database[54]. The guide oligos (SRC-3-B) were ordered at ThermoFisher Scientific and are listed in Table 2. Guide oligos were cloned into the CRISPR-Cas9 expression vector PX458 (1.1) (Addgene #48138) as described in[52]. A549 cells were sorted using BD FACSAria III (BD Biosciences, NJ, USA) 48 h after transfection and only those cells expressing GFP were seeded individually into 96 well plates. Knock out of SRC-3 was confirmed by Western-blot using Rabbit-anti-SRC-3 (5E11, Table 1). To identify indels, genomic DNA was extracted by diluting $10 \times 10^4$ cells in 30 µl of 50 mM NaOH, transferred to a tube and incubated for 10 min at 95 °C. The sample was then placed on ice and 3 µl 1 M Tris pH 8.0 was added before the sample was centrifuged for 10 min at 10,000 rpm. 3 µl of the isolated genomic DNA was used to amplify the area of interest in the SCR-3 gene by PCR (30 cycles (98 °C for 5 s, 63 °C for 10 s, 72 °C for 30 s)) using Platinum SuperFi PCR Master Mix (ThermoFisher Scientific, #12358050) and SRC-3-A forward and reverse primers listed in Table 2. The PCR product was cloned into p-Zero-blunt vector (ThermoFisher Scientific, #K270040) and at least 8 individual clones were sequenced by Sanger sequencing (DNA sequencing lab, University Hospital North Norway, Tromsø, Norway) using M13 primer listed in Table 2.

**Generation of anti-P-S857-SRC-3 antibody.** The anti-P-S857-SRC-3 antibody was raised in sheep and affinity purified on the appropriate antigen residues 852–862 of human SRC-3 [YNRAVS*LDSPV] by the Division of Signal Transduction Therapy, University of Dundee, Scotland, UK.

**Cell staining.** About $4 \times 10^4$ A549 cells were seeded on fibronectin coated coverslip in 24-well plate. Cells pretreated with either 10 µM PF-3644022 (MK2 inhibitor), SB-202190 ($p38^{MAPK}$ inhibitor) or 0.2 µl DMSO for 30 min were treated with 10 ng/ml TNF-α for 60 min. Then, the cells were fixed with 500 µl 4% paraformaldehyde for 20 min and permeabilized with 500 µl 100% methanol for 5 min. After that the cells were blocked in 5% BSA for 20 min at room temperature. The cells were incubated in anti-SRC-3 antibody (Cell signaling Technology #2126) for 1 h at room temperature. Immunostaining was performed using Alexa Fluor-568 conjugated with anti-rabbit antibody (Invitrogen, #A-10042) while nuclei were visualized by staining with 1 µg/ml DAPI. Images were captured using LSM 780 inverted confocal microscope (Zeiss) at 63X magnification. Images were acquired with ZEN Black ver. 2.3 (Carl Zeiss Microscopy) software and analysed with the Fiji software. At least 100 cells were studied for each group.

**Luciferase reporter gene assay.** For luciferase reporter gene assay, $4 \times 10^4$ $SRC\text{-}3^{WT}$ or $SRC\text{-}3^{KO}$ A549 cells were seeded in each well of a 24-well plate and left overnight. On the other day, cells were transfected with

