Involvement of the matrix proteins SPARC and osteopontin in the dynamic interaction between tumour and host cells

A thesis submitted for the degree of Doctor of Philosophy

by

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Declaration

I hereby declare that the work presented in this thesis is my own work, unless otherwise specified, and has not been submitted for any other degree.

Amir Jassim

ABSTRACT

Osteoblasts are highly active cells that are responsible for secreting bone forming components such as collagen type I and matricellular proteins that mediate collagen deposition and mineralisation. SPARC and osteopontin are matricellular proteins that are involved in bone regulation and cell-matrix interactions and are also upregulated in metastatic disease. Secretion of these proteins results in changes to the stromal environment that includes cell migration, angiogenesis, matrix degradation, matrix deposition, bone mineralisation and bone resorption. Signalling pathways not only lead to the expression of target proteins, but also have immediate early effects, for example, on cell adhesion. We asked if the ERK 1 and 2 module of the MAPK pathway was involved in the intracellular trafficking of SPARC and Osteopontin. Membrane trafficking is an essential process that ensures newly synthesised proteins pass from their site of synthesis to the extracellular environment. Using an inhibitor of ERK 1 and 2 activation (U0126), as well as siRNA directed against ERK 1 or 2 individually, a change in intracellular localisation of SPARC and osteopontin was observed in cells treated with U0126 and siRNA against ERK 2 alone, likely in or around the Golgi apparatus. Consistent with the observation above, analysis of protein secretion showed that there was a reduction of total protein secreted (30% reduction) when ERK 1 and 2 activation was prevented together or knock down of ERK 2 alone. A mechanism is proposed where ERK 2 is likely activating a substrate that is allowing SPARC and osteopontin to continue along the secretory pathway. This directly implicates ERK 2 as an important regulator of matricellular protein secretion in osteoblasts. In cancer, Ras mutations can lead to permanent activation of the MAPK pathway leading to cancer cell proliferation and survival, however, we propose another mechanism important in metastasis whereby ERK 2 activation is manipulated to facilitate secretion of matricellular proteins which can then mediate changes to the stromal environment that allow the tumour to metastasise successfully.

TABLE OF CONTENTS

TABLE OF FIGURES

1.0. Introduction

3.0. Results: Inhibitor Treatment

4.0. Results: siRNA knockdown of ERK 1 and 2

TABLES

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ABBREVIATIONS

AKT: **Ak** strain **t**ransforming

AP: **A**dapter **P**rotein

ARF: **A**DP **r**ibosylation **f**actor

ATP: **A**denosine **t**ri**p**hosphate

BMP: **B**one **m**orphogenetic **p**rotein

CMV: **C**yto**m**egalo**v**irus

COD: **C**alcium **O**xalate **D**ihydrate

COM: **C**alcium **O**xalate **M**onohydrate

COP: **Co**at **P**rotein

CTC: **C**irculating **t**umour **c**ell

DDR: **D**iscoidin **d**omain **r**eceptor

DMEM: **D**ulbecco's **M**odified **E**agle **S**erum

ECL: **E**nhanced **C**hemo**l**uminescence

ECM: **E**xtra**c**ellular **m**atrix

EMT: **E**pithelial **m**esenchymal **t**ransition

ER: **E**ndoplasmic **R**eticulum

ERGIC: **E**ndoplasmic **R**eticulum-**G**olgi **I**ntermediate **C**ompartment

ERK: **E**xtracellular-signal **R**egulated **K**inase

FAK: **F**ocal **a**dhesion **k**inase

FGF: **F**ibroblast **G**rowth **F**actor

FGFR: **F**ibroblast **G**rowth **F**actor **R**eceptor

GDP: **G**uanosine **di****p**hosphate

GEF: **G**uanine nucleotide **E**xchange **F**actor

GFP: **G**reen **F**luorescent **P**rotein

GTP: **G**uanosine **t**ri**p**hosphate

HEK293: **H**uman **e**mbryonic **k**idney 293 cells

HeLa: **He**nrietta **La**cks (immortalised cervical carcinoma cell line)

HMEC: **H**uman **m**ammary **e**pithelial **c**ells

IF: **I**mmuno**f**luorescence

ILK: **I**ntegrin **l**inked **k**inase

JNK: C-**J**un **N**-terminal **K**inase

MAPK: **M**itogen **A**ctivate Protein **K**inase

MEK: **M**APK/**E**RK **k**inase

MET: **M**esenchymal **e**pithelial **t**ransition

miRNA: **mi**cro **R**ibo**n**ucleic **A**cid

MMP: **M**atrix **M**etallo**p**roteinase

mRNA: **M**essenger **R**ibo**n**ucleic **A**cid

MSC: **M**esenchymal **s**tem **c**ell

mTOR: **m**ammalian **t**arget **o**f **r**apamycin

NLK: **N**emo **L**ike **K**inase

PBS: **P**hosphate **B**uffered **S**aline

PDGF: **Pl**atelet **D**erived **G**rowth **F**actor

PTEN: **P**hosphatase and **Ten**sin Homolog

Rab: **Ra**s related proteins in **b**rain

ROS: **R**at **O**steo**s**arcoma (referring to cell line used)

RTK: **R**eceptor **T**yrosine **K**inase

SDS: **S**odium **d**odecyl **s**ulphate

SDS-PAGE: **S**odium **d**odecyl **s**ulphate-**P**oly**a**crylamide **g**el **e**lectrophoresis

SH2/3: **S**rc **h**omology domain 2/3

SIBLING: **S**mall **i**ntegrin-**b**inding N-**lin**ked **g**lycoproteins

siRNA: **S**hort **i**nterfering **R**ibo**n**ucleic **A**cid

SMOC: **S**ecreted **mo**dular **c**alcium binding proteins

SPARC: **S**ecreted **P**rotein **A**cidic and **R**ich in **C**ysteine

Src: **S**a**rc**oma

TGF-β: **T**ransforming **g**rowth **f**actor β

RAF: **R**apidly **a**ccelerated **F**ibrosarcoma

RANK: **R**eceptor **a**ctivator of **n**uclear factor-**k**appa B

RANKL: **R**ANK **l**igand

RAS: **Ra**t **S**arcoma

RISC: **R**NA **I**nducing **S**ilencing **C**omplex

RLC: **R**ISC **L**oading **C**omplex

TRAPP: **Tr**ansport **a**ssociated **p**rotein **p**articles

UPR: **U**nfolded **p**rotein **r**esponse

VEGF: **V**ascular **E**ndothelial **G**rowth **F**actor

WB: **W**estern **B**lot

Wnt: **W**ingless **in**te**g**rated

"The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them."

-William Bragg

INVOLVEMENT OF THE MATRIX PROTEIN SPARC AND OSTEOPONTIN IN THE DYNAMIC INTERACTION BETWEEN TUMOUR AND HOST CELLS

1.0. INTRODUCTION

1.1. THE EXTRACELLULAR MATRIX

The extracellular matrix (ECM) forms the surrounding (stromal) environment of all major organs. It is not just a physical support for these organs but is essential in facilitating major signalling events as well as changing in response to various situations that arise. Such situations include cells that need to migrate, blood vessels that need to be formed and re-formation of the ECM in situations such as wound repair as well as bone resorption and mineralisation. Cells connect to the surrounding stromal environment through specialised attachments called focal adhesions. Focal adhesions are the connections of the extracellular matrix with filamentous actin inside the cell. Bridging of the ECM and cells is facilitated by cell surface receptors called integrins, discoidin domain receptors (DDRs) and syndecans (Frantz et al. 2010).

1.1.1. STRUCTURAL COMPONENTS OF THE ECM

The ECM is made up of several structural proteins, primarily collagens and proteoglycans. Fibrillar collagens are made of 3 triple helices that coil around each other to form fibrillar bundles that are laid in the ECM (Shoulders & Raines 2009). Structural components of the ECM are primarily composed of different types of collagens including collagen type I, the main collagen that is mineralised in skeletal tissue, collagens type II to XVIII forming the basement membranes of several ECM tissues as well as forming the ECM in the stromal environment around organs of the body including blood vessels, liver and kidney (Järveläinen et al. 2009).

In addition to the collagens, the ECM is composed of another set of structural proteins called proteoglycans. Proteoglycans are proteins that have glycosaminoglycan (GAG) chains covalently attached to them. Glycosaminoglycans are polysaccharides that consist of repeating disaccharides (Afratis et al. 2012).

Several proteoglycans form part of the meshwork with collagens in the ECM. Important families of proteoglycans include cell surface proteoglycans syndecans, aggrecans, testicans and perlecans (Couchman & Pataki 2012).

Additional structural proteins of the ECM include elastins, for tensile strength and facilitating the movement of the ECM without compromising its structure or integrity; fibronectin, a protein that is involved in cell attachment and binds to integrins with the capability of being stretched out as well as the ability to contract rapidly when bound to integrins, can form blood clots at sites of injury and is involved in cell migration (Frantz et al. 2010).

Laminins are also an important component of the ECM. They form part of the basement membranes along with collagen type IV. The basement membrane is the section of the ECM that separates the functional (parenchymal) cells (i.e. from an organ) to the surrounding stromal environment. Laminins are heterotrimeric proteins made of α, β and γ subunits that are deposited in the basal lamina. Like many of the ECM components, laminins do not simply offer structural support but are also capable of sending signals through the cell in response to changes in the surrounding environment (Hamill et al. 2009).

1.1.2. FUNCTIONAL PROTEINS IN THE ECM

While the proteins discussed above form the structural components of the ECM, the ECM is also a dynamic environment. Functional proteins that signal to the cell in response to changes in the stromal environment are required to mediate cell-matrix interactions. A key set of proteins that carry out these functions are the matricellular proteins. By definition, matricellular proteins solely have a functional role in the extracellular matrix and not a structural role. The concept of matricellular proteins was described in the 1970s and work on these proteins showed them to be secreted from the cell where they would carry out their functions. Several members of the matricellular family of proteins have been described. These include SPARC (reviewed in introduction, section 1.4), secreted modular calcium binding proteins (SMOCs), proteins that contain EF hands and are part of the SPARC family (Vannahme et al. 2002), osteopontin (reviewed in introduction section 1.5), part of the SIBLING family of proteins (small integrin-binding ligand N-linked glycoproteins) (Bellahcène et al. 2008), thrombospondin, tenascin, the CCN family of matricellular proteins (cysteine-rich angiogenic protein, connective tissue growth factor and nephroblastoma overexpressed protein), periostin, galectin and plasminogen activator inhibitor type I (Murphy-Ullrich & Sage 2014; Bornstein 2009).

These matricellular proteins carry out different functions in the ECM ranging from facilitating the deposition of the extracellular matrix components, to inducing the expression of MMPs for degrading the matrix in order to allow for processes such angiogenesis and cell migration. While matricellular proteins are essential in normal processes such as development and wound healing, they also play a role in cancer. The matricellular proteins and their activities have been implicated in various diseases including metastatic disease (Murphy-Ullrich 2001).

1.2. BONE MINERALISATION

Bone mineralisation is the process by which collagen is hardened and reinforced with the mineral hydroxyapatite (Nair et al. 2013). Collagen type I comprises 90% of the organic component of mineralised tissue and constitutes almost 20% of the body weight in humans. In addition to collagen, the organic component of bone is made up of matrix proteins and proteoglycans while the rest is the inorganic mineral component hydroxyapatite. The ratio of collagen to hydroxyapatite differs at different sites of the skeleton, which fine tunes strengths of the different components of the skeletal system (Bonucci 2013).

The inorganic phase of bone bares similarity to geological apatite in its chemical composition (calcium and phosphate), which was shown in 1926 as described within Rey et al. However, further work showed that the ratio of phosphate and calcium plus carbonate groups differed to that of geological hydroxyapatite (Rey et al. 2009).

The formation and mineralisation of bone is a complex process that relies on a constant crosstalk between the resorption and formation of bone. While the components of bone itself mentioned above (collagen, matrix proteins and hydroxyapatite) are the essential components of mineralised tissue, cells (namely osteoblasts and osteoclasts) are at the heart of the interplay between bone formation, resorption and repair. Osteoblasts function primarily to secrete matrix proteins and collagen type I in bone formation while osteoclasts are primarily responsible for the resorption of bone. The crosstalk between osteoblasts and osteoclasts is essential and is known as ‘bone coupling’. A lack of regulation in bone coupling leads to diseases such as osteoporosis, a disease characterised by a decrease in bone mass (Bernabei et al. 2014). Bone coupling depends on the needs of the surrounding environment and cell signalling is essential in this process. Growth factors such as RANKL and sclerostin

are secreted by osteoblasts to induce the differentiation of osteoclast precursors (OCP) into osteoclasts or to reduce osteoblastic bone formation. Mature osteoclasts release factors such cathepsin K which are directly involved in the breakdown of mineralised tissue (Charles & Aliprantis 2014).

Osteoclasts are large multi-nucleated cells that contain a ruffled border at the site of contact between the osteoclast and target bone that must be resorbed. The ruffled border exists in order to provide a large surface area that allows for the release of components that are involved in bone degradation as well as the internalisation of resorbed bone (Stenbeck 2002).

Coupling of skeletal breakdown with the formation and mineralisation of new bone is mediated by osteoblasts, the primary cells that secrete collagen type I and matricellular proteins. Mesenchymal stem cells (MSCs) are the precursor of osteoblasts. Three signalling pathways appear to be essential in osteoblastogenesis, transforming growth factor-β (TGF-β), Wnt and bone morphogenetic (BMP) signalling. These pathways turn MSCs into pre-osteoblasts, which become mature osteoblasts. Important transcription factors in the differentiation process are distal-less homeobox 5 (Dlx5), runt related transcription factor 2 (Runx2) and osterix (Osx) (Chen et al. 2012).

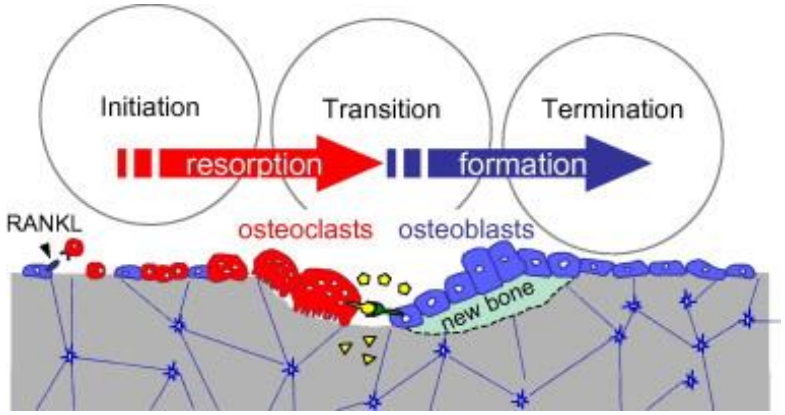


FIGURE 1.0, Osteoclast activity is followed by bone formation mediated by osteoblasts. Factors such as RANKL are important in inducing the differentiation of osteoclast precursor cells, which once mature (osteoclasts), secrete factors that degrade bone (mature osteoclast shows a ruffled border against surface of bone in image). Osteoblasts release a decoy receptor known as osteoprotegerin (OPG). OPG masks RANKL and inhibits its ability to activate osteoclasts thus terminating resorption of bone. Simultaneously, osteoclasts secrete factors such as Wnt 10b, cardiotrophin-1 and BMP-6 (Sims & Martin 2015) that induce the

differentiation of osteoblasts so that bone formation may begin through differentiation of osteoblast precursor cells into mature osteoblasts that secrete bone-forming components. At this point, osteoclasts will undergo apoptosis. Yellow pentagons represent factors released by osteocytes and osteoblasts as part of the crosstalk during the bone coupling process. Blue stars in the grey area represent osteocytes. Osteocytes are osteoblasts that have become trapped in the matrix that they secrete. These are not inactive however and it is thought they act as sensors of bone damage which then dictate the sites of osteoclast activity. Image taken directly from (Matsuo & Irie 2008).

Bone formation must replace the amount of bone lost through resorption. Osteoblasts synthesise and secrete collagen type I for the mineralisation of lost bone. It is likely that matricellular protein secretion proceeds or occurs simultaneously with collagen secretion to facilitate its mineralisation with hydroxyapatite. There are various matricellular proteins that have been reported to induce mineralisation of collagen. The evidence suggests that matricellular proteins are important not just in mineralisation of bone but resorption as well. An important observation made by researchers is that mice lacking a single matricellular protein are viable suggesting that no single matricellular protein alone is involved in bone mineralisation/resorption. Matricellular proteins involved in bone mineralisation include SPARC (role in bone mineralisation reviewed in introduction, section 1.4.2.3), tenascins, thrombospondins and dentin phospho- and sialoprotein for the mineralisation of teeth (Prasad et al. 2010; Alford & Hankenson 2006).

Matricellular proteins are likely working in conjunction with each other with respect to the mineralisation of bone. For example, SPARC null mice exhibit osteopenia and lower rates of collagen deposition. Thrombospondin knockout mice display deformities in the skeletal structure suggesting an important role in the organisation of fibrillar collagen. Taking the knockout of SPARC as an example, the development of osteopenia does not equal a complete lack of mineralisation, although demonstrably it clearly is less. This means that other matricellular proteins such as tenascin may be compensating to a certain extent for mineralisation to continue (Li et al. 2016).

Matricellular proteins important in bone resorption include osteopontin (role in bone regulation reviewed in introduction, section 1.5.2) and bone sialoprotein (BSP). Both osteopontin and BSP contain RGD (arginine, glycine, aspartic acid) motifs that bind to

integrins. Osteopontin and BSP are important in activating osteoclasts and mediating bone resorption (Alford & Hankenson 2006).

Clearly, the role that matricellular proteins play in bone regulation is essential; however, more work must be carried out to elucidate further specific functions with regards to bone homeostasis. It appears that timing of growth factor signalling, activation of specific signalling pathways and secretion of matricellular proteins is crucial in bone coupling.

1.2.1. OSTEOCYTES

Osteocytes are mature osteoblasts that have become trapped in the matrix that they have secreted. While buried in this matrix, osteocytes are not inactive and are capable of transducing signals to the surrounding environment. Osteocytes make up 95% of bone cells in adults. The fate of osteoblasts that become buried in their surrounding matrix is not clear, whereas it is thought that osteoblasts can undergo three pathways which include quiescence to become bone lining cells, apoptosis or maturation into osteocytes. Differentiation into osteocytes is characterised by changes in gene expression which include downregulation of collagen type I and upregulation of sclerostin expression (Dallas et al. 2013). The complete functions of osteocytes are not fully understood, however, it is thought that osteocytes are part of a complex signalling event in 'bone coupling' that includes communication between all bone resident cells (Graham et al. 2013). Osteocytes communicate with the surrounding environment through canaliculi, microcanals that link osteocyte lacunae (cavities in the bone matrix) and also to the bone surface, which allows signals from the osteocytes to pass to osteoblast/clasts (Aarden et al. 1994).

An important marker of osteocytes is the expression of sclerostin. Sclerostin is a secreted protein that serves to supress bone formation by signalling to osteoblasts. Sclerostin inhibits the Wnt signalling pathway in osteoblasts and prevents bone formation. Additionally, sclerostin serves as an autocrine molecule to stimulate the expression of RANKL in osteocytes which then promotes differentiation of pre-osteoclast cells into osteoclasts. Together, these functions show that sclerostin is important in mediating the degradation of bone, and it is likely that osteocytes secrete sclerostin in response to bone damage. The suppression of

bone formation through Wnt pathway inhibition in osteoblasts is a well reported function of sclerostin (Suen & Qin 2016).

The Wnt signalling pathway is a cascade of signals initiated by Wnt ligands that signal to two receptors, Frizzled and low-density lipoprotein receptor (LRP5/6). Activation of these receptors regulates β-catenin, a protein involved in cell-cell junctions but also gene transcription. When inactive, β-catenin is degraded by adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase 3 (GSK3) protein complex, collectively termed the Axin complex. Upon activation of Wnt receptors, the Axin complex is recruited to the receptors thus preventing β-catenin degradation. β-catenin accumulates and enters the nucleus where it activates transcription factors (MacDonald et al. 2009).

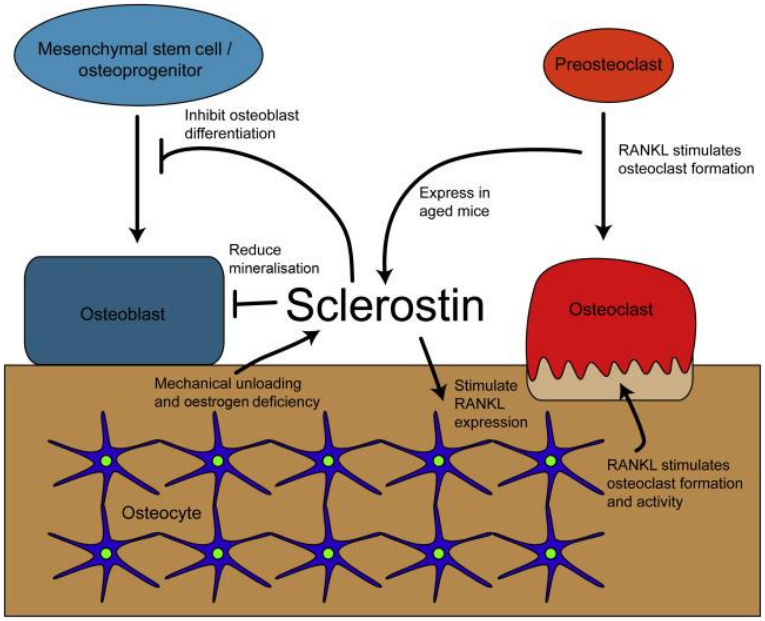


FIGURE 1.1, Sclerostin is secreted by osteocytes and promotes osteoclast differentiation. Various stress signals such as mechanical stress cause osteocytes to secrete sclerostin to prevent further bone formation in order to allow for bone repair and homeostasis. Oestrogen deficiency has been shown to increase sclerostin levels and can contribute to osteoporosis. Once secreted, sclerostin acts to inhibit bone mineralisation through its role as a Wnt pathway antagonist. Sclerostin induces the expression of RANKL which results in osteoclast differentiation. Image taken directly from (Suen & Qin 2016).

Osteocytes appear to be important in bone regulation and are constantly secreting molecules that act on osteoclasts and osteoblasts. With the characteristic traits of osteocytes

being the down regulation of proteins such as collagen I and the upregulation of proteins such as sclerostin, it seems that once osteoblasts have secreted content involved in bone mineralisation, their maturation into osteocytes causes them to become negative regulators of bone formation in response to bone damage. This response initiates signals by osteocytes to start the process of bone resorption which will subsequently follow with bone formation completing a cycle of tight regulation between osteoblasts and osteoclasts.

1.3. METASTASIS

Matricellular proteins play a key role in facilitating the metastatic process. Apart from acquiring the characteristics to invade, a primary tumour needs an ideal environment in which it can migrate to other sites. While the role of SPARC and osteopontin in metastasis are more thoroughly described in introduction sections 1.4.4 and 1.5.3 respectively, a brief summary of the metastatic process as well as the importance of matricellular proteins in this process shall be given here.

The ability for a cancer to metastasise and invade other sites of the body is a hallmark of cancer. The hallmarks of cancer (figure 1.2) are a set of 6 processes that are essential in the survival and propagation of a tumour. Since cancer is not a static process but one that is dynamic and constantly evolving, the hallmarks of cancer were created in order to provide a clear and concise understanding of cancer biology. Hallmarks include the ability for the cancer cell to lose control of the cell cycle and continue to replicate becoming immortal. Cell signalling that cannot be regulated and is constantly turned on also provides the ability for the cancer cells to keep dividing allowing for cancer cell propagation. Most crucially, and probably the most dangerous hallmark of cancer is its ability to escape its primary site and spread to other parts of the body where the cancer will continue to grow. Ninety percent of cancer deaths are caused by metastatic tumours (Hanahan & Weinberg 2011).

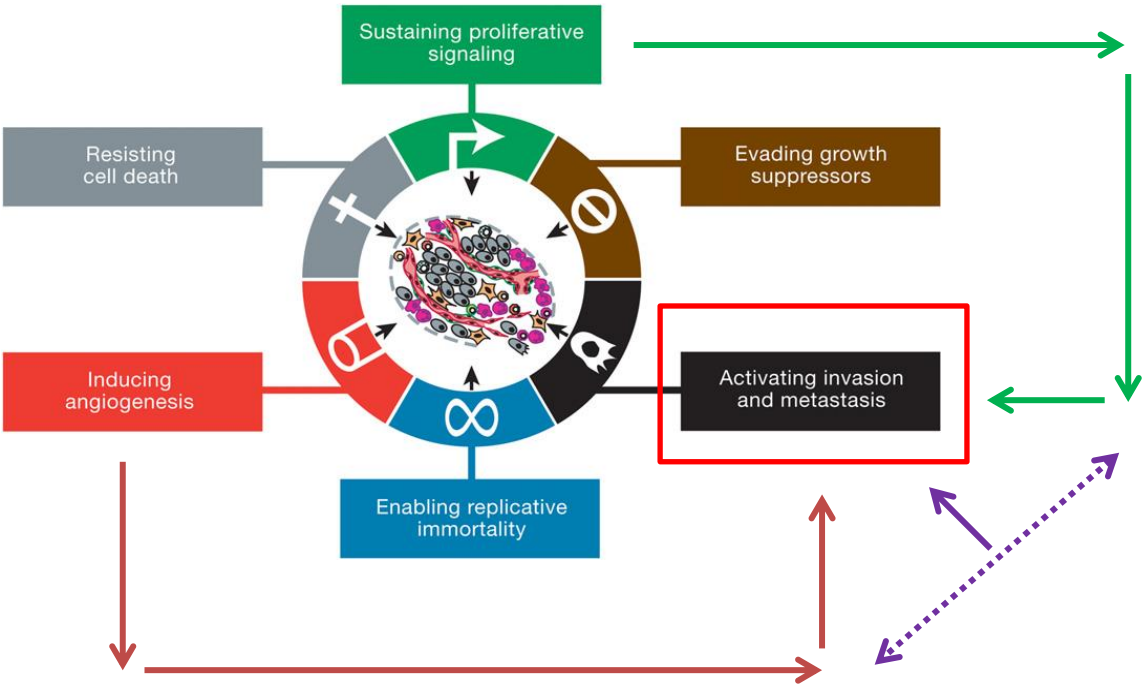


FIGURE 1.2, Essential characteristics of cancer cell survival can be divided into 6 hallmarks. 'Activating invasion and metastasis' allows for a primary tumour to establish secondary and tertiary sites. While the capability to metastasise can be seen as a separate hallmark, other hallmarks such as inducing angiogenesis (red arrows) and sustained proliferative signalling (green arrows) are essential in facilitating the metastatic process and can therefore be linked (although their importance as a separate hallmark should not be underestimated). Cell signalling and induced angiogenesis are also linked and essential in the metastatic process (purple arrow) as cell signalling in the ECM is essential in inducing angiogenic factors for the invasion of metastatic cancer cells through blood vessels. Picture taken and adapted from (Hanahan & Weinberg 2011).

While metastasis is a hallmark of cancer, the process of metastasis is complex and involves various events that lead to an invasive tumour. The process of tumour metastasis is referred to as the invasion-metastasis cascade. The invasion-metastasis cascade is the series of events required for invasion of a primary tumour. The first step in this cascade is the ability to invade through the extracellular matrix and stromal environment, followed by entrance into the blood vessels, survival in the bloodstream, successful establishment of a secondary site, survival in the new environment and finally, successful proliferation at the new site (Valastyan & Weinberg 2011).

It is not entirely understood why some cancers metastasise while others do not and it is not fully understood which tumour cells in the blood stream will establish secondary sites while others may not (Leber & Efferth 2009). However, one theme that is essential to all

metastasising tumours is the ability to invade the surrounding stromal environment in order to enter the blood/lymphatic vessels. Creating an environment ideal for metastasis is achieved through a re-structuring of the extracellular matrix to allow for the processes mentioned above. Matricellular proteins play a key role in facilitating this process (Campbell et al. 2010).

Processes that matricellular proteins have to mediate include cell detachment and migration, angiogenesis to allow for tumour dissemination, induction of matrix metalloproteinases (MMPs) to facilitate ECM degradation and cell signalling to induce the expression of proteins that carry out the functions above, but also carry out signalling events that lead to epithelial mesenchymal transition (Leber & Efferth 2009; Campbell et al. 2010).

The epithelial mesenchymal transition (EMT) is a process by which epithelial cells become mesenchymal in phenotype, therefore acquiring characteristics that allow a tumour cell to be more motile and travel to secondary or tertiary sites. Epithelial cells are highly polarised cells that form sheets and are specialised to the organ or tissue type to which they surround. They connect to the basal lamina and extracellular matrix in the surrounding stroma. They are characterised by tight junctions, held together by a set of proteins known as catenins and cadherins, as well as forming adherens junctions, connecting cells to the ECM. Additionally, epithelial cells express proteins that specifically determine epithelial polarity. A group of proteins known as partitioning defective proteins (PAR), Crumbs (CRB) and Scribble (SCRIB) localise to different parts of the cell where they are able to dictate apical and basal positions of the epithelia (Rodriguez-Boulan & Macara 2014).

Mesenchymal cells are polarised for cell movement. Apart from this, proteins such as cadherin (specifically E-cadherin) are not expressed, and as such these cells are not enriched in proteins in the same way that epithelial cells are. Furthermore, mesenchymal cells are highly motile. A dynamic actin cytoskeleton with ATP dependent motor proteins such as myosin actively works to allow for contraction and movement of these cells. Polarity for the mesenchymal cell is important in this respect to determine which part of the cell is the leading end and which is trailing. Since epithelial cells form before mesenchymal cells in embryogenesis, the EMT is an essential process in allowing for reversion of the epithelial phenotype into cells that can migrate where and when required (Hay 2005).

In cancer, this process is manipulated during the metastatic process. Epithelial cells that become mesenchymal in phenotype can migrate through the ECM, enter the circulatory system and travel to other sites before undergoing the reverse process, mesenchymal to epithelial transition (MET). In order for cancer cells to become mesenchymal in phenotype, various protein expression patterns must change. Important markers of the EMT include transcriptional repressors SNAIL and SLUG (Yoshida et al. 2009) which prevent the expression of E-cadherin, thus allowing cells tightly linked to each other to become individual sets of cells that can become motile. This involves upregulation of N-cadherin, fibronectin and vimentin, proteins important for migration as well as MMP induction in the surrounding environment to allow for cleavage of the ECM and currently formed adherens junctions (E-cadherin being a target of MMPs) (Hay 2005)

1.4. SPARC

One matricellular protein essential to normal ECM function as well as facilitating disease is SPARC.

1.4.1. STRUCTURE

SPARC (Secreted Protein Acidic and Rich in Cysteine) also known as osteonectin is a glycoprotein. SPARC is a protein that regulates cell-matrix interactions but does not form, or contribute to the structure of the extracellular matrix (Yan & Sage 1999). First described in 1981, SPARC was isolated in John Termine's lab as a calcium binding glycoprotein that appeared to be important in the mineralisation of bone (Termine et al. 1981).

SPARC is a 43Kd glycoprotein containing 280 amino acids. In humans, SPARC is located on chromosome 5 q31-33, spans 26.5 kilobases and contains 10 exons and 9 introns (Kurtul et al. 2014).

SPARC bears 70% sequence homology between the mammalian, avian and amphibian variety making it an important matricellular protein whose structure and function has remained conserved during evolution. The SPARC protein is divided into three domains (Yan & Sage 1999; Bassuk et al. 1993; Damjanovski et al. 1992):

Domain I (N-terminal, amino acids 1-52) is a highly acidic calcium binding domain that is rich in aspartic and glutamic acid residues, capable of binding 5-8 calcium molecules as well as binding to hydroxyapatite. Domain I contains the immuno-epitope where antibodies against SPARC will bind (Brekken & Sage 2000).

Domain II (amino acids 53-137) is a follistatin like domain. Follistatin is a secreted protein that inhibits a group of proteins known as activins. Activins belong to the TGF-β superfamily and activate TGF-β receptors leading to the transcription of target genes through the canonical signalling cascade. Activins were initially discovered in gonadal fluid and were found to stimulate the release of follicle stimulating hormone (FSH), however have since then been identified as important signalling proteins in many tissue types (Lotinun et al. 2012; Xia & Schneyer 2009; Wankell et al. 2001). Follistatins act as antagonists and neutralize activin and prevent it from binding to their receptors, thus inhibiting transcription of target genes (Thompson et al. 2005). Similarly, the structure of the follistatin like domain in SPARC has an inhibitory role on growth factor signalling (discussed in 1.4.2.1). The follistatin domain in SPARC has 5 disulfide linkages, contains a highly twisted β-hairpin and a pair of anti-parallel α-helices with an adjacent set of antiparallel β-sheets. Disulfide linkages are formed within the highly twisted β-hairpin and also form between the highly twisted β-hairpin and the anti-parallel α-helices that lead to domain III of SPARC (Hohenester et al. 1997).

Domain III (amino acids 138-280) contains two EF hand motifs that bind calcium with high affinity, unlike domain I, which has a lower binding affinity to calcium (Brekken & Sage 2000). EF hands are evolutionary conserved high affinity calcium binding modules. Characterised by a helix-loop-helix structure, they contain a calcium binding motif 'DxDxDG' (where D is aspartic acid, G is glycine, and x is any amino acid). Flanking this 'DxDxDG' motif are two clusters of amino acid sequences that coordinate the binding of the calcium ions to the motif, 'x, y, z' and '-y, -x, -z'. There is variation in the amino acids these two clusters are composed of but some conserved amino acid residues include phenylalanine, leucine, valine and tyrosine (Denessiouk et al. 2014; Lewit-Bentley & Réty 2000).

In SPARC, a disulphide bond links the 2 α-helices in the second EF hand to stabilise the calcium binding domain. Calcium binding in SPARC induces a conformational change in its structure that allows SPARC to bind to collagens (Delostrinos et al. 2006). Domain III binds to

collagen types I, III, IV and V in a calcium dependent manner (Maurer et al. 1997). Collagen is an essential component of the extracellular matrix and of mineralised tissue. Collagen is composed of 3 peptide chains coiled around each other to form a triple peptide helix (Sherman et al. 2015).

In SPARC, collagen binding occurs in a collagen binding 'pocket' and only domain III of SPARC is capable of binding to collagen. Collagen binding is faciliated by an α-helix present in domain 3 plus the helical loop in the first EF hand. Within this region, calcium binding exposes major residues that will bind to a recognition motif of collagen. These residues are Arginine 149, Asparagine 156, Leucine 242, Methionine 245 and Glutamic acid 246 (Hohenester et al. 2008).

1.4.2. FUNCTION

SPARC has several functions and is expressed in different cells, including bone and epithelial cells. It is associated with development, remodelling and tissue repair (Yan & Sage 1999).

1.4.2.1. INTERACTION WITH GROWTH FACTORS

One role SPARC plays is through its interaction with growth factors. SPARC has been reported to regulate the effects of growth factors involved in angiogenesis (Brekken & Sage 2000). SPARC interacts directly and indirectly with VEGF to inhibit its mitogenic effects. Binding of SPARC to VEGF directly reduces VEGFs capabilities to bind to the VEGF receptor. SPARC also blocks VEGF signalling indirectly. Treatments with VEGF and SPARC in Human Mammary Epithelial cells (HMEC) have shown reduced phosphorylation of VEGF receptors, causing inhibition of phosphorylation of ERK 1 and 2 downstream of receptor activation (Kupprion et al. 1998; Mohanraj et al. 2012).

Furthermore, SPARC interacts with platelet derived growth factor (PDGF). SPARC has been shown to bind to PDGF through domain III, but at a site that is distinct to its collagen binding domain (Motamed et al. 2002; Raines et al. 1992). SPARC also regulates Fibroblast growth factor (FGF) and has been shown to prevent phosphorylation of the FGF receptor indirectly. SPARC does not prevent binding of FGF-2 to the FGF receptor-1 and introduction of recombinant human SPARC into human dermal microvascular endothelial cells (HMVEC)

prevented activation of the ERK 1 and 2 module of the MAPK pathway which prevented DNA synthesis of HMVEC cells. Domain III of SPARC was able to exert these indirect effects on FGF-2 mediated signalling (Motamed et al. 2003).

1.4.2.2. COLLAGEN DEPOSITION

SPARC regulates collagen deposition and bone mineralisation. SPARC regulates the processing of procollagen which is bound to integrin receptors on the cell surface (figure 1.3). In the presence of SPARC, procollagen detaches from its receptor on the cell surface, cleavage of N and C-propeptides takes place, thus allowing collagen fibrils to form. When SPARC is not present, procollagen remains anchored to the cell surface receptor and collagen processing is diminished (Rentz et al. 2007). Studies on SPARC null mice show smaller and more uniform skin compared to wild type mice, as well as the development of cataracts, caused in part by a lack of SPARC-Collagen interactions (Bradshaw et al. 2003).

Proposed mechanism of collagen deposition, adapted from (Rentz et al. 2007).

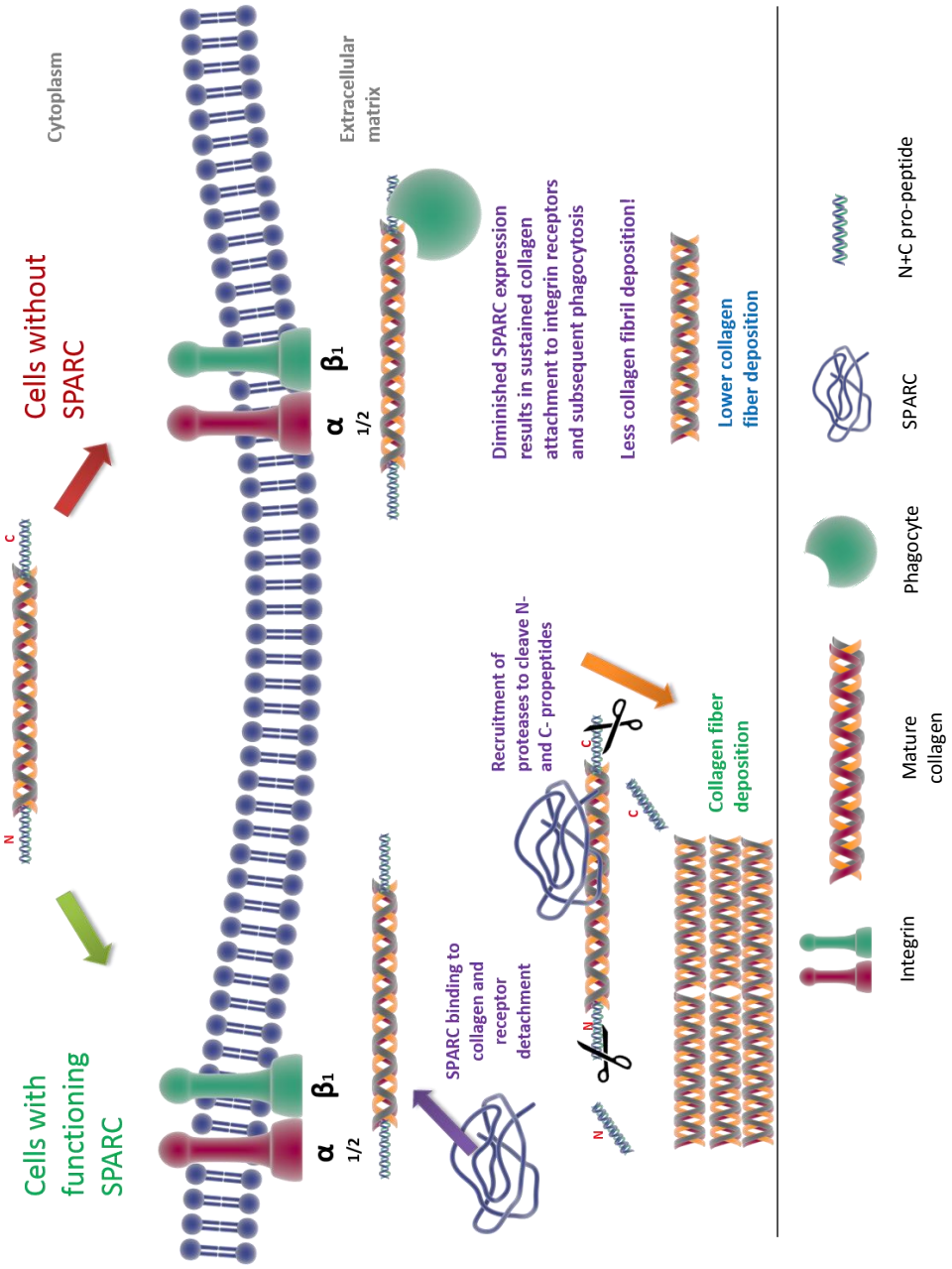


FIGURE 1.3, collagen deposition appears to require the presence of SPARC. SPARC is an important factor in taking collagen away from integrin receptors, recruiting proteases that cleave the propeptide ends of collagen and facilitating collagen deposition. Absence of SPARC keeps collagen anchored to integrin receptors, and promotes a high turnover of anchored collagen by phagocytosis.

1.4.2.3. BONE MINERALISATION

SPARCs ability to bind to calcium and hydroxyapatite (a mineral comprising of calcium and phosphate) allows it to facilitate the mineralisation of bone. These minerals bind to domain

III of SPARC where they are laid precisely in the bone's collagenous matrix which has been secreted by osteoblasts (Renn et al. 2006).

Initial evidence that SPARC was able to facilitate the mineralisation of bone came in 1981 when John Termine et al showed that SPARC was able to bind to hydroxyapatite. They found that SPARC bound with high affinity to hydroxyapatite and type I collagen and was therefore likely to be implicated in the mineralisation of bone. SPARC levels are high in mineralised tissue comprising 20-25% of the non-collagenous component of bone, and 4-6% in dentin (a component of mineralised tissue in teeth). Evidence that SPARC bound to hydroxyapatite was shown when radioactively labelled hydroxyapatite bound to collagen coated tubes in the presence of SPARC in a dose dependent manner, with binding increasing with higher levels of SPARC. Control tubes that did not contain SPARC showed no binding of radioactively labelled hydroxyapatite to collagen. Additionally, SPARC bound to phosphate and calcium with high affinity also suggesting that SPARC could facilitate the transition of these minerals into a crystal phase where it would be incorporated into the collagen lattice (Termine et al. 1981).

Work in mice has shown that knockout of the SPARC gene produced osteopenia, a precursor to osteoporosis. The most affected region resulting from loss of SPARC was trabecular bone (Ribeiro et al. 2014; Delany et al. 2000).

Trabecular bone is present in the epiphyses and metaphyses of long bones. The epiphyses and metaphyses are situated at the distal ends of long bones and serve as sites of active bone metabolism with high levels of osteoblast-osteoclast activity based on mechanical stresses applied to the skeletal system (Oftadeh et al. 2015; Clarke 2008; Matsuo & Irie 2008).

SPARC null mice had less trabecular bone at 11 weeks with a reduction of about 50% of trabecular bone by 17 weeks of age compared to wild type mice. The amount of trabecular bone by 36 weeks had reduced by 70%. In addition, the tensile strength of the femoral bone was reduced and rescue of the SPARC gene was sufficient to return the phenotype resembling wild type mice, implicating SPARC as an important regulator of bone mineralisation (Delany et al. 2000).

1.4.2.4. SPARC IN CELL MIGRATION

As a matricellular protein important in cell-matrix interactions, SPARC has been shown to be important in cell migration. Studies have shown that SPARC is a counter adhesive protein and induces cell detachment through activation of protein tyrosine kinases in bovine aortic endothelial cells (BAE) with a 60% reduction of cell spreading (Motamed & Sage 1998).

SPARC induces the disassembly of focal adhesions which induces cell rounding and spreading. It has been shown that domain II and III (follistatin like and EF hand calcium binding domains respectively) are important for cell migration (Hohenester et al. 1997).

The involvement of these domains induces changes in the intracellular distribution of cytoskeletal proteins. Work in BAE cells has shown that SPARC introduction causes vinculin (a protein involved in focal adhesions as part of a bridge between cells and the extracellular matrix) to become diffuse inside cells and to no longer localise to the cell periphery as part of focal adhesions. Addition of 0.03µM of SPARC to BAE cells was the optimal concentration required to cause disruption of focal adhesions. Diffusion of vinculin away from focal adhesions caused BAE cells to become motile (Murphy-Ullrich et al. 1995).

Work in malignant glioma has shown a mechanism by which SPARC signalling can induce cell migration. SPARC overexpression in D54 and U373MG (malignant glioma cell lines) showed that SPARC was able to induce the activation of focal adhesion kinase (FAK) and integrin linked kinase (ILK), kinases linked directly to integrins. The activation of these kinases led to downstream activation of AKT which was attributed to the invasive nature of malignant glioma cells. The study also identifies the likely interaction of domain II and III of SPARC with integrin receptors to induce cell migration although which specific integrin receptors is involved is unknown (Shi et al. 2007).

The importance of SPARC in cell migration was demonstrated by the knockdown of SPARC in glioma cells, which showed reduced migration in malignant gliomas. Knockdown of SPARC in mice showed localised glioma cell growth with no invasion past the brain, while mice expressing SPARC developed invasive tumours (Seno et al. 2009).

1.4.3. PROTEIN STRUCTURE

A schematic representation of SPARC (figure 1.4) shows the protein structure of each domain. The α-helices and β-sheets are shown with respect to how the amino acids of SPARC arrange themselves across the three domains.

Image taken and adapted from (Ribeiro et al. 2014)

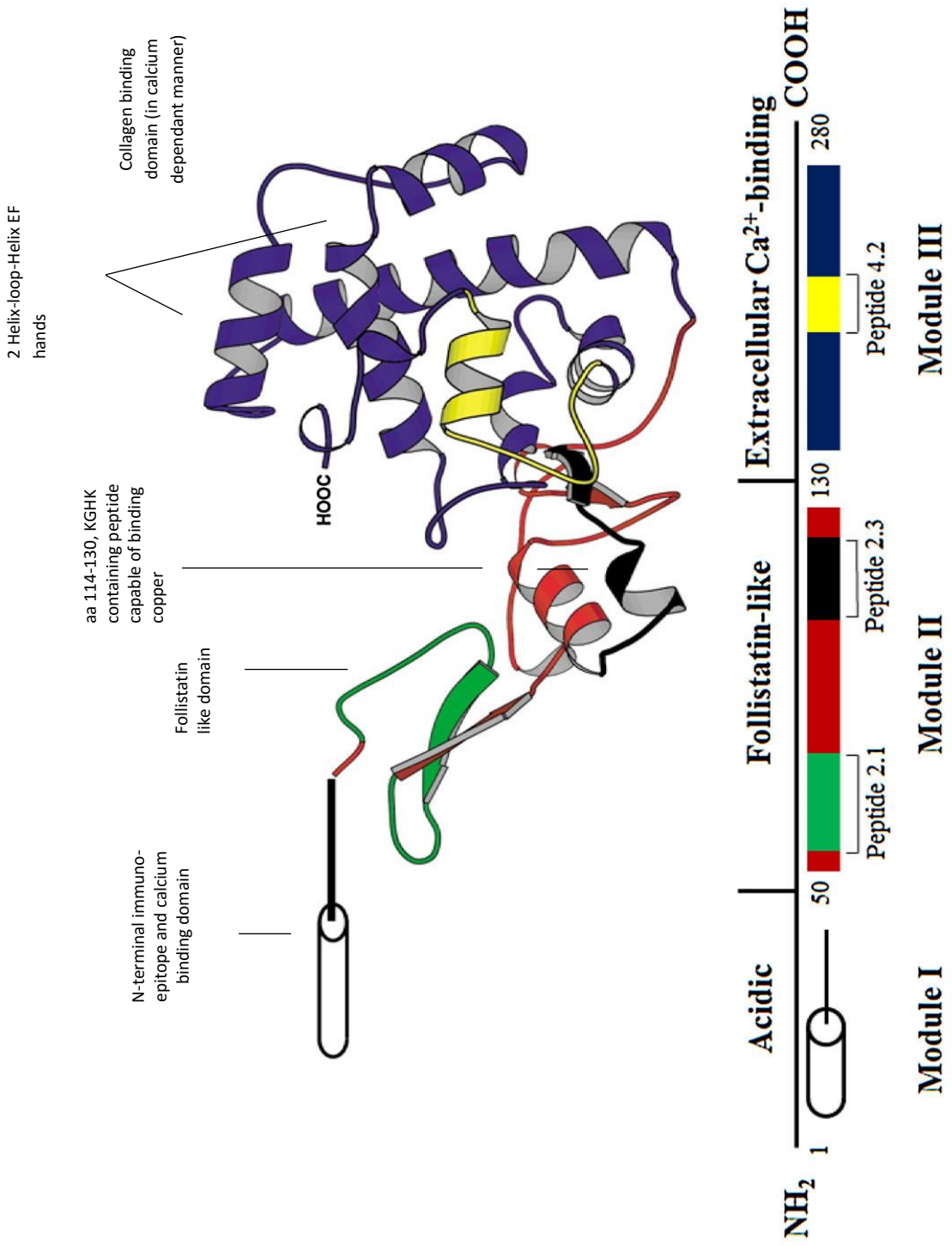


FIGURE 1.4, Protein structure of SPARC shows the three domains (written as modules) with labels highlighting functionally important sections. Domain I (Module I) represents the acidic domain of SPARC that contains the immuno-epitope for antibody binding as well as the ability for this domain to bind to calcium molecules. **Domain II** (Module II) represents the follistatin like domain that exerts an inhibitory role of SPARC towards growth factor signalling

as well as the KGHK (Lysine, Glycine, Histidine, Lysine) copper binding domain which is capable of inducing angiogenesis. **Domain III** (Module III) represents the calcium and collagen binding domain characterised by two EF hands. Collagen binding is important for matrix deposition in the ECM as well as in mineralised tissue.

1.4.4. SPARC IN CANCER

SPARC has been implicated in cancer and metastatic disease because of its involvement in formation and homeostasis of the extracellular matrix. SPARC can facilitate the movement of tumours by inducing the expression of MMPs to degrade the matrix, promoting de-adhesion of cells from the matrix thus allowing them to become motile, and promoting angiogenesis which is important for the formation of a tumour, but also its invasion.

1.4.5. SPARC AND MMPs

SPARC is able to induce the expression of matrix metalloproteinases (MMPs) through its interactions with integrins. Which combination of integrins is unknown, and whether SPARC can activate focal or integrin linked kinase is also unknown, although it is likely that the activation of these kinases will recruit proteins containing SH2 domains that will signal downstream through the MAPK pathway. MMPs are zinc and calcium dependent endopeptidases that are capable of cleaving extracellular matrix proteins (Verma & Hansch 2007).

It has been shown that SPARC upregulates MT-1-MMP and MMP2. In experiments on non-invasive glioma cell lines, exogenous addition of SPARC upregulated MMP2 and MT-1-MMP transcripts 2.2 and 2.3 fold respectively. In addition, galectin-3 (a target of these two MMPs) was found to be proteolytically cleaved when SPARC was added to the cells (McClung et al. 2007). These experiments implicate SPARC in a very important step in the invasion of a tumour, since MMPs such as MMP2 cleave collagens such as collagen IV, which is predominantly found in the basal lamina.

In the cancer microenvironment, this is an essential tool that facilitates migration of the tumour and invasion of the basement membrane is essential for allowing cells to reach the stromal environment.

1.4.6. CLEAVAGE OF SPARC

The follistatin like domain containing the KGHK sequence has been shown to stimulate angiogenesis, meaning a combination of elevated MMP expression and blood vessel formation can stimulate metastatic growth (Lane et al. 1994). The hypothesis that SPARC can be cleaved to release this peptide sequence to induce angiogenesis has been tested in vitro in bovine aortic endothelial cells (BAE). However, whether SPARC is cleaved in the tumour environment remains to be shown. It would make sense however that this is the case in metastasis (as well as SPARCs many other roles). In 2003, Sage et al showed that MMP3 was able to cleave SPARC into three smaller peptides, one of which contained the KGHK sequence, which was able to induce angiogenesis in a concentration dependent manner (Sage et al. 2003).

More recently, studies on mesenchymal stem cells showed that the abridged peptide sequence GHK (Glycine, histidine, Lysine) increased VEGF secretion in a dose dependent manner, which in turn increased endothelial cell proliferation and tubule formation. There was an indication that GHK was able to act through integrin α6β1 in order to induce expression of VEGF. Not only does this confirm that this amino acid sequence from SPARC is capable of inducing angiogenesis, but also showed its possible mechanism of action which was not known before (Jose et al. 2014).

1.4.7. SPARC AS A TUMOUR SUPRESSOR

In addition to its role as tumour promoter, SPARC has also been identified as a tumour suppressor through the vital role it plays in regulating growth factor stimulation, namely the angiogenic growth factors, VEGF, FGF and PDGF, as well as having a reciprocal feedback mechanism with TGF-β. Some cancers such as colorectal and breast cancers show that reduced SPARC expression by hyper-methylation of the SPARC promoter allows tumour progression (Arnold & Brekken 2009).