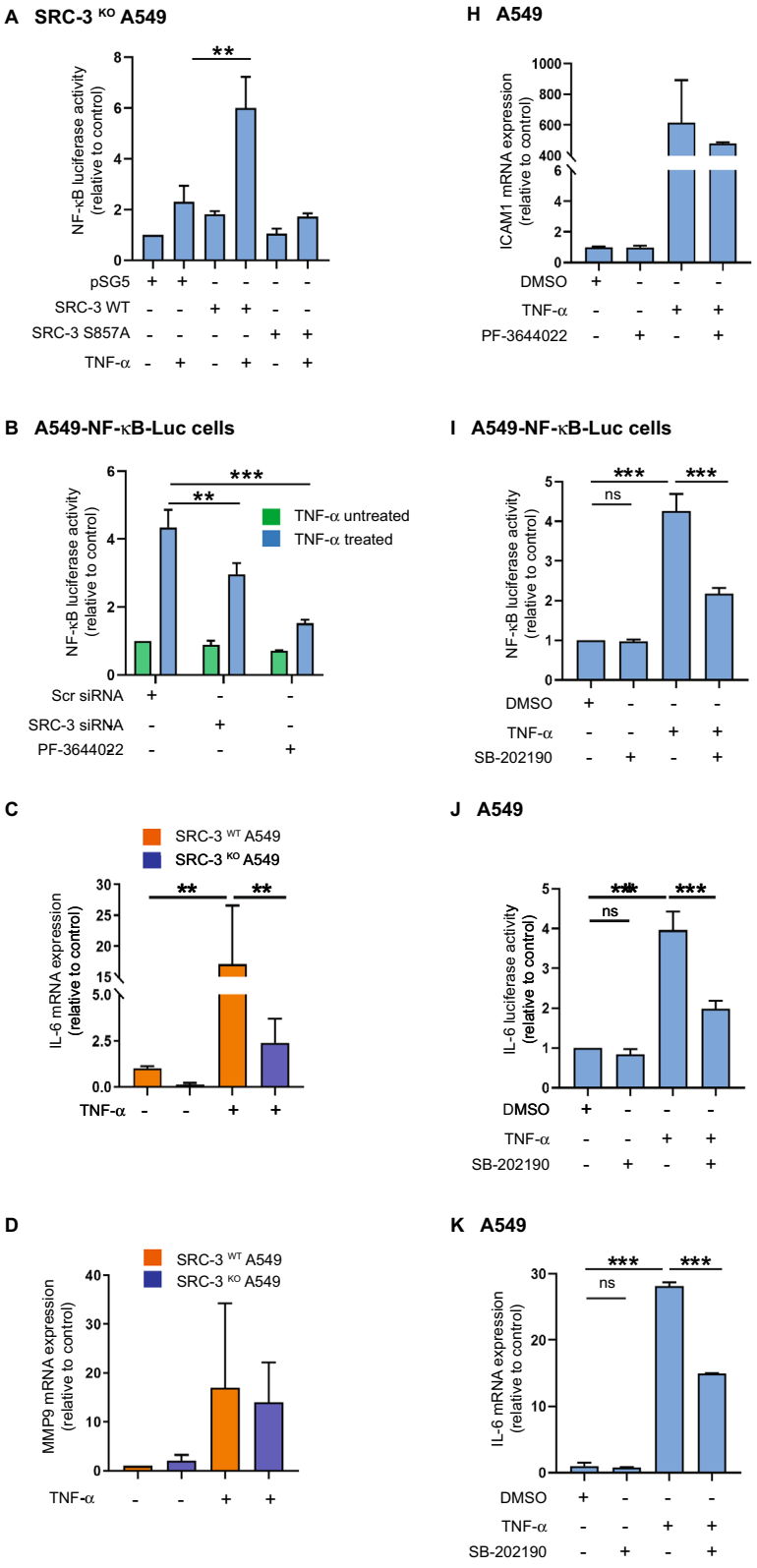
**Figure 8.** SRC-3 is required for MK2-mediated induction of IL-6 expression in response to TNF-α. (**A**,**B**) SRC-3 is involved in NF-κB activation. (**A**) SRC-$3^{KO}$ A549 cells were co-transfected with 120 ng κB-ConA-luc vector and 50 ng of either pSG5 empty vector, SRC-3 wild type (WT)-FLAG or SRC-3 S857A-FLAG vector. After 48 h TNF-α was added (if not other indicated 10 ng/ml for 5 h) before determination of luciferase activity relative to pSG5. (**B**) A549-NF-κB-Luc cells were transfected with scrambled siRNA or SRC-3 siRNA and 48 h later stimulated with TNF-α or left unstimulated. Nontransfected cells were pretreated with PF-3644022 for 30 min before TNF-α treatment. Luciferase activities are shown relative to unstimulated scrambled siRNA. (**C**,**D**) SRC-3 is involved in TNF-α-induced IL-6 expression. SRC-$3^{WT}$ and SRC-$3^{KO}$ A549 cells were stimulated with TNF-α for 2 h or left unstimulated. mRNA expression of IL-6 (**C**) and MMP9 (**D**) were determined relative to GAPDH and TFRC. Fold changes are presented relative to unstimulated SRC-$3^{WT}$ cells. (**E**,**J**) MK2 and $p38^{MAPK}$ activity are required for transcription of IL-6. A549 cells were transfected with 120 ng pGL3-IL-6-promoter vector and after 48 h treated for 30 min with 0.2 µl DMSO, 10 µM PF-3644022 (**E**) or SB-202190 (**J**) followed by stimulation with TNF-α. Luciferase activities are shown relative to DMSO. (**F**–**H**) MK2 is involved in TNF-α induced TRAF1 (**F**), IL-8 (**G**) and ICAM1 (**H**) mRNA expression. A549 cells pretreated with DMSO or 10 µM PF-3644022 for 30 min were stimulated with TNF-α for 2 h or left unstimulated. MRNA expression were determined relative to GAPDH and TFRC. Fold changes are presented relative to DMSO. (**I**) $p38^{MAPK}$ is involved in NF-κB-dependent luciferase activity. A549-NF-κB-Luc cells were pretreated with DMSO or SB-202190 and then stimulated with TNF-α or left unstimulated. Luciferase activities are shown relative to DMSO. (**K**–**M**) $p38^{MAPK}$ is involved in TNF-α-induced IL-6 (**K**) and IL-8 (**L**) but not MMP9 (**M**) mRNA expression. A549 cells pretreated with DMSO or 10 µM SB-202190 for 30 min were stimulated with TNF-α or left unstimulated. MRNA expression were determined relative to GAPDH and TFRC. Fold changes are presented relative to DMSO. Data are presented as mean ± SD (n = 3). Unpaired t-test; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P \leq 0.001$.