Recent studies have shown that exogenous addition of SPARC can inhibit cell proliferation in neuroblastoma through mediating PTEN and AKT signalling, which induces C-Jun activation. In turn PTEN levels increase, causing AKT signalling to reduce significantly, inhibiting neuroblastoma cell proliferation (Bhoopathi et al. 2012).

1.5. OSTEOPONTIN

Osteopontin is a highly conserved negatively charged protein that is phosphorylated and glycosylated. In a similar fashion to SPARC, osteopontin is a secreted glycoprotein that is involved in modulation of cell-matrix interactions (Mazzali et al. 2002).

Osteopontin is synthesised as a 32kD protein composed of 333 amino acids. The gene for osteopontin is found on the long arm of chromosome 4 at position 13. Post-translational modification can bring the protein size up to about 75kD in mammals, and the structure of osteopontin is acidic as it is rich in aspartic and glutamic acid residues. Polyaspartic residues are found on osteopontin and this allows for hydroxyapatite and calcium binding (Sodek et al. 2000).

The functions of osteopontin are diverse and include:

- Calcium oxalate regulation
- Bone regulation
- Wound repair

Unsurprisingly, some of the functions of osteopontin overlap with SPARC and like SPARC; osteopontin has also been implicated in cancer (Delany 2010).

1.5.1. CALCIUM OXALATE REGULATION

Osteopontin is involved in the regulation of calcium oxalate crystal formation in the urinary tract. Calcium oxalate is the most common compound found in kidney stones and calcium oxalate monohydrate (COM) as oppose to calcium oxalate dihydrate (COD) is by far the most common form of calcium oxalate crystals found in the urinary tract. COD crystals form but are easily soluble in urine, do not adhere to the same extent as COM and are flushed out without crystal formation in healthy individuals. Should COD not be excreted however, COD becomes COM and crystals begin to form although the process of COD to COM phase conversion is not clear. COM crystals rapidly adhere to renal tissue and fail to solubilise with urine. Crystals begin to grow leading to kidney stones (Chan et al. 2012; Grohe et al. 2006).

Osteopontin is known to inhibit the formation of COM from COD and therefore plays an important role in preventing the formation of kidney stones. Osteopontin knockout mice have been shown to develop calcium oxalate crystals when administered ethylene glycol (an oxalate precursor). In a 4 week study, wild type mice were completely unaffected with no calcium oxalate deposition, while osteopontin knockout mice had significant calcium oxalate crystal formation in the nephron. The study found that the calcium oxalate deposits in osteopontin knockout mice were COM with no COD present, and calcium oxalate crystals were found in the distal nephron and collecting ducts. Together, these results show that osteopontin is an important regulatory protein in preventing Calcium oxalate crystal formation (Wesson 2003).

1.5.2. BONE REGULATION

Like SPARC, osteopontin is involved in bone homeostasis, however, unlike SPARC it is not involved in the mineralisation of bone, but in bone resorption, particularly resorption in response to mechanical stress. Osteopontin is expressed by osteoclasts, osteoblasts and osteocytes and mechanically induced stress in calvariae (skull cap) of mice shows that osteopontin expression significantly increases in osteocytes during the bone resorption phase. Interestingly, a reduction in osteopontin expression results in a lack of bone formation (Fujihara et al. 2006; Morinobu et al. 2003).

It would be interesting to know the role of osteopontin secreted by each of the different bone cell types and if secretion leads to bone resorption each time through signalling to osteoclasts, or if expression by a different cell type results in different biological functions. For example, osteocyte expression of osteopontin might induce sclerostin expression or vice-versa to promote bone resorption. Coupling of these proteins may exist in the scenario where sclerostin might need to initiate osteoclast differentiation via induction of RANKL expression followed by signalling by osteopontin to promote osteoclast mediated bone resorption. Osteoblasts may express osteopontin to inhibit bone mineralisation through steric hindrance which may act as a signal to terminate the process of mineralisation before further signals would result in the termination of osteoblastic activity.

Osteopontin is thought to serve as a signalling molecule in the resorption of bone, with osteopontin interacting with αvβ3 integrins to induce osteoclast activity which is in line with the finding that osteopontin deficient mice exhibit reduction in bone resorption (Yoshitake et al. 1999). As well as its role in signalling and promoting osteoclast activity, osteopontin has been shown to inhibit hydroxyapatite formation and therefore calcification of bone.

Studies to identify domains in osteopontin involved in inhibition of hydroxyapatite formation have shown that phosphorylated sites are absolutely essential. These are located within amino acids 41-52 (phospho-serine 46 and 47) and amino acids 290-301 (phospho-serine 295 and 297). These sequences which also contain glutamic and aspartic acid residues suggest that the high negative net charge plus phosphorylation of serine residues is important in the inhibition of hydroxyapatite formation. It is proposed that phosphorylated residues inhibit hydroxyapatite formation by replacing the phosphate group in hydroxyapatite which then results in physical hindrance of hydroxyapatite formation resulting in no mineralisation (Neve et al. 2011; Pampena et al. 2004).

1.5.3. OSTEOPONTIN AND CANCER

Osteopontin has also been implicated in cancer, and being a matricellular protein, it has been reported to play a role in the progression and advancement of tumours. Overexpression of osteopontin has been found in various tumours such as breast, lung and colorectal cancer. Osteopontin and integrin binding have been well reported, particularly osteopontin binding to integrin αvβ3 during bone resorption by osteoclasts. Integrin upregulation may cause continuous signalling and upregulation of various proteins required to maintain tumour growth and/or facilitate metastasis. In a highly invasive breast cancer cell line (MCF-7), αvβ3 integrin expression was linked to progression of this tumour, and osteopontin binding to this integrin receptor promoted cell survival (Rodrigues et al. 2007). Angiogenesis is another hallmark of metastasis, and osteopontin has also been shown to promote angiogenesis through its interaction with αvβ3. Blocking this integrin has resulted in reduced angiogenesis although the evidence has yet to be verified, and the effect of osteopontin on angiogenesis is far less understood than it is with SPARC (Rodrigues et al. 2007).

Osteopontin has also been shown to be involved in the expression of MMP2 in GCT23 (human osteoclast like cells) although its precise mechanism of action is not fully understood. As mentioned above, osteopontin has been implicated in various cancers. Such observations have been made through gene expression profiles that show that osteopontin is upregulated in these cancers. More work is required to fully understand the roles of osteopontin in cancer and to separate its particular function with respect to metastasis and demonstrate distinct functions; nevertheless it seems that osteopontin is involved in cancer progression despite its roles not being as fully understood as its matricellular counterpart, SPARC (Rangaswami et al. 2006).

1.6. MEMBRANE TRAFFICKING

Membrane trafficking is an important biological function of the cell. Membrane trafficking ensures that synthesised proteins are packaged and sorted appropriately allowing them to reach their final destinations. Membrane sorting begins at the Endoplasmic Reticulum (ER). Proteins synthesised by ribosomes attached to the ER (rough endoplasmic reticulum) are subject to very strict checks by various proteins in the ER. Proteins begin their folding whilst they are being translated. This folding is facilitated by a group of proteins that ensure that peptides are folded correctly and do not aggregate. Even minor faults in folding will lead to newly synthesised proteins being retained in the ER until they are correctly folded and modified for later transport. Proteins assisting folding are known as chaperones and include heat shock proteins, Calnexins and Calreticulins and Thiol-disulphide oxireductases (Ellgaard & Helenius 2003).

These chaperones act also as sensors; unfolded proteins will be picked up by chaperones, whilst proteins folded in their native conformation will be free to leave the ER via COPII coated vesicles towards the Golgi complex.

1.6.1. THE SECRETORY PATHWAY

Following the activities that take place in the ER, proteins become cargo that are stored in specialised bodies known as vesicles which are responsible for delivering them to their

destinations. There are three main pathways in the secretory process facilitated by different **co**at **p**roteins. These are:

- COPI coated vesicle pathway
- COPII coated vesicle pathway
- Clathrin coated vesicle pathway

1.6.2. COPII COATED VESICLES

Each vesicular pathway requires a different set of coat and adaptor proteins that help the vesicle to form. Anterograde (forward) trafficking pathway, which operates from the ER to the Golgi network requires COPII coated vesicles. COPII coated vesicles are comprised of two hetero-dimeric coat proteins, sec13 and 31p, and sec23-24p plus a GTPase protein, Sar1p. Coat recruitment and vesicle formation is triggered by activation of Sar1p at ER exit sites (Kirchhausen 2000).

An ER transmembrane protein called Sec12p facilitates the exchange of a GDP for a GTP on Sar1p. The Sec12p GTPase is restricted to the ER membrane and is therefore the only place that will allow for COPII coat assembly (Jensen & Schekman 2011).

Activated Sar1p will then embed itself into the membrane through an amphiphatic N-terminal α-helix. This activation causes the recruitment of the Sec23-24p complex followed by the Sec13-31p protein complex. Sec23 has a dual role, as well as being a part of the COPII coat, it acts a GTPase activating protein (GAP) which is able to stimulate the hydrolysis of Sar1p-GTP into Sar1p-GDP (figure 1.5). This process occurs during fusion with the target membrane, and allows the coat proteins (Sec23-24p and Sec13-31p) to dissociate. Another protein, Sec16p, is associated specifically with the ER membrane, and is thought to be involved in Sec23-24p and Sec13-31p organisation during vesicle formation (Duden 2003).

Furthermore, it should be noted, that budding from ER-exit sites following the formation of the COPII coat does not require another protein such as dynamin (a constrictive enzyme involved in scission of the vesicle) to allow the vesicle to 'bud' of the membrane. This action is independent of any assistive protein (Jensen & Schekman 2011).

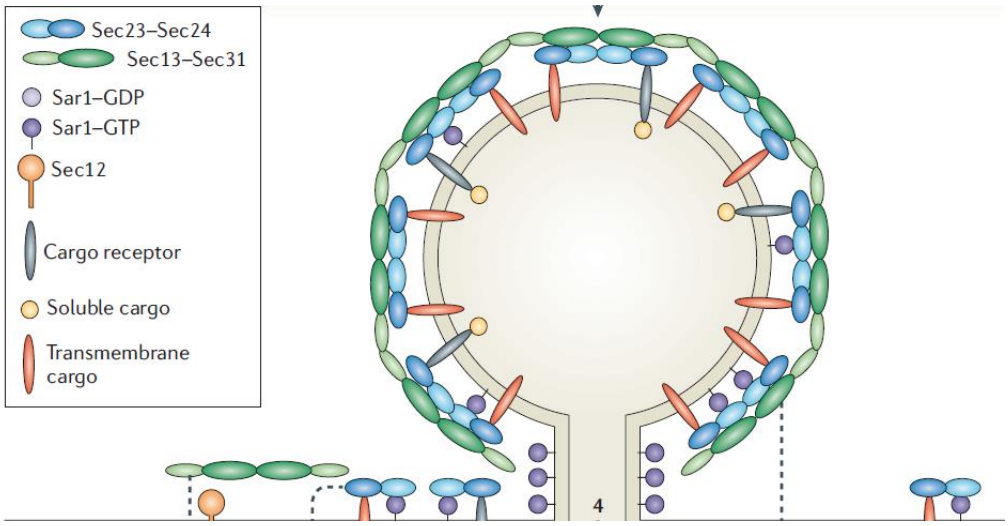


FIGURE 1.5, Assembly of COPII coated proteins requires 5 main proteins, namely Sar1p, Sec13-31p and Sec23-24p. The Sec proteins form a physical coat around the cargo while Sar1p initiates vesicle formation in an active form, Sar1p-GTP. Figure taken and adapted from (Gürkan et al. 2006).

1.6.3. COPI COATED VESICLES

COPI coated vesicles form in a similar fashion; however, their coat proteins and initiating factors are different to the ones in COPII coated vesicles. COPI coated vesicles participate in cargo transport from the Golgi complex to the ER, the pathway known as retrograde (backwards) transport. Escaped ER proteins that have been carried in vesicles along with cargo, as well as SNAREs need to be transported back to the ER. Successful retrieval is dependent on a specific amino acid sequence present on the carboxy-terminus of resident ER proteins. This sequence is KDEL (Lysine, Aspartic Acid, Glutamic acid and Leucine), or KKXX for membrane proteins where 'X' is any amino acid, but the double Lysine is essential as a retrieval signal (Cabrera et al. 2003).

KDEL and KKXX signals present on resident ER proteins are picked up by a KDEL receptor, a 7-transmembrane-domain protein. The receptor belongs to the *ERD2* gene family, and is resident in the Golgi until it picks up cargo and is shuttled back to the ER (Townsley et al. 1993).

Following the binding of KDEL sequence containing proteins to the KDEL receptor, COPI vesicle formation will take place. The COPI coat is comprised of 7 proteins. These are, α, β,β',γ,δ,ε and ξ COP collectively termed coatomer. In addition to the coat proteins, a GTPase ARF1 is responsible for coat recruitment. The ARF1 GTPase protein contains a myristoyl chain (a saturated fatty acid) which is hidden in its inactive GDP bound form. It is not known exactly what happens upon GTP activation, however, it is thought that the myristoyl group is exposed and embeds itself into the membrane allowing it to stabilise itself (Hsu & Yang 2009).

Once ARF1 is activated, the 7 protein components of COPI coated vesicles are recruited to the site of activation, and cargo is incorporated. Coat recruitment and formation causes deformation of the membrane, which allows the vesicle to bud off. Delivery to the ER is dependent on ARF1 GTP hydrolysis which promotes dissociation of the coatomer, fusion with the membrane and release of cargo (figure 1.6).

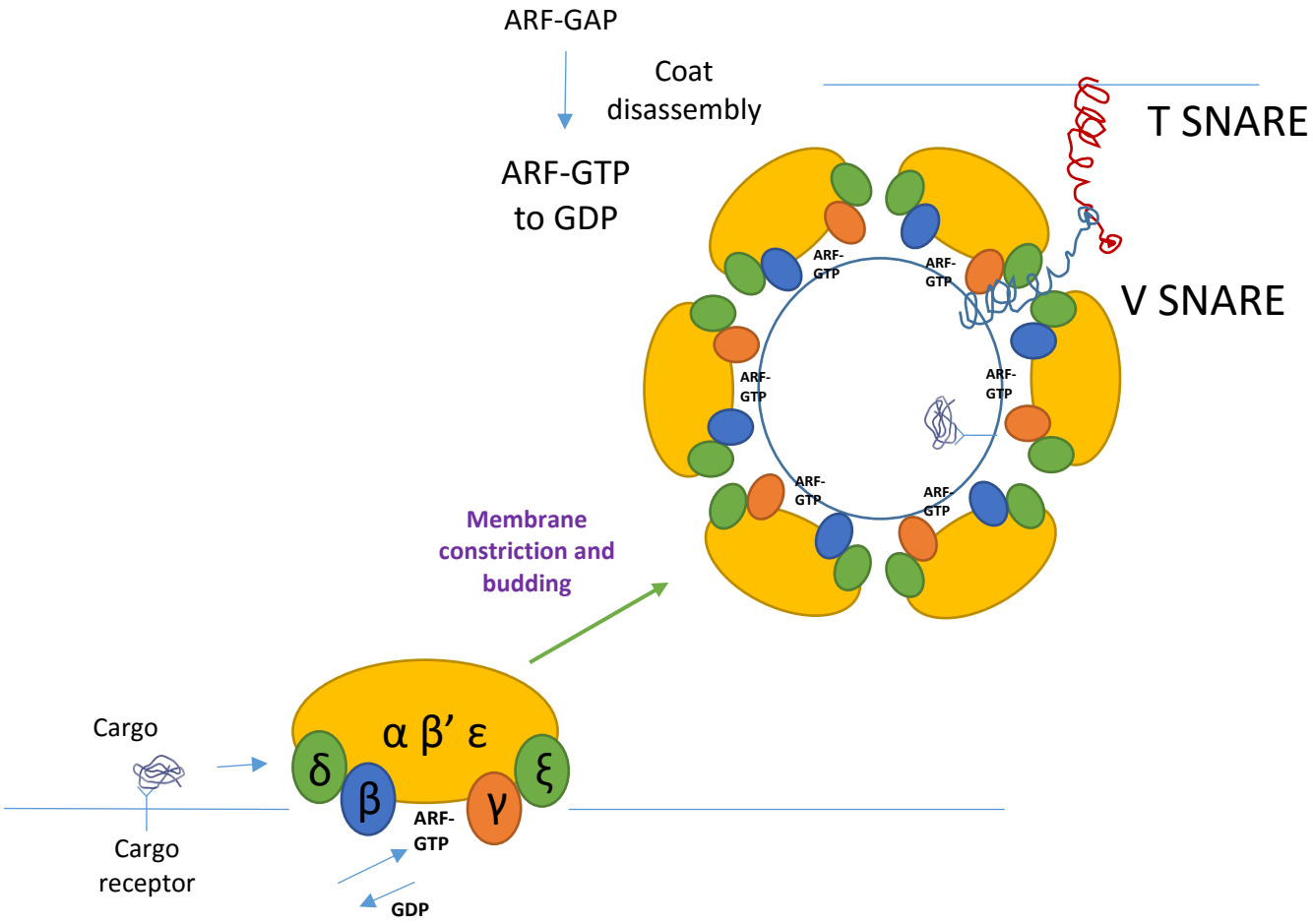


FIGURE 1.6 (adapted from Kirchhausen, 2000), COPI coated vesicles are specific to proteins containing KDEL/KKXX sequences. COPI cargo is very specific, is always bound for the ER and is characterised by the presence of KDEL/KKXX sequences. COPI coats are comprised of 7 proteins and an ARF1 GTPase which drives vesicular formation.

1.6.4. CLATHRIN COATED VESICLES

The final trafficking pathway is the clathrin coated vesicle pathway. Clathrin coated vesicles (CCVs) form from the Trans-Golgi network and form vesicles with cargo bound for the plasma membrane or extracellular space (figure 1.7). Additionally, clathrin coats are used on vesicles in the endocytosis pathway, for cargo that is to be internalised inside the cell. Like COPI coated vesicles, the initiating factor is ARF1, as well as ARF6 (another small GTPase). Clathrin is the main coat that actually forms the vesicle, and is the most abundant vesicle coat that has been identified. A group of proteins known as adapter proteins (AP) are essential for

clathrin coat formation. There are 6 AP proteins, all of which are associated with clathrin coat formation at different sites in the cell. For example, clathrin coated vesicle formation from the Trans-Golgi network is mediated by AP-1, whilst clathrin coated vesicle formation at the plasma membrane for endocytosis is mediated by AP-2 (Robinson & Pimpl 2014; Nakatsu & Ohno 2003).

Adapter proteins link the clathrin coats to the membrane where the vesicles will bud. High concentrations of clathrin at the membrane are known as clathrin coated pits, and it is from here, that vesicle formation takes place. In addition to this role, APs also recognise proteins that will be incorporated into the vesicle as cargo. Like in COPI mediated retrograde transport, cargo carries specific sequences that act as a signals for APs and allow binding. These signals are normally tyrosine and di-leucine based motifs present on the carboxy-terminus of proteins (Godlee & Kaksonen 2013)

Arrestins are another set of adaptor proteins that recruit seven-transmembrane G-protein coupled receptors into clathrin coated vesicles. The recruitment of these GPCRs to the clathrin coated vesicles promotes the internalisation of the vesicle, although its precise mechanism of action is not known (Kang et al. 2009).

Dynamin, is another essential component of the clathrin coated vesicle. Dynamin contains a G domain (a domain required for GTPase activity) which is comprised of three helices at the N and C-terminal sides of the protein. The G domains dimerise in a GTP-dependent manner upon vesicle formation and recruitment. This process is thought to cause constriction of the membrane from which the vesicle is budding, and as a result, the vesicle can be released from the membrane (Ferguson & De Camilli 2012).

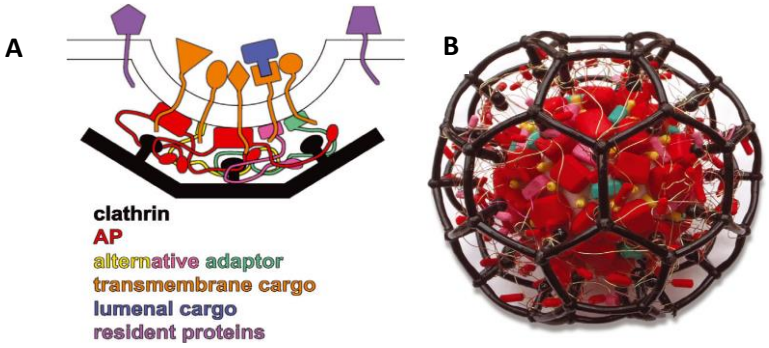


FIGURE 1.7, Clathrin coated vesicle formation is essential for post-Golgi as well as the endocytic pathway. A, A complex set of protein interactions is required for clathrin coat formation and recruitment of cargo proteins is dependent upon recognition by various different adapter proteins that facilitate CCV formation at different sites in the cell. Vesicle budding occurs via constriction of the membrane through dynamin following vesicle formation. **B,** Proposed model of a CCV covered by the triskelion structure of the clathrin protein. Pictures taken directly from (Robinson 2015).

1.6.5. SNARES AND TETHERS

The last essential component to vesicular transport is the SNARE (soluble NSF attachment protein [SNAP] receptor) family of proteins and tethers. SNARES are essential for membrane recognition and fusion. Two types of SNARES exist in the vesicle transport pathway, V-SNARES on the vesicle, and T-SNARES at the target membrane (Kulkarni et al. 2014).

Tethers act separately to SNAREs but are essential in anchoring incoming vesicles in order to allow interaction and formation of SNARE complexes. Tethers such as TRAPPs (transport associated protein particles) are large multi-subunit complexes that are involved in recognising vesicles at locations such as the cis-Golgi face and the plasma membrane. Once bound, tethers position vesicles so that SNARE complexes can form and the fusion step can take place (Miller 2007; Lowe 2000).

Both SNAREs, whether on the membrane or on the vesicle contain coiled coil domains in which α-helices coil around each other, in the same fashion coils are formed on a rope. A hydrophobic C-terminal domain will embed the SNARE into its respective membrane, and factors such as Rab (a small GTPase) and NSF, an ATPase, are required for SNARE activation and recycling of the SNARE complex. V-SNARES will carry a Rab-GTP with them during vesicle transport. A protein known as Sly1/Sec1, embedded in the membrane renders it inactive. Initial contact of a vesicle with the membrane will allow Rab-GTP to displace Sec1, allowing the two SNARES (V and T) to interact. Vesicle and membrane SNARES interact through their

coiled coil region, and 'zip' up where membrane fusion and cargo release takes place. NSF (ATPase) is then recruited to the SNARE complex, and the ATPase activity of NSF disrupts the SNARE interaction, allowing recycling of the SNARE components back to their initial compartments (Rizo & Südhof 2012; Sanderfoot 1999) .

1.7. MITOGEN ACTIVATED PROTEIN KINASE (MAPK) PATHWAY

The Mitogen-activated protein kinase (MAPK) pathway is a cascade of signals propagating through a set of serine/threonine kinases that leads to expression of genes that are involved in processes including cell proliferation, differentiation and development (Zhang, 2002). There are 7 groups of MAPKs including ERK 1 and 2 (Extracellular-signal Regulated Kinase) (figure 1.9), P38 and JNK (C-Jun N-terminal kinase) 1, 2 and 3. The ERK 1 and 2 pathway is the best characterised pathway. ERK 1 and 2 share 83% amino acid identity, and both contain an active 'TEY' (Threonine, glutamic acid and tyrosine) domain. This TEY domain is essential for kinase activity. Phosphorylation by a MAPK kinase (MAPKK) upstream of ERK 1 and 2 must take place on the threonine and tyrosine present in the kinase domain in order for ERK 1 and 2 to be activated (Cargnello & Roux 2011).

MAPK activation begins upstream by various stimuli which act on receptors such as receptor tyrosine kinases (RTK). Using an RTK as an example, ligand binding to an RTK normally induces a conformational change in the receptor which will then dimerise. Dimerisation of receptor tyrosine kinases induces auto-phosphorylation of tyrosine residues in the intracellular domain of the RTK. Phosphorylated RTKs are now binding sites for proteins that will initiate a cascade of signals downstream. These proteins contain SH2 domains (Src homology domain), such as GRB2 (growth factor receptor bound protein 2). The binding of GRB2 recruits a guanine nucleotide exchange factor (GEF), which can facilitate the exchange of a GDP for a GTP on RAS, the initiation factor of the MAPK pathway. RAS will phosphorylate RAF, a MAPK kinase kinase (MAPKKK), which will phosphorylate MEK 1 and 2, a MAPKK, which will phosphorylate ERK 1 and 2, the MAPK. ERK 1 and 2 can enter the nucleus and activate selected transcription factors (Raman et al. 2007).

1.7.1. THE DIFFERENT MAPK PATHWAYS

The MAPK pathways are divided into two groups, namely, the conventional MAPKs and atypical MAPKs (figure 1.8). Conventional MAPKs include ERK 1 and 2, P38, JNK and ERK 5. The conventional MAPK pathways are a cascade of sequential phosphorylations that start from a MAPK kinase kinase (MAP3K) which phosphorylate and activate MAPK kinases (MAP2K) which phosphorylate and activate MAPKs. Upstream activators of the individual MAPKs are unique to each MAPK, for example, MEK 1 and 2 (MAP2K) are specific to ERK 1 and 2 and will not phosphorylate other MAPKs such as p38 or ERK 5 (Avruch 2007).

ERK 5 and P38 are about 50% identical to ERK 2. However, as mentioned above, since the different MAPKs have different upstream activators, neither P38 or ERK 5 are considered different isoforms of ERK 1 or ERK 2 as the MEK kinases are highly specific to their downstream MAPK substrates (Obara & Nakahata 2010).

Less is known about the atypical MAPK pathways but they are so called because they are not activated in the similar fashion to the typical MAPKs. The atypical MAPKs are ERK 3 and 4, ERK 7 and 8 and Nemo-like kinase (NLK). These pathways are normally activated due to stress exerted on the cell (as are the typical MAPKs JNK and p38). Very few specific substrates have been identified for the atypical MAPK pathways, although it is thought that they are involved in various physiological functions. WNT-1 and 5, ligands for the WNT signalling pathway have been shown to activate the NLK MAPK pathway. More work is required to elucidate further the functions of the atypical MAPK pathways (Cargnello & Roux 2011).

MAPK Pathway	Typical/Atypical	Isoforms
ERK 1/2 (Extracellular signal-regulated kinase)	Typical	ERK 1, ERK 1b, ERK1c. ERK 2, ERK2b
P38	Typical	P38 α, β, γ and δ
JNK (C-Jun N-terminal Kinase)	Typical	JNK 1,2 and 3
ERK 5	Typical	ERK 5 a, b and c
ERK 7/8	Atypical	None known
Nemo like kinase (NLK)	Atypical	None known
ERK 3/4	Atypical	None known

FIGURE 1.8, 14 MAPKs exist across 7 individual MAPK pathways. ERK 1 and 2 remain the best characterised MAPK pathway with their function being critical to cell survival. The typical MAPKs are a conserved sequence of activations from MAP3K phosphorylation's downstream to the MAPK. The atypical MAPKs are less well understood and less is known about their activation or specific substrates.

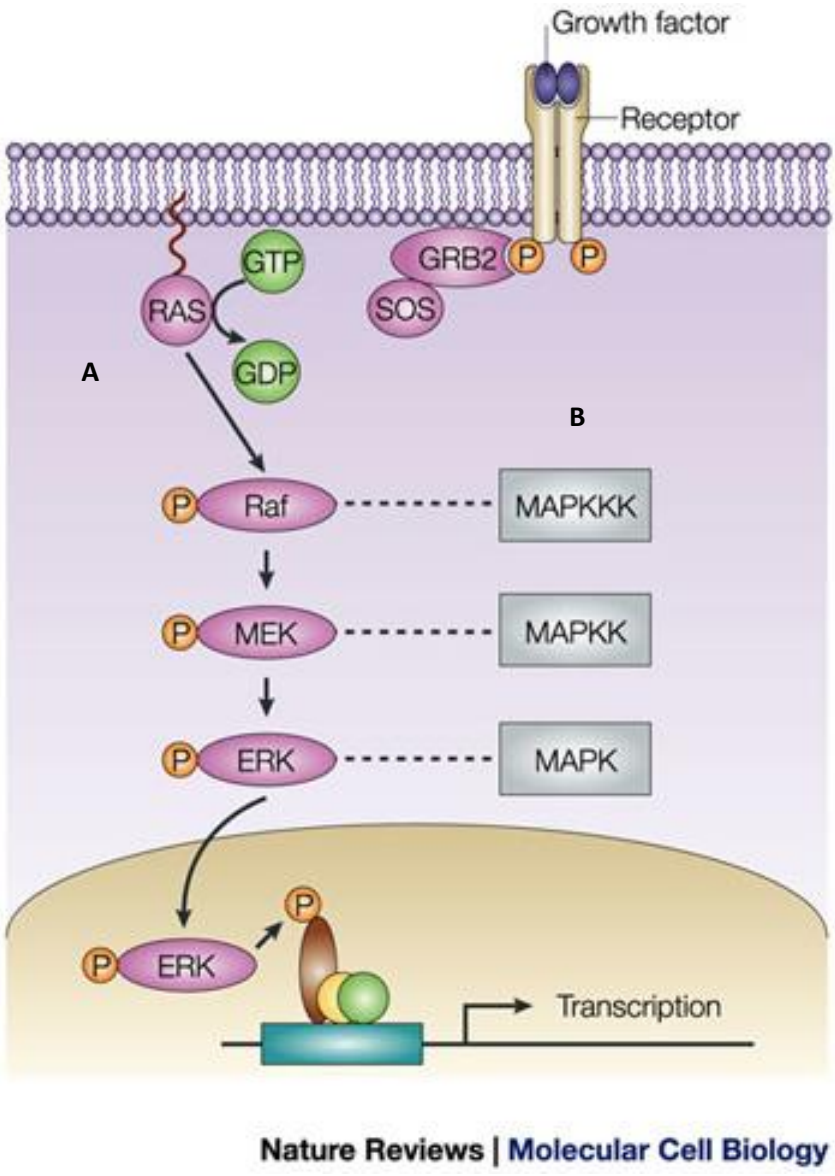


FIGURE 1.9, overview of the ERK module of the MAPK pathway. A, Stimuli outside of the cell can induce receptor tyrosine kinase (RTK) activation through auto-phosphorylation on tyrosine residues. Phosphorylation recruits proteins that begin a cascade of phosphorylations leading to the activation of ERK 1 and 2. Phosphorylated ERK can now activate transcription factors. **B,** simplified process of the MAPK pathway where extracellular stimulus leads to phosphorylation events starting with the phosphorylation of MAP3K which then phosphorylates and activates MAP2K which phosphorylates MAPK which then activates transcription factors by phosphorylating them. Image taken directly from (Kim & Bar-Sagi 2004).

1.7.2. ERK 1 AND 2 KINASES

The ERKs are MAP kinases that are activated in response to extracellular signals. Both ERK 1 and 2 share a high degree of amino acid homology with 83% sequence identity. As serine/threonine specific kinases, ERK 1 and 2 are responsible for activating their substrates through phosphorylation of these residues on target proteins (Roskoski 2012).

Kinases are responsible for transferring the γ-phosphate groups from ATP to their target substrates. Generally, kinases utilise divalent cations such as magnesium (Mg^{2+}) to coordinate water molecules that catalyse the cleavage of the γ-phosphate on ATP before the phosphate is transferred to its substrate (Matte et al. 1998).

The ERK kinases utilise a similar mechanism whereby magnesium cations coordinate the cleavage of phosphate groups with water molecules. Both kinases are divided into an N-terminal lobe and a C-terminal lobe. ERK 1 and 2 (figure 1.10) contain a small glycine rich N-terminal domain (glycine loop) in an antiparallel β-sheet conformation as well as an α-helix that is orientated differently in active or inactive states (with respect to phosphorylation of ERK). The glycine loop reaches over the adenine and will position the γ-phosphate for cleavage while a valine residue in this loop makes hydrophobic contacts with the adenine base (Taylor & Kornev 2011).

The C-terminal lobes of ERK 1 and 2 are largely α-helical and also contain a β-sheet that contains the majority of residues that are responsible for the phosphate transfer to target protein serine/threonine residues. The C-terminal lobe serves as a docking site for ERK 1 and 2 substrates. Three sets of amino acid motifs are essential for the catalytic activity carried out by the two kinases. A KDD motif (Lysine, Aspartate, Aspartate) is important for facilitating the transfer of the γ-phosphate onto target –OH serine/threonine residues , DFG (Aspartate, Phenylalanine, Glycine) is responsible for binding to magnesium molecules to coordinate the α, β and γ phosphate along with water molecules for phosphate transfer. An HRD (Histidine, Arginine, Aspartate) is located in the 'activation loop' of the kinase where Aspartate is likely acting as a base to acquire a proton from the substrate –OH residue to allow for phosphate transfer. In this activation loop, the conserved TEY (Threonine, Glutamic acid, Tyrosine) motif

is present for activation by phosphorylation from MEK 1 and 2 upstream of the MAPK pathway (Roskoski 2012; Taylor & Kornev 2011; Wilsbacher et al. 1999).

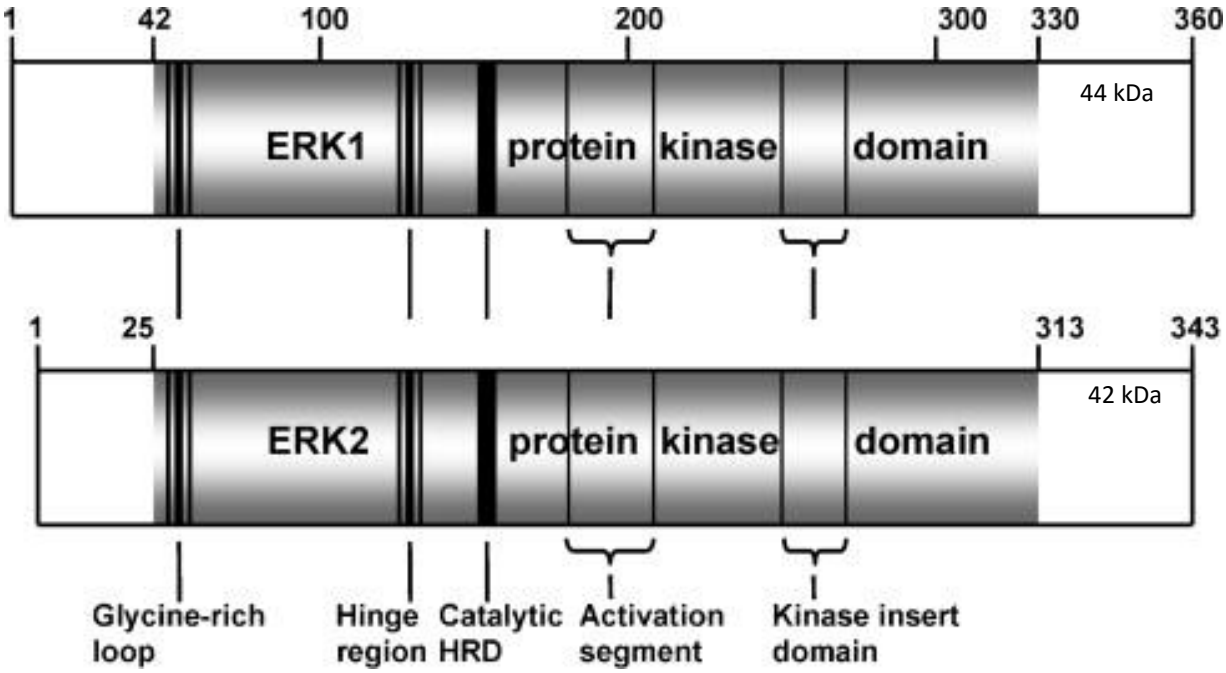


FIGURE 1.10, **Schematic representation of important regions of the ERK 1 and 2 kinases**. ERK 1 and 2 share 83% homology. A glycine rich loop on the N-terminal lobe of the kinases attach to the adenine base of ATP. A hinge region facilitates the movement of the two lobes closer or further away from each other depending on their active state (phosphorylated or un-phosphorylated). The C-terminal lobe contains important amino acid motifs that (upon activation by phosphorylation) coordinate the phosphate groups of ATP along with magnesium cations and water molecules to transfer a γ-phosphate from the ATP onto the substrate. The 'activation segment' also contains the conserved TEY motif that is phosphorylated by MEK 1 and 2 which induces the active state of the ERK kinases. The kinase insert domain is thought to be important for binding of MEK so that it may phosphorylate the TEY motif in the activation segment. Picture taken directly from (Roskoski 2012).

1.7.3. MAPK PATHWAY IN MEMBRANE TRAFFICKING

In addition to its role in a signalling pathway essential for life, MAPK may play other roles that are independent of the traditional signalling cascade that leads to transcription of target genes. There are studies that suggest that MAPK may also be involved in the processing and packaging of vesicles, and that an improper MAPK function leads to impaired vesicle formation or trafficking in general (Barry, 2012). Trafficking of peroxiredoxin 6 (Prdx6) to the

lysosomal compartment was found to be dependent on a functional MAPK pathway, and inhibition of the ERK and p38 pathways caused decreased trafficking of Prdx6, hinting that these signalling pathways are also involved in trafficking and sorting of proteins. In this study 14-3-3ε was identified as a binding partner for Prdx6, which was required for vesicular trafficking of Prdx6 to the lysosome. 14-3-3ε belongs to a family of proteins that interact with over 200 proteins containing phosphorylated serine and threonine residues (Molly Foote 2012). It appears that ERK and P38 are both required for activation of 14-3-3ε to act as a chaperone to Prdx6, as inhibition leads to a lack of entry into the vesicular pathway (Sorokina et al. 2011).

There is evidence that the MAPK pathway is also involved directly in the early secretory pathway. As well as binding partners such as 14-3-3, MAPK has been shown to phosphorylate components of the secretory pathway. MAPK and specifically ERK 2 was shown to phosphorylate sec16, an ER membrane protein that interacts with COPII components and is involved in ER export (Montegna et al. 2012).

Inhibition of ERK 1 and 2 lead to a decrease in ER exit sites. Individual knockdown of ERK 1 or 2 by siRNA showed that knockdown of ERK 2 significantly decreased ER exit sites while knockdown of ERK 1 showed little or no effect suggesting that the role of ERK 2 in this response was specific. Furthermore, re-introduction of ERK 2 increased ER exit sites. Site directed mutagenesis at threonine 415 to Isoleucine reduced the ability of ERK to phosphorylate sec16 suggesting that this could be the site of phosphorylation in sec16. Precisely what phosphorylation of sec16 achieves is unknown, however, it is likely that phosphorylation recruits sec16 to ER exit sites, serving as the site for COPII formation (Farhan et al. 2010).

AIM

Cross-talk between the stromal environment and cells is an essential form of communication. Processes such as angiogenesis, cell migration and bone regulation require this communication. Matricellular proteins like SPARC and osteopontin are at the heart of these processes and mediate cell-matrix interactions as described in the introduction. In diseases like cancer, specifically metastatic disease, proteins like SPARC and osteopontin have been shown to be upregulated to facilitate tumour invasion. We are interested in understanding how SPARC and osteopontin secretion is regulated before they themselves carry out their regulatory functions in the extracellular space to mediate cell-matrix interactions.

Our current hypothesis is that the MAPK (ERK 1 and 2) signalling pathway is involved in the intracellular trafficking of SPARC and osteopontin. Using GFP-SPARC and endogenous osteopontin, we aim to investigate the transport processes after knockdown of ERK 1 and 2 by siRNA and inhibitor treatment using the U0126 inhibitor.

Experiments planned to investigate our hypotheses are:

- Transfection of siRNA specific for ERK 1 and 2 individually into ROS cells
- Total inhibition of the MAPK pathway (ERK 1 and 2 together) using a small molecule inhibitor U0126
- Western blot analysis to measure efficiency of knockdown as well as the expression of other proteins such as SPARC and osteopontin
- Immunofluorescence staining to look at the localisation of SPARC and osteopontin inside the cell in untreated and treated conditions
- Subcellular fractionation to look at the localisation of SPARC and osteopontin to identify organelles they are located in
- ^{35}S labelling to measure the rate of secretion of matricellular and extracellular matrix proteins. If the MAPK pathway affects the trafficking of osteopontin and SPARC, this might be reflected in the amount of protein content secreted
- Cell counts to see how inhibition of ERK 1 and 2 affect the growth of osteosarcoma ROS cells over various time points.

- STRING analysis to look at protein-protein interactions to identify proteins that interact with ERK 1 and 2
- Analysis of ERK binding proteins and their ability to be phosphorylated/bind to ERK as substrates

2.0. MATERIALS AND METHODS

2.1. MATERIALS

Cell growth medium (complete DMEM)

- Dulbecco's Modified Eagle Medium (DMEM) with 1g/L glucose (Gibco, ThermoFisher Scientific, UK)
- 10% Foetal calf serum (Invitrogen, UK)
- 1% penicillin and streptomycin (Invitrogen, UK)

Lysis buffer

- 4.8% Sodium Dodecyl Sulphate (SDS) (Sigma Life Sciences, UK)
- 8% sucrose (Sigma Life Sciences, UK)
- 2M urea (Sigma Life Sciences, UK)

SDS acrylamide gel, separating layer

- Distilled H_2O (up to final volume/gel)
- 12% acrylamide mix (National Diagnostics, Fisher Scientific, UK)
- 0.375M Tris (pH 8.8) (Sigma Life Sciences, UK)
- 0.1% (SDS) (Sigma Life Sciences, UK)
- 0.1% ammonium persulfate (Sigma Life Sciences, UK)
- 0.001 % TEMED (National Diagnostics, Fisher Scientific, UK)

SDS acrylamide gel, stacking layer

- Distilled H_2O (up to final volume/gel)
- 3.75% acrylamide mix
- 0.125M Tris (pH 6.8)
- 0.1% (SDS)
- 0.1% ammonium persulfate
- 0.001 % TEMED

SDS loading buffer

- 50mM Tris. Cl (pH 6.8)
- 100mM dithiothreitol (Sigma Life Sciences, UK)
- 2% SDS
- 0.1% bromophenol blue (Fisher Scientific, UK)
- 10% glycerol (Sigma Life Sciences, UK)

Phosphate Buffer Saline (PBS) (Oxoid, ThermoScientific, UK)

- 8 g/l of sodium chloride
- 0.2g/l of potassium chloride
- 1.15 g/l of Di-sodium hydrogen phosphate
- 0.2 g/l of potassium dihydrogen phosphate

Enhanced Chemiluminescence

- 0.1M Tris pH8.8 in solution A
- 90mM Coumaric acid (Sigma Life Sciences, UK) in solution A
- 250mM Luminol (Sigma Life Sciences, UK) in solution A
- 0.1M Tris pH8.8 in solution B
- 30% hydrogen peroxide (Fisher Scientific, UK) in solution B

Reagents for immunofluorescence staining

- Citifluor antifade (Citifluor Ltd. UK)
- 5% FBS in PBS (FBS from Invitrogen, UK)

siRNA

- Flexi tube MAP kinase 1 (SA Biosciences, Qiagen, UK)
- ON-TARGETplus siRNA MAP Kinase 3 (Dharmacon, GE Healthcare, Life Sciences, UK)

Plasmid construct

- EGFP:SPARC (kindly provided by Dr Luminita Paraoan, University of Liverpool)

Transfection reagents

- Nanofectin (PAA laboratories, UK)
- HiPerfect (Qiagen, UK)
- JetPrime (PolyPlus, UK)

Permeabilisation buffer

- 20mM HEPES (Sigma Life Sciences, UK)
- 300mM sucrose
- 50mM sodium chloride (Sigma Life Sciences, UK)
- 3mM magnesium chloride (AnalaR, VWR, UK)
- 0.5% Triton x 100 (Fluka, UK)
- 10% sodium azide (Fluka, UK) in 1 ml H_2O

HES (HEPES, EDTA and Sucrose) buffer

- 20mM HEPES (Sigma Life Sciences, UK)
- 1mM EDTA (Sigma Life Sciences, UK)
- 250mM Sucrose

OptiPrep

- Iodixanol solution prepared at different percentages from a 50% Iodixanol solution diluted in water (Axis Shield, Sigma Life Sciences, UK)

Breaking Buffer

- 10mM Tris
- 1mM EDTA
- 1mM EGTA (Sigma Life Sciences, UK)
- 250mM Sucrose
- Protease inhibitor (Roche, Sigma Life Sciences, UK)

2.2. METHODS

2.2.1. TISSUE CULTURE

Rat Osteosarcoma cells (ROS 17/2.8) were maintained in T75 flasks, and supplemented with complete DMEM media. Cells were grown at 37°C in an incubator with 5% CO_2. Cells were trypsinised with 0.25% trypsin-EDTA (Sigma Life Sciences, UK) after reaching confluency and passaged to keep the cells dividing.

2.2.2. TRANSFECTION

Transfections were carried out to deliver siRNA targeting ERK 1 or ERK 2 and transfections were also carried out in order to deliver a plasmid with a DNA sequence for SPARC conjugated to green fluorescent protein. Three transfection reagents were used to transfect siRNA for ERK 2 (table 2.0). Nanofectin and HiPerfect were used during initial experiments, and JetPrime was used later on. JetPrime only was used to transfect ERK 1 siRNA. JetPrime and Nanofectin were used to transfect both GFP-SPARC and siRNA (table 2.0 and 2.1). 10-50nM of siRNA was transfected in the experiments, on cells seeded on 13mm glass coverslips (for immunofluorescence) or without coverslips for SDS-PAGE in 24 well plates or 6 well plates. Cell density was dependent on the transfection protocol. For GFP-SPARC, total DNA concentration was dependent on transfection reagent used (table 2.1). Transfections were allowed to take place over 48 hours for all transfection reagents used. After 48 hours, cells were fixed on coverslips, or cell lysates taken for proteins to be run on SDS-PAGE.

TABLE 2.0, siRNA transfection

Transfection Reagent	Cells seeded	Concentration (nM)	Transfection reagent volume (µl)	Incubation (hours)
JetPrime	24-well: 25,000 6-well: 100,000	10 and 50	24-well: 2 6-well: 4	48
HiPerfect	24-well: 25,000	5 and 10	3-4.5	48
Nanofectin	24-well: 30,000	10 and 20	2.5-3.3	48

TABLE 2.1, DNA transfection

Transfection Reagent	Cells seeded (24-well)	Total DNA (µg)	Transfection reagent volume (µl)	Final volume of transfection mixture added to well (µl)	Incubation time (hours)
JetPrime	25-40,000	0.5	2	55	48
Nanofectin	50,000	1	3.2	50	48

Another method used to transfect cells was electroporation with the Neon transfection apparatus (Invitrogen, UK). Electroporation was carried out to transfect the GFP-SPARC plasmid. Electroporation uses an electric pulse that passes through the cells causing the plasma membrane to open slightly revealing holes through which the plasmid can pass through. ROS cells were trypsinised and re-suspended in a 50 ml vial. 100,000 cells would be seeded onto 24 well plates (per well) on 13mm glass cover slips, or in the absence of cover slips for running protein samples on SDS-PAGE. ROS cells were counted on a counting chamber and media containing total number of cells required for electroporation was transferred into a new vial, centrifuged for 5 minutes at 1500 RPM and re-suspended in 1 ml of PBS. Once re-suspended in PBS, cells were centrifuged once more for 3 minutes at 1500

RPM. PBS was aspirated and cells re-suspended in 10 µl per 100,000 cells of re-suspension buffer. DNA was then added to the cells in re-suspension buffer and the solution transferred into the electroporator (1400V, 20ms and 1 pulse). Cells were then incubated for 48 hours.

2.2.3. WESTERN BLOT

Following experiments on cells grown in tissue culture, cell lysates were taken and pipetted into an Eppendorf tube containing glass wool. Samples were centrifuged at 13,000 RPM for 5 minutes, and supernatants were then collected and stored in fresh Eppendorf tubes at -20˚C.

On the day of western blotting, cell lysates were thawed and 10 µl of cell lysate was added to 2µl of 5x SDS sample buffer. Once cell lysate was mixed with SDS sample buffer, samples were boiled for 5 minutes and then centrifuged for 3 minutes at 13,000 RPM. Samples, along with 5µl of pre-stained protein marker 'color plus' (New England BioLabs Inc., UK) were loaded into the wells of a 12% SDS acrylamide gel. Gels were prepared on the day of running SDS-PAGE and run on a mini-PROTEAN® vertical electrophoresis tank (BioRad, UK). Once loaded, samples were run for one hour at 25mA/gel constant current.

After an hour, a membrane 'sandwich' was prepared with a cassette containing (in order) sponge, filter paper, acrylamide gel with separated proteins, nitrocellulose membrane, filter paper and sponge. With the nitrocellulose membrane superimposed on the gel, the cassette was closed, placed in the tank, and an ice pack and magnetic stirrer added into the tank, in order to ensure the transfer buffer stayed cool. The transfer was run for one hour at 100V constant voltage.

Following the transfer phase, the nitrocellulose membrane was transferred into a petri dish and stained with 5% Ponceau S in acetic acid (Sigma Life Sciences, UK) for 3 minutes. After 3 minutes, the membrane was washed in deionised water to reveal the protein bands. The membrane was wrapped in saran wrap, and scanned for later reference. After scanning, the membrane was returned into the petri dished and washed twice for 10 minutes (5 minutes per wash) with PBS-Tween. After the two washes, 5% Marvel milk powder in PBS-Tween was added onto the membrane and the membrane blocked for an hour.

Following the blocking phase, the membrane was washed for 10 minutes (5 minutes per wash) in PBS-Tween, and a primary antibody (table 2.2) was added onto the membrane and left on an orbital shaker in the cold room at 4°C overnight.

TABLE 2.2, Antibodies

Antibody	Primary/Secondary	Host	Dilution WB	Dilution IF	Company
Anti-Osteopontin (MPIIIB10)	Primary	Mouse	1/500	1/200	Developmental studies Hybridoma Bank
Anti-SPARC	Primary	Mouse	1/2000	-	Haematologic technologies Inc.
Anti-GM130	Primary	Mouse	1/1000	-	BD Biosciences
Anti-Rab27	Primary	Rabbit	1/1000	-	Synaptic Systems
Anti-ERGIC-53	Primary	Mouse	1/250	-	Enzo Life Sciences
Anti-α-tubulin	Primary	Rat	1/1000	-	Abcam
Anti-ERK 1 and 2	Primary	Rabbit	1/1000	-	Cell Signaling Technology
Anti-phospho-ERK 1 and 2	Primary	Rabbit	1/1000	-	Cell Signaling Technology
Anti-COPI	Primary	Mouse	1/1000	-	Sigma
Anti-ERp72	Primary	Rabbit	1/1000	1/100	Cell Signaling Technology
Anti-LAMP-1	Primary	Rabbit	1/1000	1/500	Abcam
Anti EEA	Primary	Mouse	-	1/100	BD Biosciences
HRP conjugated anti-rabbit	Secondary	Goat	1/3000	-	Bio-Rad Laboratories
HRP conjugated anti-mouse	Secondary	Goat	1/3000	-	Bio-Rad Laboratories
HRP conjugated anti-rat	Secondary	Goat	1/5000	-	Sigma
Alexa Fluor® 488/546 anti-mouse	Secondary	Goat	-	1/100	ThermoFisher Scientific
Alexa Fluor® 488/546 anti-rabbit	Secondary	Goat	-	1/100	ThermoFisher Scientific
Alexa Fluor® 488 conjugated Helix pomatia agglutinin	-	-	-	1/200	Invitrogen

WB: Western blot **IF:** Immunofluorescence

The primary antibodies were then saved for later use, and membranes washed for 10 minutes (5 minutes per wash) in PBS-Tween. HRP-conjugated secondary antibodies (table 2.2) were put onto membrane in 5% milk in PBS-Tween. Membranes were left on orbital shaker for 45 minutes at room temperature.

After 45 minutes, membranes were washed three times, twice at 5 minutes, and once at 15 minutes. Enhanced chemiluminescence solutions were made in two separate solutions, A, and B. After final wash, membranes were taken to the developing room, where solutions A and B were mixed together and placed on membrane for 5 minutes. After 5 minutes, membranes were drained on tissue paper, wrapped in saran wrap, and developed. The initial exposure was for one minute and then adjusted according to signal density.

Densitometry analysis was carried out by scanning developed films, and then relative signal intensity was quantified using ImageJ.

Each lane was normalized against a protein that is constantly expressed. α-tubulin was selected as the loading standard of choice unless specifically stated otherwise. In order to make a comparison of protein expression/activation between treatments, the density value of the treated/control cell was divided by the density value of its respective α-tubulin. The calculation is as follows:

Normalization= Density value from control cell/Density value from constitutively expressed protein

2.2.4. IMMUNOFLUORESCENCE STAINING

Prior to immunofluorescence, ROS cells were seeded on 13mm coverslips in 24 well plates. Seed density was dependent on transfection/experiment protocol, but typically, between 25,000-60,000 cells were seeded on one coverslip. Following tissue culture, seeded cells were allowed to adhere and grow for up to 48 hours (depending on the protocol for a procedure) before fixation. Media was aspirated from the wells, and washed three times with PBS. 4% Paraformaldehyde (TAAB Laboratories, UK) in PBS was then added onto the cover slips (about 200µl for a 13mm coverslip) for 10 minutes at room temperature. After 10

minutes, coverslips were washed three times with PBS, leaving the last wash in the well in order to keep the coverslip moist until immunostaining procedures were carried out.

PBS was aspirated from the coverslips, and cells were permeabilised for 5 minutes in permeabilisation buffer. After 5 minutes, permeabilisation buffer was aspirated, and coverslips washed 3 times with PBS. Cells were then blocked for 30 minutes in PBS-FCS wash buffer. After 30 minutes, coverslips were transferred into an incubation chamber with wet tissue to prevent evaporation and parafilm to hold the coverslips. 30µl of PBS-FCS wash buffer containing a primary antibody directed against a specific protein (list of antibodies in table 2.2) was added onto the cells. Cells were incubated for 30 minutes at room temperature. After 30 minutes coverslips were drained of primary antibody, dipped into FCS-PBS wash buffer, and then transferred back into 24 well plate containing PBS-FCS wash buffer to be washed for 30 minutes. Coverslips were then transferred back into the incubating chamber with PBS-FCS wash buffer containing secondary antibody (antibodies used in table 2.2) and probes for DNA (Hoechst 33342 (1/10,000), Invitrogen, UK). In some experiments, an actin cytoskeleton probe (phalloidin 488/568 [1/200], Fisher Scientific, UK), marker of the Golgi apparatus (Helix Pomatia Agglutinin 488 [1/200], Invitrogen, UK) or a marker of the endoplasmic reticulum, rhodamine labelled concanavalin A (1/1000, Vector Labs, UK) was also used and incubated with secondary antibodies. Secondary antibodies were allowed to bind in the dark for 30 minutes. Coverslips were then transferred back into 24-well plates for 30 minutes to be washed, before they were mounted on superfrost (Fisher Scientific, UK) slides, cells side facing up, with citifluor antifade added onto the coverslips. Cell containing coverslips were covered with a large coverslip to prevent dehydration whilst viewing under a fluorescence microscope. Generally, about 100 cells were analysed per coverslip per treatment when looking at localisation of proteins or co-localisation of proteins with compartments. Individual experiments were carried out more than once and specific numbers of each experiment carried out are detailed in the respective results chapters.

2.2.4.1. IMAGE ACQUISITION AND ANALYSIS

Cells were imaged using a fluorescence microscope (Leica DM4000, UK). Exposure and gain used was kept the same for all treatments carried out in an experiment. Once captured,

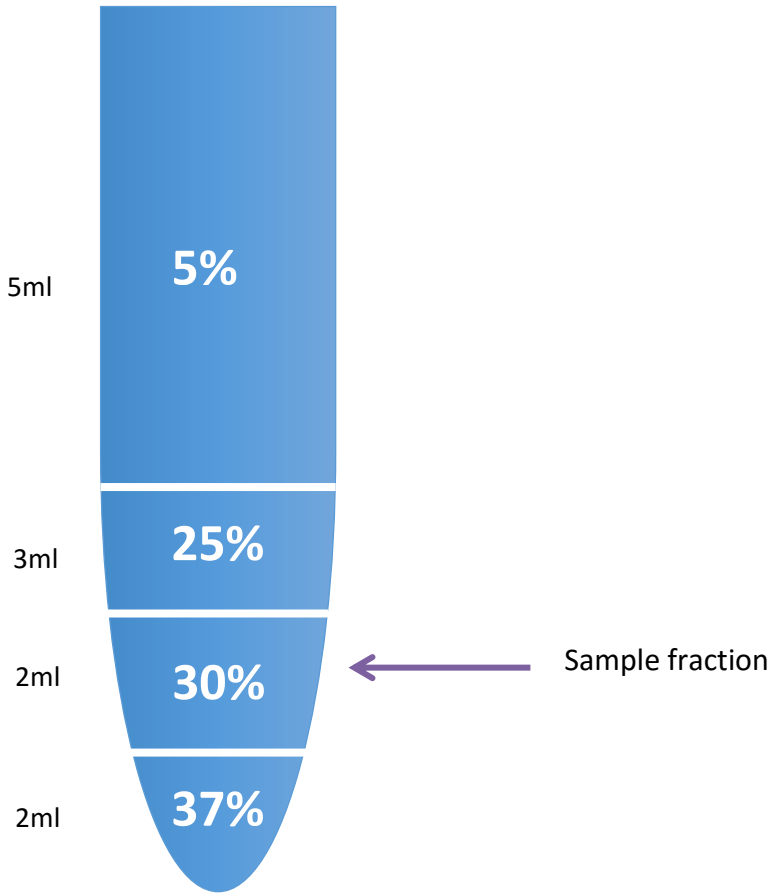
images were converted to the TIFF format before being analysed in ImageJ. Representative cells were cropped before being used for presentation.

Co-localisation was measured using the Leica Microsystems LAS AF software. Graphs were produced of intensity of fluorophores versus distance. Peaks that overlapped perfectly (i.e. green and red channels peak overlap) at the same location inside the cell were deemed co-localised. Peaks that did not overlap did not occupy the same space and therefore were not counted as co-localised.

2.2.5. SUBCELLULAR FRACTIONATION

Subcellular fractionation was carried out in order to separate compartments within the cell to show the localisation of proteins of interest. ROS cells were grown to confluency in three T75 flasks to achieve a final cell number of approximately 12 million cells. Once cells were fully confluent (90-100%), media was aspirated and cells washed with PBS three times at room temperature. After PBS washes, a further wash step was carried out with ice cold wash buffer (10mM Tris pH 7.5) for one minute. Wash buffer was aspirated and 500 µl of breaking buffer was added into one flask. Cells were scraped with a cell scraper before breaking buffer and cell suspension was collected and transferred into second T75 flask. Again, cells were scraped with a cell scraper before process was repeated for the third flask too. Breaking buffer and cell suspension was collected from third flask and transferred to labelled 2ml Eppendorf tube. 500 µl of fresh breaking buffer was added to first flask and process above was repeated one more time in order to collect as much cell suspension as possible. Cells were passed through a ball homogenizer (EMBL) with cell suspension passing through a very narrow (0.8mm) opening in order to permeabilise the cells. Cells were passed between two (15 ml) syringes (4 strokes). Lysate suspension was collected and added back into Eppendorf tube. Cells were centrifuged (Heraeus microcentrifuge) at 1000g (3500RPM) for 10 minutes. After 10 minutes, supernatant was collected and spun for a further 20 minutes at 8000g (10,000 RPM). Pellet from first spin (1000g) was re-suspended in 1ml breaking buffer and kept on ice. After the 8000g spin, supernatant was discarded carefully in order to ensure pellet remained intact. Pellet was then re-suspended in 1 ml breaking buffer and 50% OptiPrep was added in order to achieve a final density of 30% OptiPrep. Breaking buffer was

added to this suspension to bring volume up to 2ml. Eppendorf tube was vortexed to ensure proper mixing. OptiPrep gradient was then assembled on a 14ml Beckman SW-40 centrifugation tube as follows:



Once assembled, the gradient with sample was centrifuged for 16 hours at 150,000g on a Beckman SW-40 swinging rotor.

After centrifugation, 1ml samples were slowly and carefully taken from the top creating 12 fractions. 10µl of each fraction was run on a 12% SDS gel in order to check where proteins were localised (see 'Western Blot' section 2.2.3 in Materials and Methods).

2.2.6. PROTEIN SECRETION ASSAY

Protein secretion using labelling of radioactive ^{35}S methionine/cysteine (PerkinElmer, UK) mixture was carried out in order to look at the total amount of secreted protein. 150,000 ROS cells were seeded in each well of a 6 well plate and incubated at 37°C and 5% CO_2 for 48 hours. On the day of the experiment, cells were incubated with methionine free DMEM (Gibco, ThermoFisher Scientific, UK) plus 10% dialyzed FBS (Invitrogen, UK) for 15 minutes at 37°C and 5% CO_2. After 15 minutes, 'pulse' labelling of the cells with ^{35}S began. 110µCi of ^{35}S was added to each well (1ml total DMEM/well). Incubation with ^{35}S took place for 15 minutes at 37°C. After 15 minutes, ^{35}S DMEM was recycled, cells washed with PBS at room temperature and 1ml DMEM containing non-radioactive methionine (75mg/5ml) (Sigma Life Sciences, UK) added to each well initiating the 'chase'. A 0 time point was taken immediately. 1 ml of DMEM (supernatant) was saved in an Eppendorf tube and then 500µl of RIPA (Sigma Life Sciences, UK) buffer was added to the cells to lyse the cells. The chase period took place for one hour in which appropriate wells were treated with the MAPK inhibitor U0126 or following siRNA treatment for ERK 1 or ERK 2. After one hour, supernatants were saved into appropriate Eppendorf tubes, wells washed twice with ice cold PBS and cell lysates taken on ice with RIPA buffer.

10µl of each sample (supernatants and cell lysates) were spotted onto 3M filter paper (Ge Healthcare, Life Sciences, UK) and 10µl of 10mg/ml BSA was then added on top of each sample. Filter disks were then transferred into 10% ice cold Trichloroacetic acid (TCA) (Sigma Life Sciences, UK) and incubated at 4°C for 10 minutes. After 10 minutes, filter disks were transferred into 5% boiling TCA (maintained at 100°C on heating block) for 10 minutes. Filter disks were then washed with acetone for 15 minutes (2 washes) before being placed on benchcote to air dry. Each disk was individually placed into 4 ml scintillation vials (4ml screw cap, Fisher scientific, UK) and 4ml scintillation fluid (Perkin-Elmer-ultima Gold) added into each vial.

Vials were placed into a scintillation counter (Tri-CARB 2100TR, liquid scintillation analyser, united technologies Packard).

The scintillation counter counted the amount of radioactivity counts per minute by measuring the light created in the scintillation fluid in response to energy emitted by ^{35}S. In order to calculate how much protein was present in the supernatant (secreted protein content) the counts per minute in the supernatant was divided by the counts per minute in the cell lysate *plus* the counts per minute in the supernatant (total counts per minute inside and outside the cell) and then multiplied by 100 to calculate a percentage.

Example: counts per minute in cell lysate= 98150.30, counts per minute in supernatant for corresponding lysate=1795.33

Total protein secretion= 1795.33 / 98150.30+1795.33 (99945.66) * 100 = <u>1.8%</u> protein content present in supernatant

2.2.7. STATISTICS

Analysis of variance for all data was determined by calculating standard deviation using Microsoft Excel (Microsoft Inc.). A paired 2 tailed Student's t-test was used to determine significance between two sets of data where only two treatments were compared, normally control or untreated control versus a treated group of cells. The null hypothesis was rejected if a p-value of less than 0.05 was achieved and was calculated using Microsoft Excel. One way Anova analysis with a post-turkey test was carried out in order to analyse significance across multiple experimental conditions. Prism (GraphPad software Inc.) was used to design all graphs presented in this thesis.

3.0. RESULTS: Inhibitor Treatment

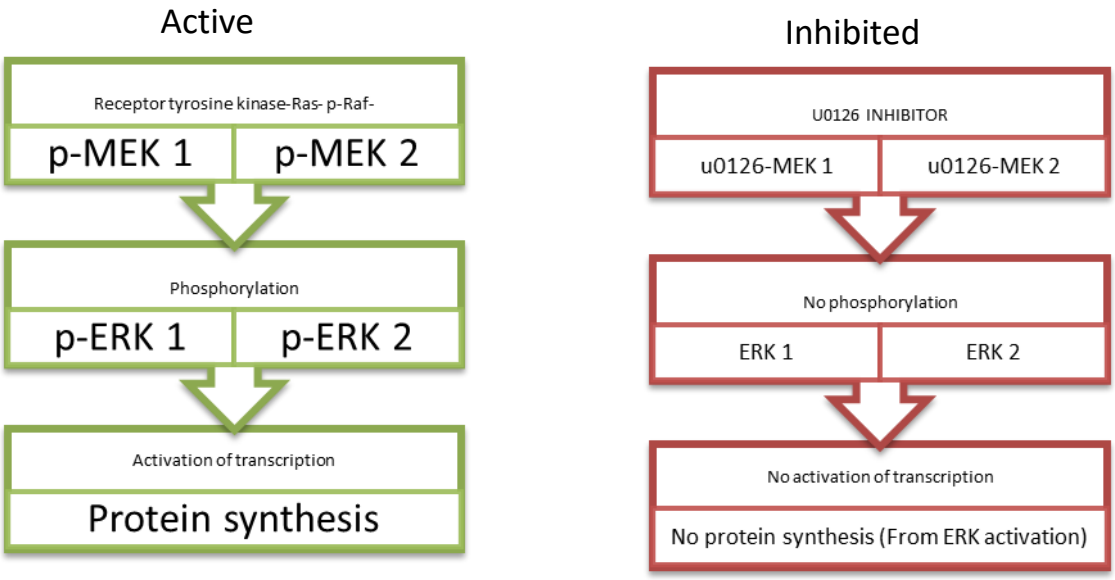
3.1. INTRODUCTION

To investigate the role of MAPK in protein secretion, we targeted the MAPK pathway with an inhibitor called U0126. U0126 inhibits Map Kinase Kinases (MAPKK) (MEK 1 and 2) that lie upstream of ERK 1 and 2. This inhibition means that ERK 1 and 2 cannot be activated (Marampon et al. 2009). To undertake these experiments, cells were either transiently transfected with GFP-SPARC or endogenous expression of osteopontin and SPARC was observed with a monoclonal anti-osteopontin antibody and polyclonal anti-SPARC antibody, which required antibody validation. Co-localisation of osteopontin and GFP-SPARC with various intracellular compartments after inhibitor was assessed with immunofluorescence staining and subsequent peak intensity analysis.

3.1.1. U0126 INHIBITOR

The U0126 inhibitor (1,4-diamino-2,3-dicyano-1,4-bis [2-aminophenylthio]butadiene) is a small molecule inhibitor that specifically inhibits MEK 1 and MEK 2 (a MAPKK) upstream of the ERK kinases (Ferrell & Bhatt 1997).

U0126 binds selectively (and with high affinity) to MEK 1 and MEK 2 but at a site distinct to its 'TEY' phosphorylation domain, in other words, MEK 1 and MEK 2 can still be phosphorylated, but their kinase activity is blocked by U0126 meaning they cannot phosphorylate the 'TEY' domain on ERK 1 and ERK 2, essentially shutting down the ERK module of the MAPK pathway (figure 3.0) (Favata et al. 1998).



‘P-’= Phospho

FIGURE 3.0, The U0126 inhibitor prevents phosphorylation of ERK. Binding of the U0126 inhibitor to MEK does not prevent phosphorylation of MEK, but inhibits MEK, resulting in inhibition of the ERK 1 and 2 pathway. The pathway on the left hand side (green) is the uninterrupted ERK MAPK pathway. In each step, MEK and ERK are phosphorylated (eg, p-ERK) eventually leading to transcription or activation of ERK substrates. Once the U0126 inhibitor is introduced (red), binding to MEK prevents phosphorylation of ERK resulting in no transcription or activation of ERK substrates.

Another MEK inhibitor (PD98059) could have been used, however, it has been reported that it binds to MEK with weaker affinity compared to the U0126 inhibitor and higher concentrations of PD98059 are required to achieve MEK inhibition compared to the U0126 inhibitor. The U0126 inhibitor has been shown to have off-target effects. Off-target effects are effects a drug might have on other biological processes other than the intended one. The U0126 inhibitor has been shown to prevent the activation of the ERK 5 pathway in HeLa (human epithelial cervical cancer) cells, a pathway activated by cell stress. The likely explanation for such an off target effect is that ERK 5 bares similarity to ERK 2 and is also activated on a ‘TEY’ domain by a MEK upstream of ERK 5 (MEK 5). The similarity probably allows for a certain extent of inhibition by the U0126 inhibitor (Mody et al. 2001).

Additionally, the U0126 inhibitor has also been shown to prevent the activation of phospho s6-kinase, (a downstream target of the mTOR [mammalian target of rapamycin] pathway) in human embryonic kidney cells (HEK293). The likely explanation for this off target effect is that as well being a downstream target of the mTOR pathway, phospho s6-kinase is also a MAPK associated protein kinase (MAPKAPK), in other words, a kinase that can be activated by MAPKs and in the specific case of phospho s6-kinase, ERK 1 and 2. 11 mammalian MAPKAPKs exist and have been shown to be activated by various stimuli. In the process of inhibiting ERK 1 and 2 activation, phosphorylation of MAPKAPKs would be lost and this might result in less activation of proteins such as s6-kinase (Moens et al. 2013; Naegele & Morley 2004).

3.2. RESULTS

3.2.1. MEK INHIBITION BY U0126

To establish the effect of U0126 in ROS cells, treatments were carried out in a time dependent manner (30 minutes and 2 hours respectively). The western blot results with an antibody directed against phosphorylated ERK showed that 30 minutes of inhibitor treatment (10µM) was sufficient to inhibit MEK 1 and 2 and thus phosphorylation of ERK 1 and ERK 2 (figure 3.1).

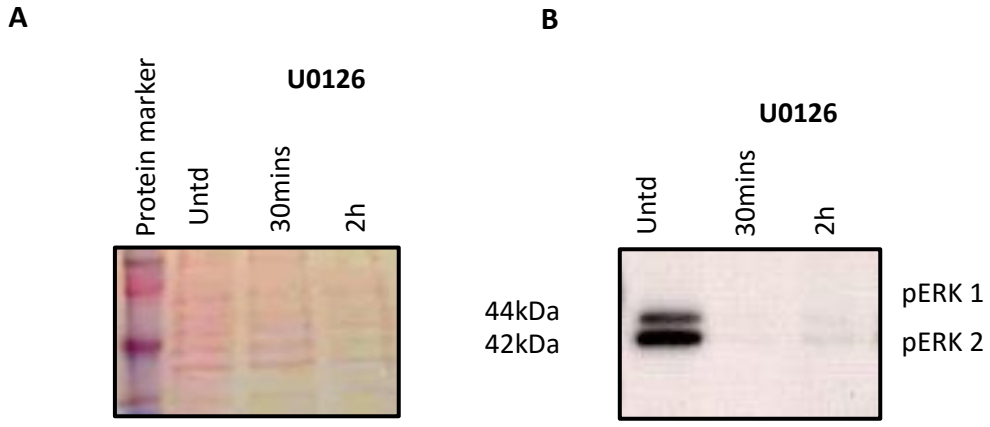


FIGURE 3.1, Treatment of ROS cells with the U0126 inhibitor shows MEK inhibition by 30 minutes of treatment. A: Ponceau S staining shows protein loading of the cell lysates run. **B**: Western blot analysis shows no detectable bands at 30 minutes and 2 hours of treatment

compared to an untreated control when looking at phosphorylated ERK. Treatment for 30 minutes appears to be a sufficient time point to prevent ERK activation.

3.3. IMMUNOFLUORESCENCE STAINING AFTER INHIBITION OF ERK 1 AND 2 ACTIVATION

We looked at the localisation of GFP-SPARC and osteopontin when the ERK 1 and 2 module of the MAPK pathway was inhibited in order to analyse if ERK 1 and 2 have an effect on protein localisation/transport in ROS cells. The antibody against endogenous osteopontin worked well, however, we did not have similar success with an endogenous SPARC antibody (figure 3.2).

3.3.1. USE OF ANTIBODIES TO LOOK AT ENDOGENOUS SPARC

Commercial antibodies directed against SPARC were tested on the ROS cell line that we were using to test our hypothesis. We tried two SPARC antibodies (anti-osteonectin, 5031 and AON-1 Haematologic technologies Inc. USA and Santa Cruz Biotechnology, USA respectively) but no significant staining was seen under the microscope for immunofluorescence staining. These antibodies, however, worked well in western blot analysis (figure 3.19, section 3.6). This was useful for checking the presence of SPARC in ROS cells, but not for localisation analysis in immunofluorescence. We purchased one more antibody in a bid to look at endogenous SPARC, a polyclonal SPARC antibody from Insight Biotechnology Limited (Wembley, London). The differentiating characteristic with this antibody compared to other SPARC antibodies was that it was reactive with rat cell lines. We initially started with this antibody by carrying out an immunofluorescence experiment to check for localisation of SPARC in the cell (figure 3.2). Unfortunately, the staining was non-specific, and much of staining was indiscriminately spread out across the cell.

3.3.2. ANTIBODY TITRATIONS

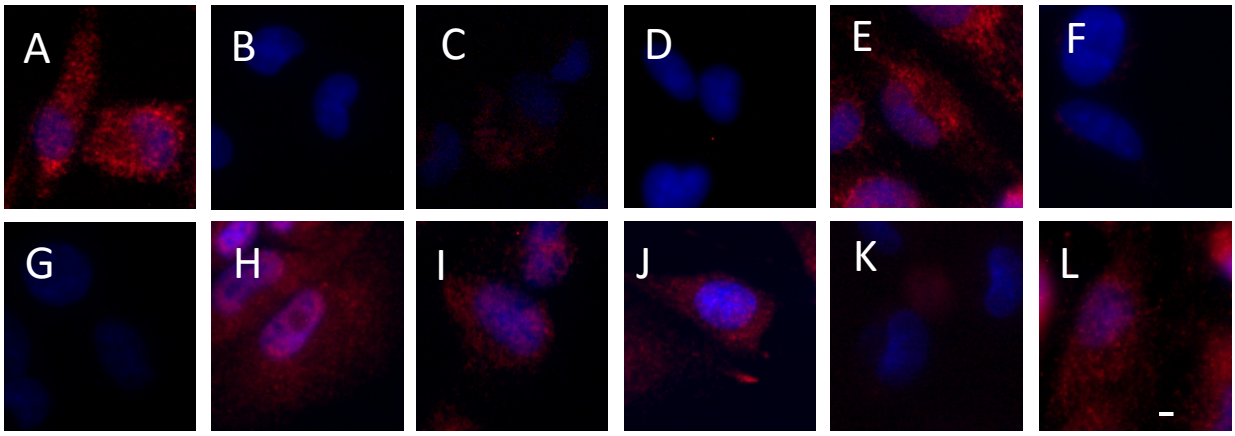
Antibody titration is a useful technique to optimize the concentration of an antibody required for an experiment. This can also save on the amount of antibody required if you find that using less still gives the same or better results than the recommended concentration that is included in the protocol for a particular antibody.

We performed an antibody titration for SPARC. The way this was carried out was to take 1µg of the antibody and add it to the incubation buffer PBS-FCS. 60µl was used as 30µl would be taken out of this stock concentration and diluted with a further 30µl to half the concentration and so on. 30µl was sufficient to cover a 13mm coverslips and stain all cells (Table 3.0)

Concentration 1µg		**0.5µg**	**0.25µg**	**0.125µg**
Dilution: 1:100		1/200	1/400	1/800
Volume: 60µl	30µl taken	30µl from previous dilution added to 30µl dilution buffer	30µl from previous dilution added to 30µl dilution buffer	30µl from previous dilution added to 30µl dilution buffer

TABLE 3.0, Antibody titration took 1 µg and halfed the concentration each time. The concentration was diluted up to 0.125µg from 1µg, and with these concentrations antibodies could be added to cells to check to see which concentration was the best (figure 3.2).

Following the antibody titration, immunofluorescence staining was carried out to look at SPARC inside the cell.



Red: 'SPARC'
Blue: DNA

FIGURE 3.2, Immunostaining with the SPARC antibody shows non-specific staining. A-F, Methanol and acetone fixation, with **A** and **B** being methanol alone, fixed for 1 minute and **B** is a negative control with only the secondary antibody added. At this point, it is apparent that the secondary antibody itself is not binding non-specifically but is binding to the SPARC

antibody. **C** and **D**, cells fixed with 100% acetone for 5 minutes with **D** being a negative control. It seems as if acetone fixation does not favour SPARC binding as there is much less binding compared to other images. It may be that acetone fixation is denaturing the protein/epitope to which the antibody will bind. **E** and **F** are a combination (50/50) of methanol and acetone fixation for 5 minutes with **F** being a negative control. It appears that fixation does not affect SPARC binding and there is clear labelling, although the problem is still arising where there appears to be non-specific staining. **G–L**, 4% paraformaldehyde fixation for 10 minutes at room temperature and permeabilisation for 5 minutes in 0.5% triton X does not appear to solve the problem and there appears to be non-specific staining throughout. **G** is a negative control with **H-K** being an antibody titration, beginning with 1µg/µl and diluting up to 0.125 µg/µl in **K**. **L** follows the same conditions above but the concentration is 0.3 µg and 0.1M calcium was added also. This does not appear to improve specificity. Scale bar: 50µm.

The staining appeared non-specific as it was consistently dispersed throughout the cytoplasm. There was much less of a perinuclear localisation and the red 'spots' appeared to be thick and a little longer in length than might be expected with a clearly defined punctate, higher resolution vesicular pattern. It seems very unlikely therefore that this is specific staining. Following on from the unsuccessful staining experiment, the antibody was tested in a western blot to look at the presence of SPARC (figure 3.3).

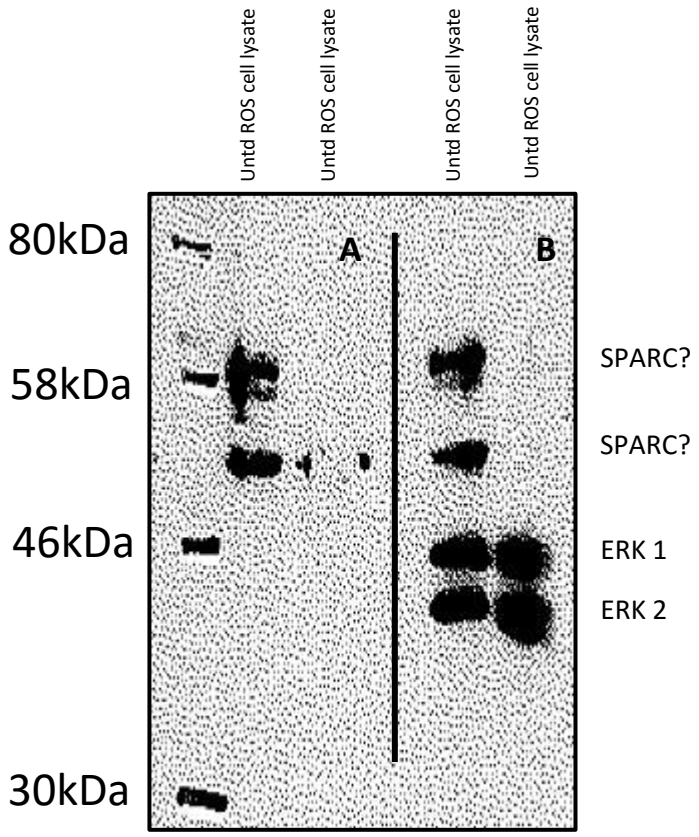


FIGURE 3.3, SPARC antibody (polyclonal anti-SPARC from Insight Biotechnology limited) does not detect SPARC. A, SPARC only. Bands for SPARC are present at around 50 and 60 kDa. While SPARC is a 43kDa peptide, there is no evidence that SPARC has isoforms bigger than this, nor is there any evidence that SPARC dimerises and therefore it is unlikely that proteins resolving above 43 kDa are going to be specific to SPARC. **B,** Looking for SPARC and MAPK shows a clear separation between the SPARC antibody and the MAPK antibody (ERK 1, p44 and ERK 2, p42 respectively). The SPARC antibody, if specific to SPARC would resolve in the same region as MAPK (ERK 1 and 2).

The protein was recognised at a molecular weight larger than 43kDa (molecular weight for SPARC), showing that the antibody purchased was not specific to SPARC but for another protein although precisely which is unknown. MAPK (p44 and p42 kDa) was run essentially as a positive control, as SPARC would have resolved in this region on the gel. It is clear the absence of a band here indicates that the antibody is not for SPARC. In hindsight, it might have been wiser to carry out western blot analysis first before any time was spent on immunostaining just to verify that we had purchased an antibody specific to SPARC.

3.4. LOCALISATION OF OSTEOPONTIN AND GFP-SPARC

After failure of the commercial antibody to detect SPARC, a different strategy was used to localise SPARC after MAPK inhibition. An expression plasmid containing GFP-SPARC under

the cytomegalovirus (CMV) promoter was transfected into ROS cells. We decided to test 3 time points, 1, 2 and 6 hours. We carried out an initial treatment of 30 minutes. The results after 30 minutes of MEK inhibition were inconclusive in this experiment as GFP-SPARC transfection was very low in our untreated control cells and were therefore unable to make any valid comparisons (data not shown). Nanofectin was used to transfect GFP-SPARC into ROS cells in this experiment.

3.4.0.1. OSTEOPONTIN ANTIBODY VALIDATION

Antibodies against endogenous osteopontin were used to look at the localisation of this matricellular protein under different conditions in the cell, treated or untreated. Antibody validation was carried out in order to ensure that there was specific staining of this antibody as it would be used extensively throughout this project (figure 3.3a). Immunofluorescence staining was carried out to look at cells that had been stained with the osteopontin antibody, and cells that had not been stained with the osteopontin antibody to check for non-specific binding of the secondary antibody.

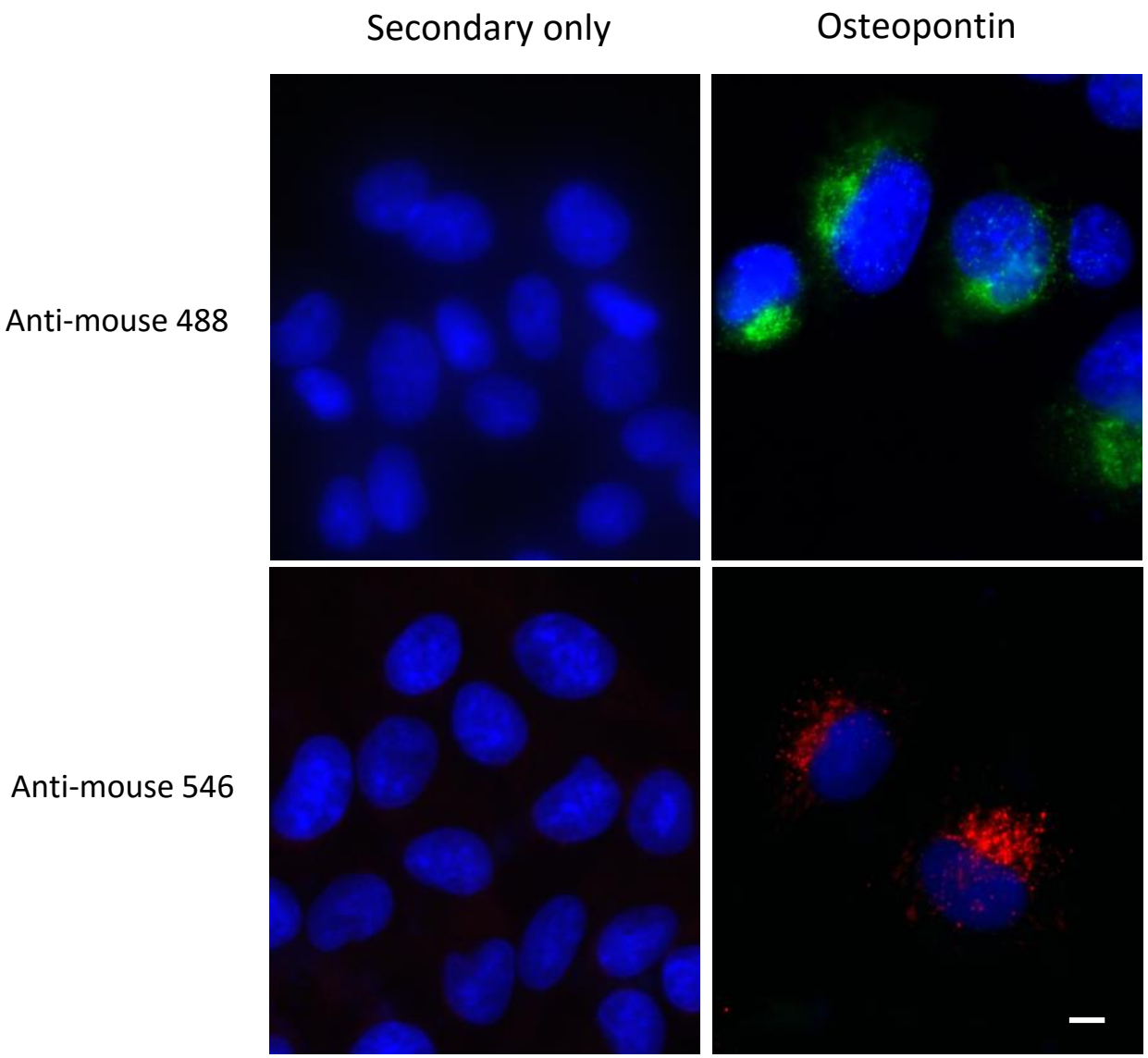


FIGURE 3.3A, The osteopontin antibody (MPIIIB10) is specific and secondary antibody only controls do not show non-specific staining. ROS cells grown on glass coverslips were fixed and incubated either with monoclonal mouse anti-osteopontin antibody or with mouse serum. Secondary AlexaFluor488 or 546 anti-mouse antibodies were used to reveal antibody binding. Controls without primary antibody show no observable green or red staining suggesting that there is no non-specific binding while samples incubated with the anti-osteopontin antibody showed clear perinuclear staining. Scale bar: 50µm.

Having treated cells with U0126 for 1, 2 and 6 hours respectively, fluorescence microscopy was carried out to look at the localisation of GFP-SPARC (figure 3.4). Using Hoechst (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloridetrihydrate), a nucleus (DNA binding) probe and fluorescently labelled Phalloidin (an actin cytoskeleton probe), localisation of GFP-SPARC as well as differences in

the morphology of the actin cytoskeleton was analysed. This would give preliminary clues as to whether inhibiting MAPK was having an effect on the trafficking of GFP-SPARC.

Please note that all immunofluorescence experiments and western blots were carried out at least 2 times (independent experiments) and in duplicates unless specifically stated otherwise.

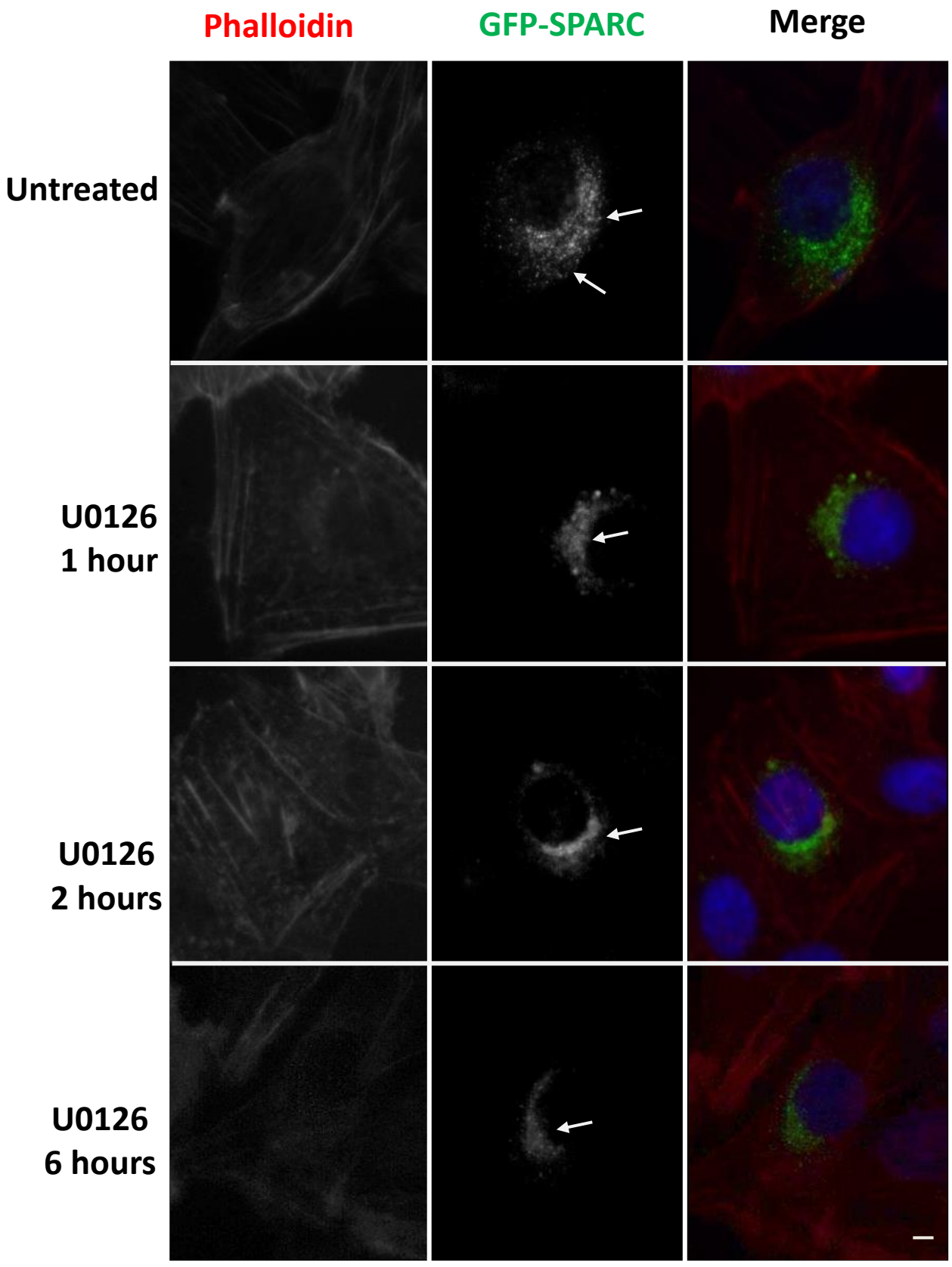


FIGURE 3.4, Treatment of ROS cells with the U0126 inhibitor appears to change the localisation of GFP-SPARC molecules. Successful inhibition of MEK 1 and 2 activity seems to

have an effect on the localisation of GFP-SPARC molecules. There appears to be 'entrapment' of GFP-SPARC molecules within the perinuclear region of the cell from 1-6 hours (arrows). Electroporation was used to transfect the GFP-SPARC containing plasmid in this experiment (n=2). Scale bar: 50μm.

There appeared to be clustering and entrapment of GFP-SPARC in a perinuclear region (U0126 treated cells), and GFP-SPARC vesicles appear to be more localised here (the majority) with far less apparent forward trafficking compared to the untreated control cells. Bright GFP-SPARC molecules appear to be located in the ER/Golgi region, indicating that there is dispersion of the protein between these organelles, as well as a diffuse pattern of punctate green vesicles throughout the cell indicating secretion (arrows, figure 3.4). This may suggest that the MAPK pathway could play a part in the trafficking of SPARC out of the ER/Golgi as knockdown of the pathway causes a sustained entrapment in a perinuclear region. Further experiments are needed to analyse and elucidate the mechanism behind this observation. Staining the actin cytoskeleton is a good measure of protein localisation as this should represent the entire shape and size of the cell. There also appears to be less GFP-SPARC in the 6 hour sample. At this point, it is unlikely that GFP-SPARC expression is being influenced by downregulation of the MAPK pathway (as a result of U0126 treatment), but is rather a result of something else.

To confirm this observation and to see if this effect might be limited to GFP-SPARC, we carried out the same experiment but looked at endogenous osteopontin (figure 3.5). Cells were treated with the U0126 inhibitor for 1, 2 and 6 hours before indirect immunofluorescence was performed using an anti-osteopontin antibody plus Hoechst for DNA and Phalloidin for the actin cytoskeleton.

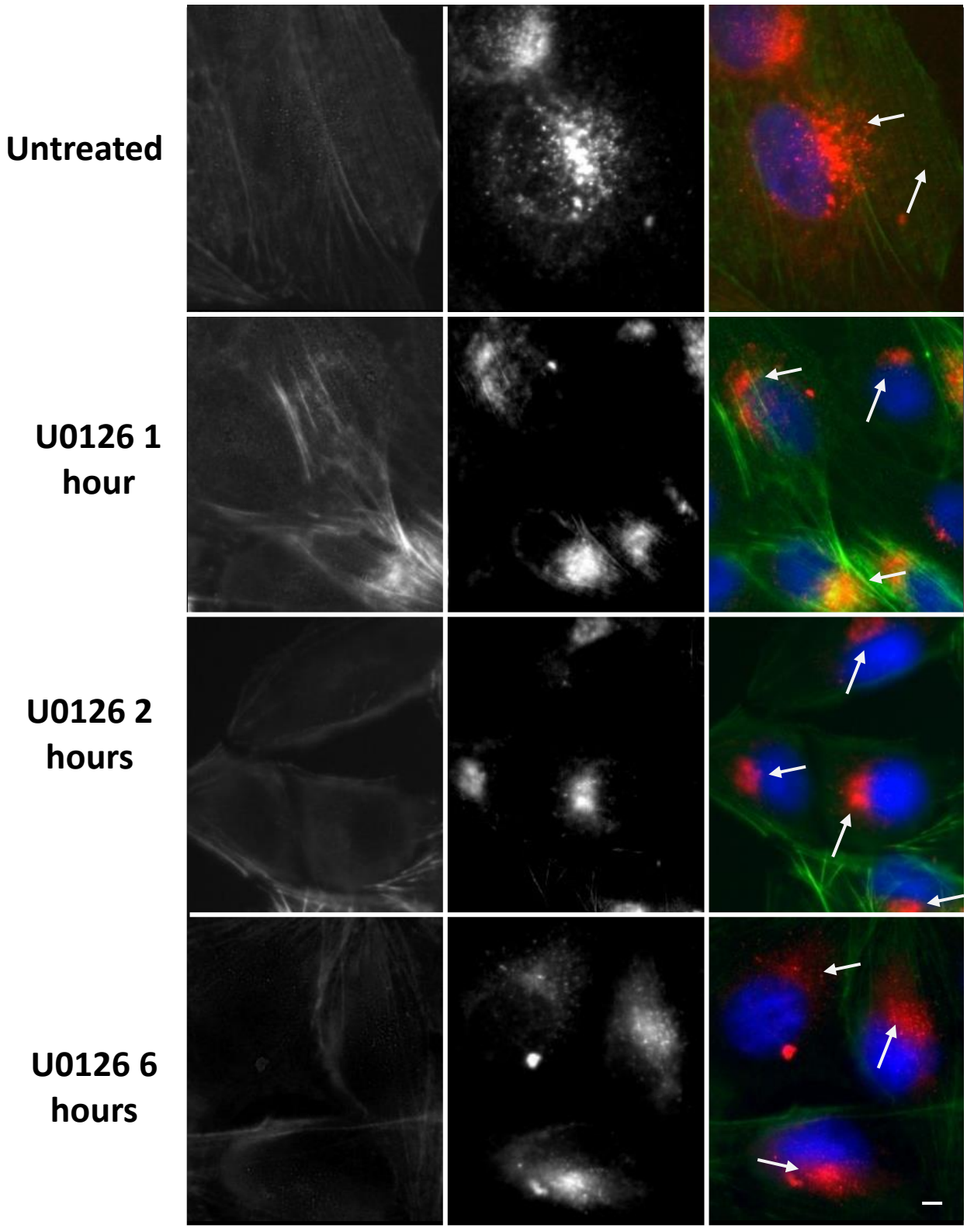


FIGURE 3.5, The U0126 inhibitor also affects the trafficking of osteopontin. Similar to the localisation of GFP-SPARC, we also observed a diffuse pattern of osteopontin vesicles indicating secretion (arrows). Cells treated with the U0126 inhibitor for 1, 2 and 6 hours

showed a sustained retention in a perinuclear region (arrows). As with GFP-SPARC, we do not know specifically where this retention is taking place, but it appears that inhibition of MAPK may be involved in an important step in the trafficking of multiple matricellular proteins. Scale bar: 50µm.Scale bar: 50µm. n=2.

While we were not sure precisely what MAPK was doing in the trafficking of GFP-SPARC and osteopontin, where these proteins were being 'retained' was the next question. No difference in the morphology of filamentous actin can be seen between untreated cells and cells treated with the U0126 inhibitor. Therefore, we do not think that the differences in trafficking are being caused by cytoskeletal changes. For this reason we decided to stain various organelles and compartments in order to further isolate where this retention might have been taking place.

3.4.1 LOCALISATION OF GFP-SPARC AND OSTEOPONTIN WITH CELLULAR COMPARTMENTS

As we observed an apparent retention of osteopontin and GFP-SPARC in a perinuclear region, we decided to stain the endoplasmic reticulum to see if clustering of these two matricellular proteins was taking place here. Again, cells were treated with the U0126 inhibitor for 1, 2 and 6 hours before immunofluorescence staining was carried out (figure 3.6).

We used two probes for looking at the endoplasmic reticulum, Concanavalin A and ERp72. Concanavalin A is a lectin and binds to proteins that contain α-D-glycosyl and α-D-mannosyl attachments which are mainly found in the in the endoplasmic reticulum (Schneider & Sievers 1981).

ERp72 is a resident enzyme in the endoplasmic reticulum that is involved in breaking down disulphide bonds when proteins are improperly folded and therefore acts as a retention protein (Forster et al. 2006).

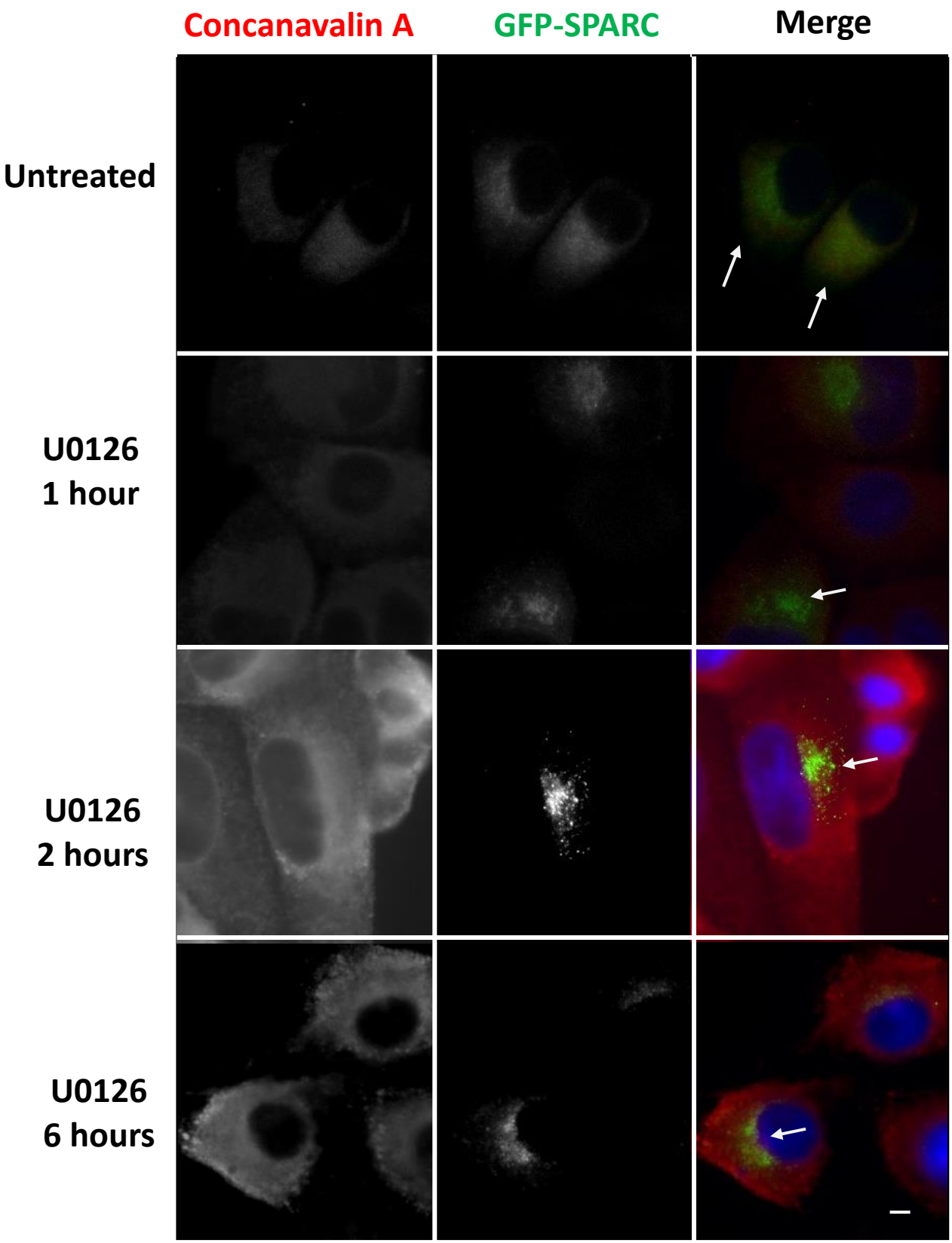


FIGURE 3.6, GFP-SPARC does not appear to co-localise with the endoplasmic reticulum. We checked to see if there was any co-localisation of GFP-SPARC with the ER (according to staining with Concanavalin A) in case inhibition of the MAPK pathway caused protein

retention in the ER. Comparing the untreated control cells to cells treated with the U0126 inhibitor for 1-6 hours, there does not appear to be co-localisation of GFP-SPARC with the endoplasmic reticulum (arrows). The retention or clustering effect that we saw previously is present in cells treated with the U0126 inhibitor confirming the observation that inhibition of the MAPK pathway affects trafficking. Electroporation was used to transfect the GFP-SPARC containing plasmid in this experiment. Scale bar: 50μm. n=2

We carried out the same experiment to look at the co-localisation of osteopontin with Concanavalin A and also did not observe any apparent co-localisation (figure 3.7).

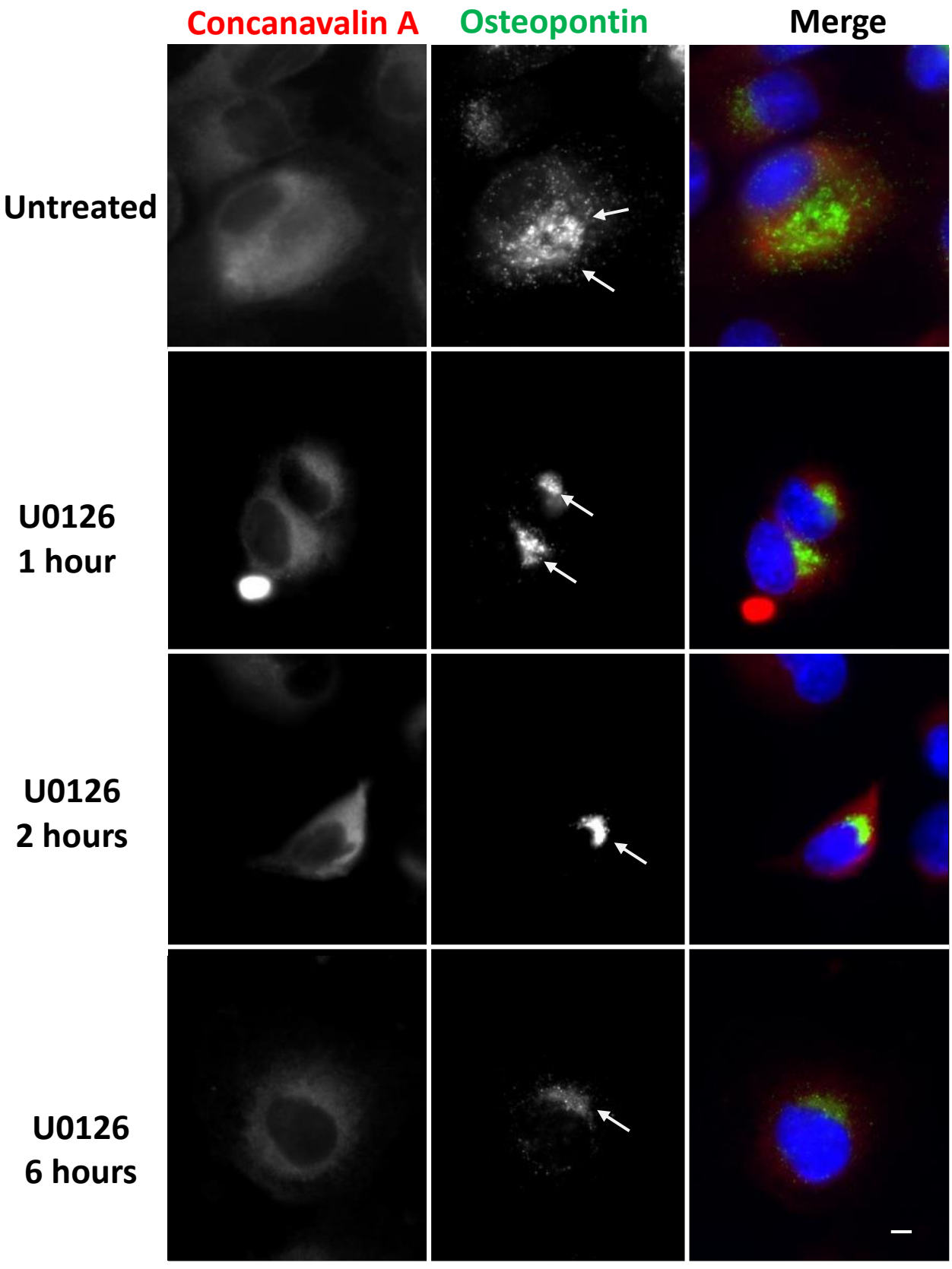


FIGURE 3.7, Osteopontin does not co-localise with the endoplasmic reticulum. The pattern of the untreated cells is consistent. A diffuse pattern of osteopontin vesicles indicates secretion out of the cell (arrows). Cells treated with the U0126 inhibitor appear clustered

and indicate some sort of retention (arrows), but as there is no co-localisation of osteopontin with Concanavalin A (untreated control and treated cells), it is unlikely that the retention is taking place in the endoplasmic reticulum. Scale bar: 50µm. n=2

Osteopontin and Concanavalin A co-localisation was quantified. The following graphs (figure 3.7a) are representative of all cells that show a given intracellular localisation (whether untreated or treated with U0126 inhibitor). The data given will represent two time points only (untreated and 1 hour U0126) as an effect was observed within one hour.