120 ng of κB-ConA-luc or pGL3-IL-6-pro vector along with other required expression vectors for 48 h. After necessary treatment, cells were lysed and luciferase activity was determined using Pierce Firefly Luciferase Glow Assay Kit (ThermoFisher Scientific, #16177) or Luc-Screen (ThermoFisher Scientific) according to the manufacturers` instructions. The luminescence was measured using a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany).

**Expression of GST-CID-SRC-3 in *E. coli*.** GST fusion proteins were expressed in *E. coli* (BL21) as described in[25].

**In vitro protein kinase assay.** Generation of recombinant ERK3 is described in[25] and ERK3 specific activity was determined as described in[55] using myelin basic protein (MBP) as a substrate. Recombinant active p38α, ERK2, MK5 and MK2 were purchased from MRC PPU reagent and services, Dundee, UK. For in vitro protein kinase assay, the active kinase was incubated with wild type GST-CID-SRC-3 fusion protein (GST-CID-SRC-3 WT) or mutant GST-CID-SRC-3 fusion protein (GST-CID-SRC-3 S857A) and 60 µM ATP in 50 µl kinase buffer (50 mM Tris HCl pH 7.5, 0.1 mM EGTA, 1 mM sodium vanadate, 1 mM DTT, 10 mM $Mg(CH_3COO)_2/MgCl_2$). The reaction was carried out at 30 °C for 5–30 min and terminated with LDS Sample Buffer (ThermoFisher Scientific #NP0008) and Sample Reducing Agent (ThermoFisher Scientific #B0009) and finally analyzed by Western-blotting. For in vitro kinase assay using radioactive ATP, 1 µCi [$\Upsilon^{32}$P] ATP (Amersham, Little Chalfont, UK) was added in the ATP mix. Phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography using the phosphorimager Fuji BAS-5000 (Fujifilm Life Science, Tokyo, Japan).

**Western-blot.** Total cellular extract was obtained by lysis of the cells in MKK lysis buffer (50 mM Tris/HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1% (w/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate and 0.27 M sucrose) containing protease inhibitors cocktail tablets (Roche, Mannheim, Germany, #04693132001). The cellular lysate was centrifuged at 13,000*g* for 10 min at 4 °C. Protein concentration was determined using Pierce Bradford Assay Kit (ThermoFisher Scientific #23246) and was denatured by heating for 10 min at 70 °C along with LDS Sample Buffer (ThermoFisher Scientific #NP0008) and Sample Reducing Agent (ThermoFisher Scientific #B0009). For Western-blot analysis, equal amounts of protein were separated by running it on 4–12% Bis–Tris Gels (Invitrogen # NW04122BOX) for 35 min, at 200 V, 120 mA in MES SDS Running buffer (Invitrogen #NP0002-02). See Blue Plus2 Prestained Standard (Invitrogen #LC5925), Super Signal Molecular Weight protein ladder (ThermoFisher Scientific, #84785,) and MagicMark XP Western Protein Standard (Invitrogen, #LC5602) were used as molecular weight markers. Then the proteins were transferred at 30 V, 150 mA for 2 h to Odyssey nitrocellulose membranes (LI-COR Biosciences, NE, USA #926–31092) using blotting buffer (48 mM Trisbase, 384 mM glycine and 20% methanol). After that, the membrane was blocked for 1 h using Odyssey blocking buffer (PBS) (LI-COR #927-40000) followed by incubation in respective primary antibodies overnight at dilution as mentioned in Table 1 and washed thrice with 1XTBST for 15 min then incubated with IRDye secondary antibodies (LI-COR Biosciences) in 1XTBST for 1 h. The mem-


A SRC-3 KO A549
NF-κB luciferase activity (relative to control)
pSG5
SRC-3 WT
SRC-3 S857A
TNF-α
B A549-NF-κB-Luc cells
TNF-α untreated
TNF-α treated
Scr siRNA
SRC-3 siRNA
PF-3644022
C
SRC-3 WT A549
SRC-3 KO A549
IL-6 mRNA expression (relative to control)
D
MMP9 mRNA expression (relative to control)
H A549
ICAM1 mRNA expression (relative to control)
DMSO
I A549-NF-κB-Luc cells
SB-202190
J A549
IL-6 luciferase activity (relative to control)
K A549
IL-6 mRNA expression (relative to control)
ns