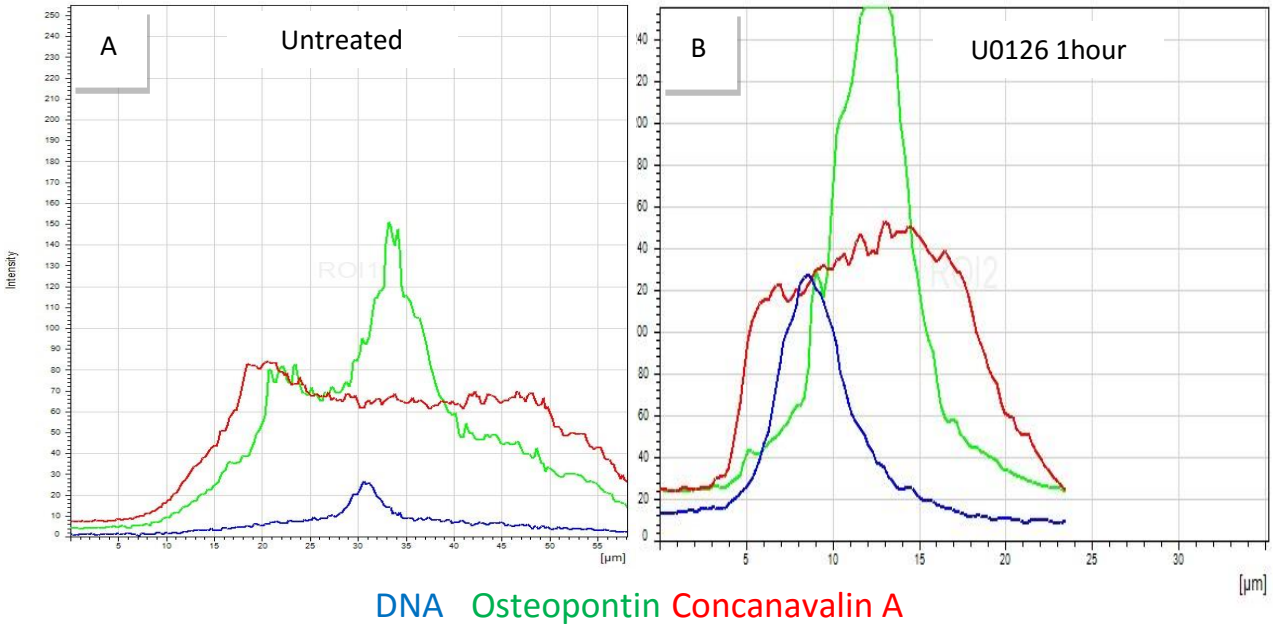


FIGURE 3.7A, Representative intensity analysis of osteopontin and Concanavalin A (ER) shows no co-localisation between these two molecules and is therefore unlikely to be the area of entrapment (x-axis=µm, y-axis=intensity). A and B, There is no co-localisation between Concanavalin A and osteopontin. Co-localisation would be indicated by the peaks of two fluorophores overlapping with one another as they lay precisely in the same location on the image and therefore represent two fluorophores that occupy the same space and also overlap as the intensities coincide with one another. Looking at this graph, osteopontin does not co-localise with the ER.

Neither GFP-SPARC nor osteopontin appeared to co-localise with the endoplasmic reticulum by staining with Concanavalin A. To confirm this observation, co-localisation of osteopontin and ERp72 (a resident ER protein) was tested (figure 3.8).

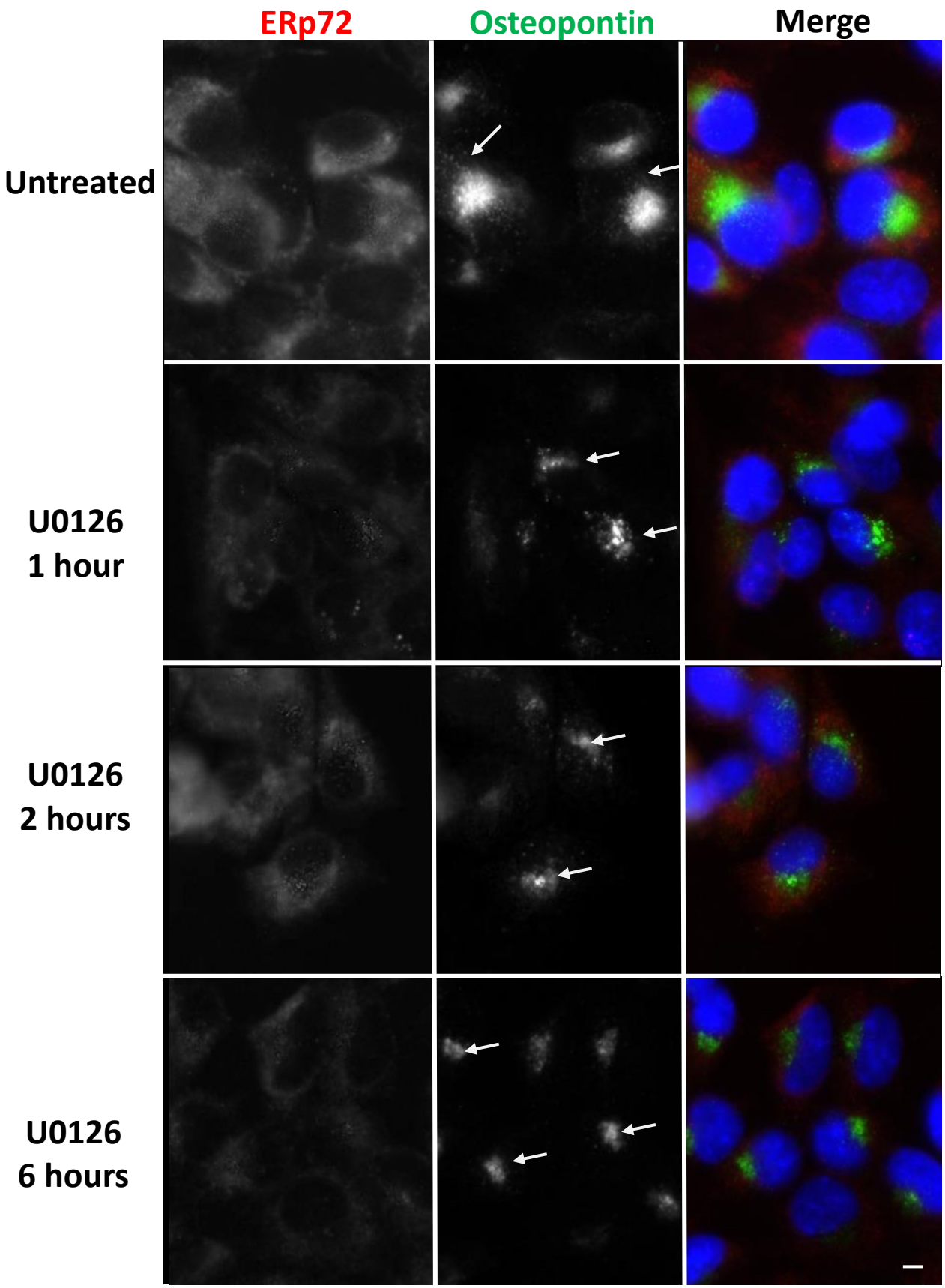


FIGURE 3.8, Staining of ERp72 with osteopontin shows similar results to staining cells with Concanavalin A and no co-localisation between the endoplasmic reticulum and

osteopontin can be observed. The same effect can be observed in terms of osteopontin trafficking when cells are treated with the U0126 inhibitor from 1-6 hours compared to the untreated control cells (arrows). Like Concanavalin A, there is no apparent co-localisation of osteopontin with ERp72 confirming the observation that there is no co-localisation of osteopontin (or GFP-SPARC) with the endoplasmic reticulum. Scale bar: 50μm. n=2

We did not observe any co-localisation of osteopontin or GFP-SPARC with the endoplasmic reticulum. Since entrapment/retention of the matricellular proteins was in a perinuclear region, we then decided to look at co-localisation of osteopontin with the Golgi apparatus. We stained the cells with fluorescently labelled helix pomatia agglutinin (HPA), which selectively binds to terminal α-N-acetylgalactosaminyl residues in the Golgi apparatus (figure 3.9).

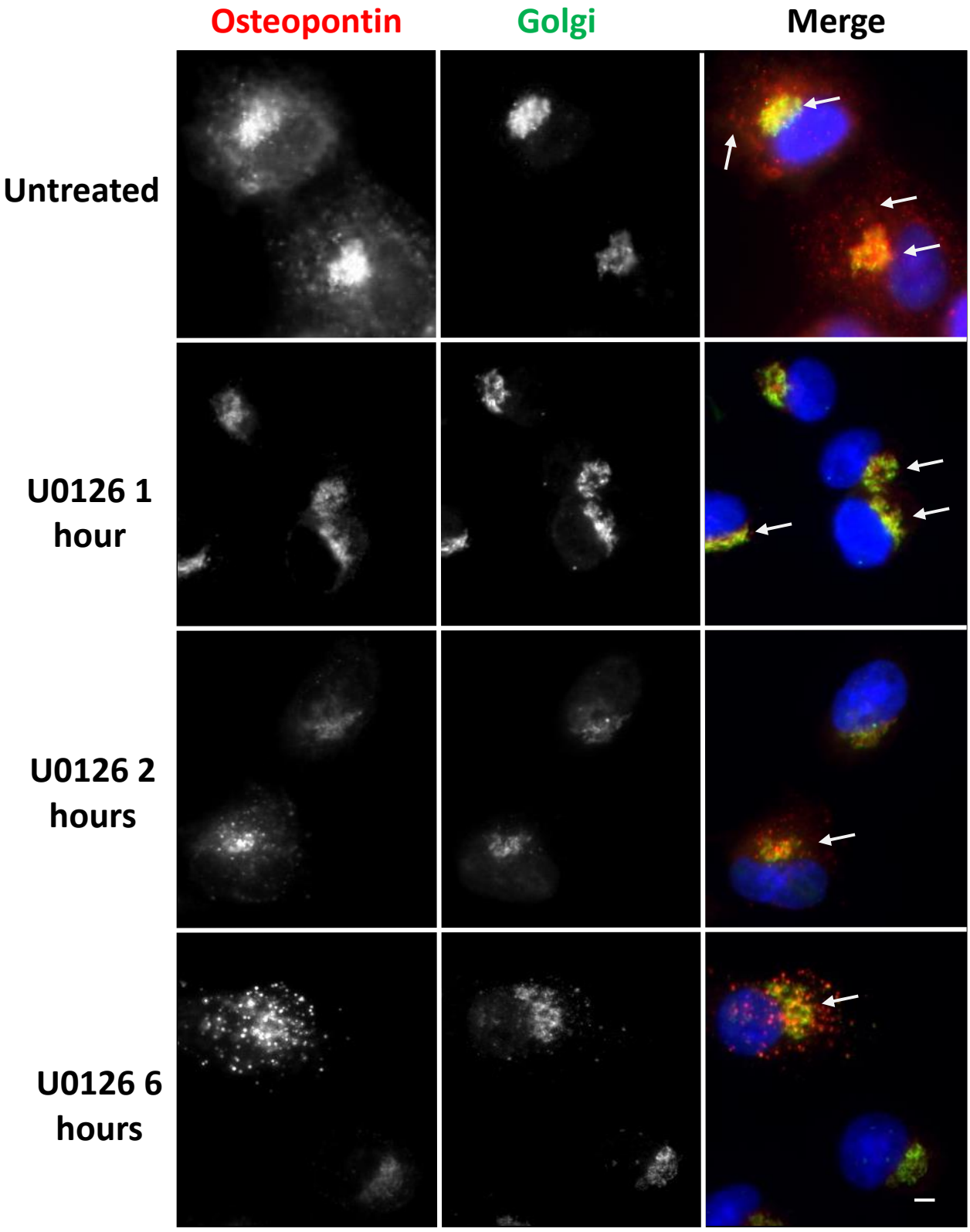


FIGURE 3.9, Retention and clustering of osteopontin appears to take place around the Golgi apparatus. Osteopontin in the untreated control cells appears to show co-localisation with the Golgi apparatus. Again, a diffuse pattern of punctate red osteopontin vesicles can be seen post-Golgi (arrows). When cells are treated with the U0126 inhibitor from 1-6 hours,

the retention or clustering effect is seen, but appears to overlap precisely where the Golgi staining has taken place (arrows). While there does not appear to be co-localisation, it is difficult to say at this point and there appears to be more co-localisation in cells treated for 2 and 6 hours. Scale bar: 50 µm. n=3

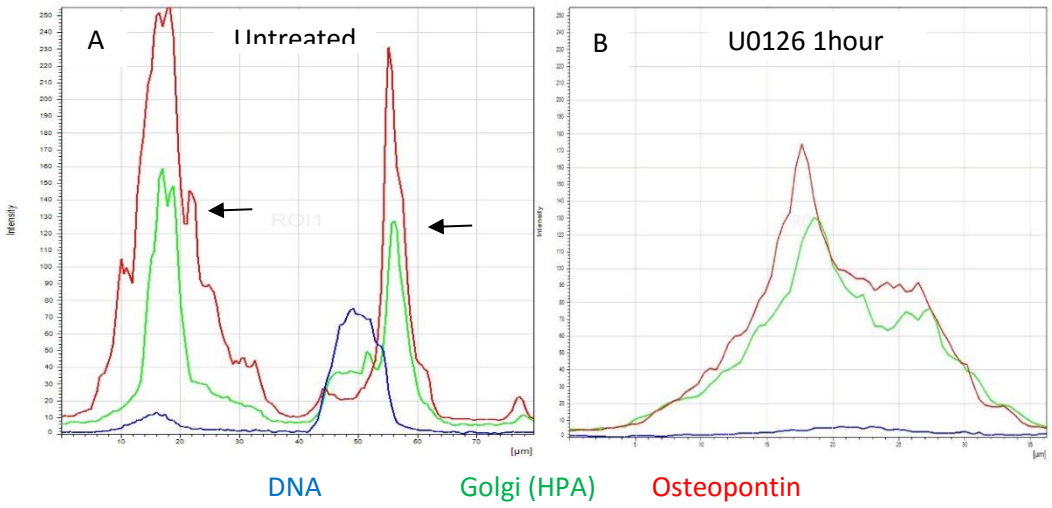


FIGURE 3.9A, Representative intensity analysis of osteopontin and HPA (Golgi apparatus) shows co-localisation between osteopontin and the Golgi in the untreated cells and less in cells treated with the U0126 inhibitor (x-axis=µm, y-axis=intensity). A, There is co-localisation between the Golgi and osteopontin. Two independent cells on the graph (arrows) show the peaks of the green and red channel overlapping perfectly and in precisely the same spot confirming co-localisation. **B,** There is a shift in the distance of the green and red channels. There appears to be some overlap (co-localisation) in the cell represented by the graph but the shift indicates there is much less as the peaks are not aligned. This is in agreement with results observed in immunofluorescence staining.

The immunofluorescence staining experiment for GFP-SPARC and the Golgi apparatus could not be carried out because efficiency of transfection of GFP-SPARC became very poor. However, we did carry out an immunofluorescence staining to look at localisation of GFP-SPARC and an early endosomal marker (figure 3.10). Although transfection efficiency had reduced, cells that were transfected did not appear to show strong co-localisation with early endosomes.

Please note that immunofluorescence staining for GFP-SPARC and EEA was carried out once in duplicate.

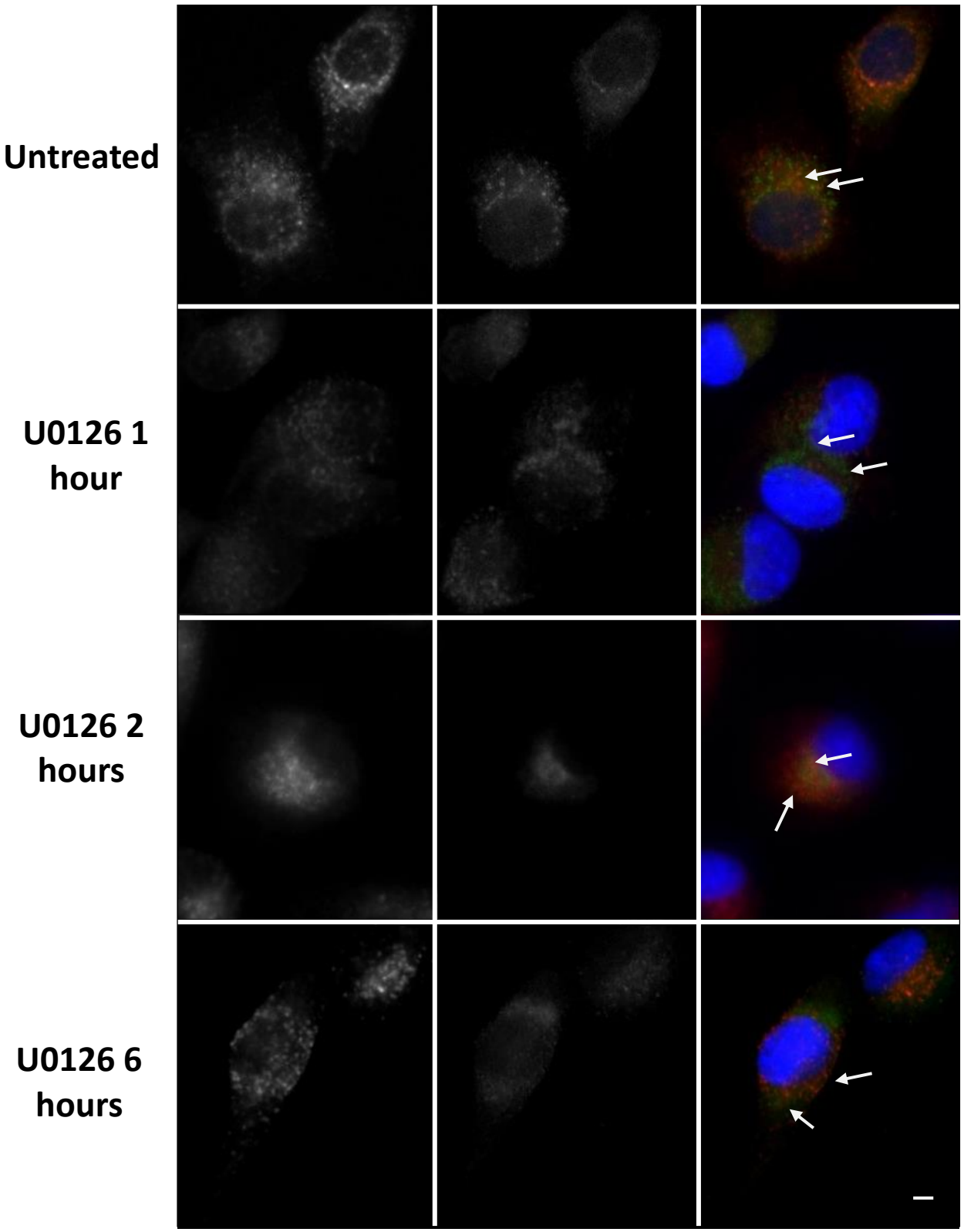


FIGURE 3.10, GFP-SPARC does not co-localise with early endosomes, although GFP-SPARC expression is very low. It is difficult to draw any conclusion based on the immunofluorescence images of GFP-SPARC and the early endosomes, although there is no

co-localisation between GFP-SPARC and early endosomes in the images (arrows), and there is no difference in co-localisation between untreated and treated cells. Electroporation was used to transfect the GFP-SPARC containing plasmid in this experiment. Scale bar: 50μm. n=1

Finally, we looked at the localisation of endogenous osteopontin and LAMP-1 (a lysosomal marker). There is evidence that lysosomes also act as secretory compartments and not just protein degrading compartments (Blott & Griffiths 2002).

For this reason, we decided to see if osteopontin was co-localising with lysosomes, and to see if the retention might have been taking place in this compartment (figure 3.11).

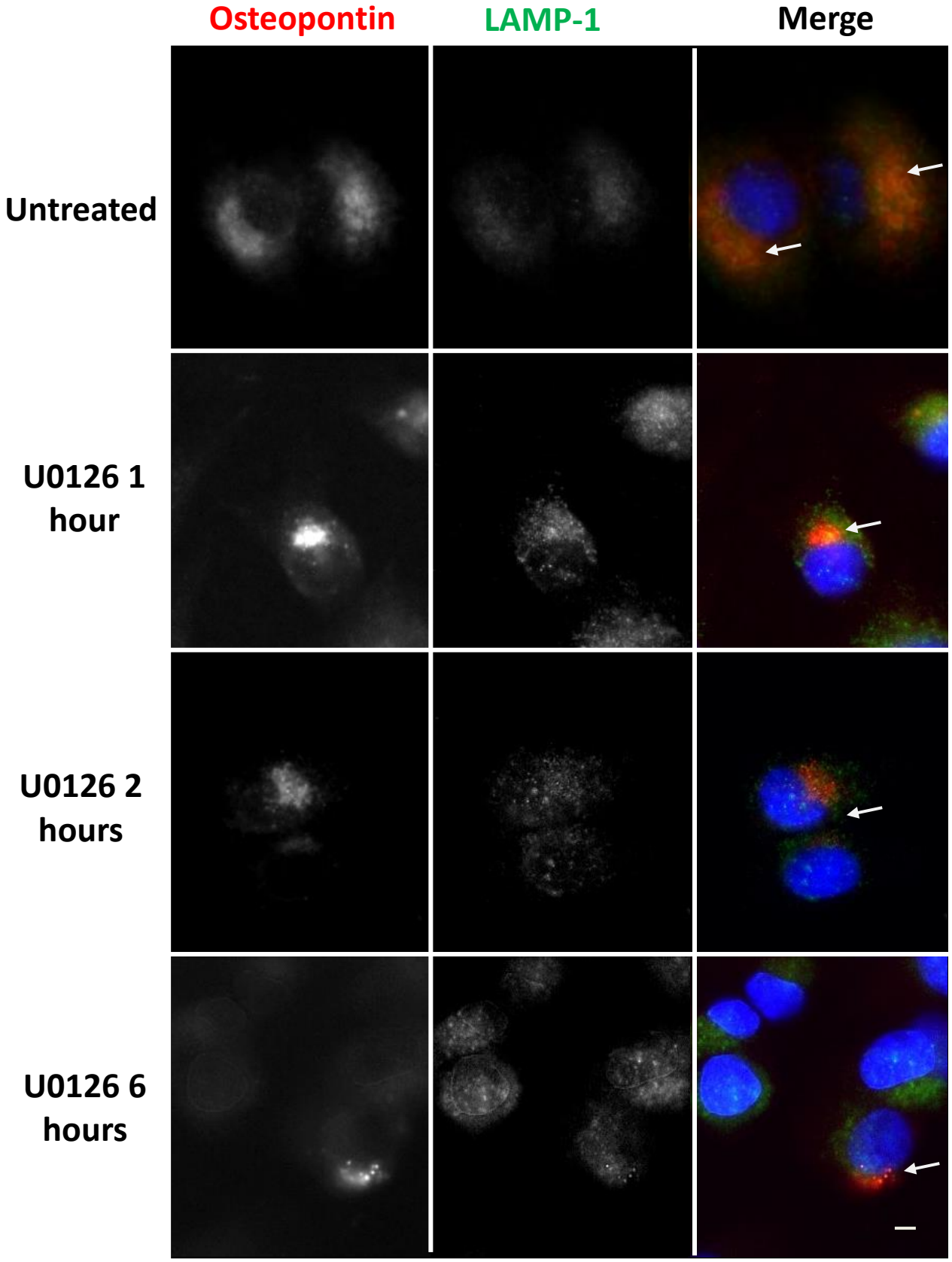


FIGURE 3.11, there does not appear to be any co-localisation of osteopontin (and GFP-SPARC) vesicles with LAMP-1 when MAPK is inhibited. There didn't appear to be a strong merge between LAMP-1 and osteopontin in the untreated cells although there are some

parts that may suggest co-localisation (arrows). Treated cells do not show any co-localisation of osteopontin with the lysosomes and it is unlikely that the retention is taking place in lysosomal compartments. Scale bar: 50µm. n=2

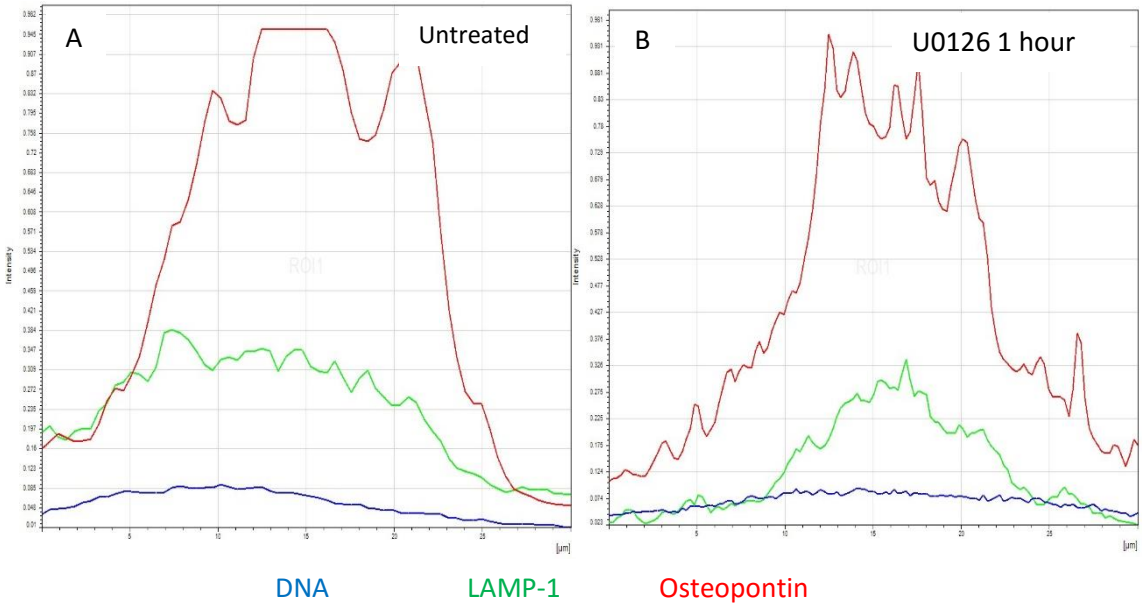


FIGURE 3.11A, Representative intensity analysis of osteopontin and LAMP-1 (lysosomal compartments) shows no co-localisation between osteopontin and the lysosomes in the untreated and U0126 treated cells (x-axis=µm, y-axis=intensity). A and B, There is no co-localisation between the lysosomes and osteopontin. Quantification of co-localisation by intensity analysis confirms the observation in immunofluorescence staining showing that osteopontin does not co-localise with the lysosomes in untreated or treated conditions.

Having looked at the co-localisation of osteopontin and GFP-SPARC with different cellular compartments, it appears likely that the retention is taking place around the Golgi-apparatus. Please note, that the transfection efficiency of the GFP-SPARC plasmid was variable. It is important to take into consideration that the efficiency is different among different experiments and there were many cells that did not take up (or express) the GFP-SPARC plasmid. The extent of plasmid uptake might also have some effects on the expression and trafficking of GFP-SPARC and may therefore show effects on trafficking that may not necessarily be affected by MEK inhibition.

3.4.2 USE OF A 'NORMAL' OSTEOBLAST MODEL AS A CONTROL

Comparing the rate of trafficking of osteopontin and GFP-SPARC in the ROS cell line should be compared to a normal control set of osteoblastic cells. Unfortunately, there is no rat model of normal osteoblasts besides primary cells which we did not have access to, so we decided to use the MC3T3 cell line which resembles a more normal osteoblastic cell. The MC3T3 cell line is a pre-osteoblastic cell line with the potential to differentiate into mature osteoblasts. The cell line is derived from mouse calvaria (skull cap) and although we could not measure the rate of secretion in normal rat osteoblasts, looking at secretion of normal mouse osteoblasts might still provide clues as to the contribution of MAPK to membrane trafficking.

We ran an experiment in which we had cells treated for 1, 2 and 6 hours with the U0126 inhibitor. We wanted to compare the secretion patterns we were seeing in the ROS cell line with secretion patterns in the MC3T3 cell line. Initially we carried out an indirect immunofluorescence where we targeted osteopontin with an antibody, followed by dye tagging with an Alexa Fluor conjugated secondary antibody against mouse (the host that the primary antibody was raised in). The staining for osteopontin did not appear to look like the staining in the ROS cells. While this may be perfectly normal as no two types of cells are going to show precisely the same results, the staining seemed non-specific and we were not convinced that this was target specific binding. We then decided to run a secondary only control. Cells were seeded at the same density as the U0126 inhibitor treatment carried out. Cells were not treated with the inhibitor as this was a troubleshooting experiment. Cells were fixed in 4% paraformaldehyde followed by the protocol for immunofluorescence (as described in 'materials and methods', immunofluorescence staining section 2.2.4). We followed the protocol as had been previously carried but did not add a primary antibody, staining only with 'Hoechst' a blue DNA binding dye, green 'Phalloidin', an actin cytoskeleton (filamentous actin) binding dye and a secondary antibody (Alexa Fluor 546 red anti-mouse). The result we obtained confirmed our previous suspicion that the red staining was not specific to osteopontin (figure 3.12). Without the antibody, it appears the secondary anti-mouse antibody is binding non-specifically to an organelle or other component of the cell. Furthermore, the staining was non discriminant and one might mistake red staining towards

the periphery of the cell for secretory or endocytic vesicles containing osteopontin. For this reason, immunofluorescence images taken with the U0126 inhibitor were discarded as no meaningful conclusion could be drawn considering there was non-specific staining. With no endogenous SPARC antibody, we were unable to look at the secretion of these two matricellular proteins. With this in mind, it should be noted that this is a limitation of the project and a comparison of a normal osteoblastic control is essential in comparing how ERK 1 and 2 contribute to trafficking in non-cancerous cell lines.

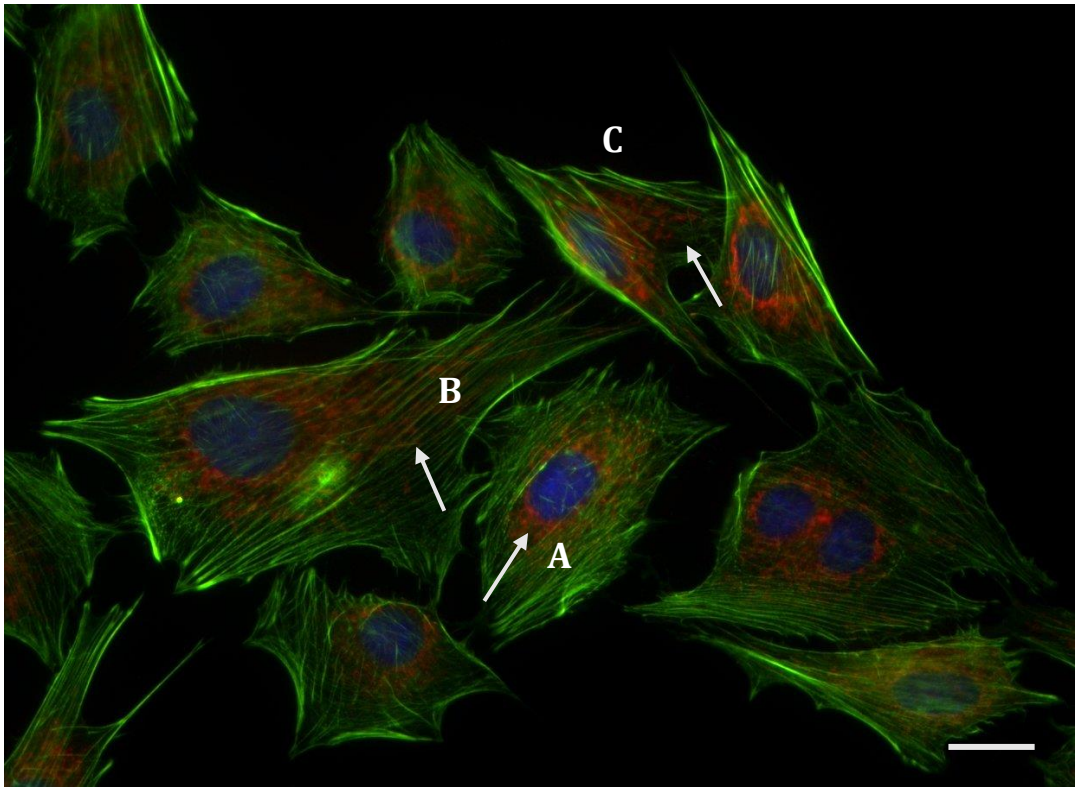


FIGURE 3.12, staining the MC3T3 cells with a secondary antibody only (without primary) shows non-specific staining. We observed non-specific staining when we stained cells with a secondary antibody only. It is clear by the red staining that there is non-specific binding, and that binding is occurring in a perinuclear region, **A**, and further out in the cytoplasm, **B** and **C.** This gives the false illusion of specific staining as osteopontin (and GFP-SPARC) dynamics correspond with what is shown in figure 3.4 untreated and 3.5 untreated. Scale bar: 50µm.

3.4.3 MONO-PHASIC EFFECT OF INHIBITING MAPK WITH U0126

Experiments were run for a longer time point (72 hours) to see how long the U0126 inhibitor could sustain MEK inhibition and also to see if osteopontin trafficking might return to normal after MEK inhibition had worn off. Before immunostaining for the 72 hour time point, a western blot was run to check phosphorylation of ERK 1 and 2 (figure 3.13) to check how long the inhibitor was working without further addition (10µM of U0126). At the same time, cell counts were checked for the time points to see how the U0126 inhibitor affected the growth of ROS cells. ROS cells were treated with the U0126 inhibitor for 72 hours, counting cells or taking cell lysates at 6, 24, 48 and 72 hours.

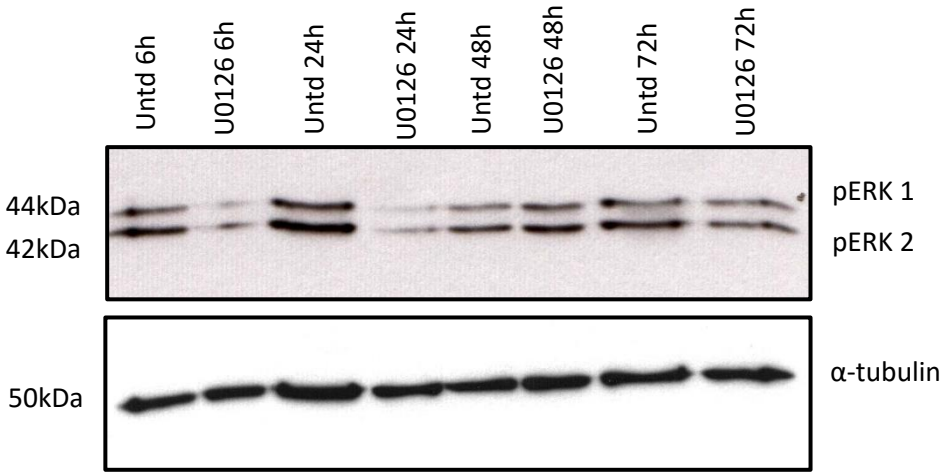


FIGURE 3.13, Western blot shows levels of phosphorylated ERK during the 72 hour time course. Inhibition of MEK 1 and 2 by the U0126 inhibitor is successful up to 6 hours (with bands slowly becoming visible at 6 hours of treatment). Compared to the untreated controls for the respective time points, ERK phosphorylation is very low yet present at 24 hours before full phosphorylation is seen at 48 and 72 hours. It is likely that by 24 hours, the U0126 inhibitor becomes ineffective, before MEK is fully able to phosphorylate ERK 1 and 2 by 48 hours although full phosphorylation of ERK may take place before the 48 hours.

Having run the western blot, cell counts (figure 3.14) showed that the total number of cells decreased over the time the inhibitor was active, with an increase in cell number once the inhibitor had worn off after 24 hours.

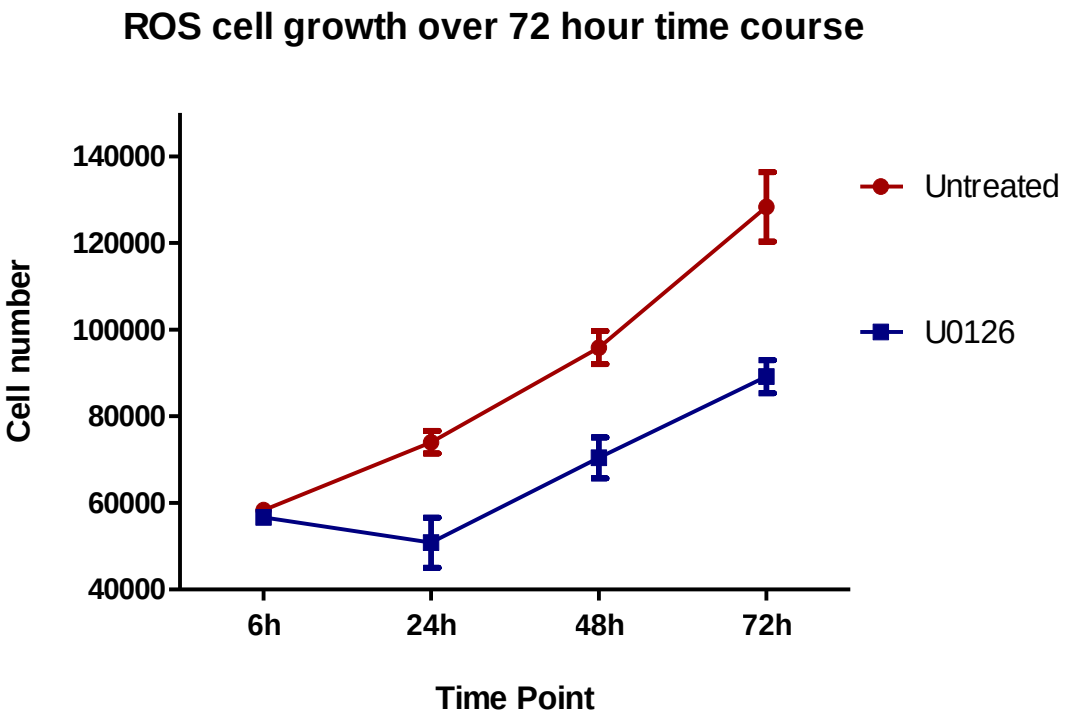
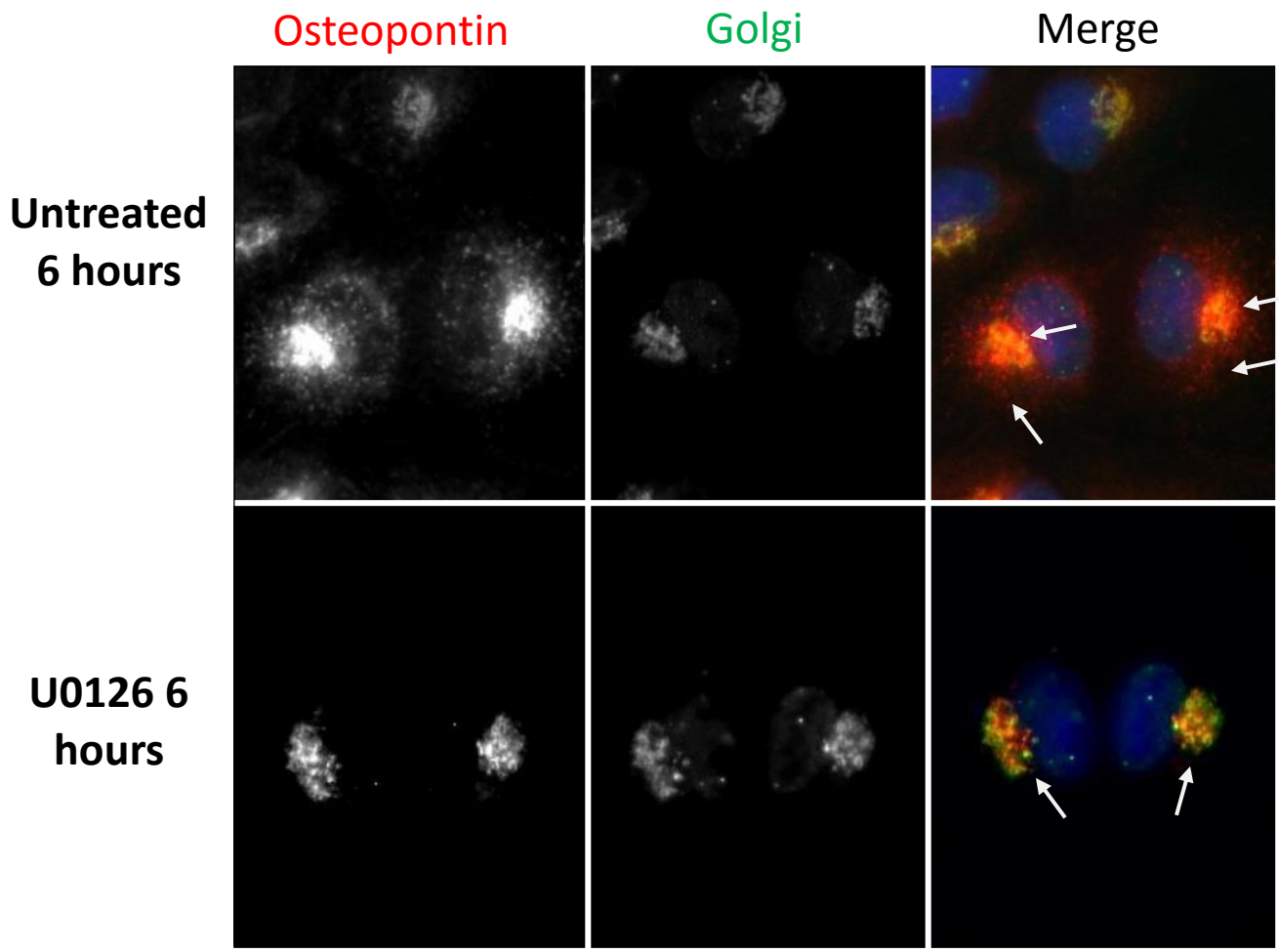


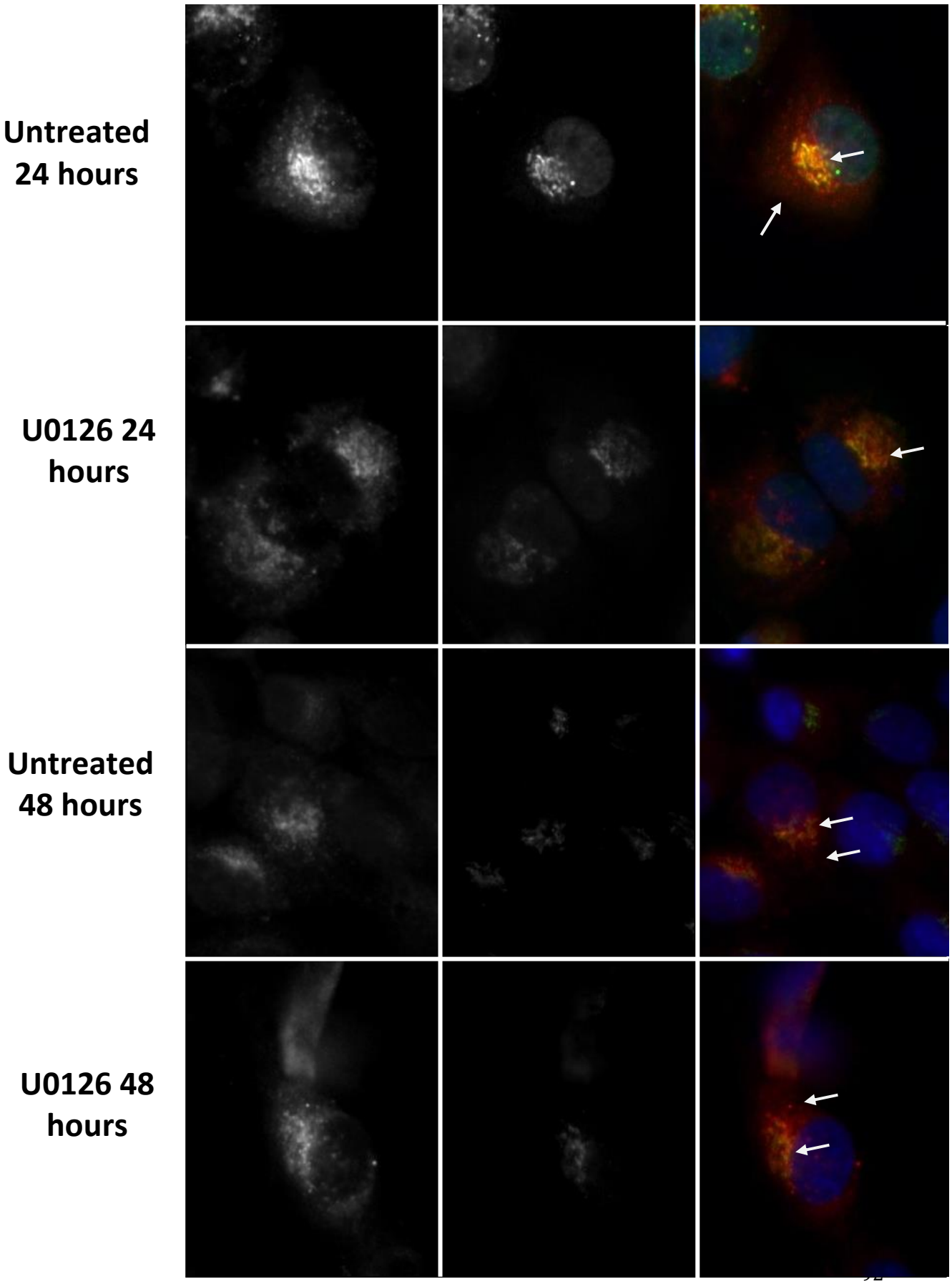
FIGURE 3.14, Number of Cells treated with the U0126 inhibitor decreases before increasing again after MEK inhibition no longer takes place. Untreated cell numbers increase after point of seeding (red line) and continue to grow over the entire time course. When cells are treated with the U0126 inhibitor, a reduction of cells is seen up to 24 hours due to the inhibition of MEK 1 and 2 and subsequent lack of phosphorylation on ERK 1 and 2 (blue line) although this reduction is not significant. After 24 hours when phosphorylated ERK levels are rescued, the cells begin to grow once more demonstrating not only the effect that inhibition of the MAPK pathway has on cell growth, but the potential therapeutic effects of the inhibitor on ROS cells. **Error bars represent SD.** n=1

Please note that cell count experiment was carried out once in triplicate and this experiment must be repeated multiple times to verify the result obtained

The untreated and treated cells were grown in parallel. Cells were counted after 6, 24, 48 and 72 hours. Cell numbers decreased within 24 hours of treatment with the inhibitor. This is consistent with phosphorylated ERK levels in western blots as the inhibitor begins to wear off around the 24 hour mark, and bands can be seen for phosphorylated ERK. Full rescue of phosphorylated ERK is seen at 48 and 72 hours and this is reflected in the graph as the number of cells start to increase once more.

According to the western blot and the cell number growth chart (figure 3.13 and 3.14 respectively), we expected that after 24 hours, we might observe a re-initiation of secretion of endogenous osteopontin due to the U0126 inhibitor no longer being effective. To analyse localisation of the protein after U0126 treatment over 72 hours, cells were stained with an antibody directed against osteopontin, the DNA binding dye Hoechst 33342 to reveal the nucleus and HPA for the Golgi apparatus (figure 3.15) and Concanavalin A (figure 3.16) for the endoplasmic reticulum.