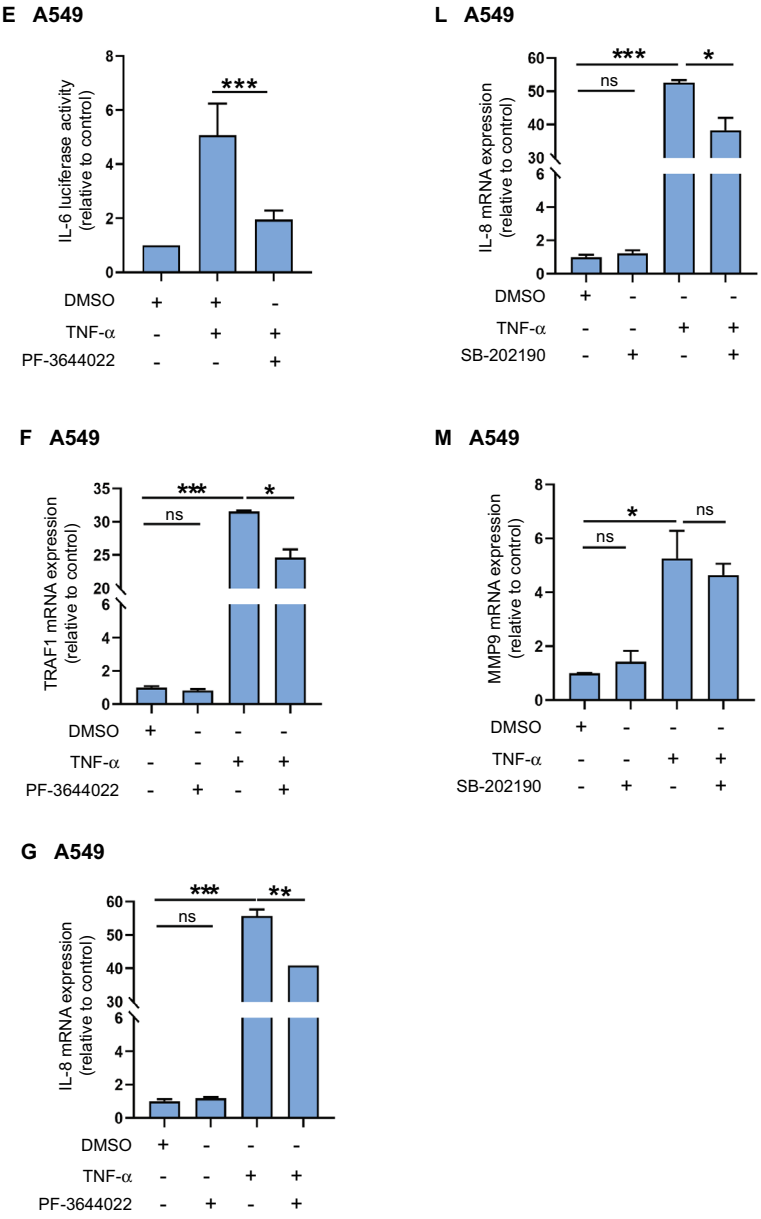



**Figure 8.** (continued)

| | Antibody | Source | Identifier | Dilution |
|---|---|---|---|---|
| 1 | Mouse-anti-AIB1 | BD transduction laboratories | 611,105 | 1:1,000 |
| 2 | Rabbit-anti-SRC-3 (5E11) | Cell signalling Technology | 2,126 | 1:1,000 (WB)<br>1:200 (immunostaining) |
| 3 | Rabbit-anti-NCoA-3 (M-397) | Santa Cruz Biotechnology | sc-9119 | – |
| 4 | Rabbit-anti-ERK 2 (C-14) | Santa Cruz Biotechnology | sc-154 | 1:1,000 |
| 5 | Mouse-anti-phospho p44/42 MAPK (ERK1/2) (T202 Y204) | Cell Signaling Technology | 9,106 | 1:1,000 |
| 7 | Rabbit-anti-MK2 | Cell Signaling Technology | 3,042 | 1:1,000 |
| 8 | Rabbit-anti-phospho-p38 MAPK (T180/Y182) | Cell Signaling Technology | 9,211 | 1:1,000 |
| 9 | Rabbit-anti-phospho-MK2 (T334) | Cell Signaling Technology | 3,041 | 1:1,000 |
| 10 | Rabbit-anti-p38 MAPK | Cell Signaling Technology | 9,212 | 1:1,000 |
| 11 | Mouse-anti-FLAG | Sigma-Aldrich | F1804 | 1:1,000 |
| 12 | Rabbit-anti-actin | Sigma-Aldrich | A2066 | 1:1,000 |
| 13 | Rabbit-anti-GST (Z-5) | Santa Cruz Biotechnology | sc-459 | 1:1,000 |
| 14 | Mouse-anti-PRAK (A-7) (MK5) | Santa Cruz Biotechnology | sc-46667 | 1:1,000 |
| 15 | Mouse-anti-MAPK6 (ERK3) | Abnova | H00005597-M02 | 1:1,000 |
| 17 | Sheep-anti-P-SRC-3-S857 | Custom made by Division of Signal Transduction Therapy, (DSTT), University of Dundee, Dundee, UK | | 1:1,000 |
| 18 | Goat-anti-mouse AF 800 | Invitrogen | A32730 | 1:10,000 |
| 19 | Goat-anti-mouse AF 700 | Invitrogen | A21036 | 1:10,000 |
| 20 | Goat-anti-rabbit AF 800 | Invitrogen | A32735 | 1:10,000 |
| 21 | Goat-anti-rabbit AF-700 | Invitrogen | A21038 | 1:10,000 |
| 22 | Rabbit-anti-sheep DyLight 800 | Invitrogen | SA5-10060 | 1:10,000 |
| 23 | Donkey-anti-sheep AF 680 | Invitrogen | A-21102 | 1:10,000 |
| 24 | Donkey-anti-rabbit AF 568 | Invitrogen | A-10042 | 1:4,000 |
| 25 | Mouse-anti-IKK-α (B-8) | Santa Cruz Biotechnology | sc-7606 | 1:1,000 |
| 26 | Rabbit-anti-IκB-α (C-21) | Santa Cruz Biotechnology | sc-371 | 1:1,000 |
| 27 | Mouse-anti-HSP27 | Millipore | MAB88051 | 1:1,000 |
| 28 | Rabbit anti-phospho-HSP27 (S82) | Cell Signaling Technology | 2,401 | 1:1,000 |