Untreated 24 hours
U0126 24 hours
Untreated 48 hours
U0126 48 hours

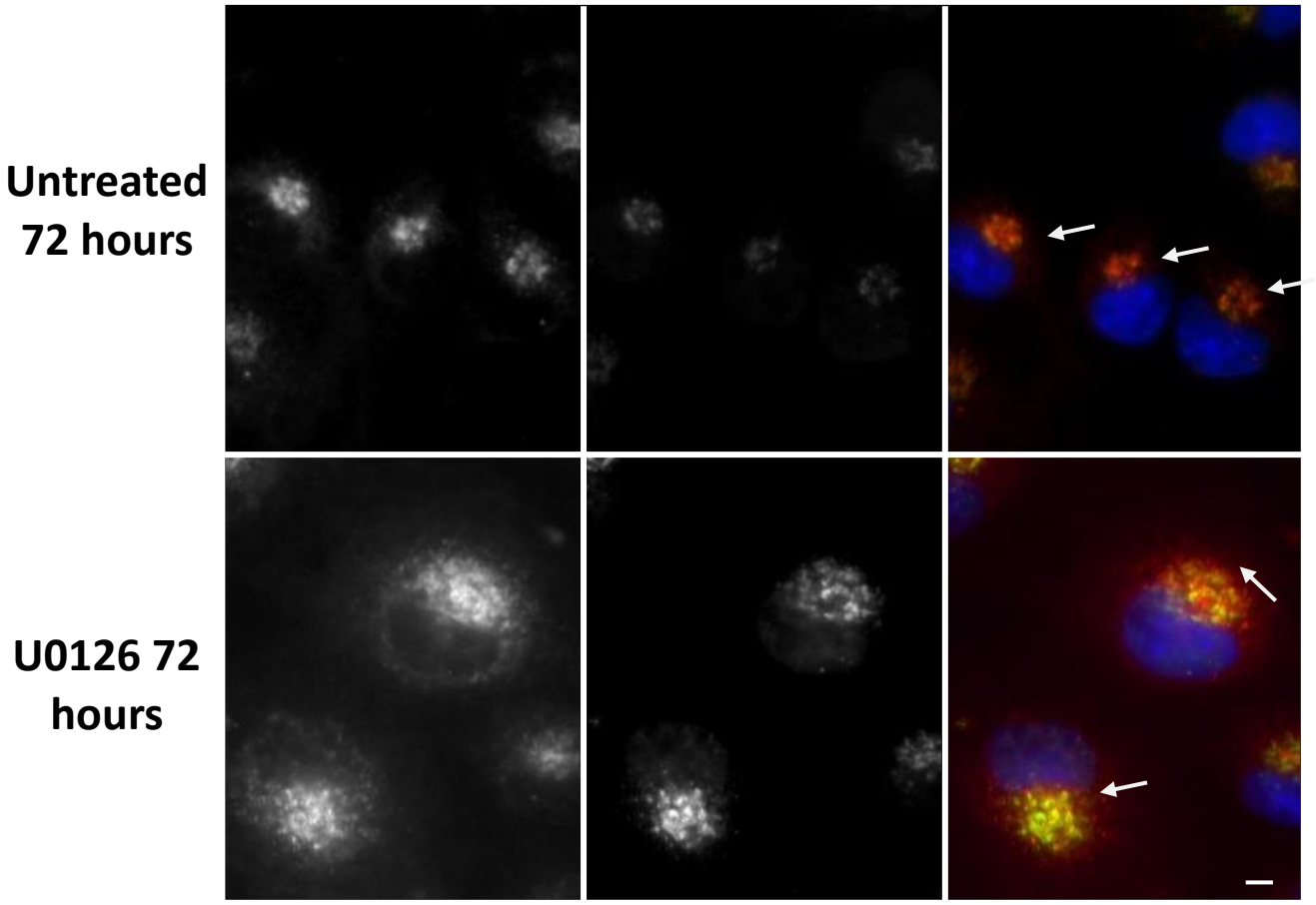
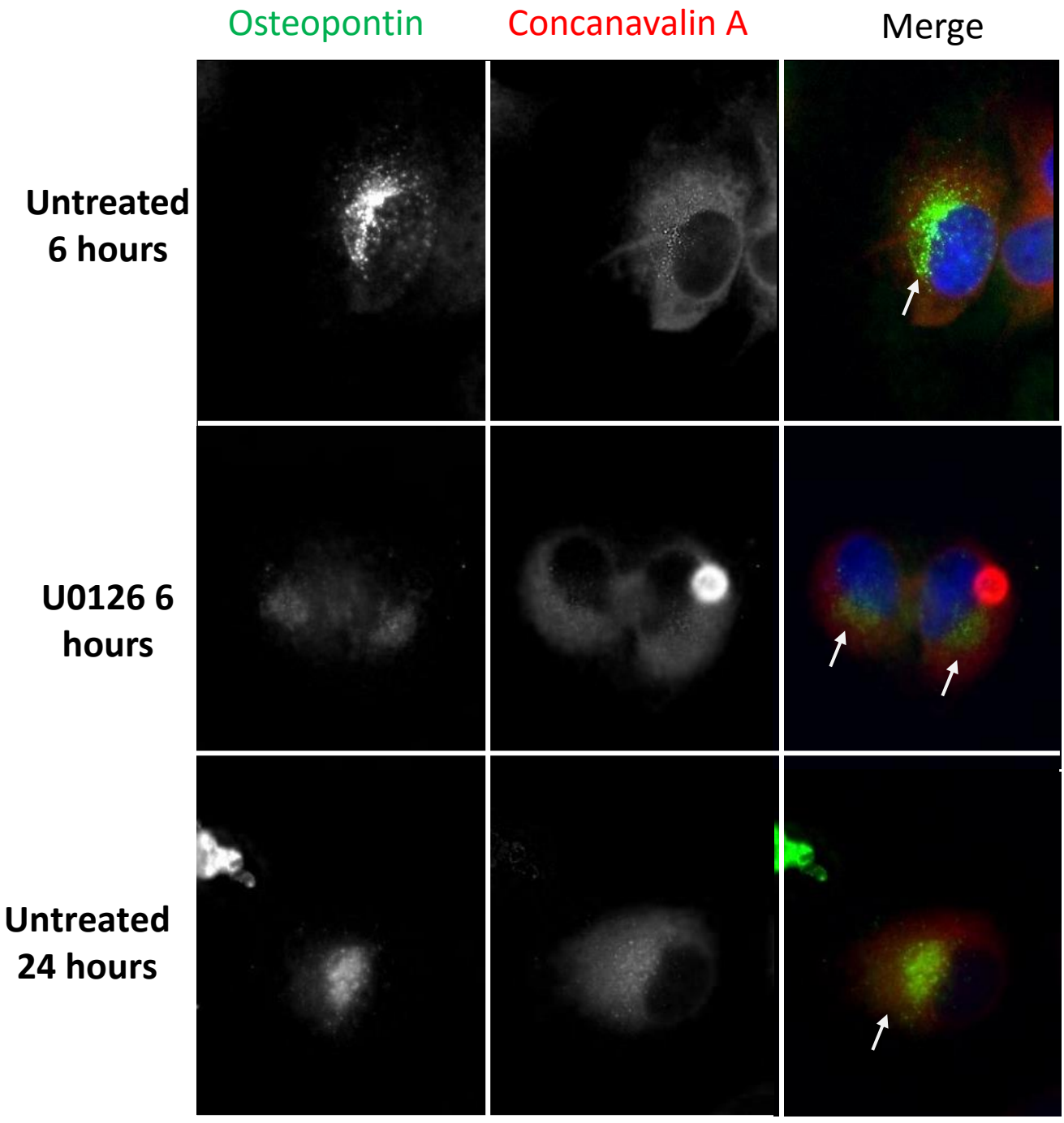


FIGURE 3.15, Immunostaining of osteopontin and the Golgi apparatus over 72 hours shows a mono-phasic effect on osteopontin trafficking. As predicted, an effect is seen at 6 hours of treatment with the U0126 inhibitor, with retention of endogenous osteopontin compared to the untreated control at 6 hours (arrows). Once the inhibitor begins to wear off after 24 hours (untreated and U0126 24-72 hours) trafficking of osteopontin appears to take place normally, with no retention or clustering seen the way it is at 6 hours (arrows). Scale bar: 50µm. n=3

We carried out the same experiment but stained the cells with Concanavalin A to check osteopontin localisation in the endoplasmic reticulum. A study in 2010 by Farhan et al showed that ERK 2 affected Endoplasmic reticulum exit sites (ERES) which subsequently led to reduced ER-Golgi transport. In this study, ERES numbers reduced after 24 hours and we asked if there might be a similar reduction in ROS cells. The hypothesis is that we might see co-localisation of osteopontin and Concanavalin A as there would be less trafficking out of the ER if MAPK inhibition was reducing ERES. Note that, while we carried out the experiment

over the 72 hour time course, data will be shown up to the 48 hour time point as this is the time point of interest (figure 3.16).



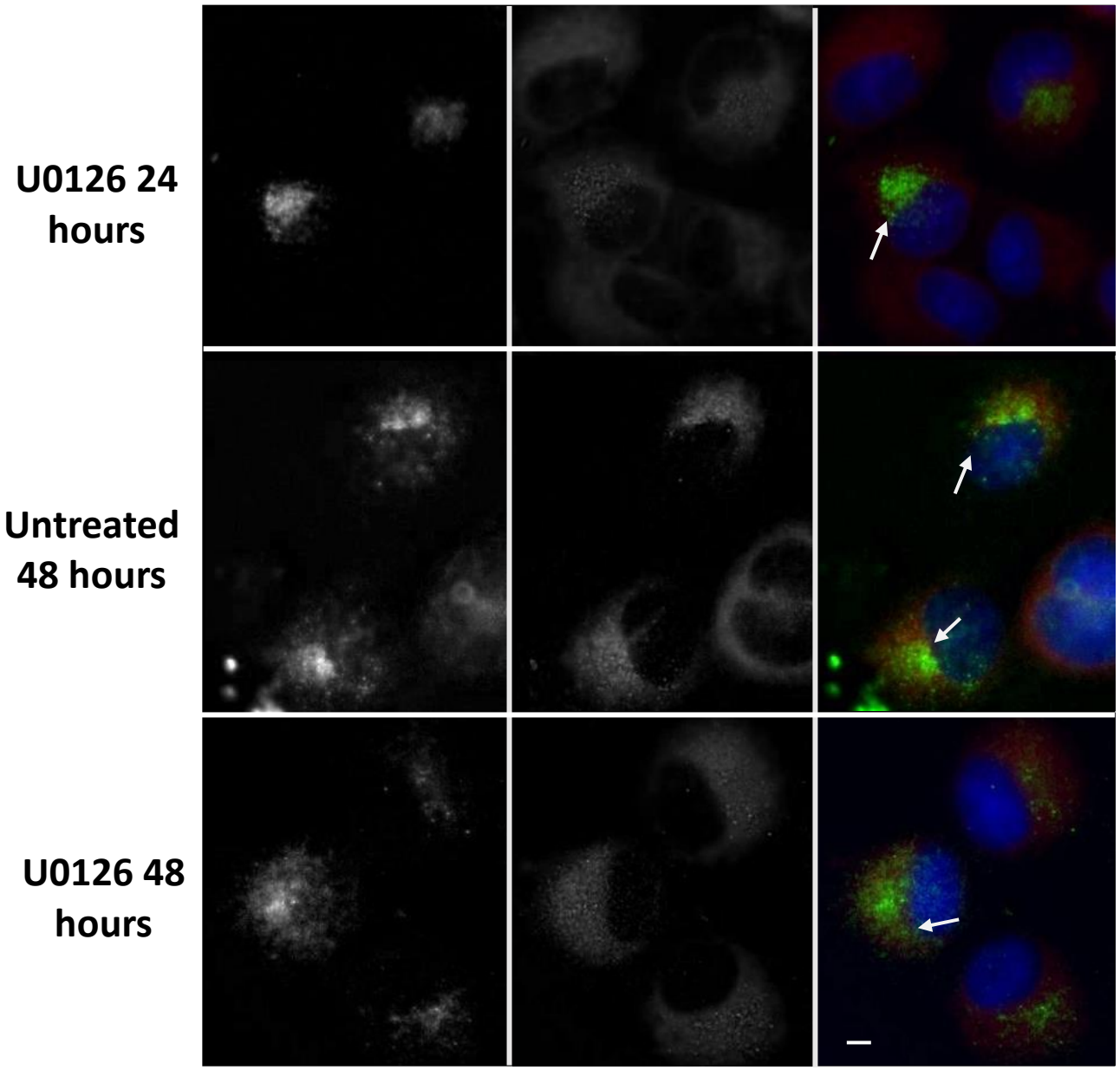


FIGURE 3.16, Inhibition of the MAPK pathway does not cause retention of osteopontin in the endoplasmic reticulum at 24 hours. There is no apparent co-localisation between osteopontin and the endoplasmic reticulum with the cellular distribution of osteopontin being similar to the 6 hour experiment (figure 3.7). At 24 hours, there is no difference in distribution of osteopontin, nor is there any co-localisation with the ER. The same applies for 48 hours, but this is expected as phosphorylated levels of ERK are back to basal levels by this point. Scale bar: 50µm. n=3

Experiments looking at longer time points with the U0126 inhibitor confirmed that once the inhibitor had stopped inhibiting MEK 1 and 2, secretion of osteopontin resumed.

3.5. U0126 WASHOUT

Research carried out has shown the rapidly reversible nature of the U0126 inhibitor (Subramanian & Morozov 2011).

For this reason, we attempted to carry out a U0126 washout experiment in order to see if osteopontin secretion could be rescued after inhibition of the MAPK pathway.

Please note, U0126 washout experiment was carried out twice in duplicate.

To achieve reversal of MEK inhibition, cells were seeded, allowed to adhere and grow for 48 hours in normal complete DMEM.
On the day of treatment, U0126 inhibitor was added to the appropriate wells on a 24 well plate. Following 1 hour of treatment, untreated samples as well as samples treated were fixed and cell lysates taken. 4 coverslips that had also been exposed to the U0126 inhibitor were washed as follows:

- Media aspirated
- Rinsed twice with warm PBS
- Replaced with fresh, non-U0126 containing media
- Plate left in incubator

After 15, 30, 45 and 60 minutes, cells were fixed along with cell lysates taken for respective time points.
We observed a rapid reversal of MEK inhibition with ERK phosphorylation resuming by the 15 minute wash out time point (figure 3.17). The same effect was also reflected in the localisation of osteopontin when we carried out immunofluorescence staining and stained cells for endogenous osteopontin and the Golgi apparatus (figure 3.18).

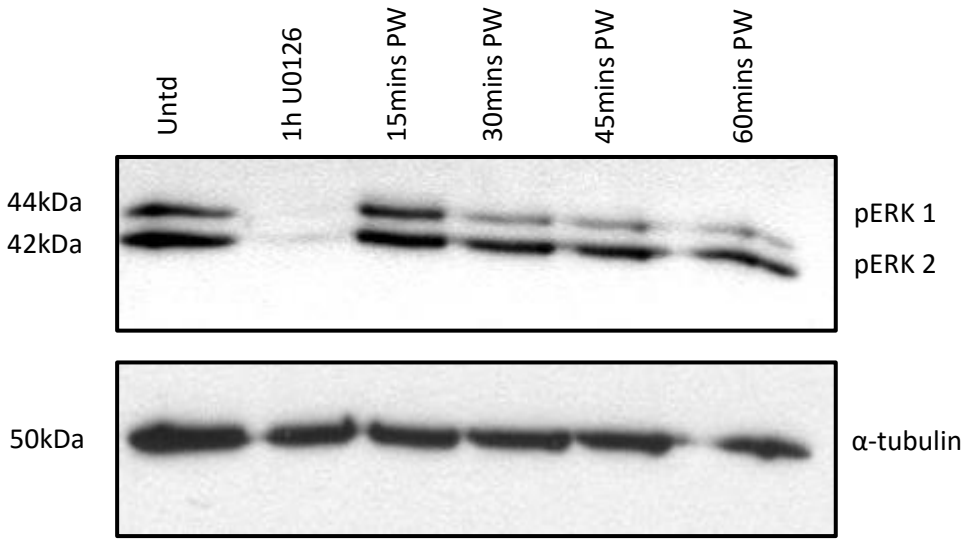
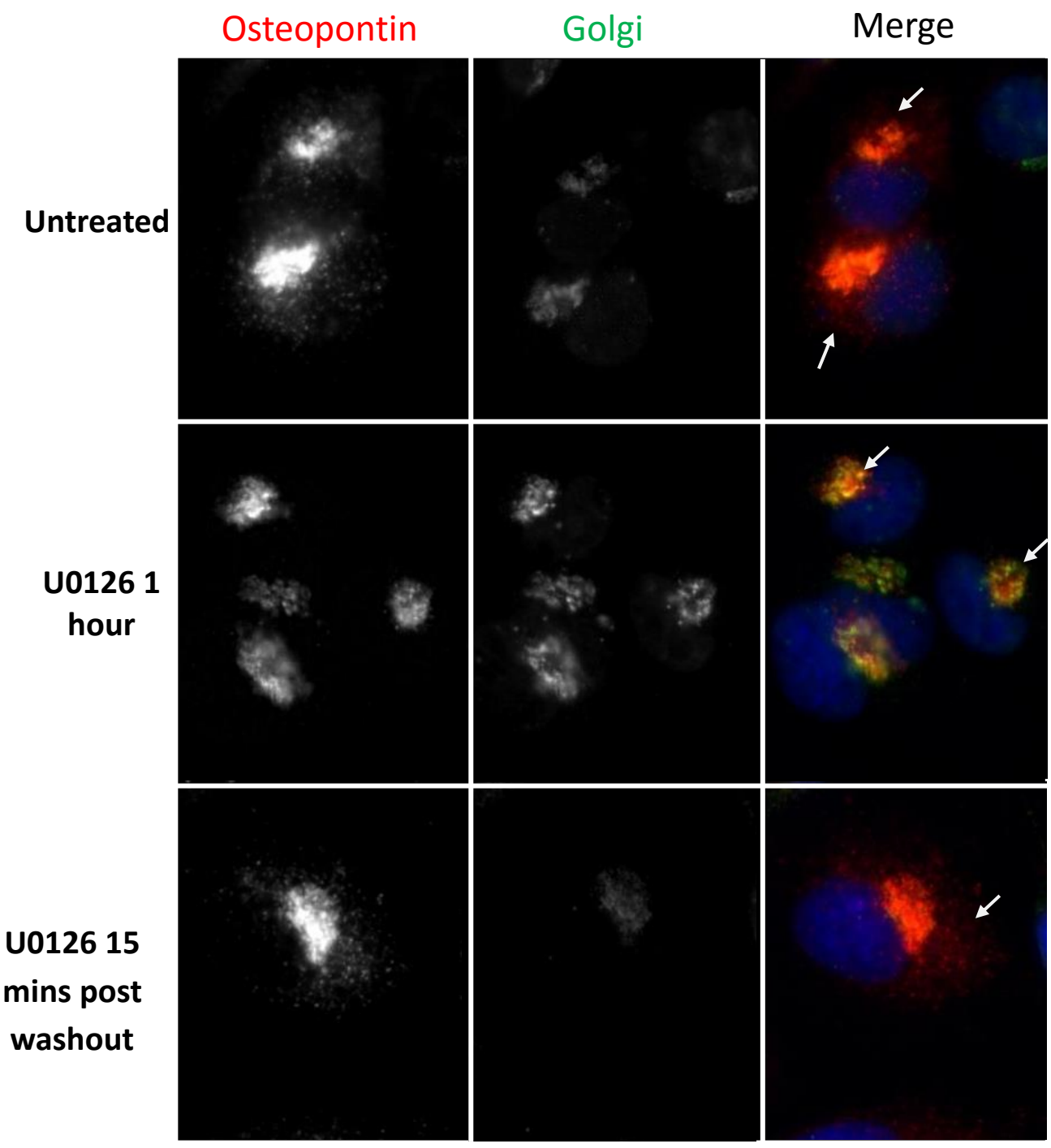


FIGURE 3.17, Effects of the U0126 inhibitor are rapidly reversible within 15 minutes of washout (PW=post washout). Western blot analysis shows successful MEK 1 and 2 inhibition after 1 hour with no phosphorylated ERK being detected. Following U0126 washout (15-60 minutes pw) there is a presence of 2 bands (phospho ERK 1, [p44] and 2, [p42] respectively). After 15 minutes of washout, ERK 1 activation levels are highest with a slight reduction in ERK 1 activation from 30-60 minutes although this may be normal as more protein has been detected in the first well (shown by α-tubulin). n=2

Very briefly, phosphorylated ERK 1 levels are lower from 30-60 minutes post-washout. Looking at α-tubulin, there does appear to be more total protein content in the untreated cell sample. Protein loading appears consistent from 1h U0126 inhibition to 60 minutes post-washout. There may be a surge of phosphorylation following rescue of ERK 1 and 2 activation in order to compensate for a lack of phosphorylation in 1 hour and it may be that ERK activation returns to basal levels after this (bearing in mind phosphorylated ERK 1 is lower than phosphorylated ERK 2 in ROS cells (discussed in discussion section 3.7). The total protein loaded may explain why there appears to be less phosphorylated ERK 1 in untreated cells post-washout. Alternatively, while rapidly reversible, there may be some residual inhibition by U0126 that is already inside the cell. Since phosphorylated ERK 1 levels are lower in ROS cells, it is plausible that residual U0126 would be more evident on ERK 1 than ERK 2.

We carried out an immunofluorescence staining to look at osteopontin localisation during the washout experiment (figure 3.18). Cells on coverslips treated at the same time as the western blot experiment shown above were fixed. We predicted that we would observe the entrapment we were seeing after an hour of inhibition of ERK 1 and 2 activation, with secretion resuming once MEK inhibition was diminished.

We observed a dispersed intracellular pattern of osteopontin within 15 minutes of ERK 1 and 2 re-activation. The advantage we had with this particular experiment was that the effects of the U0126 inhibitor are rapidly reversible and therefore a good way of observing the intracellular trafficking pattern of osteopontin. Untreated cells show co-localisation with the Golgi as well as evidence for osteopontin vesicles post-Golgi implying that secretion of the protein is taking place. Similar to the observations in previous experiments, inhibition of ERK 1 and 2 activation showed retention around the Golgi apparatus and fewer osteopontin vesicles post-Golgi. Following U0126 washout after 15 to 60 minutes, there is apparent co-localisation with the Golgi plus evidence of osteopontin post-Golgi set for secretion similar to that of the untreated control cells.


Osteopontin
Golgi
Merge
Untreated
U0126 1 hour
U0126 15 mins post washout

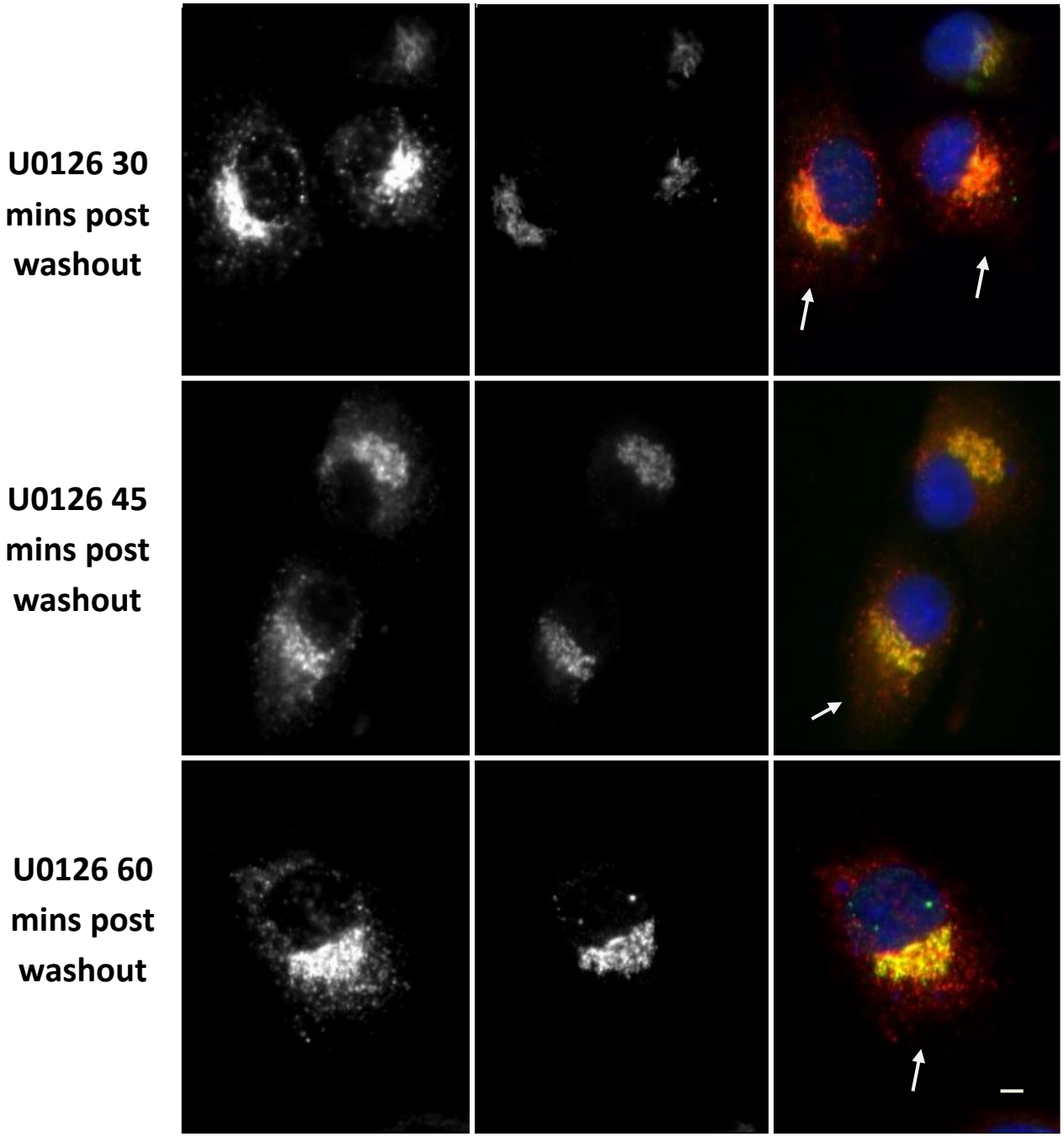


FIGURE 3.18, Washing out the U0126 inhibitor 1 hour after treatment shows secretion of osteopontin at various time points following washout. A difference can be seen between the **untreated** cells and the cells treated with the U0126 inhibitor for **1 hour**. This is in agreement with previous experiments carried out that show entrapment of osteopontin and GFP-SPARC in a perinuclear region following inhibition of MEK 1 and 2 (arrows). **15-60 minutes**, following washout of the U0126 inhibitor, there appears to be anterograde (forward) trafficking of osteopontin within 15 minutes of ERK 1 and 2 activation (arrows). This trafficking appears to be sustained throughout the time points up to 60 minutes. Scale bar: 50µm. n=2

Reversal of U0126 inhibition showed that osteopontin trafficking was indeed affected by the MAPK pathway as there was a clear and distinct difference in osteopontin localisation at the point of inhibition compared to untreated control cells, and also after washout of the inhibitor with a re-initiation of osteopontin trafficking post-Golgi.
In order to confirm that the effects we were seeing were truly a result of trafficking and not downregulation of SPARC and osteopontin, we carried out western blot analysis across all time points to look at relative expression of proteins.

3.6. PROTEIN EXPRESSION

The ERK 1 and 2 module of the MAPK pathway are fundamental in the expression of target proteins required for cell survival and overall cellular activity (Mebratu & Tesfaigzi 2009).

We asked if inhibition of ERK 1 and 2 was actually not affecting the trafficking of SPARC or osteopontin, but affecting the expression of target genes and therefore showing us the effect we observed, not because ERK 1 and 2 are involved in the trafficking process, but absence of active (phosphorylated) ERK 1 and 2 is downregulating the expression of SPARC and osteopontin.

Experiments were carried out under the same conditions as were carried out for immunofluorescence, in fact, western blots were run to accompany immunofluorescence experiments and not separately, thus ensuring effects seen under the microscope were likely to be due to the inhibition of ERK 1 and 2 (or ERK 1 and 2 individually for siRNA, chapter 4.0). Treatments carried out were as follows: Untreated control, 1, 2 and 6 hours inhibition with the U0126 inhibitor. Expression of several proteins was looked at (figure 3.19) to check for any differences.

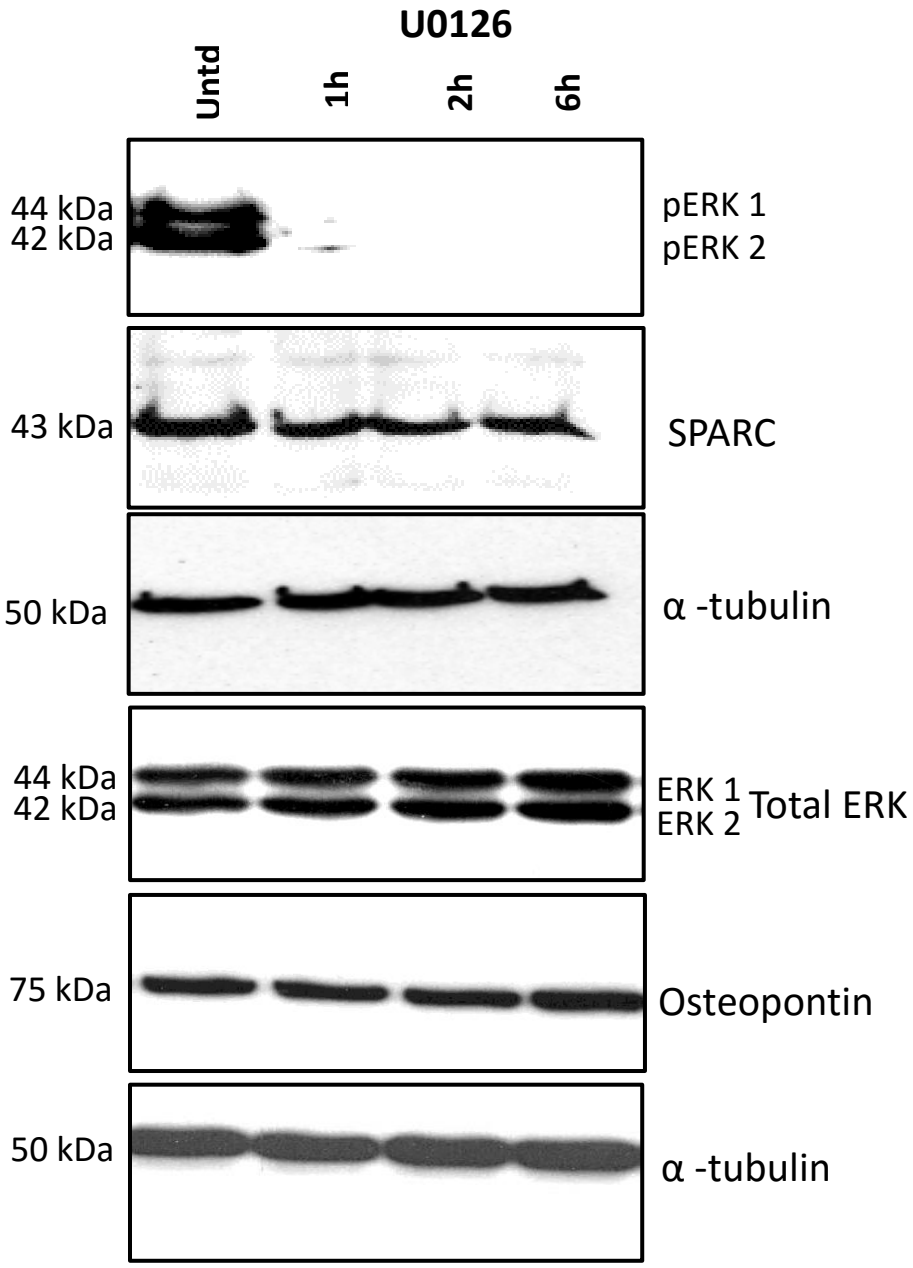
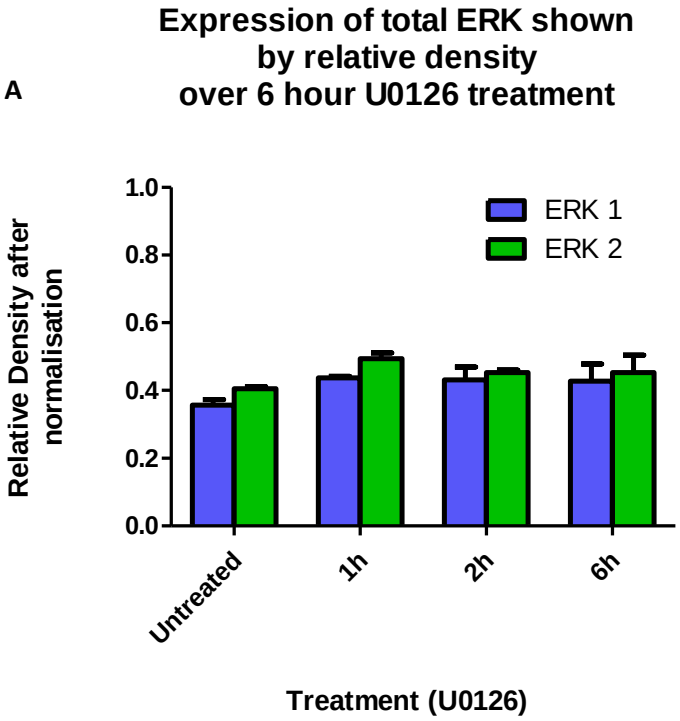


FIGURE 3.19, Expression of proteins in the ROS cell line shows phosphorylated ERK is not present when inhibited (1-6 hours), and other proteins show relatively similar expression patterns between untreated and treated cells. We used antibodies targeting various proteins as shown above. Following inhibition of MEK 1 and 2 with the U0126 inhibitor, activated (phosphorylated ERK) is no longer present with the absence of bands for ERK 1 and 2 indicating this. SPARC (antibody AON5031) and osteopontin expression levels are relatively

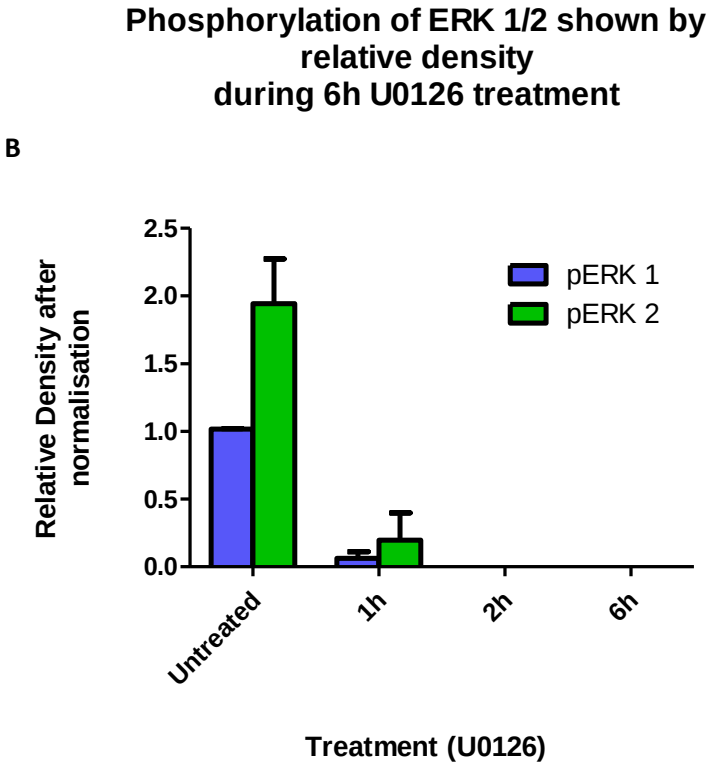
similar, and it is unlikely that differences seen are due to downregulation of SPARC or osteopontin expression, as the difference is not significant (see figure 3.20).

Relative density of the bands shown in figure 3.19 for each sample was compared and graphs were produced to show the expression differences according to the density of the bands and the comparison between untreated and treated cells after normalisation to the housekeeping protein α-tubulin (figure 3.20).

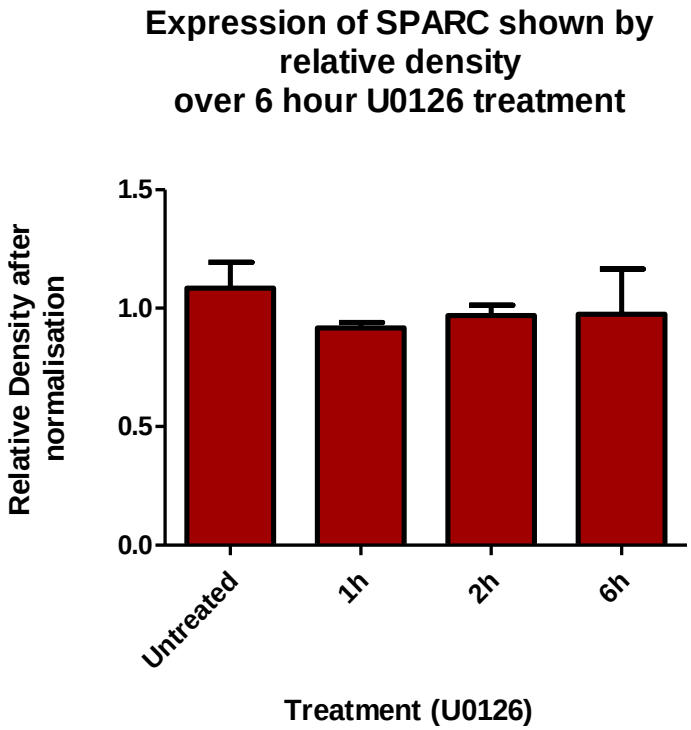
A


Expression of total ERK shown by relative density over 6 hour U0126 treatment
Relative Density after normalisation
1.0
0.8
0.6
0.4
0.2
0.0
ERK 1
ERK 2
Untreated
1h
2h
6h
Treatment (U0126)

B


Phosphorylation of ERK 1/2 shown by relative density during 6h U0126 treatment
Relative Density after normalisation
2.5
2.0
1.5
1.0
0.5
0.0
pERK 1
pERK 2
Untreated
1h
2h
6h
Treatment (U0126)

C


Expression of SPARC shown by relative density over 6 hour U0126 treatment
Relative Density after normalisation
1.5
1.0
0.5
0.0
Untreated
1h
2h
6h
Treatment (U0126)

D

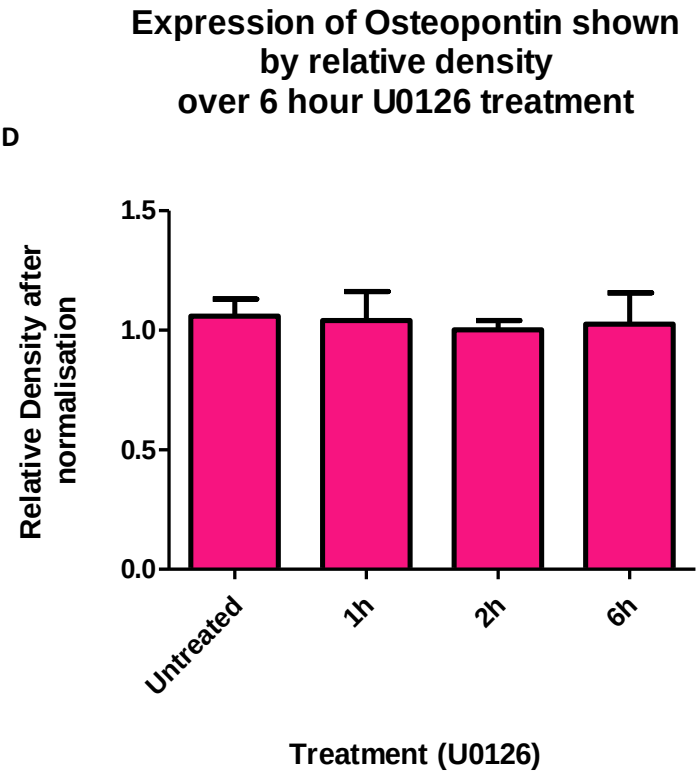

Expression of Osteopontin shown by relative density over 6 hour U0126 treatment
Relative Density after normalisation
1.5
1.0
0.5
0.0
Untreated
1h
2h
6h
Treatment (U0126)

FIGURE 3.20, Expression of total ERK, SPARC and osteopontin does not fluctuate significantly. All error bars indicate standard deviation (SD). A, Levels of total ERK do not change significantly over the time course, although there is an increase in total ERK 1 and 2 levels at 1 hour. This may be a side effect of MEK inhibition with an increase in ERK 1 and 2 expression to try and compensate for the lack of phosphorylation, as time points 1-6 hours show slightly higher levels of total ERK 1 and 2 (although this is increase is not significant according to a one way Anova test apart from untreated ERK 1 versus 1h U0126 ERK 2 [*P<0.05], but not between untreated ERK 2 and 1h U0126 ERK 2). **B,** The U0126 inhibitor is extremely effective, and by one hour there is very little phosphorylation of ERK 1 and 2 with no detectable bands during treatment of ROS cells from 2-6 hours. **C and D,** Expression of SPARC and osteopontin does not appear to be significantly affected by MEK inhibition with no significance between all time points for both SPARC and osteopontin as determined by a one way Anova test, n=3.

Having carried out western blot analysis and showing that there was very little difference in protein expression of SPARC and osteopontin over the 6 hour U0126 time treatment, a cell count was carried out to see if MAPK inhibition over 6 hours would affect cell survival (figure 3.21).

Please note that cell count experiment was carried out once in triplicate.

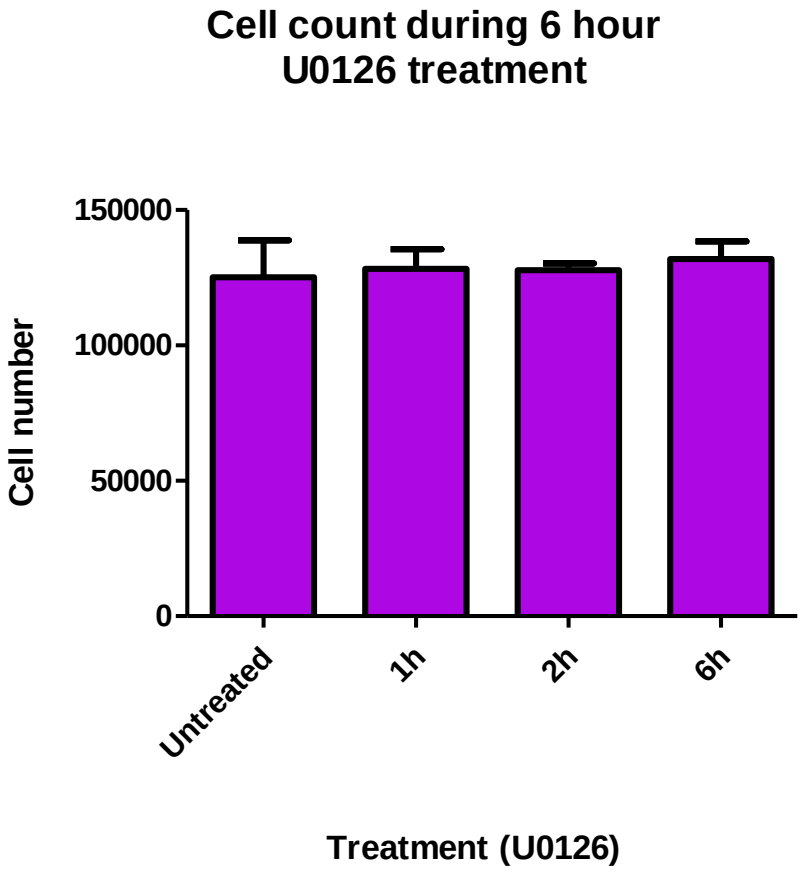


FIGURE 3.21, Cell count over 6 hour U0126 treatment shows little difference in cell number between treatments. Error bars represent SD. The effect of osteopontin and SPARC retention during the U0126 inhibitor treatment is likely to be an effect on early events in trafficking and not on cell survival. During the 6 hour time treatment, there is no significant difference in cell number when ERK 1 and 2 activation is inhibited. There is no significance between all time points as determined by a one way Anova test. n=1

Western blot and densitometry analysis determined that the effects we were seeing in the localisation of SPARC and osteopontin were truly caused by the effects on trafficking when MAPK is inhibited, and not by an effect on the expression of SPARC and osteopontin. We also paid some attention to the off target effects of the U0126 inhibitor to be sure that what we were seeing was as a result of MEK inhibition and not any off target effects on other proteins.

3.6.1. OFF TARGET EFFECTS OF THE U0126 INHIBITOR

There is evidence that the mTOR pathway is also affected by the U0126 inhibitor. For this reason, phosphorylated s6-kinase, a substrate for mTOR complex 1, which is phosphorylated before activating transcription factors of target genes was checked to exclude potential involvement of the mTOR pathway in the trafficking of SPARC or osteopontin. Activation of phosphorylated s6-kinase is unchanged between the untreated cells and cells treated with the U0126 inhibitor for 1 and 2 hours (figure 3.22 and 3.23). There is a reduction however at 6 hours, and it seems that the U0126 inhibitor is having an effect here. Nevertheless, activation of phosphorylated s6-kinase is still evident, and the inhibitory effect is not substantial enough to deplete active s6-kinase entirely (Martin et al. 2001).

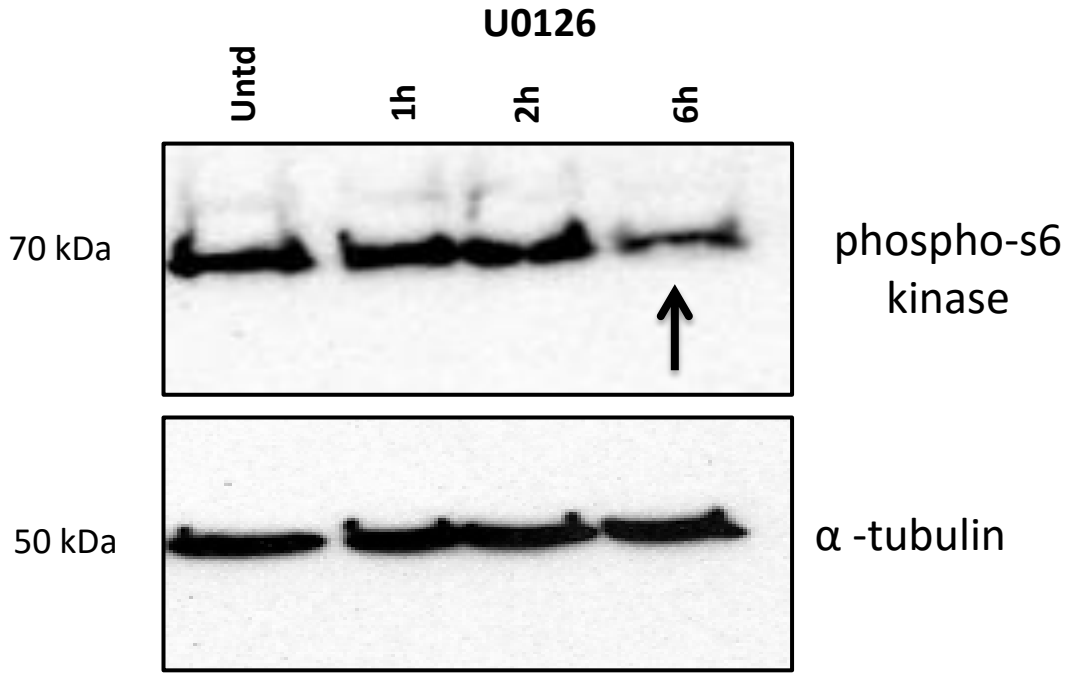


FIGURE 3.22, Activation of phospho-s6 kinase remains consistent up to the 2 hour point and reduces at 6 hours after treatment with U0126 (arrow).

Multiple U0126 treatments could not effectively reduce phospho-s6 kinase activation at 6 hours. In some instances the lack of phosphorylated s6-kinase was reduced by about 32% while in other 6 hour treatments there was less of an effect on activation of phospho s6-kinase at 6 hours. Activation of this kinase was always relatively equal at 1 and 2 hours of treatment (figure 3.23).

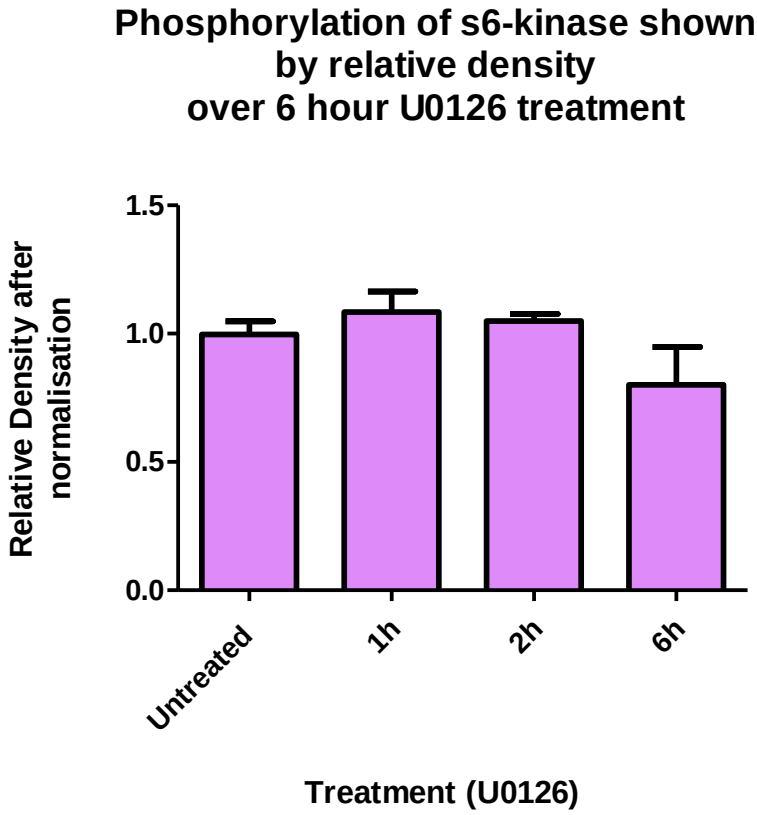


FIGURE 3.23, Phospho s6-kinase in treated ROS cells does not show a significant change in expression, and is unlikely to be the reason for the effect seen on SPARC and osteopontin trafficking when the U0126 inhibitor is administered into the cell. Expression of phospho-s6 kinase remains relatively similar up to 2 hours with the U0126 inhibitor when compared to the untreated control cell with a slight reduction in activation at 6 hours. This reduction is not significant compared to the untreated control cell, nor is the reduction consistent with more activation in some instances over others. It is unlikely therefore that this reduction is contributing to the trafficking of SPARC and osteopontin. Differences between all samples are not significant according to a one way Anova test. **Error bars indicate SD.** n=2

There is evidence also that phospho-s6 kinase activation is reduced under the influence of the U0126 inhibitor within the time course of 1 hour. Studies in HEK293 cells showed that there was a reduction in phospho-s6 kinase activation at 15 and 30 minutes before activation began once more at the 60 minute time point (Naegele & Morley 2004).

We decided to replicate this experiment in order to rule out any involvement before one hour. Since phospho-s6 kinase expression resumed at 60 minutes in the study cited above (Naegele & Morley 2004), there was no way of knowing what was happening prior to an hour in the ROS cell line, and if reduction of phospho-s6 kinase might also affect trafficking within an hour if down regulated (figure 3.24).

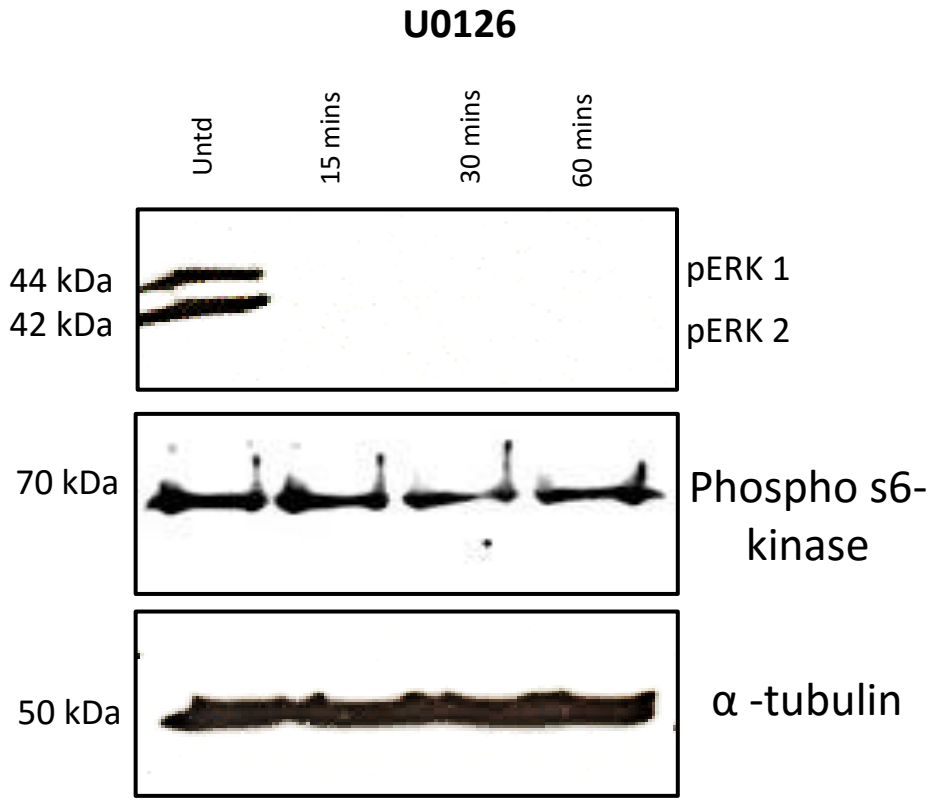


FIGURE 3.24, 60 minute treatment with the U0126 inhibitor shows no effect on the activation of phospho s6-kinase. Phospho-ERK bands are absent from 15-60 minutes in the ROS cell line and accompanying this blot is activation of phospho-s6 kinase in the 1 hour time point, 15, 30 and 60 minutes. Unlike the expression in HEK293 kidney cells (Naegele & Morley 2004), it seems that phospho-s6 kinase activation is unaffected by the U0126 inhibitor. Please note that this experiment was carried out multiple times, however, due to poor western blot results, this figure is the best representative image and no quantification has been carried out (n=1).

Similar to the protein expression profile above, we carried out western blot analysis for the 72 hour U0126 treatment (figure 3.25) to see if osteopontin and SPARC expression might be

affected over a longer time point. The same proteins as the 6 hour U0126 inhibitor treatment were targeted with the exception of phospho-s6 kinase.