**Table 1.** List of antibodies. BD transduction laboratories, NJ, USA; Santa Cruz Biotechnology, CA, USA; Cell Signaling Technology, Danvers, MA, USA; Abnova, Taipei City, Taiwan; Invitrogen, CA, USA, Sigma-Aldrich. If not indicated, the antibody was used for Western-blotting (WB).

brane was washed thrice with TBST for a total of 15 min. Finally, fluorescent images of the blots were acquired on Odyssey Sa detection system (LI-COR Biosciences).

**Immunoprecipitation.** For immunoprecipitation, $2 \times 10^6$ H1299 cells were seeded in a 100 mm dish. The cells were lysed and centrifuged as described in Western-blot section. The lysate obtained was cleared with activated Pierce Agarose resin (ThermoFisher Scientific). 2 mg of clarified lysate was incubated with 2 µg Mouse-anti-FLAG (Sigma-Aldrich F1804) or Rabbit-anti-NCoA-3 (M-397) (Santa Cruz Biotechnology sc-9119) antibody (Table 1) overnight at 4 °C. Then 30 µl Protein G agarose (Millipore #16-266) was added to it and incubated for 60 min at 4 °C. The mixture was then transferred to spin column (Sigma-Aldrich #SC1000) and centrifuged. The spin column was washed twice with 500 µl ice cold MKK lysis buffer then with 50 mM TRIS chloride pH 7.5. The immunoprecipitated protein was eluted by heating at 70 °C for 10 min in 60 µl LDS Sample Buffer (ThermoFisher Scientific #NP0008) and Sample Reducing Agent (ThermoFisher Scientific #B0009). The denatured protein was separated by SDS PAGE, transferred and incubated with anti-SRC-3 and anti-P-S857-SRC-3 antibody as described earlier.

**RNA extraction, reverse transcription, quantitative real-time PCR.** Total RNA was obtained by lysing the cells with RLT Plus Buffer (Qiagen, Venlo, Netherlands #74136) supplemented with 1 M DTT (40 µl/ml) followed by extraction with RNeasy Plus Mini kit (Qiagen #74136) according to the manufacturer's instructions. Quantity and purity were determined using NanoDrop spectrophotometer (ThermoFisher Scientific). 1 µg of total RNA was reverse transcripted to cDNA using the High capacity cDNA reverse transcription kit (ThermoFisher Scientific #4368813) supplemented with RiboLock RNase inhibitor (ThermoFisher Scientific #EO0381) (2 U/µl). Quantification of mRNA expression was determined using Light cycler 96 (Roche). Primer pairs for qRT-PCR were purchased from Sigma-Aldrich and are listed in Table 2. 2 µl cDNA was amplified for 40 cycles (95 °C for 15 s, 60 °C for 1 min) in a 20 µl Power UP SYBR green master mix (ThermoFisher Scientific #25741) containing 200 nM of each primer. The relative expression of the target gene was normalized to the average expression of the two reference genes TFRC and GAPDH using the $2^{-\Delta\Delta Ct}$ method[56].