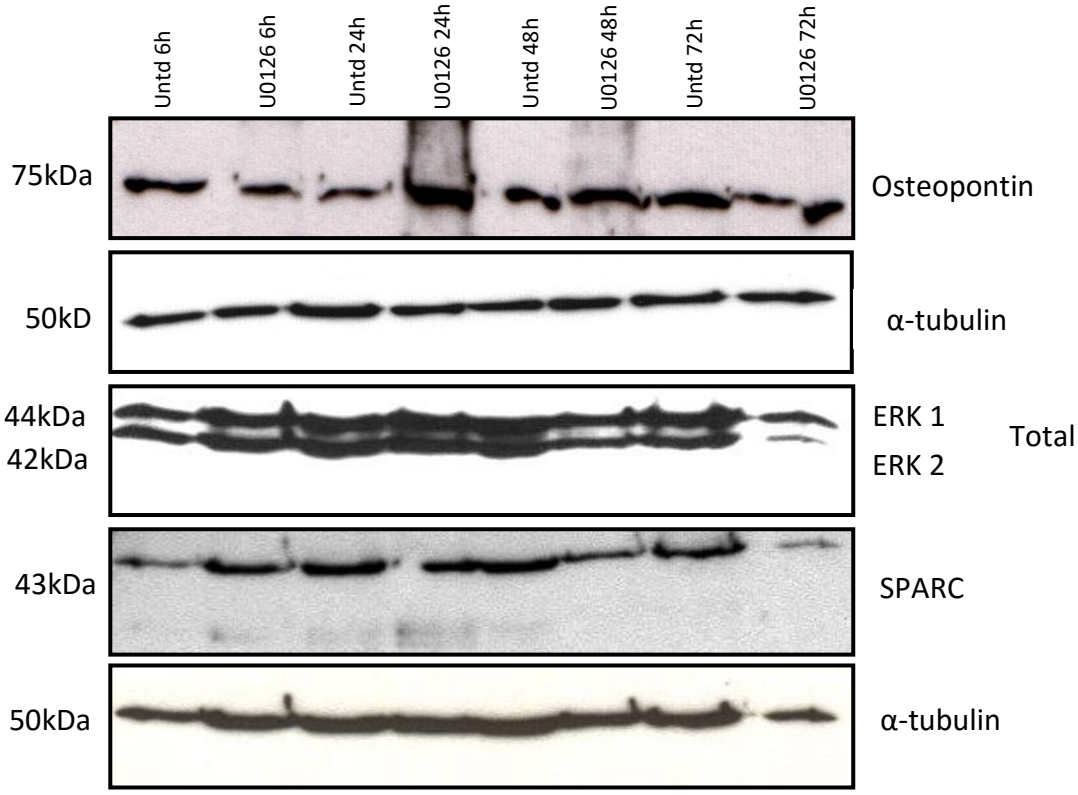
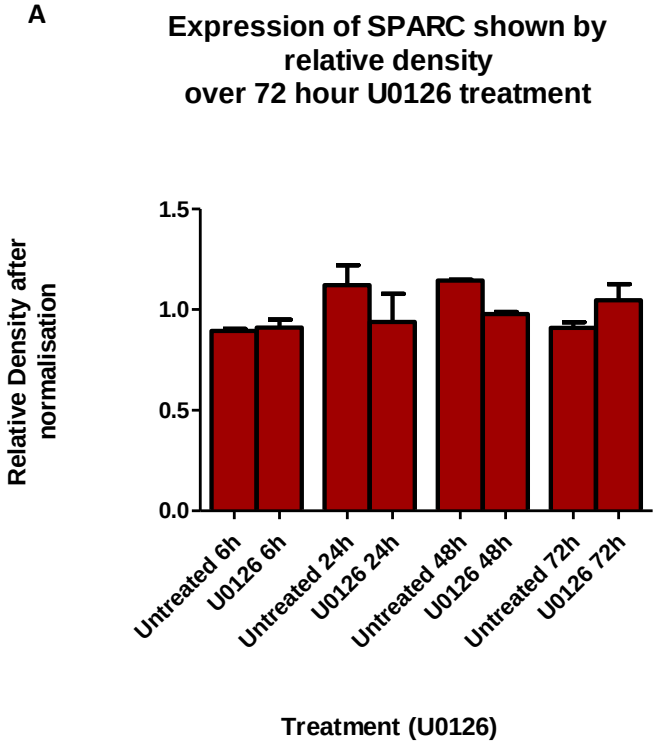


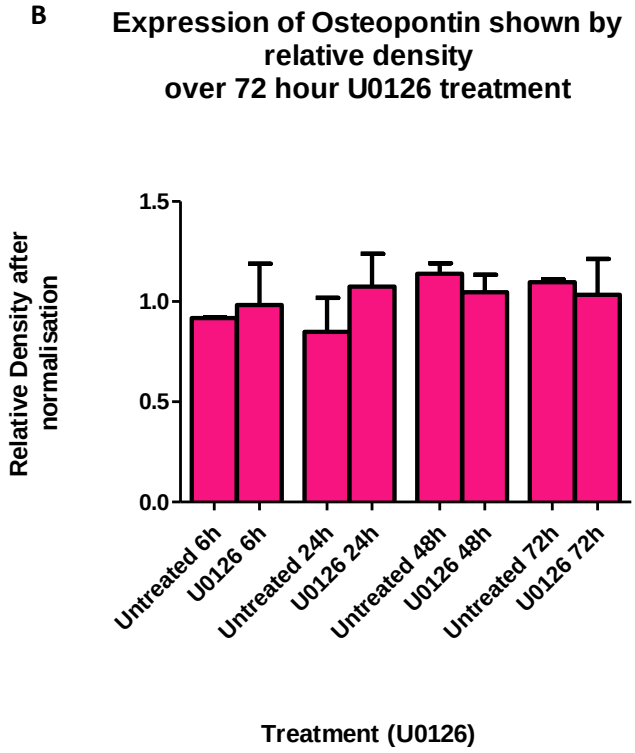
FIGURE 3.25, 72 hour treatment showing expression of SPARC, osteopontin and total ERK over the time course. There are slight differences in loading levels of the cell lysates with some samples appearing to show more loading than others, however, it is evident that SPARC and osteopontin are expressed throughout and up to the point of 24 hours (while the inhibitor is active) and there does not appear to be major differences in expression of these two matricellular proteins. n=2

We quantified the bands for the 72 hour western blots above in order to look at protein expression differences (figure 3.26).

A


Expression of SPARC shown by relative density over 72 hour U0126 treatment
Relative Density after normalisation
1.5
1.0
0.5
0.0
Untreated 6h
U0126 6h
Untreated 24h
U0126 24h
Untreated 48h
U0126 48h
Untreated 72h
U0126 72h
Treatment (U0126)

B


Expression of Osteopontin shown by relative density over 72 hour U0126 treatment
Relative Density after normalisation
1.5
1.0
0.5
0.0
Untreated 6h
U0126 6h
Untreated 24h
U0126 24h
Untreated 48h
U0126 48h
Untreated 72h
U0126 72h
Treatment (U0126)

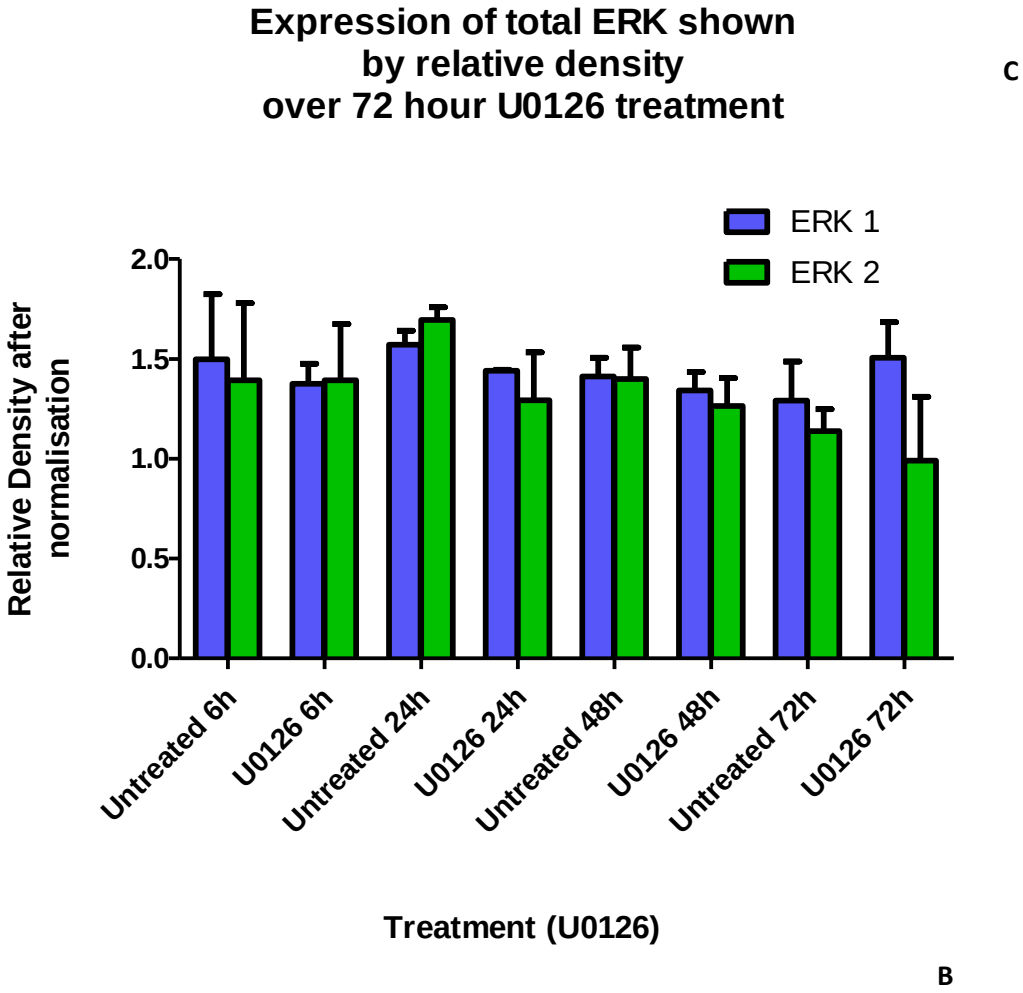


FIGURE 3.26, Expression of SPARC, osteopontin and total ERK shows some variation in expression levels, but these differences are not statistically significant. All error bars indicate SD. A and B, SPARC expression shows some fluctuation between the different time points with untreated levels for SPARC being higher at 24 and 48 hours. Untreated SPARC versus 6h U0126 SPARC does not show any difference indicating that there may be some influence of SPARC expression by MEK inhibition, but this is not effective until 24 hours although the apparent down regulation is not high enough to affect expression significantly. The reverse is true for osteopontin with U0126 treated cells having higher levels of osteopontin from 6-48 hours indicating no effect on expression of osteopontin by the inhibitor. **C,** Total ERK levels do not fluctuate significantly although there is some variance at the later time points (72 hours). There is no apparent difference between ERK 1 and 2 levels, with the biggest difference being seen in the levels of phosphorylated ERK. One way Anova test does not show any significance (**A-C**) between any of the groups in each graph. n=2

We were not convinced that there was a significant difference in the expression of SPARC and osteopontin through all U0126 treatments. It is likely that the effects we are seeing in the localisation of osteopontin and SPARC in immunofluorescence staining is due to the involvement of ERK 1 and 2 in the trafficking of these two matricellular proteins.

3.7. DISCUSSION

We investigated the effect that the ERK 1 and 2 module of the MAPK pathway might have in the intracellular trafficking of the matricellular proteins SPARC and osteopontin. The process of trafficking is well understood in terms of coat assembly and destinations associated with different types of vesicles. What is not fully understood are the factors that might be involved in the trafficking of certain types of proteins, factors such as signalling pathways in response to extracellular stimuli, whether they are specific factors or multiple different factors that can influence trafficking. We were interested in understanding the role of the ERK 1 and 2 modules of the MAPK pathway in the trafficking of matricellular proteins in osteoblasts. This might help understand how matricellular proteins are secreted in response to signals that induce bone mineralisation, cell migration or metastasis in cancer. Our initial immunofluorescence staining results suggested that these processes are likely to be very tightly regulated from induction of proteins involved in processes above, to the signals required to push them out of the cell.

We used the small molecule inhibitor U0126 to inhibit MEK mediated phosphorylation of ERK 1 and 2. Activation of ERK 1 and 2 was rapidly prevented (within 30 minutes) and the fast acting nature of this inhibitor helped us look at the early effects of inhibition of the MAPK pathway in the trafficking of GFP-SPARC and osteopontin.

Initial experiments staining the actin cytoskeleton along with either GFP-SPARC or endogenous osteopontin gave us an idea of what the pattern of matricellular protein trafficking looked like, along with what happened when cells were inhibited. Within an hour, we saw a tight clustering, or retention of GFP-SPARC and osteopontin compared to the untreated control cells. The ERK 1 and 2 module of the MAPK pathway has been implicated in the forward trafficking of proteins. The data for ERK 1 and 2 and trafficking is sparse, nevertheless, the data that does exist suggests that there may be involvement of these two kinases in the trafficking process. Work carried out in NIH3T3 (fibroblast) cells showed that the MAPKs p38 (and ERK 1 and 2 to a lesser extent) were important in activating and inducing the shuttling of SAC1 (a phosphoinositide phosphatase) from the Golgi apparatus to the endoplasmic reticulum allowing phosphatidylinositol-4-phosphate (PI(4)P, phosphoinositide

lipid) to promote forward trafficking of proteins out of the Golgi. When in the Golgi apparatus, SAC1 would inhibit PI(4)P and stimulation of p38 and ERK 1 and 2 activation would cause rapid shuttling of SAC1 into the ER where PI(4)P could promote forward trafficking. Specifically what p38 and ERK 1 and 2 are doing is not clear, but it seems they are involved in regulating forward trafficking in response to mitogens (Blagoveshchenskaya et al. 2008).

Recent work carried out by the same group showed that 14-3-3, a protein commonly used as a chaperone in the trafficking pathway was required for sorting of SAC1 into COPII coated vesicles for transport back to the Golgi apparatus. As 14-3-3 is a substrate for ERK 1 and 2, there is a possibility that the MAPK pathway may also be involved in this process (Bajaj Pahuja et al. 2015).

Indeed we observed a change in the intracellular distribution of osteopontin and GFP-SPARC when MEK was inhibited although we were not sure as to what the kinases might be doing to cause this effect.

Furthermore, ERK 1 and 2 have also been implicated in the endocytosis pathway with the ERK kinases being involved in endosomal recycling of intracellular cargo such as class I major histocompatibility complex (MHCI) (Robertson et al. 2006).

There is work that has looked at possible roles that SPARC may play inside the cell (despite being a predominantly secreted matricellular protein). Studies have shown that SPARC can translocate into the nucleus (when cytoplasmic SPARC levels are low) in murine lens epithelial cells implicating SPARC with a possible regulatory role inside the cell, although it is not known if cytoplasmic SPARC is as a result of internalised SPARC after secretion or SPARC that remains in the cytoplasm after synthesis (Yan et al. 2005).

The internalisation of SPARC in skeletal muscle progenitor cells (Skm-PCs) has been shown to increase with age in Skm-PCs taken from rats. Cells taken from aged rats show a higher level of internalised SPARC. SPARC is thought to play an important role in preventing adipogenesis via integrin α5. Skm-PCs from aged rats showed a higher level of internalised SPARC which also correlated with higher levels of Rab7, a GTPase associated with late endosomes suggesting lysosomal degradation. Furthermore, these cells were shown to have an increased adipogenic potential versus Skm-PCs from younger mice. With this data,

although situation specific, it seems SPARC is degraded via the clathrin mediated endocytosis-lysosomal degradation route (Nakamura et al. 2014).

SPARC has also been shown to interact with the scavenger receptor stabilin-1 and contains a stabilin-1 binding site. Interaction of SPARC with stabilin-1 increases its uptake and subsequent clearance from the stromal environment of endothelial cells. The identification of such a receptor and method of internalisation demonstrates that SPARC (and likely other matricellular proteins) clearance is tightly regulated once they have carried out various roles in the extracellular space (Workman & Sage 2011).

Recent work has also shown that SPARC can mediate the internalization of other ECM proteins such as collagen, fibronectin and vitronectin when extracellular calcium levels are high in fibroblasts. This implicates SPARC as a physical chaperone, and gives SPARC a functional role in how it may mediate some of its cell-matrix interactions (Chlenski et al. 2011).

The studies cited above show that SPARC can shuttle inside and outside the cell upon stimuli such as calcium levels, or SPARC shuttling into other compartments of the cell based on intracellular SPARC levels, but there is no work looking at the degradation of these proteins implicating SPARC in roles that were previously unknown. Recycling of SPARC therefore may follow the traditional route of clathrin mediated endocytosis, but only in carrying out a secondary function such as its role as a chaperone, before it might be recycled through the endosomal pathway into the extracellular space once more. Alternatively, it may simply enter the lysosomal/degradation pathway.

It is unlikely that any of the effects that were seen upon MAPK inhibition were due to a change in the endocytic pathway, and it appeared that the aggregation of GFP-SPARC and osteopontin was taking place early on, more likely through its passage through the anterograde (forward) trafficking pathway.

To identify possible organelles or sites in which GFP-SPARC and osteopontin were being retained, co-immunostaining was undertaken. This would help us narrow down where this effect was taking place. Since the apparent retention was taking place in a perinuclear region, we looked first at the endoplasmic reticulum (ER) and the Golgi apparatus. Staining with

Concanavalin A and ERp72 (2 probes for the endoplasmic reticulum) did not show any co-localisation of GFP-SPARC and osteopontin with the ER. Retention in the endoplasmic reticulum might suggest that proteins have not been folded correctly and therefore do not enter ER exit sites to be incorporated into COPII coated vesicles. This lack of co-localisation led us to believe that there was no problem with protein folding (or an involvement of ERK 1 and 2 in this process) in ROS cells, and that the entrapment was not taking place here. There is no evidence that ERK 1 and 2 shuttle into the endoplasmic reticulum as part of one of their functional roles although they have been implicated in the unfolded protein response (UPR) (Darling & Cook 2014). Two mechanisms for the involvement of the MAPK pathway in the UPR have been shown, the first is by signalling to promote the activation of transcription factors (not limited to ERK 1 and 2 but also p38 and JNK MAPKs) that promote cell survival in response to ER stress as well as activating a substrate (ATF6) known to become activated in response to unfolded proteins in the ER, which shuttles from the ER to the Golgi apparatus, is proteolytically cleaved by S1P and S2P (two Golgi resident proteases), is phosphorylated by p38 (and maybe other MAPKs) and then promotes the transcription of ER stress response proteins (Darling & Cook 2014).

Given our data as well as work that has been published about how MAPKs are involved in ER processes such as folding, it is unlikely that ERK 1 and 2 are doing anything to cause retention of SPARC and osteopontin in the ER.

SPARC retention in the endoplasmic reticulum has been reported in patients suffering from Pseudoachondroplasia (PSACH), a disease that causes dwarfism and irregularities in the long bones. A mutation in cartilage oligomeric matrix protein (COMP) causes retention of proteins in the endoplasmic reticulum in chondrocytes, and SPARC was found to be a protein that was retained in the ER of long bones of PSACH patients (Hecht & Sage 2006).

Again, we did not see a deficiency of this form caused by inhibition of MEK and therefore concluded that the effects we were seeing were post ER. We decided to stain the Golgi apparatus to look at co-localisation with this compartment. The results were interesting. We saw the entrapment effect taking place specifically where the marker for the Golgi apparatus was. In some instances we saw the same effect (retention and clustering) but with co-localisation with the Golgi apparatus and in others we saw an overlap of osteopontin where

the Golgi was located, but no apparent co-localisation. This suggested that the effects might be more pronounced in some cells than others, *but what is the effect?* It is possible that ERK 1 and 2 may be involved in the phosphorylation of factors in the Golgi that allow SPARC and osteopontin to proceed along the secretory pathway (post-Golgi secretion). It has been shown that ERK 1 and 2 localise to the Golgi apparatus, although the specific function for ERK 1 and 2 at this organelle is not fully known. The ERK kinases phosphorylate GRASP65 (a 65kDa protein required for Golgi disassembly during mitosis as well as re-assembly after mitosis) at serine 277 in order to induce Golgi unstacking in preparation for mitosis (Tang et al. 2012; Bisel et al. 2008).

A study in 2004 showed that ERK 3 translocates temporarily to the Golgi on its way to the nucleus. Why this occurs is not known, but allows for the possibility that ERK 3 may also have a functional role in the transport process (or other processes) in the Golgi apparatus before it continues into the nucleus (Bind et al. 2004).

It is possible that we may be seeing something similar. Of course, ERK 1 and 2 localisation has not been looked at by us (although given the amount of ERK substrates which are normally widely dispersed throughout the cell), it would be difficult to make any conclusions based on localisations of ERK, nor do we know if MAPK has a direct or indirect effect on the protein localisation we are seeing. Nevertheless, these studies provide an interesting insight into what could be occurring. It would be difficult to tell what ERK 1 and 2 were activating in order to promote forward trafficking of matricellular proteins, but the immunofluorescence data suggests that it is likely there is involvement of a factor prior to/in the Golgi apparatus that is preventing the trafficking of osteopontin and GFP-SPARC when ERK 1 and 2 are not activated.

Another interesting observation that has been made recently is that COPII coated vesicles can be phosphorylated and phosphorylation is required for proper fusion of COPII coated vesicles at the cis-Golgi face for release of cargo. Hrr25p, a nucleus and Golgi localised kinase is responsible for phosphorylating the sec23-24 hetero dimer coat subunit of COPII coated vesicles, that then allows for proper fusion of these vesicles with SNAREs at the cis-Golgi face. This prevents back fusion of COPII coated vesicles with the ER. Phosphorylation of three sites on Sec23 (thr555, ser742 and thr747) were identified, and inhibition of Hrr25p prevented

capture of COPII coated vesicles with TRAPP1 (a tethering factor) and subsequent fusion via SNARE interaction. Since ERK 1 and 2 are serine/threonine kinases, there may be some involvement in vesicle phosphorylation, or phosphorylation of other sites present in tethering factors in order to induce fusion of osteopontin and SPARC containing vesicles with the Golgi membrane (Lord et al. 2011). The topic of tethering factors and membrane fusion will be discussed in more detail later (4.0 section 4.4).

We looked also at the co-localisation of osteopontin with LAMP-1 (a marker of lysosomal compartments). Lysosomes can also secrete content in osteoblast cells as is the case in Primary cultured mouse osteoblast-like cells (POBs), where RANKL, a ligand that activates pathways in response to processes such as apoptosis and bone remodelling is secreted from POBs from lysosomal compartments. Vps33a (a protein involved in the transport of components from the trans-Golgi to post-Golgi compartments was found to be important in mediating transport of RANKL to the lysosomes and inhibition led to an accumulation of RANKL in the Golgi apparatus. Degradation of RANKL was found to be dependent on the proteasomal pathway and not the lysosomal pathway further showing that the lysosomes were utilised as secretory compartments and not just a degradation compartment (Kariya et al. 2009).

The fact that RANKL is selectively degraded via another mechanism shows the specificity and selectivity of transport of different proteins in osteoblast cells, that is to say, it's a very specific process and not a random use of the lysosome as a secretory compartment. Further to this study, it was found that Rab27, a GTPase involved in the secretion of proteins was activating effector proteins to facilitate fusion with the plasma membrane, and release of RANKL was reduced as a result of a lack of fusion when Rab27 was knocked down by siRNA (Kariya et al. 2011).

We asked if osteopontin took a similar route out of the cells, and if inhibition of MAPK affected any co-localisation. There was no convincing data to tell us that lysosomes were utilised as secretory compartments in our osteosarcoma model telling us that it was unlikely a pathway that was utilised in the release of osteopontin (and possibly SPARC). Untreated cells did not show any co-localisation with the compartments with a few apparent merged spots. These were sparse and rare in the untreated cells, and could simply indicate

endogenous osteopontin that is being degraded. Cells treated with U0126 showed the same retention effect under the influence of the inhibitor, but no co-localisation with LAMP-1.

We looked at the effect of the U0126 inhibitor over a 72 hour time point. We saw a decrease in the total number of cells over a 24 hour time point. This coincided with the effectiveness of the U0126 inhibitor, with western blot analysis showing activated ERK levels to be depleted up to the point of 24 hours where light bands can be seen, and full activation of ERK 1 and 2 being present at 48 hours. The number of cells reflects this change in activation of ERK 1 and 2. We saw a decrease of about 6000 cells over a 24 hour time period before cells started to grow steadily again. We also carried out cell counts over the 6 hour time course and did not see a difference in the number of cells, meaning the effects of the inhibitor on cell survival are likely to take effect after 6 hours. It is not surprising to see that the inhibitor has this effect on cell survival. The MAPK pathway is essential for cell survival, and activation by many ligands is crucial in driving fundamental cellular processes (both in normal and cancer cells) (Lu & Xu 2006).

Studies have looked at the effects of knocking down the MAPK pathway in cells and have observed detrimental effects on cell survival implicating this pathway as a universally utilised mechanism for driving cellular processes that apparently cannot be compensated for sufficiently by other pathways (Todd et al. 2014; Gailhouste et al. 2010).

It seems the same is true in ROS cells, but this is also positive as it points to a potential therapeutic target in the pathway itself. U0126 has been used primarily as an in vitro reagent to look at the effects of the MAPK pathway in cancers and other cellular processes but has not been involved in clinical trials. However, 3 MEK inhibitors have entered clinical trials with two at phase I (PD184352 and AZD6244) and PD0325901 entering phase II clinical trials. All of these drugs cause diarrhoea, nausea and rashes, and the percentage of people that show stable disease (i.e. a tumour that does not continue to progress likely as a result of the drug) is variable. Additionally, some patients only show a partial response to treatment with the MEK inhibitors. Some tumours showed up to a 71% successful MEK inhibition in tumour samples (Wang et al. 2007).

This is a promising avenue for research, and more potent MEK inhibitors are being looked at to increase efficacy of drugs entering trials. We see just in 24 hours the negative effects that the U0126 inhibitor has on cell survival in the ROS osteosarcoma cell line.

We observed a mono-phasic effect on secretion of osteopontin over the 72 hour time course. We saw the consistent entrapment of osteopontin defined by U0126 inhibition before re-initiation of osteopontin secretion after 24 hours, showing that rescue of ERK 1 and 2 activation can allow osteopontin to continue through the trafficking pathway, and is therefore likely the cause of this entrapment of osteopontin and GFP-SPARC we were seeing at 1-6 hours of inhibition. We checked to see if there might be retention of osteopontin in the endoplasmic reticulum over a longer time period than 6 hours. Research has shown that down regulation of ERK 2 via siRNA reduces sec16 levels which represent points of COPII coated vesicle formation sites, and as such enrichment of sec16 at the ER membrane represents ER exit sites (ERES). At 24 and 48 hours of ERK 2 inhibition ERES numbers were reduced by 30% and Sec16 was found to be a substrate of ERK 2 but not ERK 1 (Farhan et al, 2010). Furthermore, it has been found that Sec16 appears to be an important substrate of other MAPKs such as ERK 7, which was found to negatively regulate ERES through its interaction with Sec16 in situations where the cells (Schneider 2 [S2, drosophila melanogaster cell line]) were starved (and ERK 7 would become stabilised) demonstrating the role ERK 7 plays in the stress response (Zacharogianni et al. 2011).

We determined this not to be the case in ROS cells from the time of 1-6 hours and therefore discounted this as a mechanism by which osteopontin and GFP-SPARC were being retained. We did not quantify Sec16, however reduced ERES might suggest that less osteopontin and GFP-SPARC would exit the ER. Since we did not observe this through co-localisation analysis or total protein quantification, it is unlikely that ERES are reduced. Note that western blot quantification is the entire cell lysate, however, bands for osteopontin and SPARC were observed in their mature form and not smaller forms suggesting proper folding and exit out of the ER. However, as there is still a certain extent of knockdown at 24 hours when we treat the cells with the U0126 inhibitor, we asked if a reduction of ERES might take place in the cells at the later time points, and thus cause retention of osteopontin in the endoplasmic reticulum. Like our data from 1-6 hours, we did not see co-localisation of osteopontin in the

ER at the 24 hour time point. We conclude that (while further work should be carried to confirm this) ERK 1 and 2 does not affect ERES in the ER of the ROS cell line meaning that the likely mechanism of action of the MAPK pathway in forward trafficking in osteoblasts is very specific, but separate to mechanisms that other people have reported in different cell lines.

Following this experiment, we carried out a short time point (1 hour) U0126 washout experiment. The effects of the inhibitor are rapidly reversible, and we could take advantage of this in confirming the trafficking effects of MAPK inhibition. Washing cells with warm PBS and simply replacing with non-U0126 containing media was enough to cause rapid activation of ERK 1 and 2 (within 15 minutes). Localisation of osteopontin showed entrapment and clustering as was observed initially, but re-initiation of forward trafficking of osteopontin inside the cell took place within 15 minutes, telling us that this effect MAPK has is an early cytoplasmic event that can be rapidly reversed upon phosphorylation of ERK 1 and 2.

The rapid reversal of the effects of U0126 have been reported in the exocytosis of synaptic vesicles where ERK 1 and 2 inhibition enhances synaptic vesicle release from mouse cortical neurons within 20 minutes of treatment. Washout of U0126 was able to reverse the effects on synaptic vesicle release (Subramanian & Morozov 2011).

Studies carried out in mouse embryonic fibroblasts showed that ERK activation was required for entry into the mitotic cycle (G1) and that activation was required to induce the expression of cyclin D1 (a protein required for cell cycle progression). Inhibition using the U0126 inhibitor downregulated mRNA expression of cyclin D1 while washout of the inhibitor would reverse this effect (Villanueva et al. 2007).

While the second study did not look specifically at trafficking, it does highlight the fact that not only is the U0126 inhibitor reversible, but reversal of the inhibitor successfully leads to normal cellular function associated with ERK 1 and 2, and we also observed this in the intracellular localisation of osteopontin confirming the involvement of MAPK pathway in the forward trafficking of at least osteopontin.

Western blot analysis of various proteins was needed in order to confirm MEK inhibition as well as the expression levels of osteopontin and SPARC.

Starting with ERK, total ERK levels did not fluctuate significantly across the time points. There appeared to be a slight (although statistically insignificant) increase of total ERK 1 and 2 over the 1-6 hour U0126 inhibition. This may be a compensatory mechanism whereby the cell senses that ERK activation is not taking place and might sense that more ERK is required to bring back ERK activation. However, as the increase was very small, it could be that there is no compensatory expression of ERK, especially at lower time points. The small increase could be explained simply by the fact that there was more total ERK inside the cell at that time.

A decrease of Atg7 (a protein involved in autophagy) resulted in a decrease of phosphorylated ERK levels in Atg7 $^{-/-}$ mice liver. This decrease resulted in an increase of total ERK levels demonstrating that there may be a mechanism that induces the expression of ERK in response to sustained inhibition of ERK activation (Martinez-Lopez et al. 2013).

There are two things to say about the extent of ERK activation (phosphorylation). The first is to say that despite the fairly even expression of total ERK 1 and 2 (in any given cell condition, whether it be untreated or treated), the phosphorylation of ERK 1 and 2 are not even. We quantified the difference of ERK 2 phosphorylation to be about 1.8 times higher on average than that of ERK 1 (figure 3.20, b). We will seek to look at specific differences in the trafficking of endogenous osteopontin when either ERK 1 or ERK 2 are inhibited individually by siRNA in the following chapter. While we cannot make any conclusions at this point, the difference of ERK 1 and 2 phosphorylation in the ROS cell line (and possibly by extension to osteoblasts and fibroblasts in general) is likely to be significant whether it is important in the trafficking process or in many other processes. Such a high level of phosphorylation of ERK 2 compared to ERK 1 is likely to put more of the workload on ERK 2. Why specifically this is the case is not known since ERK 1 and 2 share 83% homology and are thought to be readily interchangeable (although this issue will be discussed in more detail in the following chapter). Work in NIH 3T3 (fibroblast cells) showed that ERK 1 activation was 4 times less than that of ERK 2 (and the same was seen for total ERK levels), although we did not make the same observation for total ERK levels. Eliminating ERK 1 in cells does not appear to show a difference in cell proliferation, while eliminating ERK 2 does. Again, this could be due to the fact that phosphorylated ERK 2 levels are much higher and therefore it bares more of the work load (Lefloch et al. 2008).

Is the difference in phosphorylation limited to cells that are mesenchymal in phenotype? Data mining did not yield any useful information, and it seems it isn't something that has been looked at widely (for epithelial cells and mesenchymal cells although the data that does exist has looked at epithelial cells). It would be interesting to see if this were a consistent difference amongst all cell types or if ERK 1 persists more in some cells.

A paper published this year (2015 at the time of writing) looked at total ERK expression across various species. Since ERK kinases are highly conserved and their activation (TEY) domain is also highly conserved, antibodies targeting this domain (for phosphorylated ERK) could be targeted across multiple species with high degrees of efficiency. The African clawed frog and the chicken expresses one ERK gene, the protein that is encoded is ERK 2. ERK 1 did not exist in these two species, and while two genes express two ERK proteins (ERK 1 and 2) in mammals, ERK 2 could have persisted along the evolutionary time scale as the more important ERK kinase in the MAPK pathway. This could explain the difference in activation of ERK 2 in the ROS cells (and maybe in others). Furthermore, differences in the ERK 1 gene occur at sites away from the function of the kinase, and with ERK 1 being the larger protein, this could explain the selection of ERK 2 solely in some species. ERK 2 is absent in squamites however (lizards and snakes), and while it is unknown why this particular difference occurs (although it has been shown that the ERK 1 gene [*MAPK3*] evolves faster than the ERK 2 gene [*MAPK1*]), the theory of ERK 1 and 2 having interchangeable roles predominates (Buscà et al. 2015).

Finally, SPARC and osteopontin expression were not affected significantly by the reduction of ERK 1 and 2, both at the 6 hour time points and the 72 hour time points. This led us to believe that the expression of SPARC and osteopontin are not dependent on ERK 1 and 2 kinases although this should be confirmed with a 72 hour time point where ROS cells are continually treated with the U0126 inhibitor to maintain inhibition of MEK. This strengthens our hypothesis that the MAPK pathway is affecting trafficking and not the expression of SPARC or osteopontin.

We are confident in concluding in this chapter that we see an effect on the trafficking of GFP-SPARC and osteopontin when ERK activation is prevented. We saw an entrapment or retention of these two matricellular proteins in a perinuclear region, with the likely clustering

taking place just prior to or in the Golgi apparatus. In the following chapter I will seek to specify which of the two kinases may be causing this effect (if there is a difference between the two) before using other techniques to identify the retention organelle as well as what ERK may be doing to cause this effect.

4.0. RESULTS: siRNA KNOCKDOWN OF ERK 1 AND 2

4.1. INTRODUCTION

Short interfering RNA (siRNA) are small strands of RNA about 20-25 nucleotides in length that bind with 100% specificity to target mRNA and cause its degradation, leading to silencing of a specific gene. The advent of RNA interference (RNAi) has revolutionised molecular biology and helped to elucidate the functions of genes. RNAi was accidently discovered in 1990 by Carolyn Napoli et al, in an attempt to overexpress Chalcone Synthase (CHS), an enzyme involved in the pigmentation of petunia petals. In attempting to overexpress this enzyme by exogenous addition of the *CHS* gene, the researchers observed a block in anthocyanin production (a member of the flavonoid family that appear in various colours such as blue and red), giving the petals a white colour without pigment. They saw that 42% of plants produced this phenotype and subsequently found that CHS mRNA had been reduced 50-fold (Tonon et al. 2010; Napoli et al. 1990).

Since then, various studies have shown that the introduction of double stranded RNA whose sense or antisense sequences were complimentary to specific genes would effectively silence them (but only when introduced as double stranded RNA and not sense or antisense sequences alone) (Romano & Macino 1992).

Further work on RNAi revealed a pathway that leads to the silencing of target genes in a simple and elegant process. RNAi works by activating a complex known as RISC (RNA Inducing Silencing Complex) which will lead to the cleavage (in the case of siRNA) of target mRNA (figure 4.0). The introduction of double stranded non coding RNA (whether exo/endogenous) starts a pathway that involves recognition, cleavage in to 20-25 nucleotide fragments, unwinding, incorporation into the RISC complex and finally gene silencing. Upon introduction, the double stranded RNA sequence forms a complex with 3 proteins, RDE-4, R2D2 and dicer. RDE-4 recognises the double stranded RNA sequence which then recruits R2D2 which will coordinate assembly of the siRNA complex with dicer, facilitating the cleavage of the double stranded RNA in to small 20-25 nucleotide fragments. This complex is known as the RISC loading complex (RLC) (Carthew & Sontheimer 2009).

Once incorporated into the RLC, the double stranded nucleotide is transported to another protein known as Argonaute (an endonuclease) where the RNA inducing silencing complex (RISC) is formed.

It is not fully understood how the double stranded RNA fragment is unwound. There is evidence that RNA Helicase A (RHA) is important in this process, with evidence of its presence in the RISC complex in HeLa and HEK293 cells (Robb & Rana 2007).

However, recent work has shown that RHA incorporation into the RISC complex is not essential for siRNA silencing and that the RISC complex is capable of silencing genes when RHA is downregulated. Using siRNA techniques, RHA depletion (85% knockdown) did not disrupt the siRNA downregulation of PTEN (Phosphatase and Tensin Homolog) in HeLa cells (Liang & Crooke 2013).

It seems unwinding may not necessarily be taking place in the RISC complex and/or by RHA itself but by another helicase. In any case, it has been established that once unwound, the anti-sense 'guide' 3'-5' strand is incorporated into the RISC complex while the sense 'passenger' 5'-3' strand is degraded. A protein known as C3P0 (component 3 promoter of RISC) assists in taking the passenger strand away and facilitating its degradation, allowing incorporation of the guide strand that will lead to cleavage of target mRNA (Wilson & Doudna 2013; Liu et al. 2009).

Once unwound, the guide strand is loaded onto the RISC complex where Argonaute will lead the cleavage of target mRNA. A domain on Argonaute known as the PAZ domain will recognise the 3' overhang of the guide strand (a 2 nucleotide overhang) while the middle domain of Argonaute will hold on to the 5' end. The strand is coordinated such that every nucleotide is paired with 100% complementarity to the target mRNA sequence. Cleavage of the mRNA target takes place between siRNA nucleotides 10 and 11 (Bouasker & Simard 2009).

Cleavage of the phosphodiester backbone is facilitated by a Magnesium (Mg^{2+}) cation. Magnesium will stabilise and position a water molecule to carry out a nucleophilic attack (donating electrons) breaking the phosphodiester backbone, thus cutting the mRNA

sequence. Argonaute is crucial in this entire process and is therefore the most important component of the RISC complex (Schwarz et al. 2004).

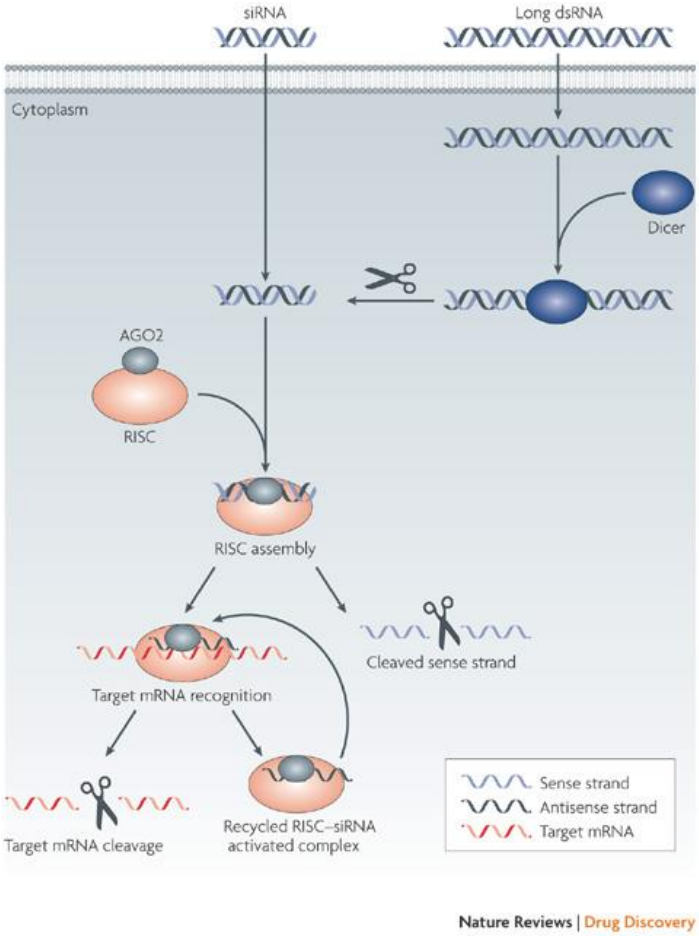


FIGURE 4.0, Illustration shows the pathway that leads to degradation of target mRNA by siRNA. Recognition of siRNA followed by incorporation into the RNA inducing silencing complex (RISC) is required for Argonaute to facilitate the cleavage of a target mRNA strand. Image taken directly from (Whitehead et al. 2009).

Another pathway leading to gene silencing is micro RNA (miRNA) however, this process is less specific. miRNAs do not require binding to target mRNA with full complementarity like siRNA does and for this reason, it is thought that single miRNAs can silence multiple genes. Argonaute is still essential in facilitating the miRNA targeted silencing, however, unlike siRNA, miRNAs prevent translation simply by being bound and not by leading to degradation of the target strand in the same way that siRNA does (Carthew & Sontheimer 2009). In order to prevent silencing of non-targeted genes, siRNA is ideal to specifically induce the

degradation of ERK 1 or ERK 2. The highly specific nature of siRNA mediated gene knockdown would ensure that any effect observed would likely be a result of the inhibition of the respective ERK kinases as oppose to any other gene.

To further address the hypothesis that the MAPK pathway was having an effect on the secretion of SPARC and osteopontin, siRNA knockdown of MAPK protein ERK 1 and ERK 2 (p44 and p42 respectively) was performed in order to see the effect that down regulation of these proteins individually has on forward trafficking. Cells were transfected with siRNA against ERK 1 or ERK 2 and the localisation of osteopontin and the Golgi apparatus was observed.

4.2. RESULTS

4.2.1. siRNA APPROACHES, ERK 2

siRNA knockdown was performed several times, with inconsistent results for ERK 2 (figure 4.1). Two transfection reagents were initially tested and gave different percentages of knockdown. The transfection reagents used were Hiperfect by Qiagen and Nanofectin by PAA laboratories. Initially, Nanofectin offered the most efficient knockdown, so Nanofectin was used as the transfection reagent of choice. However, these results were not reproducible later on, and knockdown efficiency was never as good as in the initial experiment.

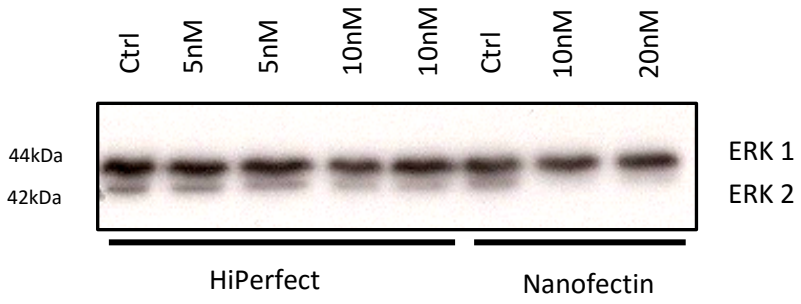


FIGURE 4.1, Western blot analysis illustrates the degree of knockdown of ERK2 (Lower band) after 48 hours. siRNA concentrations were fixed as labelled in the image. Different transfection reagent volumes were used to see if more transfection reagent would improve extent of knockdown. For each concentration treatment for HiPerfect, one concentration was diluted with 3µl and 4.5µl of HiPerfect respectively as a range was recommended according to the manufacturer's data sheet. For the Nanofectin treatments, the amount of transfection reagent used was up-scaled with an increase in concentration, again according to recommendation by the manufacturer's data sheet. Nanofectin appeared to give the greatest extent of knockdown of ERK 2 compared to HiPerfect.

The intensity of each band in figure 4.1 was measured. Before calculating percentage differences, normalization was carried out by using total ERK 1 as a loading control as it was expressed at equal amounts.

Cells treated with siRNA using Nanofectin as the transfection reagent shows greatest knockdown efficiency towards ERK 2. Cells treated with siRNA and HiPerFect show some extent of knockdown of ERK 2, but the most significant knockdown is clearly seen in the cells

transfected with Nanofectin, with 10nM of siRNA being sufficient to cause 71% knockdown of ERK 2. 20nM of siRNA also shows a similar degree of knockdown (68%) and proves to also be more effective than HiPerFect.

As Nanofectin had the best knockdown efficiency, we used this transfection reagent, however, later experiments could not reproduce the same efficiency of knockdown as Nanofectin did initially.

We used a different transfection reagent called JetPrime. JetPrime would be used to transfect ERK 2 siRNA oligos in the same way Nanofectin and HiPerfect were used. JetPrime transfection worked very well and results consistently gave 90% + knockdown (figure 4.3). Western blot results are consistent, and the following western (figure 4.2) corresponds with the immunofluorescence picture in figure 4.4.

Please note that all immunofluorescence experiments and western blots were carried out at least 3 times (independent experiments) and in duplicates unless specifically stated otherwise.

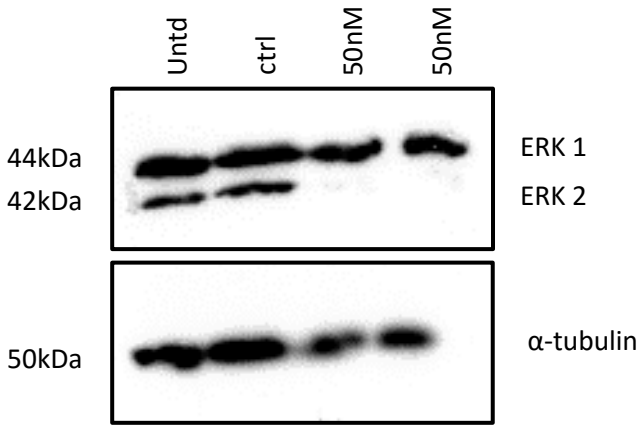


FIGURE 4.2, Knockdown of ERK 2 using JetPrime is consistently effective and cells treated with 50nM of siRNA for 48 hours give at least 90% knockdown of the target mRNA. Two replicates of cells treated, both with 50nM of siRNA are identical treatments, but carried out in duplicates in order to verify knockdown in each independent experiment. Analysis of siRNA treatment of experiments in this figure gives 97 and 99% knockdown of ERK 2 respectively. Treated cells are compared to a control cell, and the untreated cells were

seeded in order to see if there were differences in expression between control treated cells and untreated cells as well as the pattern of trafficking in immunofluorescence staining.

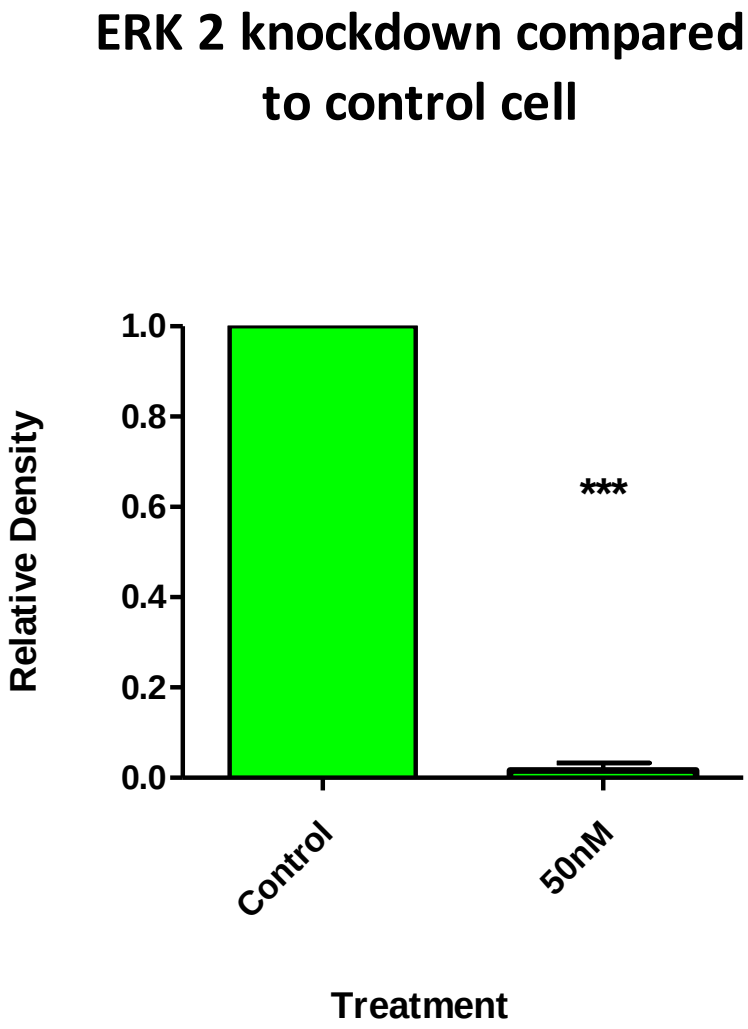


FIGURE 4.3, Knockdown of ERK 2 in siRNA treated cells after 48 hours is significant when compared to control cells. Cells transfected with 50nM siRNA for 48 hours show a mean difference of 98% knockdown (**error bars represent SD** ***P<0.001) consistent across all experiments carried out (n=3). Standard deviation for control is not present as the control cell western blot normalisation was made 1 to represent 100% of the value in order to see the percentage difference of treated cells compared to control cells.

We monitored the localisation of endogenous osteopontin. If any differences are seen in the localisation of matricellular proteins in immunofluorescence imaging, it is possible that ERK 2 may be playing a role in the difference. Cells were transfected with 50nM siRNA against

ERK 2 for 48 hours. Having treated cells with the U0126 inhibitor (chapter 3.0) and observing a difference in localisation of GFP-SPARC or osteopontin and the Golgi apparatus, we decided to carry out immunofluorescence staining of the Golgi apparatus to see if the same difference in localisation could be observed (figure 4.4).

After 48 hours of treatment, cells were fixed and stained for osteopontin, the Golgi apparatus and DNA.

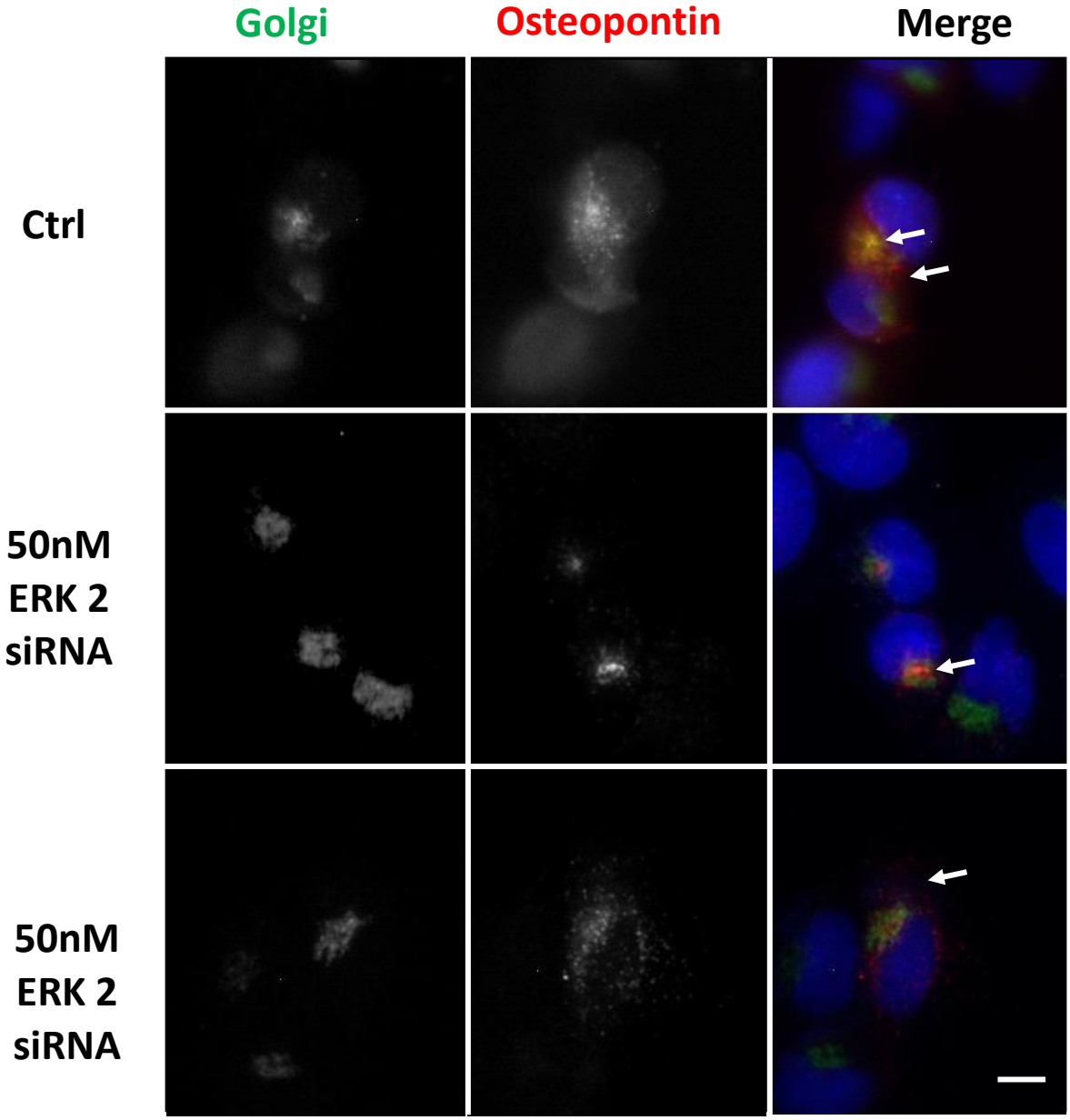


FIGURE 4.4, Knockdown of ERK 2 appears to affect localisation of osteopontin with the Golgi apparatus. Looking at the control cells, osteopontin seems to traverse the Golgi

without a problem and there are indications of vesicles travelling away from the Golgi shown by the arrow, indicating post-Golgi trafficking. Further to this, a mix of osteopontin in the Golgi, cause the two fluorescent dyes (green and red) to merge revealing a yellow tinge (arrows). Red vesicles appear more localised with few vesicles inside the area marked by HPA lectin (Golgi). Furthermore, there is very little overlap between the two fluorophores indicating that osteopontin may accumulate at a site distinct from the Golgi complex. Scale bar: 50µm.