| Target gene | Primer | Purpose | Nucleotide sequences | Source |
|---|---|---|---|---|
| IL-6 | F | RT qPCR | GCAGAAAAAGGCAAAGAATC | Sigma-Aldrich |
| | R | | CTACATTTGCCGAAGAGC | |
| IL-8 | F | RT qPCR | GTTTTTGAAGAGGGCTGAG | Sigma-Aldrich |
| | R | | TTTGCTTGAAGTTTCACTGG | |
| MMP9 | F | RT qPCR | AAGGATGGGAAGTACTGG | Sigma-Aldrich |
| | R | | GCCCAGAGAAGAAGAAAAG | |
| TRAF1 | F | RT qPCR | CTTTCCTGTGGAAGATCAC | Sigma-Aldrich |
| | R | | ACTTGGCAGTGTAGAAGG | |
| ICAM1 | F | RT qPCR | ACCATCTACAGCTTTCCG | Sigma-Aldrich |
| | R | | TCACACTTCACTGTCACC | |
| GAPDH | F | RT qPCR | CTTTTGCGTCGCCAG | Sigma-Aldrich |
| | R | | TTGATGGCAACAATATCCAC | |
| TFRC | F | RT qPCR | AAGATTCAGGTCAAAGACAG | Sigma-Aldrich |
| | R | | CTTACTATACGCCACATAACC | |
| IL-6-prom | F | Promoter PCR | CCGGGTACCTCCAAGGCAGACTCTGAG | Sigma-Aldrich |
| | R | | GGCCAAGCTTCATCTCCAGTCCTATATTTATTGGGGG | |
| M13 | R | PCR | CAGGAAACAGCTATGAC | Sigma-Aldrich |
| SRC-3-A | F | PCR | AGGAAGGGGAAGGTAAGAGCTA | Sigma-Aldrich |
| | R | | CACAGGGTTTGATGGAAATGTT | |
| SRC-3-B | F | CRISPR sgRNA | GCAATCTTGTATGATCTGTG | Life Technologies |
| | R | | CACAGATCATACAAGATTGC | |
| SRC-3-C | F | siRNA | CAGUAUAUCGAUUCUCGUUtt | Ambion life technologies |
| | R | | AACGAGAAUCGAUAUACUGgg | |
| SRC-3siR | F | Mutagenesis | CCATGCAGAAACCCCCGTCTACCGCTTCTCGTTGGC TGAT | Sigma Aldrich |
| | R | | ATCAGCCAACGAGAAGCGGTAGACGGGGGTTTCTGC ATGG | |
| IKK-α | F | siRNA | GAAGGAUCCAAAGUGUAUAtt | ThermoFisher Scientific |
| | | | UAUACACUUUGGAUCCUUCgg | |

**Table 2.** List of oligonucleotides. (Life Technologies, CA, USA) (Ambion life technologies, Carlsbad, CA, USA). The same siRNA against ERK3, MK5 and scrambled siRNA were used as reported in[25].

**Statistics.** Values are presented as mean ± SD of at least three replicates. Data were analyzed with unpaired t-test using GraphPad prism software version 8.2.1 (CA, USA). Differences between groups were considered to be significant with $P$-values < 0.05.

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## Author contributions

H.B., I.M., H.L.W. and O.-M.S. planned the experiments; A.S., H.K., G.K., H.L.W., H.B. and O.-M.S. performed the experiments and analyzed the data; M.G. provided MK2/3 knock out (KO) MEF and BMDC cells lines, A.S., H.B., I.M., M.G., H.L.W. and O.-M.S. wrote the manuscript; H.B., I.M., H.L.W. and O.-M.S. supervised; O.-M.S. conceptualization; O.-M.S. funding acquisition; O.-M.S. project administration. All authors reviewed and approved the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

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**Paper II**

**A role of the p38$^{MAPK}$-MK2-SRC-3 signaling axis in the sensitivity to doxorubicin in triple negative breast cancer cell**

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**Paper III**

**A Pilot study using RNA sequencing to identify genes regulated by phosphorylated SRC-3 S857 and the p38MAPK-MK2-SRC-3 signaling pathway in triple negative breast cancer cells**

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