It appears that there is an effect on the secretion of osteopontin when ERK 2 expression is targeted. This observation was interesting as it hinted that there was a possibility that the role of the ERK kinases on secretion of matricellular proteins might be specific to one type of ERK. We decided to look at the effect of osteopontin localisation when we targeted ERK 1 expression. In addition to immunofluorescence staining we also carried out western blot analysis to look at the expression of osteopontin, SPARC and phosphorylated ERK.

4.2.2. siRNA APPROACHES, ERK 1

Little is known about the individual roles for ERK 1 and 2 and the evidence is poor. Much of the literature reports on the interchangeable and compensatory effects the ERK proteins have for each other with regards to activation of target genes (Frémin et al. 2015).

ERK 1 siRNA was transfected into ROS cells using the JetPrime transfection reagent. We used the recommended starting concentration of siRNA (10 and 50nM) and followed the standard protocol provided by JetPrime. Using this method, we were able to achieve a high percentage of knockdown when we transfected the cells with 50nM of ERK 1 siRNA (figure 4.5 and 4.6).

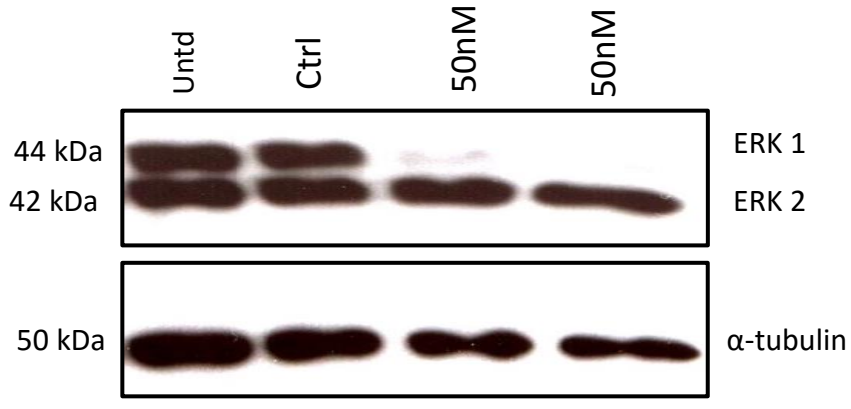


FIGURE 4.5, Knockdown of ERK 1 using JetPrime is effective and consistently achieves a high degree of knockdown. An absence of bands when targeted with a total ERK antibody

shows the knockdown of ERK 1 after 48 hours of incubation with siRNA in ROS cells. Treated cells are compared to a control cell, and the untreated cells were seeded in order to see if there were differences in expression between control treated cells and untreated cells as well as the pattern of trafficking in immunofluorescence staining.

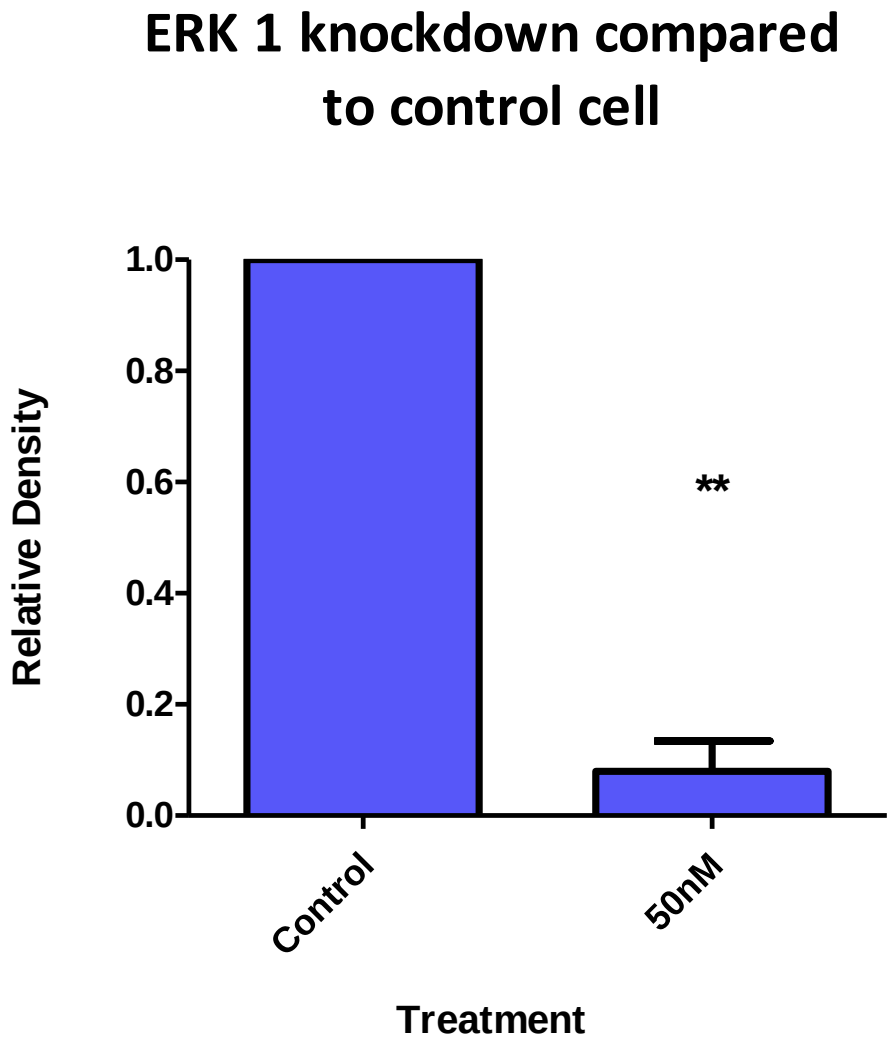


FIGURE 4.6, Total ERK 1 levels are reduced by an average of 93% in ERK 1 siRNA treated cells. We treated cells with siRNA against ERK 1 and were able to achieve a high degree of knockdown of total ERK 1 compared to the control cell (**error bars represent SD** ** P<0.01). Again, like ERK 2 siRNA, because of the high degree of knockdown, if any differences are observed in immunofluorescence staining, then it is likely that it will be due to knockdown of ERK 1. Standard deviation for control is not present as the control cell western blot normalisation was made 1 to represent 100% of the value in order to see the percentage difference of treated cells compared to control cells. n=3

Like the treatment with ERK 2 siRNA, following transfection of ERK 1 siRNA, localisation of osteopontin inside the cell was investigated (figure 4.7). We stained the cell for osteopontin, the Golgi apparatus and DNA.

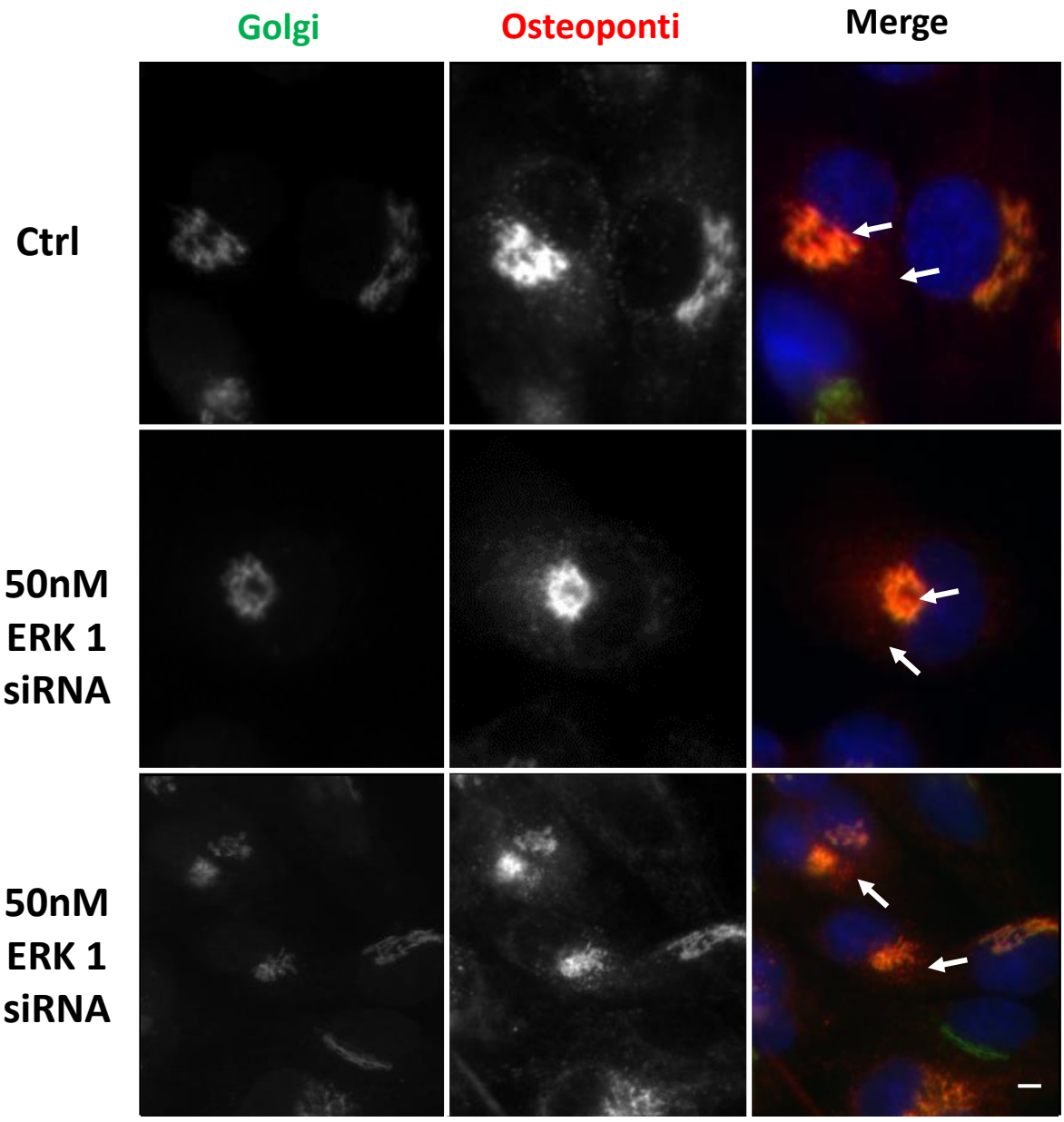


FIGURE 4.7, localisation of osteopontin does not appear to change compared to the control cells. Control cells treated with a control siRNA and cells treated with siRNA directed against ERK 1 show the same perinuclear localisation of osteopontin (red punctate dots [osteopontin containing vesicles] appearing diffuse throughout the cell). This data suggested there was no apparent difference in osteopontin secretion between control and treated cells. Scale bar: 50µm. n=2

In order to confirm that the effects seen with ERK 1 and ERK 2 siRNA were likely to be effects on trafficking, we carried out western blot analysis to check for the expression of various proteins (figure 4.8) after 48 hours. It was important to check the total protein expression of osteopontin and SPARC as we were knocking down ERK 1 or ERK 2. Since the ERK kinases activate transcription factors, there might be a possibility that SPARC and osteopontin are down regulated.

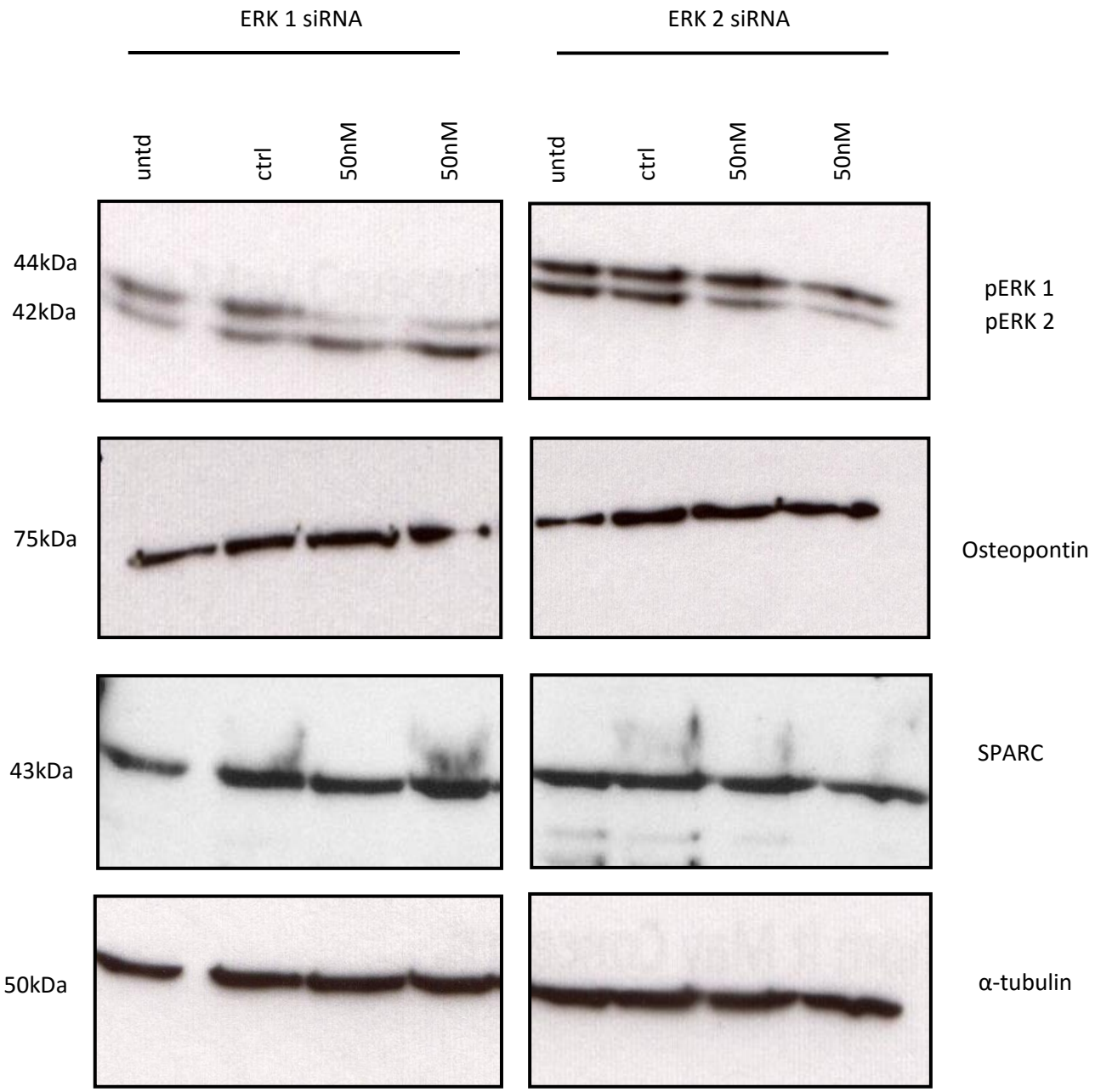
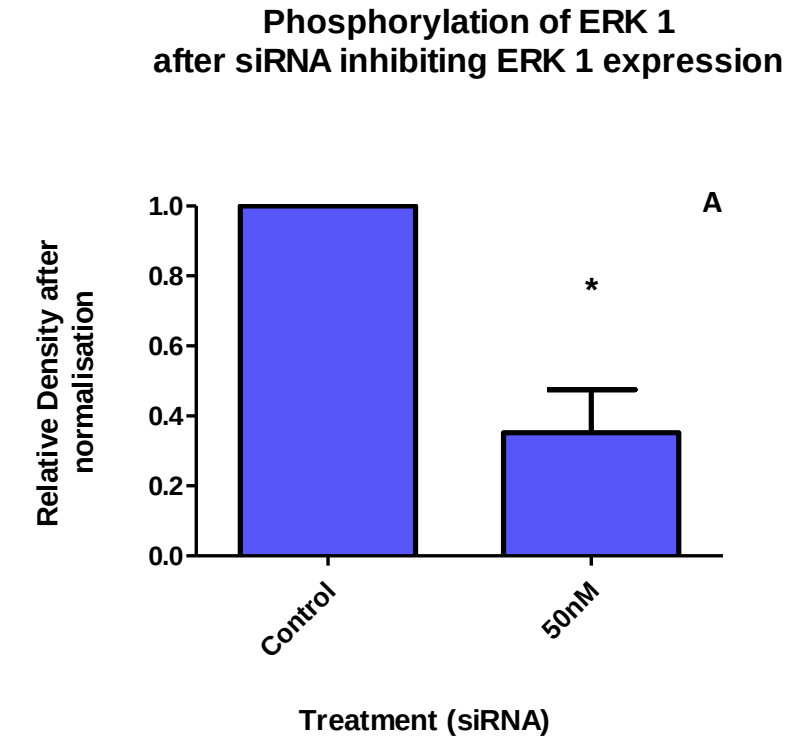
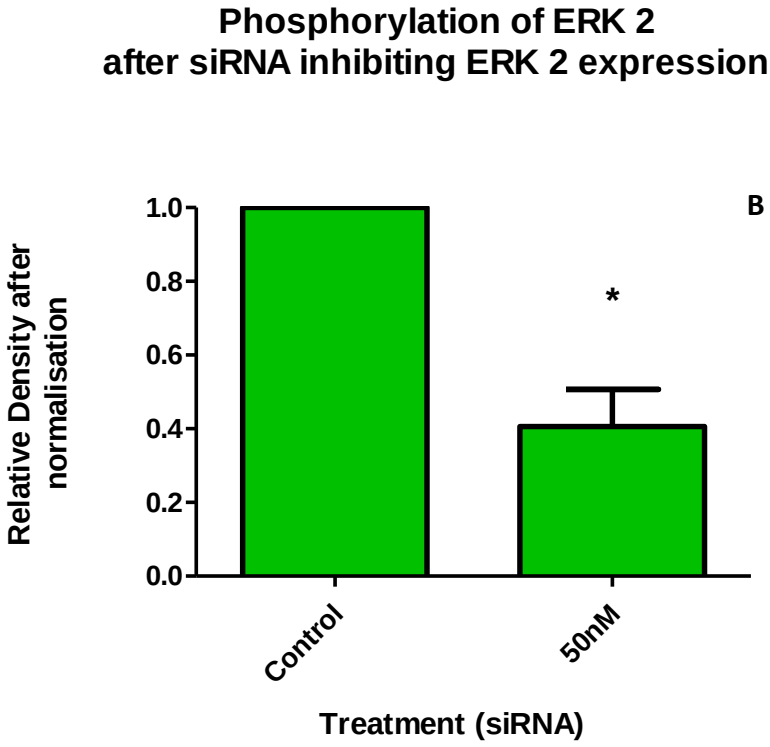
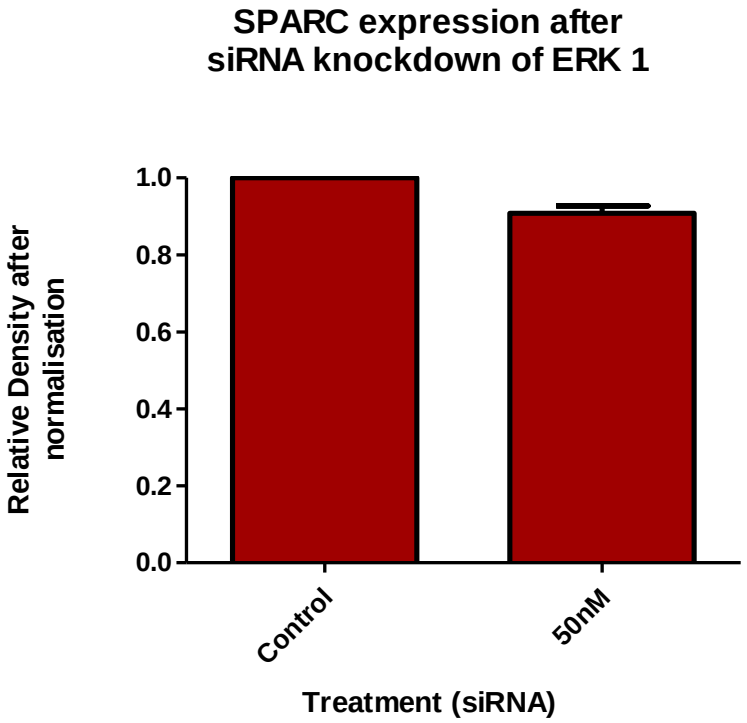


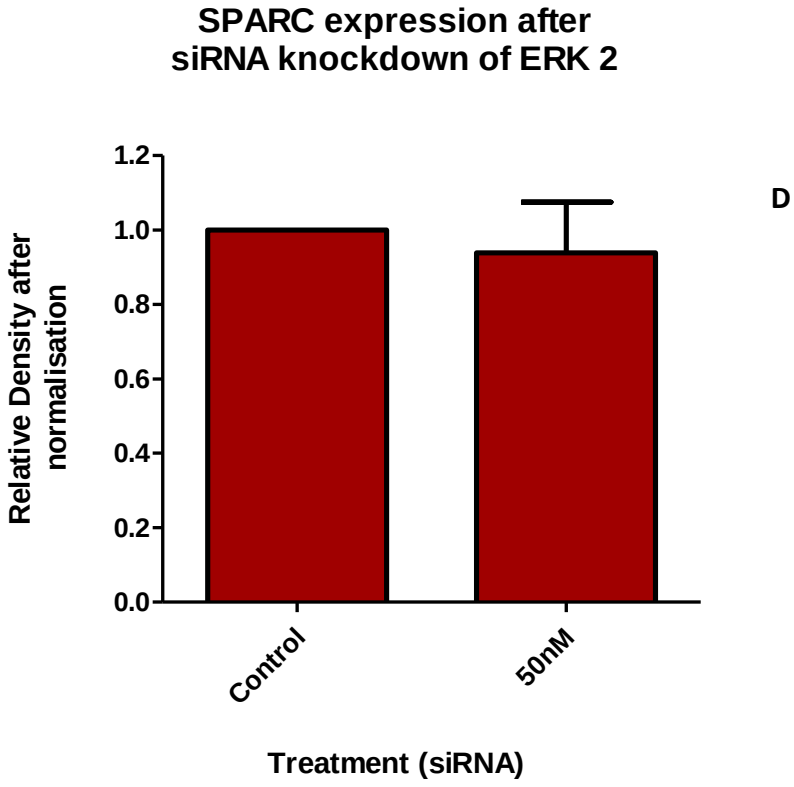
FIGURE 4.8, Western blot analysis shows expression of SPARC and osteopontin as well as activation of ERK, showing that there is residual phosphorylation of ERK 1 and 2. ROS cells were treated either with siRNA against ERK 1 or ERK 2 for 48 hours. Cells were then lysed and proteins resolved on 12% SDS-PAGE. Western blotting with anti-SPARC (AON5031) and anti-osteopontin (MPIIIB10) antibodies and subsequent densitometry analysis showed no significant change in the expression of SPARC or osteopontin after siRNA knockdown of either ERK. The presence of residual phosphorylated ERK is important. While there may be a high degree of knockdown of total ERK (shown in fig 4.2 and 4.5 for these samples), any previously activated ERK kinase would continue to have activity inside the cell. (n=2)

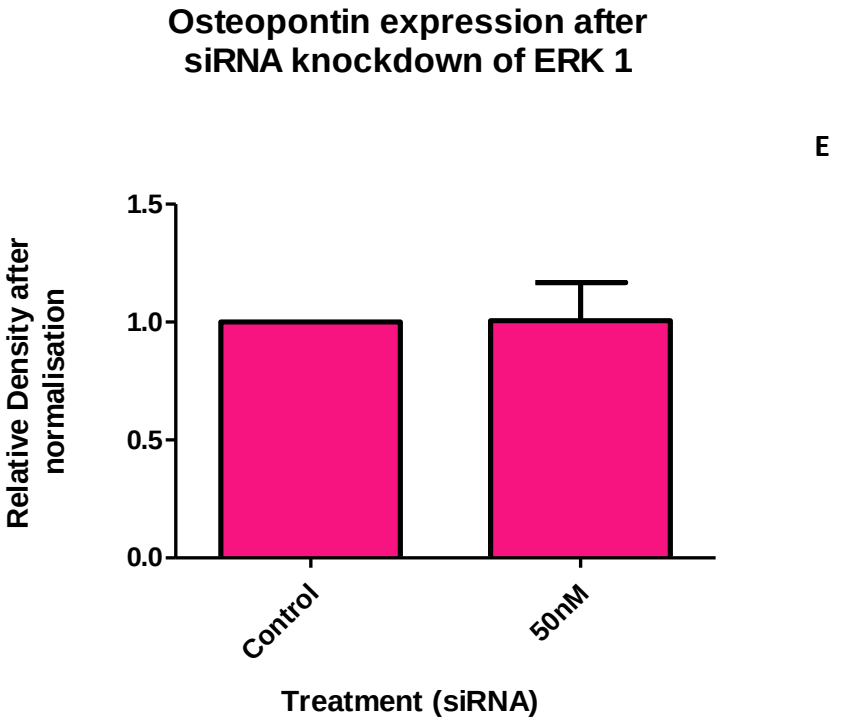
Densitometry analysis shows the quantification of the western blot bands in control cells compared to cells treated with siRNA for ERK 1 or ERK 2 (figure 4.9).


Phosphorylation of ERK 1
after siRNA inhibiting ERK 1 expression
A
Relative Density after normalisation
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
50nM
Treatment (siRNA)


Phosphorylation of ERK 2
after siRNA inhibiting ERK 2 expression
B
Relative Density after normalisation
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
50nM
Treatment (siRNA)


SPARC expression after siRNA knockdown of ERK 1
C
Relative Density after normalisation
1.0
0.8
0.6
0.4
0.2
0.0
Control
50nM
Treatment (siRNA)


SPARC expression after siRNA knockdown of ERK 2
D
Relative Density after normalisation
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
50nM
Treatment (siRNA)


Osteopontin expression after siRNA knockdown of ERK 1
E
Relative Density after normalisation
1.5
1.0
0.5
0.0
Control
50nM
Treatment (siRNA)

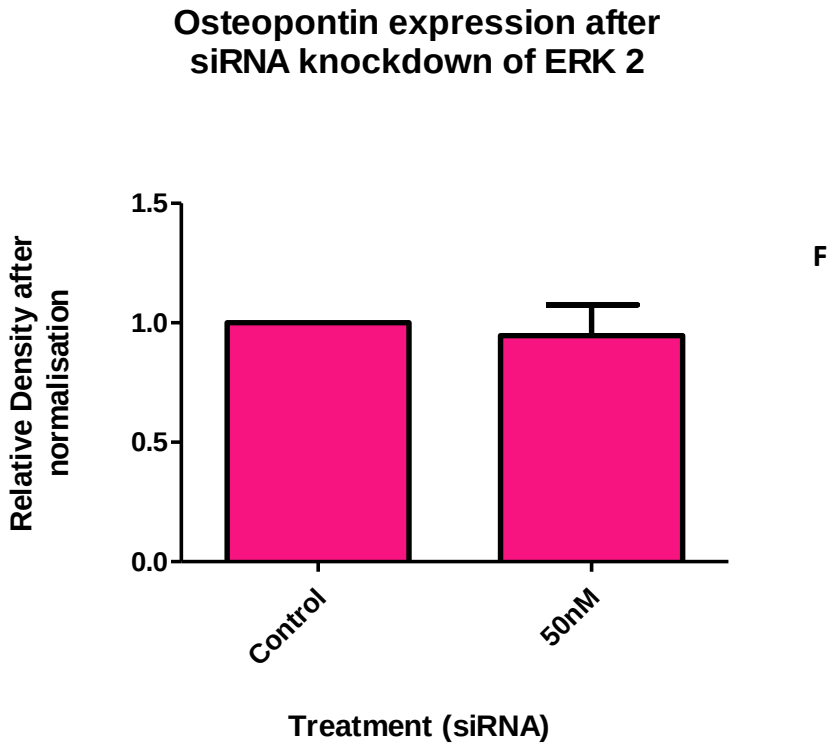

Osteopontin expression after siRNA knockdown of ERK 2
F
Relative Density after normalisation
1.5
1.0
0.5
0.0
Control
50nM
Treatment (siRNA)

FIGURE 4.9, **Densitometry analysis of western blots comparing expression of SPARC, Osteopontin and activation of ERK 1 and 2 between control cells and cells treated with siRNA. All error bars indicate SD.** Whilst degree of knockdown of total ERK is high, there is residual phosphorylated ERK (**A** and **B**). Phosphorylated ERK is 65 and 60% lower for ERK 1 and 2 respectively (*P<0.05) compared to control cells. SPARC and osteopontin expression (**C**-**F**) do not change significantly when cells are treated with siRNA for ERK 1 or ERK 2. n=2

4.3. CELL SURVIVAL WHEN ERK 1 AND 2 ARE KNOCKED DOWN WITH siRNA

We decided to see what effect ERK 1 and 2 knockdown had on cell survival (figure 4.10). If ERK 1 and 2 had redundant roles, maybe there would be sufficient compensation by the other kinase so that overall cell growth in culture was not affected. Interestingly we saw that the cell count decreased over a 48 hour period when ERK 2 was knocked down as oppose to ERK 1.

Please note that cell count was carried out once in triplicate.

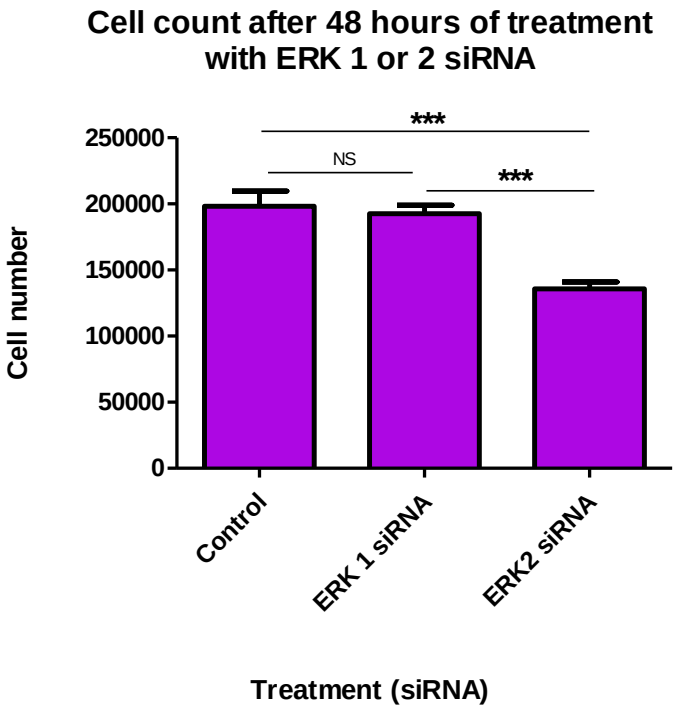


FIGURE 4.10, **Knockdown of ERK 1 by siRNA after 48 hours does not significantly affect cell growth while knockdown of ERK 2 by siRNA causes a reduction in cell growth.** We observed an average reduction of total cell number by 62,500 cells when we treated cells with siRNA

against ERK 2 (**error bars represent SD** ***P<0.001) and a reduction of total cell number by 56667 between ERK 1 and ERK 2 siRNA treated cells (***p<0.001). Cells treated with siRNA against ERK 1 did not show a significant difference against control cells. n=1

The reason for the difference in cell number is speculative. We decided to quantify the amount of phosphorylated ERK when we treated cells with ERK 1 or 2 siRNA (figure 4.11). We focused on the opposing kinase here, for example, phosphorylation of ERK 2 was checked after treatment of siRNA directed towards ERK 1.

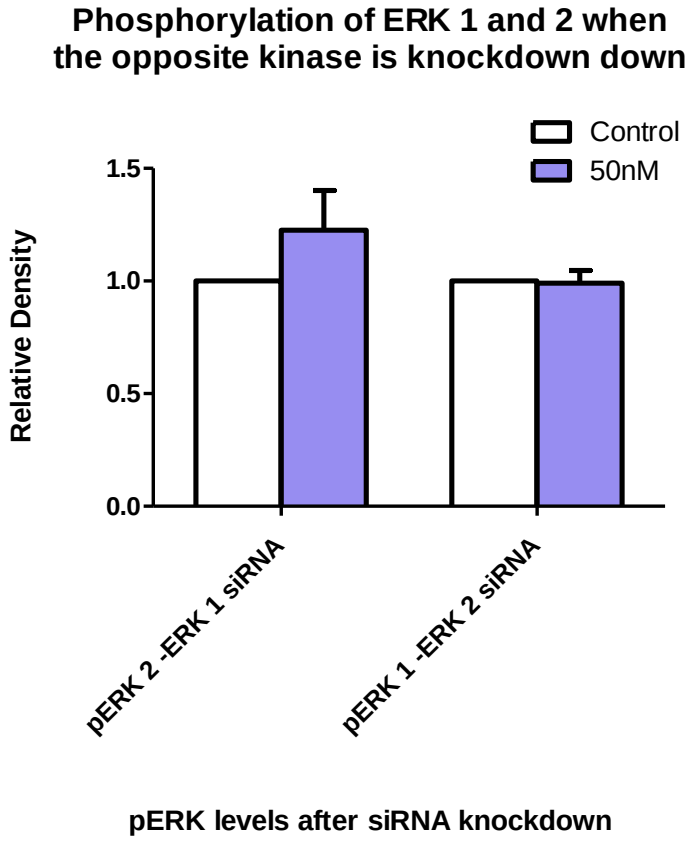


FIGURE 4.11, **Phosphorylation of ERK 2 (from figure 4.8) increases when ERK 1 is knocked down by siRNA and phosphorylation of ERK 1 is unchanged when ERK 2 is knocked down by siRNA. Error bars indicate SD** (n=1)**.**

An analysis of two treatments gave us an idea of the extent of phosphorylation of ERK 1 or 2 when the opposite kinase was knocked down. While this should be verified with more

treatments, the initial observation is that ERK 2 phosphorylation appears to increase in some instances while ERK 1 activation during ERK 2 knockdown is unaffected.

4.4. DISCUSSION

To substantiate the findings obtained with the MEK inhibitor U0126 on osteopontin trafficking, we prevented the expression of ERK 1 or ERK 2 individually through siRNA silencing. We only looked at osteopontin trafficking because we were unable to investigate endogenous SPARC as antibodies did not work in immunofluorescence staining, as mentioned in chapter 3.0., section 3.3.1. Since we had observed an effect on osteopontin and GFP-SPARC trafficking when ERK 1 and 2 activation was inhibited with the U0126 inhibitor, an effect seen on osteopontin trafficking by siRNA treatments might also apply to SPARC trafficking.

The immunofluorescence results observed were interesting for both ERK 1 and 2 siRNA treated cells. We saw that cells treated with siRNA against ERK 1 did not appear to show a significant difference compared to control cells; in fact it was difficult to distinguish the trafficking patterns of osteopontin between the two. We stained the cells with a marker of the Golgi apparatus and observed co-localisation of osteopontin with the Golgi apparatus. This would be considered normal however and not limited to osteopontin but all proteins that undergo further modification which would take place in the Golgi apparatus. Since osteopontin is heavily modified (synthesised as a 32 kDa peptide and brought up to around 75kDa through post-translational modification) this co-localisation with the Golgi apparatus would be expected (Boskey et al. 2012).

Not only is osteopontin heavily modified, but different modifications such as different states of phosphorylation mean that interactions with integrin receptors lead to different events as well as different interactions with molecules such as hydroxyapatite. A highly phosphorylated form of osteopontin has been shown to reduce cell adhesion through αvβ3 integrins (Christensen et al. 2012) demonstrating the importance of post translational modification of proteins like osteopontin. In this respect, co-localisation of osteopontin with the Golgi apparatus should be expected as part of the forward trafficking of this molecule.

Diffuse punctate red dots represent osteopontin vesicles that were moving towards the cell periphery. Again, this would be expected as osteopontin is usually not retained in the cell upon modification as it carries out the majority of functions in the extracellular space (the same is true for SPARC).

The pattern and distribution of osteopontin inside the cell was completely different in cells treated with ERK 2 siRNA. We observed a tight clustering or retention of osteopontin in a perinuclear region, a pattern that was also observed when we treated cells with the U0126 inhibitor. We did not see a diffuse pattern of punctate osteopontin across the cytoplasm of the cell and the most striking difference compared to control cells (and also to the ERK 1 siRNA treated cells) was that localisation of osteopontin was either perinuclear but distinct from the Golgi apparatus, or in the Golgi apparatus but with no other staining towards the cell periphery. This observation agrees with what was observed when we treated the cells with the U0126 inhibitor and stained them with a marker of the Golgi apparatus. Our conclusion based on this observation is that the effect we see on trafficking of osteopontin and (possibly) SPARC secretion appears to be ERK 2 specific. Some of the cells observed show less of a clustering effect in a perinuclear region. The diffuse punctate cytoplasmic pattern is still not identical to the control cells and it may be the case that these vesicles are being affected by the lack of ERK 2 in other places in the cytoplasm (as might be the case by tethering complexes at the plasma membrane, discussed further down), or the extent of knockdown may not be so high in this observed cell. Western blots showed the extent of knockdown with cells treated with ERK 1 or 2 siRNA, however, immunofluorescence coverslips were plated similarly during the same experiment, and there was no way of verifying the specific extent of knockdown in these cells. The individual roles for ERK 1 and 2 are not clear to this day, and much of the evidence points to interchangeable functions both kinases have with respect to activation of transcription factors (Lefloch et al. 2008).

However, ERK 2 knockout in mice is lethal, and offspring do not make it to term due to severe abnormalities in the labyrinthine layer of the placenta (the layer containing vessels where maternal blood containing nutrients is passed onto the foetus), implicating ERK 2 as an essential regulator of placental development. Lethality occurred at embryonic day 11.5 in development and the labyrinthine layer appeared very thin and foetal blood vessels were

difficult to observe. ERK 1 knockout mice did not display this abnormality and were otherwise healthy compared to wild type mice. ERK 2 rescue was sufficient to save the embryo. Specifically what ERK 2 might be doing is unclear, but it was proposed that there may be involvement of ERK 2 specifically in the formation of blood vessels in the labyrinthine layer, a role ERK 1 could not compensate for (Hatano et al. 2003).

It is not clear what ERK 1 or 2 might be doing in their individual functions and it may well be beyond activation of transcription factors. Saba El-Leil et al were able to verify the study above by showing that ERK 2 knockout in embryonic stem cells did not allow the formation of the ectoplacental cone and extra-embryonic ectoderm (a platform for the growth of trophoblasts and a derivative of the outer most layer of the embryo respectively) which therefore could not support a healthy foetus (Saba-El-Leil et al. 2003).

Again, the specific role for ERK 2 here is speculative. It would be interesting to see the mechanism by which ERK 2 is able to cause this effect. A more recent study looking at the role of differentiation of embryonic stem cells also found that ERK 2 null cell lines were unable to differentiate when stimulated by fibroblast growth factor 4, an observation that would be similar to the in vivo study carried out by Hatano et al and Saba El-Leil et al. The effects of embryonic stem cell differentiation in vitro were not looked at when ERK 1 was knocked out and ERK 2 was active, and while it corroborates in vivo observations, it would be useful to see what happens when ERK 1 is knocked out in the cell line to confirm that differentiation would be unaffected (Na et al. 2010; Kunath et al. 2007).

We carried out western blot analysis to look at the total expression of ERK, SPARC and osteopontin. We also looked at levels of phosphorylated ERK following knockdown of ERK 1 and 2. The efficiency of knockdown for ERK 1 and 2 treated cells was very high, with a mean knockdown of 93% and 98% for ERK 1 and ERK 2 respectively. Following from this, we looked at the activation of ERK. While the degree of knockdown of total ERK was very high, there was residual activated ERK. Quantification of phosphorylated ERK levels in siRNA treated cells showed a 65 and 60% reduction for ERK 1 and 2 respectively. This reduction was significant, but also meant that there was a consistent residual pool of phosphorylated ERK in the cell even in the absence of total ERK. This is easily explained by total expressed ERK that existed inside the cell prior to transfection with siRNA. The residual ERK activation should correlate

with some total ERK expression, but total ERK levels were very low when we treated cells with ERK 1 or ERK 2 siRNA. It may be possible that the total ERK antibody does not detect this residual phosphorylated ERK inside the cell, possibly due to ERK interactions with their substrates that may be masking an antibody binding epitope.

It may also be the case that some translation of ERK 1 and 2 existed even when we treated cells with siRNA as we never observed 100% knockdown, or, there may have been some cells that did not take up the siRNA thus displaying normal levels of ERK kinase activity.

Given this observation, ERK expression may not be a continual event (in terms of time), and much of the emphasis and effort for the ERK proteins centres more on its kinase activity and regulation of this activity. Work carried out on the kinetics of ERK phosphorylation in HeLa cells showed that ERK would be rapidly phosphorylated by MEK (MEK-ERK interaction having a half-life of $t_{1/2}$ 7.8 seconds) and that phosphorylation of ERK lasted 88 seconds before being de-phosphorylated. A model is proposed in this study that shows a cycle of constant phosphorylation and de-phosphorylation based on activity inside the cell (Fujioka et al. 2006).

With this in mind, it should be noted that phosphorylated ERK would survive and go through cycles of phosphorylation and de-phosphorylation as long as the residual total ERK was present.

This will have consequences for the trafficking of osteopontin and SPARC as any involvement in the forward trafficking process might still be present with residual ERK activation. Nevertheless, we observed with ERK 2 knockdown an effect on cells that was distinguishable from the secretory pattern of osteopontin in control cells.

We did not observe a significant difference in the expression of osteopontin and SPARC with cells treated with siRNA for ERK 1 or 2. As mentioned above, much of the literature looks at the interchangeable role of ERK 1 and 2 with respect to activation of transcription factors. While it is clear to see that osteopontin and SPARC expression are not dependent on either ERK 1 or ERK 2 alone, there may be compensation by the other active kinase in order to activate transcription factors that will induce the expression of osteopontin and SPARC. Not much is known about the precise regulation of SPARC or osteopontin expression. It has been

shown that activin, a member of the TGF-β family of ligands as well as fibroblast growth factor (FGF) in combination is able to induce the expression of SPARC in Xenopus (clawed frog) (Damjanovski et al. 1998).

Furthermore, it has been shown that activation of the TGF-β pathway can induce SPARC expression since protein secretion assays and immunoprecipitation assays showed that an increased level of TGF-β correlated with elevated levels of SPARC (Wrana et al. 1991).

Recent work has confirmed that SPARC expression correlates with higher levels of TGF-β in bovine luteinising granulosa cells, although this effect was observed at 48 hours, so it would be interesting to see if a sustained knockdown of either ERK 1 or 2 (in the ROS cell line) would cause a decrease of SPARC or osteopontin after 48 hours. Additionally, in this study, in bovine luteal cells, vascular endothelial growth factor A (VEGFA) was able to increase slightly the expression of SPARC, while fibroblast growth factor would decrease the levels of SPARC in a time dependent manner (1-9 days for both ligands). It is interesting that both ligands show an effect since the ERK 1 and 2 module of the MAPK pathway are a main target of VEGF and FGF downstream of receptor activation. Since we did not observe this effect, it is likely that different regulatory mechanisms may exist in different cell types (Joseph et al. 2012).

The role that ERK 2 is having in the forward trafficking of osteopontin and SPARC is speculative at this point. Further work in chapter 5.0 will aim to further isolate specifically where osteopontin and SPARC may be retained when MAPK is inhibited. Since ERK 1 and 2 are kinases, it would be normal to assume that they are likely activating substrates or effector proteins that will then allow osteopontin and SPARC to continue through the anterograde pathway. The problem with this hypothesis is that ERK 1 and 2 have over 200 substrates (Roskoski 2012).

There is now software available to look at protein-protein interactions (again this will be investigated in chapter 5.0.); however, pinpointing specifically which protein/s ERK interacts with directly or indirectly to cause the specific effect we see in our cells is difficult even when filtering proteins we may think are not relevant in the process. It is likely that the effect seen is by a phosphorylation event caused by ERK 2 (based on siRNA/immunofluorescence observations). There is no evidence that the ERK kinases act as chaperones themselves, as

this may also be another possible theory, but if the effect on osteopontin and SPARC is mediated via a chaperone, it is likely that ERK 2 is phosphorylating a target substrate that then allows the chaperone to facilitate SPARC and osteopontin trafficking through the anterograde pathway. More evidence of ERK activating chaperone proteins includes the activation of 14-3-3 to sequester HSF1 (heat shock factor 1) which then regulates the expression of heat shock proteins (Wang et al. 2003).

A possible mechanism by which ERK 2 may be involved in forward trafficking is through activating tethering complexes. A recent study published found that Exo70, a subunit of the exocyst complex (a tethering factor at the plasma membrane) was a substrate for ERK 2 (ser250). Phosphorylation of Exo70 was found to be essential for exocytosis and site directed mutagenesis (S250A) meant that ERK 1 and 2 could not phosphorylate Exo70. Furthermore, an ERK 2 mutant (one that was unable to bind to ATP) also meant that Exo70 could not be phosphorylated. Researchers were not able to test ERK 1 alone, but speculated ERK 1 was likely able to phosphorylate Exo70 as well. Immunofluorescence staining found that trafficking of vesicular stomatitis glycoprotein (VSV-G) to the plasma membrane was 5 fold lower than that of control cells (Ren & Guo 2012).

This study shows a possible mechanism by which ERK kinases may contribute to the forward trafficking of proteins. While they did not look individually at the effect of ERK 1 on Exo70 phosphorylation, it is possible that the roles may be interchangeable. If this were the case, it would point to a mechanism that requires compensation by the other kinase in the absence of either kinase. This may be a conserved process, while the effect we observed on osteopontin secretion seemed to be specific to ERK 2 alone.

An experiment that should be carried out is rescue of ERK 1 or ERK 2 KO depending on which was knocked down. The introduction of an ERK 1 or ERK 2 containing plasmid after 48 hours of siRNA transfection could be done with normal transfection reagents or ideally with a lentiviral system. According to the results observed in this chapter, the rescue of ERK 2 expression should reverse the phenotype of osteopontin accumulation in a perinuclear region and resemble the pattern of trafficking similar to that observed in control or untreated cells. The rescue of ERK 1 expression should not affect the trafficking of osteopontin as no difference was seen between ERK 1 siRNA treated cells and control cells. The main caveat of

this experiment is that timing of the rescue transfection as well as the development of a siRNA resistant expression plasmid would need to be undertaken which was not possible within the time frame of this project. Notwithstanding this would be an important experiment to demonstrate the specificity of ERK 2 in the forward trafficking of osteopontin and SPARC, as well as confirming the observation made in the U0126 washout experiment.

Summing up this chapter, I would like to discuss the implication of knocking down one kinase and the effect this has on the cell. Redundant roles for each kinase do not appear to exist in the osteosarcoma model under investigation here as we observe differences in cell survival as well as an effect on trafficking when ERK 2 and not ERK 1 is knocked down. The difference in cell number does not affect the expression of SPARC and osteopontin significantly enough to be the cause for the lack of forward trafficking. Instead, expression of these two matricellular proteins is proportional to the number of cells as there is not a significant difference against the control cells in ERK 2 knockout cells. We sought to look at the difference in activation of various transcription factors during siRNA treatment of ERK 1 or 2 (if any differences exist) as well as with the U0126 inhibitor. Research has shown important transcription factor substrates for ERK 1 and 2 (Murphy et al. 2004). We thought that if there was redundancy between the two kinases with respect to activation of transcription factors, that there would not be a difference in their level of activation. Unfortunately we could not acquire the antibodies for these transcription factors, but it goes without saying that this experiment is essential and should be carried out in order to confirm the effect ERK 2 is having in the osteosarcoma model is a cytoplasmic role, or that its absence cannot allow the cell to survive through its role in the nucleus. This highlights the possibility that ERK kinases have different and distinctive roles in the nucleus and the cytoplasm especially when the timing of the effect is taken into consideration. The effect observed after ERK 2 downregulation with siRNA treatment took place after 48 hours; however it is unclear when the downregulation takes full effect. On the other hand, looking back at the previous chapter (Chapter 3 section 3.5), a washout of the U0126 inhibitor showed rapid reversal in the dynamic of osteopontin trafficking within 15 minutes, a time point unlikely to impact the transcription of target genes.

It also appears to be the case that ERK 2 phosphorylation levels increase when ERK 1 is knocked down, while ERK 1 phosphorylation levels are unchanged when ERK 2 is knocked down. We expected that ERK 1 activation would also increase when ERK 2 was knocked down. The reason for this is that basal levels of phosphorylated ERK 2 were quantified to be about 1.8 times higher than that of activated ERK 1. Therefore, in response to knockdown of ERK 2, phosphorylated ERK 1 should increase to compensate not only for the lack of ERK 2, but for the quantity of ERK 2 that is no longer activated (assuming this higher level of activation correlates with a bigger workload). Another explanation for why ERK 1 did not increase is that the higher basal levels of phosphorylated ERK 2 *do not* correlate with a higher workload for ERK 2, and this could explain why ERK 1 was not phosphorylated in response to down regulation of ERK 2, however why this happened, and which theory is correct is unknown. Nevertheless, it appears that there is some form of compensation with an increase of phosphorylated ERK 2 when ERK 1 is knocked down. Further quantification of phosphorylated ERK levels should be carried out to verify the amount of phosphorylated ERK during siRNA treatment. Western blot analysis may have an impact on the result if a certain threshold is achieved during exposure of the film to the membrane. This may over saturate one or more of the samples and as a result, true differences in ERK activation might not be detected. Carrying out indirect immunofluorescence staining and quantifying the intensity of phosphorylated ERK levels would confirm observations made in western blot analysis as well as allow for the quantification of a large number of cells.

Investigations in the following chapter will aim to look at protein-protein interactions using STRING analysis to look at possible candidates for ERK 2, subcellular fractionation to isolate where SPARC and osteopontin are located in the cell and radioactive labelling of methionine and cysteine to measure the total content of protein secreted from the cell.

5.0. SUBCELLULAR FRACTIONATION, RADIOACTIVE ASSAYS AND STRING ANALYSIS

5.1. INTRODUCTION

This chapter will aim to look at the specifics of osteopontin and SPARC localisation. We observed a change in localisation that appeared to be ERK 2 specific with relation to the forward trafficking of osteopontin and (possibly) SPARC in ROS cells. Immunofluorescence staining showed that this effect was likely taking place in a perinuclear location in or around the Golgi apparatus. Subcellular fractionation was used to further isolate the cellular compartment where osteopontin and SPARC are located in the cell. ^{35}S labelling was used to confirm that this change in trafficking caused by inhibition of ERK 1 and 2 is leading to less protein secretion. Finally, STRING analysis, a software designed to look at protein-protein interactions was used to try and identify what ERK 2 may be interacting with in order to prevent forward trafficking of SPARC and osteopontin.

5.1.1. SUBCELLULAR FRACTIONATION

Subcellular fractionation has been widely used as a technique in the field of membrane trafficking as a way of isolating and confirming where proteins are located with respect to the intracellular trafficking pathway and the different compartments involved (Rangel et al. 2013).

The technique centres on several sedimentation steps to separate cellular components, followed by fractionation on a gradient of varying densities. These two methods are known as differential centrifugation and density gradient centrifugation respectively.

Differential centrifugation is a method of separating parts of the cell such as the nuclear and cytoplasmic compartments based on centrifugation at different speeds. This is a somewhat more crude way of separating cellular compartments and it does not discriminate between organelles or compartments that are contained within nuclear or cytoplasmic fractions. To further separate components of the cell and isolate compartments, density gradient centrifugation should be carried out after differential centrifugation. Density gradient centrifugation is a constructed gradient based on varying densities. The solution used can differ from sucrose to OptiPrep (Iodixanol) (Lee et al. 2010).

Iodixanol is the density gradient that we used. Iodixanol is a compound that is iso-osmolar, non-toxic and does not interfere with the cells or intracellular components (Smith et al. 1997). 50% Iodixanol has a density of 1.27g/ml. Gradients can be constructed at varying percentages to produce fractions that have lower densities (table 5.0), thus allowing different cellular compartments to settle at different fractions (Graham 2002).

% Iodixanol (OptiPrep)	Density g/ml
50	1.27
40	1.22
30	1.17
20	1.12
10	1.07

TABLE 5.0, the percentage of Iodixanol solution in water determines the density of Iodixanol. The density of solution decreases as the concentration of Iodixanol decreases. This will cause different cellular compartments to settle at their corresponding densities.

The density of the organelles is an important factor when constructing a gradient as it will determine where the organelle will settle on the gradient. Table 5.1 is a table of the densities of various organelles in the cell.

Organelle	Density g/ml
Golgi	1.14
Endoplasmic Reticulum	1.20
Plasma Membrane	1.12
Mitochondria	1.18
Lysosomes	1.12

TABLE 5.1, the density of various organelles shows where they should settle on the Iodixanol gradient. There appears to be overlap between the lysosomes and plasma membrane however, since the density of the Golgi apparatus and endoplasmic reticulum is different, there should be a cleaner separation between these two organelles (Lodish et al. 2000).

A centrifuged sample (from cell lysates from control and treated flasks) is incorporated into a constructed gradient of varying densities and is centrifuged at high gravitational forces in order for components of the cell to settle at lighter parts of the gradient if they are lighter, or migrate towards the heavier densities if they are heavier components. Following centrifugation, 0.5-1ml fraction samples are collected and SDS-PAGE is run. Antibody detection can then look for target proteins as well as markers of various compartments or organelles to see what is present with the protein of interest.

5.1.2. PROTEIN SECRETION ASSAYS

Protein secretion assays will be carried out in order to confirm that the clustering or retention of osteopontin and SPARC also correlates with less overall protein secretion. Radioactively labelled amino acids are a useful tool for measuring the intracellular and extracellular protein content. The technique is also known as the 'pulse-chase' assay and works on a basic principle. Cells are starved of the amino acid that will be introduced into the cell (radioactively labelled amino acid). The time of starvation is sufficient such that amino acid incorporation is likely to be taken over by the radioactively labelled amino acid and not the endogenous non labelled amino acid. Once the radioactively labelled amino acid is introduced into the cells, the 'pulse' phase begins. This is the point at which the radioactively labelled amino acids are being incorporated into newly synthesised proteins

(which are now radioactive) hence the term 'pulse'. Following a set time point of the pulse phase, the media containing the radioactively labelled amino acid will be removed and normal media containing non-radioactively labelled amino acid is introduced into the cell to allow for protein synthesis to take place with non-radioactively labelled amino acids. What remains is a pool of proteins that are radioactively labelled and the synthesis of proteins with a non-radioactive amino acids commences the 'chase' phase of the procedure, as the radioactively labelled proteins are essentially chased out of the cell. Treatments on the cells would take place at this point (if they haven't already over a longer time period as might be the case with siRNA). The length of the chase period depends on the time of the treatment, but it should be sufficient to allow the cell to carry out functions like secrete proteins so that differences can be seen between treated and control cells (Hou et al. 2013).

Detection of protein secretion is carried out by the addition of scintillation fluid to the sample (a fluid that is excitable by the energy emitted by the radioactivity which results in the excitation state of the scintillation fluid producing light) where the amount of radioactivity will be measured by a scintillation counter (a machine that measures the amount of light produced by the energy emitted by the radiation from the protein sample). The amount of light emitted should be reflected in the amount of radioactivity in the protein content (i.e. more radioactivity means more light and vice versa) (Erchinger et al. 2015).

The term 'radioactively labelled amino acids' is used loosely in this introduction. The reason for this is that different amino acids along with the different radioactive isotope of various elements can be used in this technique. Radioactive labels, also known as radioactive tracers (Gest 2005) are conjugated to amino acids. Various radioactive isotopes are utilised. Common elements used in molecular biology include ^{35}S, a radioactive isotope of Sulphur, ^{14}C, a radioactive isotope of carbon and ^{32}P, a radioactive isotope of phosphorus (Alberts et al. 2002).

Common amino acids that are conjugated onto these elements include methionine and cysteine. Both amino acids readily conjugate with the radioactive isotopes such as Carbon and sulphur. There are advantages of using Sulphur 35 over other radioactive isotopes. Sulphur 35 has a half-life of 87 days making disposal easier and it will be incorporated into amino acids readily and will not conjugate also with molecules like RNA and DNA like some

radioactive isotopes of carbon might. Sulphur 35 is also resistant to TCA precipitation and does not degrade allowing for an accurate measurement of radioactivity when being counted in a scintillation counter (Chen & Casadevall 1999).

5.1.3. STRING ANALYSIS

Finally, STRING analysis will be used to look at protein-protein interactions. STRING analysis is a software available online (version 10.0 at time of writing) to look at known interactions between different proteins. Having observed retention of SPARC and osteopontin in a perinuclear region and depending on the results obtained in subcellular fractionation, we will look at known interactions between ERK 1 and 2 and proteins that might be involved in trafficking to this area. This is a powerful tool that I will use to try to establish how the ERK kinases affect intracellular trafficking of matrix proteins.

5.2. RESULTS

5.2.1. SUBCELLULAR FRACTIONATION

In order to further investigate where SPARC and Osteopontin are located when ERK 1 and 2 activation is inhibited, subcellular fractionation was carried out. Subcellular fractionation is a technique used to separate cellular organelles from the cytoplasm based on their density. We used an Iodixanol gradient to carry out the separation.

Please note that the fractionation experiment was carried out twice (independent experiments).

Initially we tested gradients that are designed to separate lysosomal compartments from the Golgi and ER. These gradients consisted of 6 fractions and were constructed as follows: 27, 22.5, 19, 16, 12 and 8% Iodixanol. Osteopontin was consistently localised to one fraction when carrying out western blot analysis co-localising with a Golgi marker. As this was the case (and the gradient consisted of 6 fractions, making it more error prone to construct), we opted for a slightly modified version of the gradient consisting of four steps (37, 30, 25 and 5% Iodixanol where the sample is incorporated into the 30% solution (figure 5.0)) (Schmidt et al. 2009), which gave good resolution.

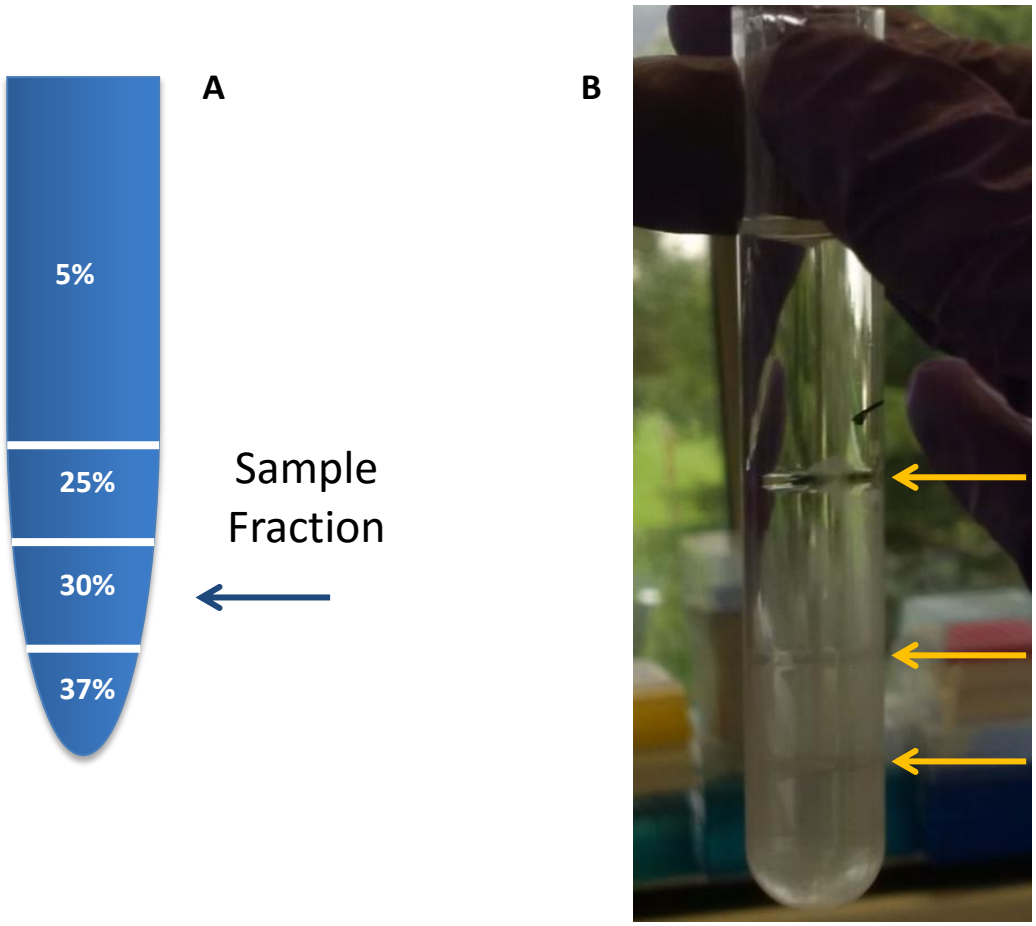


FIGURE 5.0, Illustrations of density gradient constructed. A, Density gradient that we would construct with the various densities of Iodixanol labelled. Heavier compartments in the cell should settle at 37-25% Iodixanol while lighter compartments should migrate towards the 25-5% densities. Sample fraction is added to the 30% density Iodixanol. **B,** Density gradient constructed showing the separation between the different Iodixanol gradients (yellow arrows) which correspond to illustration drawn in picture A.

Once samples had been prepared (untreated or U0126 1 hour) and incorporated into the gradient, centrifugation took place overnight at 150,000g. Fractions were run on SDS-PAGE and analysed by checking localisation of specific protein markers as well as osteopontin and SPARC (figure 5.1). We saw the presence of a Golgi marker (GM130) and an ER marker (ERp72) in the same fraction as SPARC and osteopontin. Interestingly, the U0126 treated fractions showed osteopontin and SPARC at a greater density than was observable in the untreated set of fractions (figure 5.1). This indicated some form of retention in a heavier compartment when ERK 1 and 2 activation was inhibited (agreeing with what was observed in immunofluorescence imaging).

We collected approximately 500-1000 µl of each sample (13 fractions in total) and SDS-PAGE was run (1 gel for the untreated sample, and 1 for the U0126 1 hour treated sample). The results for the gels run with all 13 samples are not shown, however, we observed that in both untreated and treated gels, SPARC and osteopontin were both present in fraction 6 of the entire fraction set. Following this, we decided to run fractions 6-9 to see which markers were present in these fractions. A Bradford assay was carried out to quantify protein content of fractions 6-9 for the untreated and U0126 treated fractions to ensure that any differences seen were due to actual differences in protein content and not due to protein concentration loaded on the gel (table 5.2).

	Fraction number	Concentration (µg/ml)
Untd	6	193.67
	7	213.67
	8	153.67
	9	137.00
U0126	6	110.33
	7	400.33
	8	245.33
	9	290.33

TABLE 5.2, Concentration of total protein content differs amongst the different gradient fractions. Western blot analysis would be carried out by running each fraction after being adjusted so that the level of loading was equal across each sample (110.33µg/ml).

We adjusted the concentration of each sample to the lowest concentration measured in the table (fraction 6 for U0126 treatment, 110.33 µg/ml). Once the concentration of all fractions had been adjusted to 110.33 µg/ml, fractions 6-9 for untreated and U0126 samples, SDS-PAGE was run. In western blot analysis, presence of SPARC and osteopontin in fraction 6 was confirmed and subsequently the presence of specific organelle protein markers was investigated (figure 5.1).

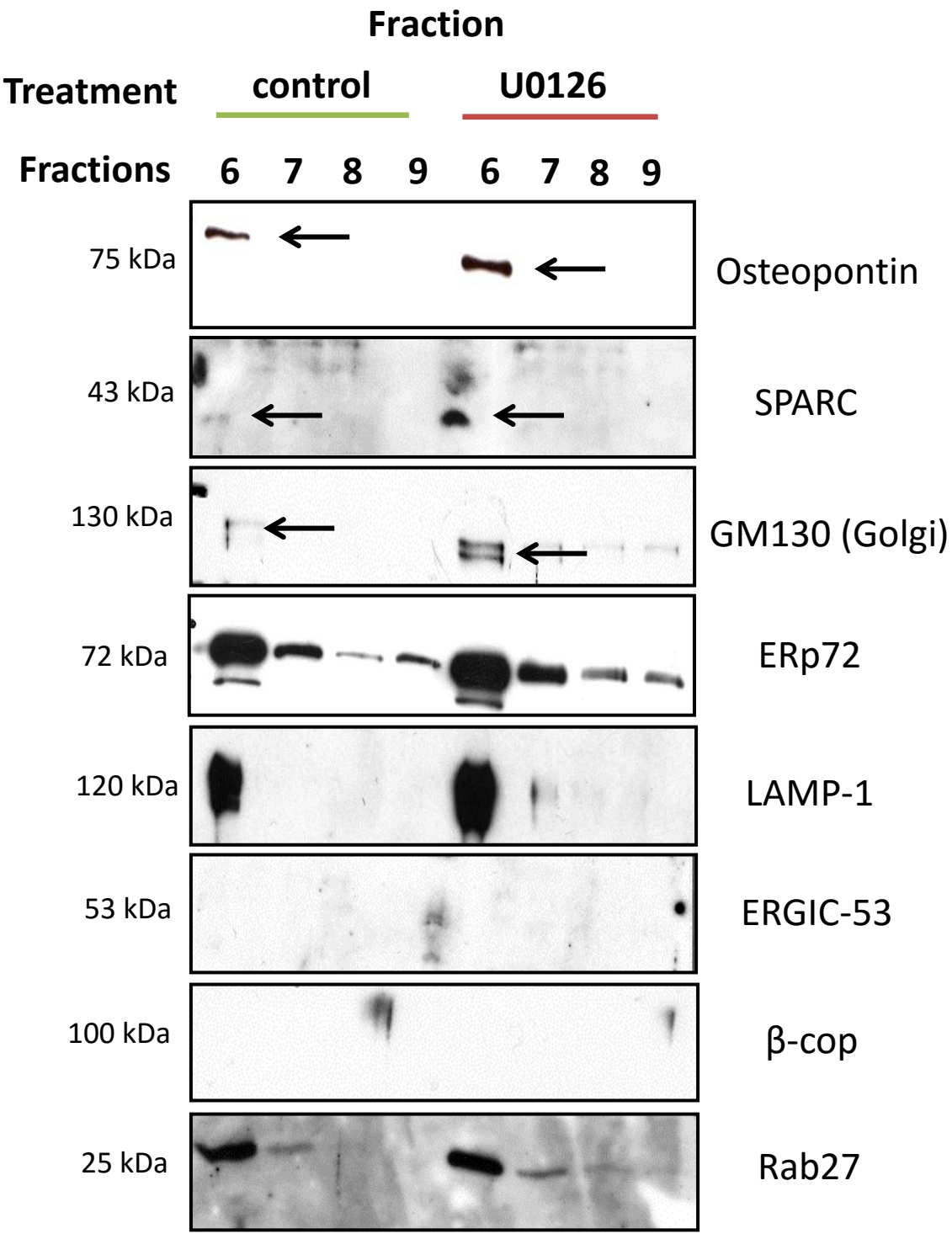


FIGURE 5.1, Specific organelle marker proteins indicate localisation of these compartments/organelles in fraction 6-9 and their relationship to SPARC and osteopontin localisation in control and U0126 (1 hour) treated cells. ROS cells were treated with U0126 for one hour and subsequently lysed with a ball homogeniser to keep intracellular organelles intact. Samples were loaded on a 5 to 35% iodexanol step gradient and spun at 150,000g

overnight. 1ml fractions were taken and 110.33µg per fraction was loaded on SDS-PAGE. Western blotting for organelle markers and SPARC/osteopontin revealed that they are both located in the same fractions (6-9), which also contains Golgi, ER and vesicular markers. In U0126 treated samples the SPARC/osteopontin localisation is shifted to a lighter fraction. SPARC and osteopontin occupy the same fraction as the Golgi marker (GM130) and it is likely that this is where retention is taking place in the treated fraction. ERp72 (ER marker) is present in various fractions. ERGIC-53 (a marker of the ER-Golgi intermediate compartment) is not present in these fractions while β-cop (COPI coated vesicle marker) is present in fraction 9. Rab27, a marker of secretory vesicle is present across multiple fractions. This figure represents multiple blots run separately at different times, but using samples from the same experiment as well as loaded to equal concentrations following Bradford assay total protein quantification. n=2

The fractionation experiments yielded interesting results. The presence of higher amounts of SPARC and osteopontin in the U0126 treated fractions was an interesting observation. Consistent with the immunofluorescence staining, we saw that COPI coated vesicles were localised in fraction 9, separate to osteopontin and SPARC giving further confirmation that it was highly unlikely that the retention was taking place in the ER, nor was there any retrograde (backwards) trafficking of osteopontin and SPARC into the ER as COPI coated vesicles are responsible for transport from the Golgi to the ER. Next, we asked if the retention might have taken place in the ER-Golgi intermediate compartment (ERGIC). ERGIC (also known as vesicular-tubular structures) is a compartment in between the ER and Golgi apparatus that is maintained by fusion of multiple COPII (and COPI) vesicular components that accumulate before fusion with the Cis-Golgi. COPI coated vesicles travelling back to the ER as well as SNARES also pass through the ERGIC. ERGIC-53 is a lectin that recognises carbohydrates on proteins bound for the Golgi apparatus (Appenzeller-Herzog & Hauri 2006; Appenzeller et al. 1999).

We did not observe co-localisation of SPARC/osteopontin with ERGIC-53 as there was no ERGIC-53 present in these fractions. Rab27 was present in fractions 6-9 in both untreated and treated cells, although the signal for Rab27 in untreated fraction 8 and 9 was difficult to detect.

The observation that SPARC and osteopontin bands were more dense in the U0126 treated sample could be explained by retention taking place in the perinuclear region (probably the

Golgi apparatus) similar to what was observed in immunofluorescence staining. Should this be case, there should be less secretion of these two matricellular proteins as well. With this in mind, we carried out ^{35}S labelling of total cellular protein by incorporation of radioactive methionine/cysteine mix. Total protein secretion in untreated and treated cells (both for the U0126 inhibitor treatments and siRNA treatments) was measured by scintillation counting after pulse chase and TCA precipitation.

5.2.2. ^{35}S LABELLING OF ROS CELLS TO MEASURE PROTEIN SECRETION

Using a radioactive isotope of Sulphur (^{35}S) incorporated into methionine and cysteine; we can measure the amount of radioactivity inside and secreted from cells in treated and untreated samples. This will give us an indication of the extent of the effect of inhibiting the MAPK pathway in the secretion of the matricellular proteins we were interested in.

Before we carried out this experiment, we did attempt to look at SPARC and osteopontin secretion in media from cells plated in 6 well plates. Carrying out TCA precipitation and spinning down the total protein content in the media was not sufficient for anti-SPARC/osteopontin antibodies to detect SPARC and osteopontin that was secreted. Unfortunately, the amount of SPARC and osteopontin antibody we have access to is limited and it was not possible to undertake a radioimmunoassay. The experiment was thus carried out measuring total protein content.

Please note that ^{35}S labelling of ROS cells for U0126 and siRNA treatments was carried out at least 3 times (independent experiments) and in triplicate.

The first condition that was tested was treatment of cells with the U0126 inhibitor for one hour. The U0126 inhibitor was added during the 'chase' phase of the assay (see materials and methods section 2.2.6 for details of assay) before being incubated at 37°C for one hour. Following one hour, the chase was stopped and the assay completed before radioactivity was measured by a scintillation counter.

The results for the inhibitor treatment agreed with what was observed in the immunofluorescence staining experiments as well as the fractionation experiments. The total protein content secreted from the cells in the untreated wells was calculated to

represent 100% of total protein content. Treated cells were then divided by the 100% total of the untreated samples to calculate how much higher or lower the protein content was in the treated samples. The reason this was carried out was because radioactivity can differ based on experimental conditions such as numbers of cells, so there could be variation between experiments. Since the ratio of total content secreted from cells is what we are interested in, setting the untreated cell protein content to 100% would allow us to see if the difference in protein secretion is constant across multiple experiments regardless of the result obtained by the scintillation counter (figure 5.2).

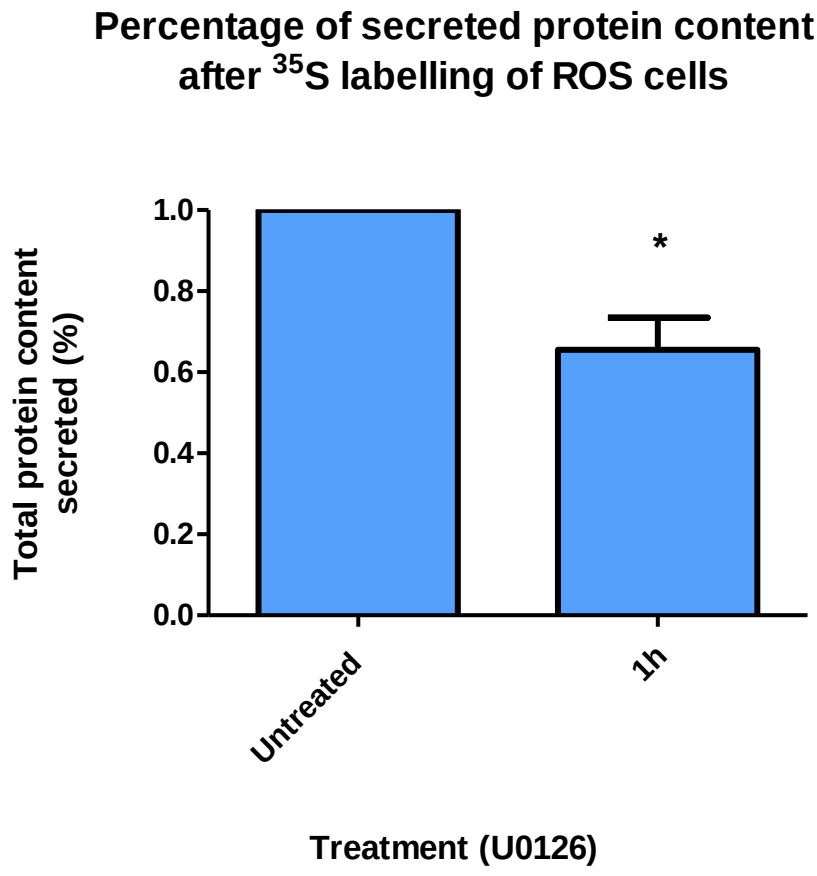


FIGURE 5.2, Treatment of cells with the U0126 inhibitor for 1 hour shows a reduction in the total protein secreted compared to the untreated cells. Consistent with previous observations, the radioactive assay shows that there is less secretion when ERK 1 and 2 activation is inhibited. This reduction is significant (**error bars represent SD** *P<0.05) and the total protein content in the treated cells is quantified to 66% total protein in the supernatant. n=3

The total protein content in the supernatant of inhibitor treated cells was 66% of that of untreated cells. This means that there is a reduction of 34% in protein secretion when ERK 1 and 2 activation is prevented in the ROS cell line. We did not carry out time points 2-6 hours due to reagent and equipment limitations. However, as we observe a similar effect in immunofluorescence staining, we would predict that we would see a similar reduction in the total protein content in cells treated for 2-6 hours.

Following on from inhibitor treatment, ROS cells were treated with siRNA for ERK 1 and ERK 2 individually to see what effect siRNA knockdown would have on protein secretion. Given the observation that osteopontin localisation had changed when we treated cells with siRNA against ERK 2 (and it showed an effect similar to that of the U0126 inhibitor), we expected to see a reduction in secretion when we treated cells with siRNA against ERK 2. We did not anticipate any significant change of protein secretion in cells treated with siRNA against ERK 1.

Cells were treated with siRNA against ERK 1 or 2 48 hours before the protein secretion assay. On the day of the experiment, cells were labelled with ^{35}S methionine for 15 minutes, before the chase for one hour.

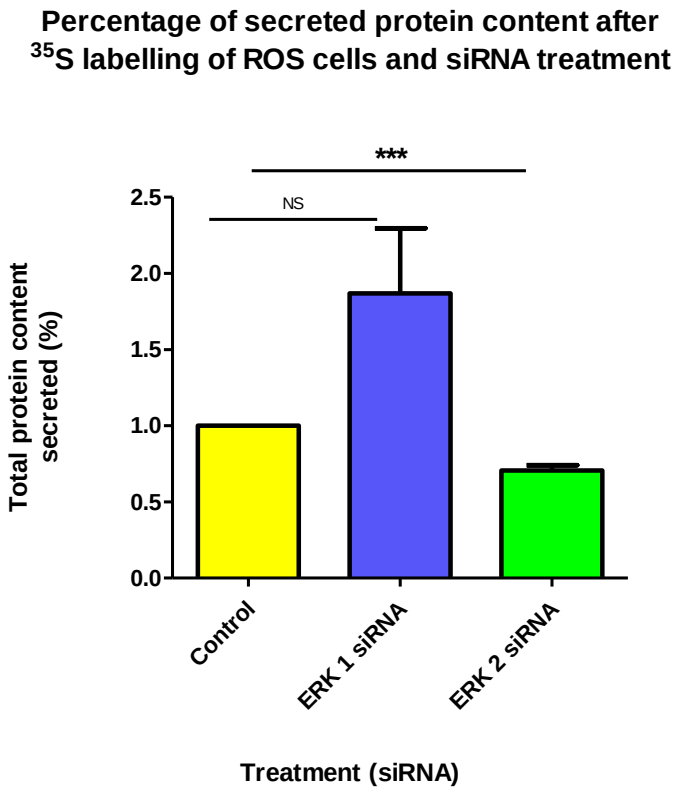


FIGURE 5.3, Total protein content decreases with ERK 2 siRNA treatments but increases with ERK 1 siRNA treatments. Consistent with observations made in immunofluorescence staining, treatment of cells with siRNA against ERK 2 reduces protein secretion. A significant reduction in total protein content (**error bars represent SD** ***P<0.001) of 30% is observed. Treatment of ROS cells with ERK 1 siRNA shows no significance as some experiments show an increase in protein secretion while others show secretion close to control levels. n=3

We could replicate the reduction of total protein content secreted with cells treated with the U0126 inhibitor and cells treated with siRNA against ERK 2. This confirms that most likely, the ERK 2 kinase is responsible for the retention of SPARC and osteopontin preventing secretion of these two matricellular proteins. The radioactive assay is not limited to SPARC and osteopontin however, and the true reduction in protein content secreted could be different with regards to osteopontin and SPARC.

Treatment of ROS cells with siRNA against ERK 1 produced highly variable results. There was a sharp increase in the total amount of protein secreted in some experiments and very little difference to control cells in others. We anticipated that the level of protein content would

be similar to that of the control cells in all experiments based on what we observed in immunofluorescence staining. A possible reason as to why total protein secretion increased in some cases will be discussed later.

The data acquired over the course of this study points to the implication of the MAPK pathway in the forward trafficking of the matricellular proteins osteopontin and SPARC, and the results obtained are convincing. Having found that the effect appears to be ERK 2 specific, we turned our attention to what ERK 2 might be doing to allow these two matricellular proteins to proceed along the secretory pathway. STRING analysis was employed to investigate ERK interactions with possible substrates that might lead to the retention and lack of secretion observed.

5.2.3. STRING ANALYSIS FOR PROTEIN-PROTEIN INTERACTIONS

Inhibition of the ERK 1 and 2 module of the MAPK pathway affects trafficking of GFP-SPARC and endogenous osteopontin. Localisation studies showed that the effects appeared to lead to entrapment of SPARC and osteopontin in a perinuclear region. When ERK 1 and 2 activation is inhibited (or ERK 2 alone with siRNA), co-localisation of osteopontin with the Golgi apparatus appears reduced as compared to untreated cells. To identify possible proteins involved in the MAPK dependent activation of trafficking proteins, a STRING database search for protein-protein interactions between *MAPK3* and *1* (gene name for ERK 1 and ERK 2 respectively) and other proteins known to be involved in trafficking was started.

The STRING database (version 10.0 at the time of use) is a database of known and predicted protein-protein interactions including direct and indirect interactions.

Parameters were set to at least 500 interacting partners in order to ensure that wide arrays of known interactions were identified. Once the search was completed, ERK 1 and 2 ‘nodes’ were at the centre of a network, with interacting partners spreading from the ERK 1 and 2 nodes. Coloured lines connecting the ERK proteins and their interacting partners identify the type of interaction, for example, phosphorylation.

The interacting partners for ERK 1 and 2 are extensive and the entire data set will not be shown as many of the proteins are signalling pathway activators or phosphatases as well as

transcription factors. To focus the search, the interacting partners were filtered by their known biological function. The STRING database offers the possibility to filter for cell signalling, activation of transcription, modulation of organelle structure and trafficking among many other biological functions. Note that these biological functions are with respect to ERK 1 and 2 and how *they* influence these biological functions through their interaction with their binding partners.

Our area of interest was in the interacting partners involved in trafficking, however, there were various trafficking categories from regulation of transport in general, to more specific transport involvement such as negative or positive regulators of transport. The filters selected were as follows:

- Regulation of transport
- Regulation of intracellular transport
- Positive regulation of protein transport
- Negative regulation of protein transport
- Cytoplasmic transport
- Vesicle mediated transport
- Retrograde transport
- Regulation of synaptic vesicle transport
- Intra-Golgi vesicle mediated transport
- Golgi to plasma membrane transport
- ER-Golgi vesicle mediated transport
- Vesicle mediated transport along microtubule track

The 'regulation of transport' filter selected proteins that were involved in the transport process in general and more specific filters such as 'positive regulation of protein transport' would filter proteins within the category of regulation of transport. The network for 'regulation of transport' is therefore shown in figure 5.4. According to our observations in immuno-imaging, it would appear that our main area of interest would be within the following categories:

- ER-Golgi vesicle mediated transport

- Intra-Golgi vesicle mediated transport
- Cytoplasmic transport
- Vesicle mediated transport
- Retrograde transport
- Golgi to plasma membrane transport

While attention was paid to these categories, it is by no means conclusive and would not necessarily yield positive results, nor would it 'definitely' contain the interaction we were interested in, nevertheless, all of these results should be considered. ERK 1 and 2 may have indirect effects on the trafficking of SPARC and osteopontin. The implication of this is that the ERKs may not be directly interacting with one factor, but through multiple, for example, initiating the phosphorylation of one protein which then binds to and chaperones another, as is the case with 14-3-3 and the localisation of peroxiredoxin to the lysosome (Sorokina et al. 2011). In this situation, simply looking at the interacting partners for ERK 1 and 2, even when specifically selecting trafficking roles would not suffice. For this reason, a series of cellular compartment and protein classes were also built. The classes built were as follows:

- Golgi class network with GM130 (cis-Golgi marker) at the centre of the network
- TRAPPC4, a component of the TRAPP tethering factors that are responsible for tethering COPII coated vesicles bound for the Golgi and positioning them appropriately for interaction with SNAREs and subsequent fusion with the cis-Golgi.
- Cog7, a subunit of the conserved oligomeric Golgi complex required for transport within the Golgi, again serving a class of proteins associated specifically with the Golgi
- Uso1, a vesicle docking protein in Golgi transport

The intention of building classes of networks around these structure and protein subunits would be to see the involvement of ERK 1 and 2 in these regions, and if they tie in with any hits associated with ERK 1 and 2 interacting partners and their role in transport as mentioned above. This would also give us indirect interactions that ERK 1 and 2 may be having and influencing the activity in these areas. We chose to include tethering factors in the class hits as there appears to be less co-localisation in the Golgi apparatus in some instances. For this

reason, tethering factors should be included as there is a possibility that any activation (or lack of) by ERK 1 and 2 might influence the positioning and fusion of vesicles with membrane compartments and thus cargo release to the Golgi.

We did not observe co-localisation of SPARC or osteopontin with the endoplasmic reticulum and therefore decided to exclude a class of ER proteins as it is unlikely that the effect we are seeing is taking place here. Additionally, as osteopontin and SPARC are observed in the cytoplasm (dispersed in untreated cells and clustered in U0126 treated cells), it is unlikely that there is an issue with ER packaging into COPII coated vesicle and release from ER-exit sites.

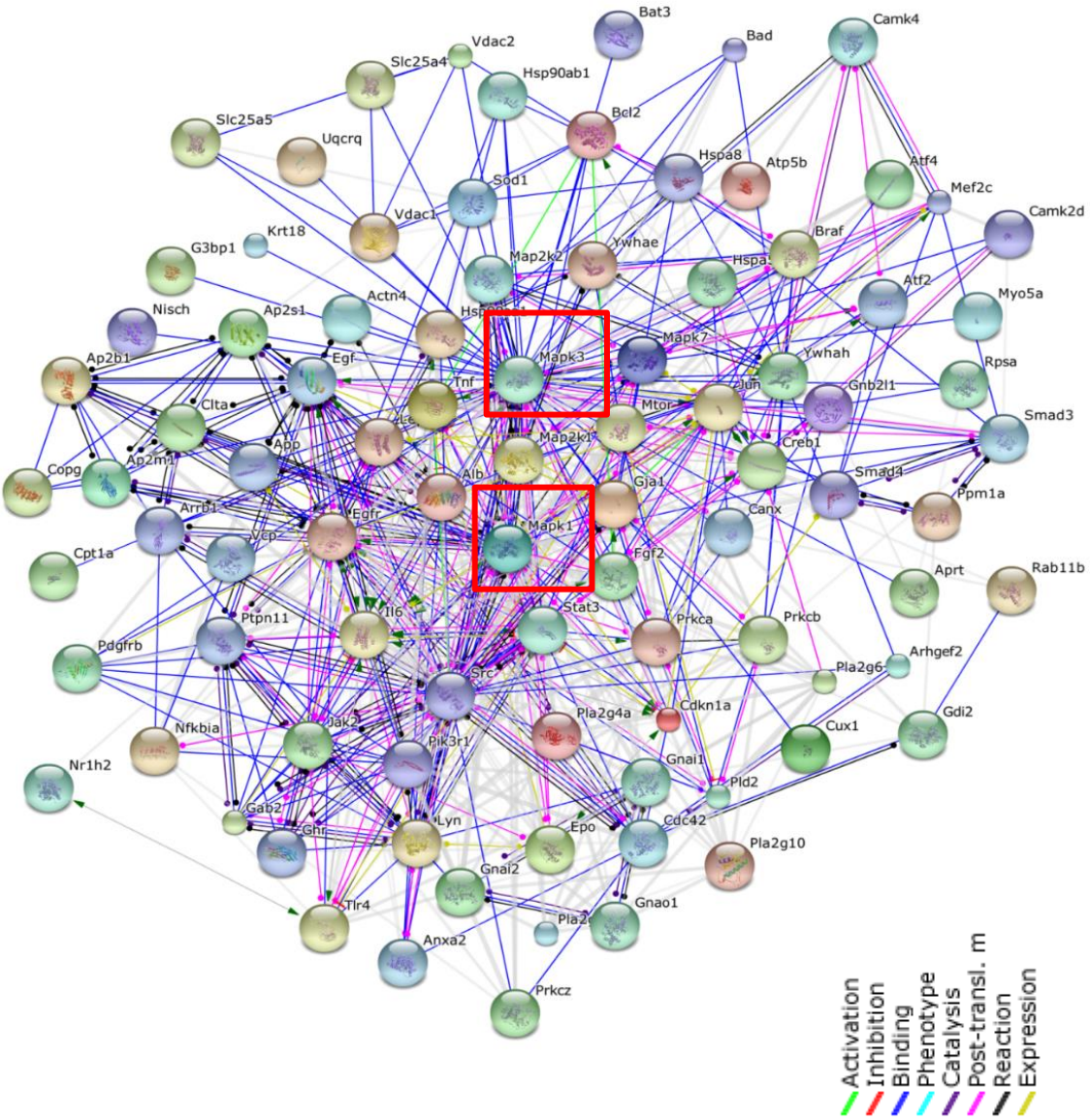


FIGURE 5.4, Initial search for ERK 1 and 2 interacting partners identifies various proteins involved in trafficking. Key below the figure defines what type of interaction is involved. A search for ERK 1 and 2 interacting partners identified over 200 proteins. A search filter for 'regulation of transport' produced the results in this figure. Many of the proteins do not necessarily function in ER-Golgi transport but function in processes such as signalling that lead to expression of proteins involved in transport and are not directly involved themselves. Nevertheless, the more the results are filtered, the more likely we will establish relevant hits. Red boxes represent ERK 1, top red box and 2, bottom red box (*MAPK3* and *1* respectively).

In order to further refine and filter this category of interacting partners and to include any potential indirect linkages, specific class and protein networks (as outlined above) were made (figure 5.5-5.8). ERK 1 and 2 were also introduced into the network. Note that all searches were carried out specifically for the rat species *Rattus Norvegicus.*

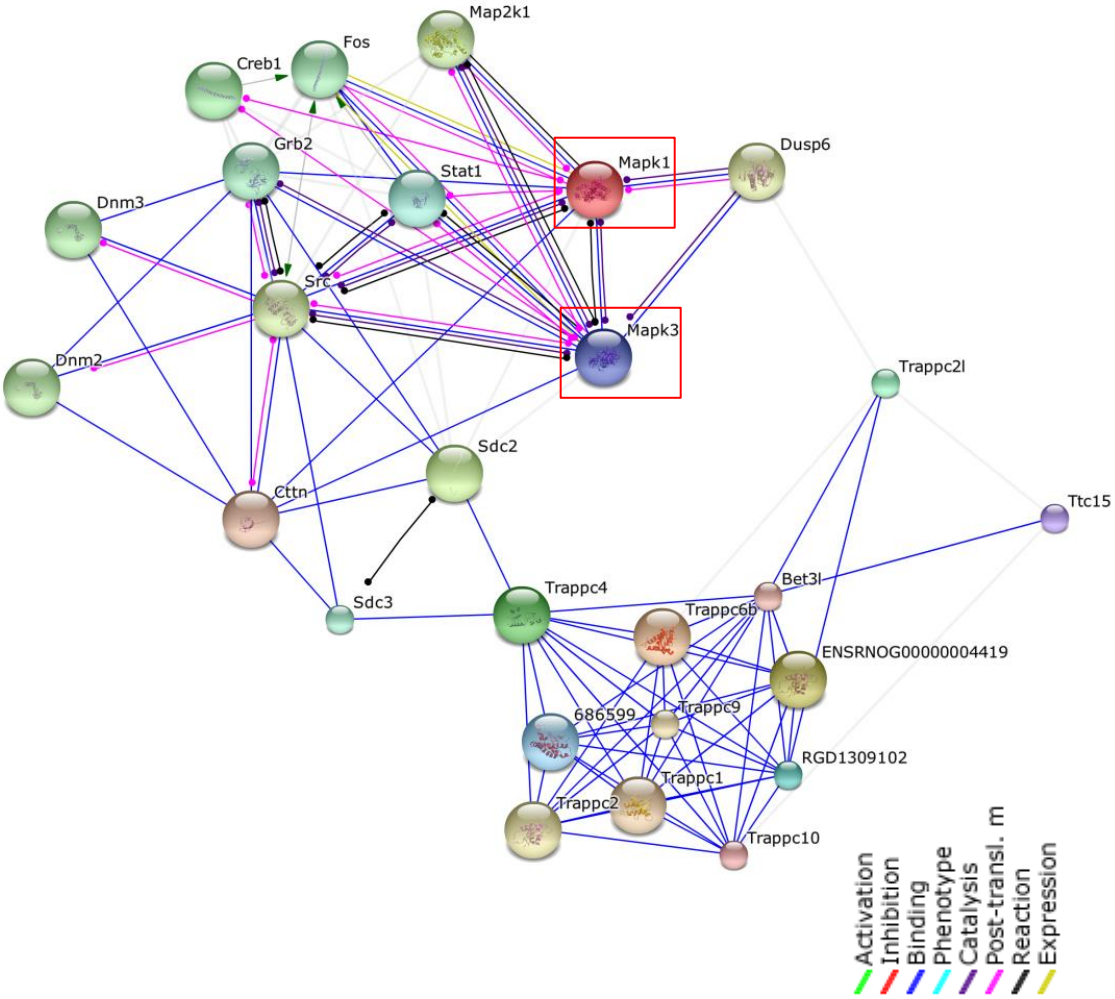


FIGURE 5.5, Trappc4 (Transport associated protein particle), a subunit of the TRAPP tethering complex shows indirect interactions with ERK 1 and 2. ERK 1 and 2 both interact with *Src* and *Cttn* which is linked to Trappc4 through *Sdc* 2 and 3.

The only possible mechanism by which ERK 1 and 2 may influence the tethering of osteopontin and SPARC is through activation of either Src (an SH2 and 3 containing signalling molecule), and *Cttn* (Cortactin, a protein involved in the rearrangement and polymerisation of the actin cytoskeleton, particularly at the cell periphery) which might then interact with the *Sdc* proteins (syndecans 2 and 3, proteoglycans involved in processes such as rearrangement of the actin cytoskeleton) which may activate the Trappc4 tethering complex to initiate recognition and tethering of COPII coated vesicles. A possible mechanism of action for the proteins linked to the ERK kinases will be discussed in more detail in the discussion.

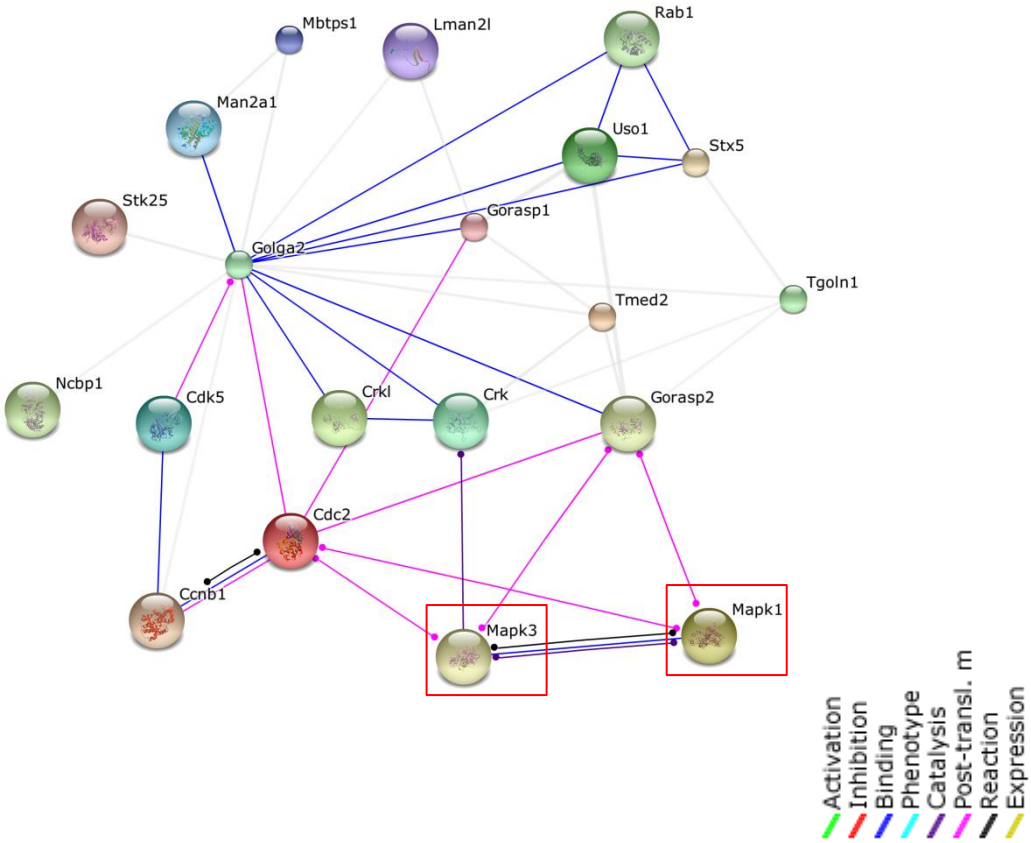


FIGURE 5.6, *Golga2* (gene name for GM130) is linked indirectly to ERK 1 and 2 through multiple proteins. It may be the case that connections to CDC2 and CDK are going to induce fragmentation of the Golgi during mitosis. The *MAPK1* (ERK 2) connection with *Gorasp2* could facilitate activation of GM130 which is a tethering factor at the Cis-face of the Golgi apparatus.

GM130 (*Golga2*) is a tethering factor, therefore there is a possibility that an indirect activation of proteins that interact with GM130 could lead to activation of the tethering factor and MAPK inhibition may disable activity of the tethering factor in ROS cells. While it isn't recognised by STRING, there is a possibility that GM130 may also be a direct substrate for ERK 2 which might then lead to activation of the tethering factor. The reason for this is that GM130 contains an ERK binding domain which may hint at an interaction between ERK and GM130 (figure 5.6). A lack of activation might cause the retention that we have observed if COPII coated vesicles are unable to fuse with the Cis-Golgi.

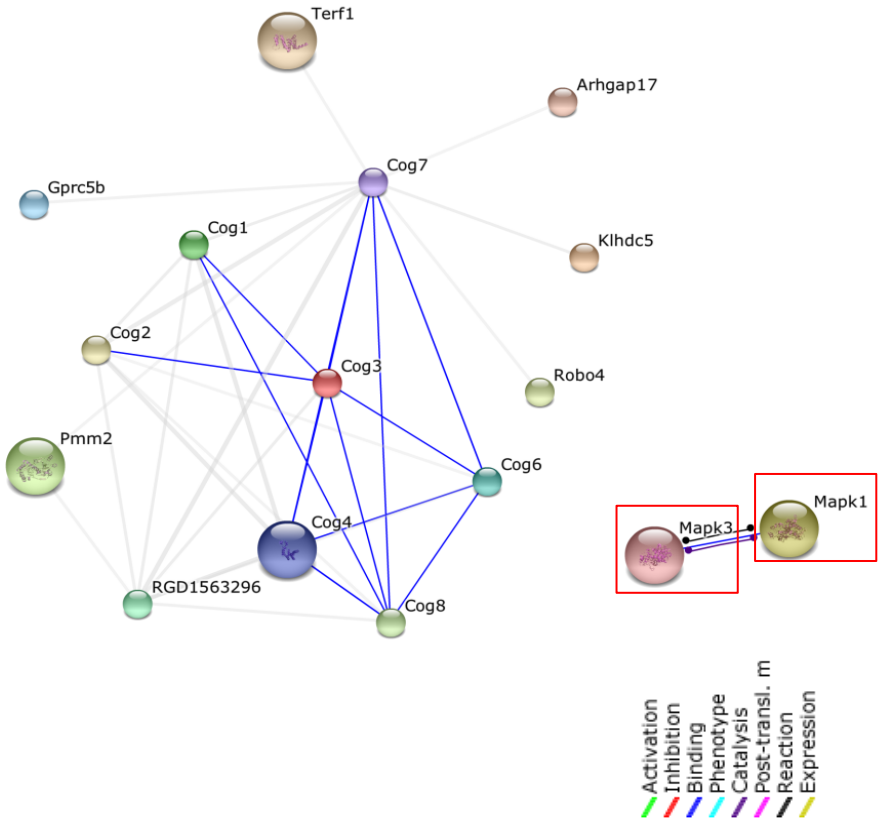


FIGURE 5.7, No direct or indirect (known) interaction exists between the ERK kinases and the Cog subunits. Cog (sub units of the conserved oligomeric Golgi complex) is part of a complex involved in retrograde transport of proteins through the Golgi apparatus.

No known interactions have been identified with the Golgi oligomeric complex. As this protein complex is involved in retrograde (backwards) transport, it is unlikely causing the effect that we are observing. Nevertheless, direct or indirect (currently unknown) interactions between MAPKs and the Cog subunits of the complex may exist to facilitate trafficking through the Golgi complex (Struwe & Reinhold 2012).

Finally, we looked at Uso1 (p115 in humans), a protein which like GM130, is involved in vesicle docking and tethering with the Cis-Golgi. The network that STRING produced for this protein and ERK 1 and 2 is more extensive, and it seems there are more indirect interactions or links between ERK 1 and 2 and Uso1 (figure 5.8) which may be significant in our study.

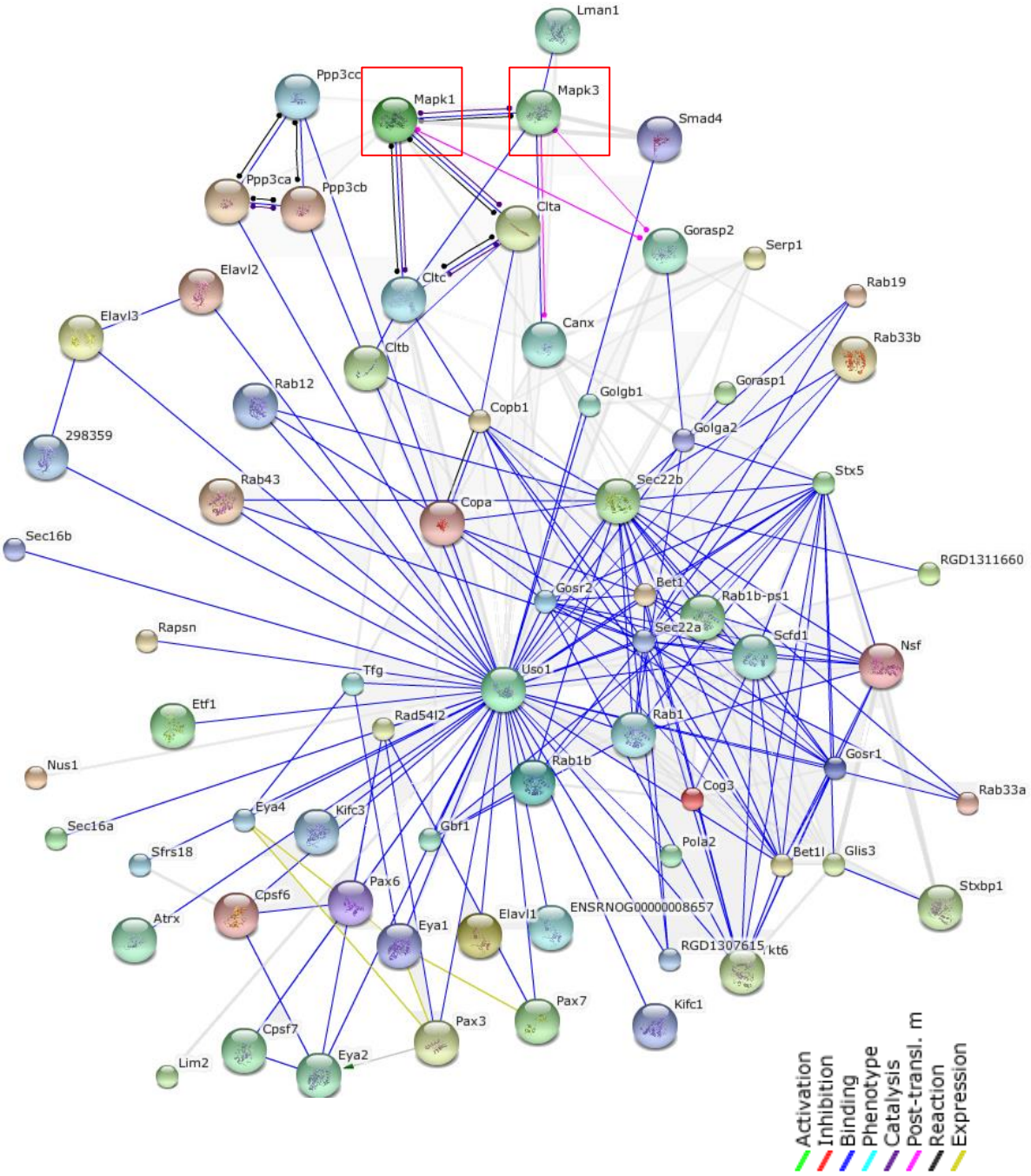


FIGURE 5.8, Uso1 shows more interactions that are linked to ERK 1 and 2. Since the effect observed in immunostaining experiments with siRNA treatments appeared to be ERK 2 specific, the links between *MAPK1* (ERK 2) and Uso1 should be considered over those between ERK 1 and Uso1 in this network. Possible candidates for ERK 2 include the genes *Cltc*, *Cltb* and *Clta*. This is a very interesting observation as these 3 genes encode for clathrin light chain A and B (*clta* and *cltb* respectively) and clathrin heavy chain 1 (*cltc*).

Looking at the links between ERK 2 and its connecting proteins, it is unlikely that the indirect connection to Uso1 is responsible for the retention of SPARC and osteopontin. Uso1 has been

shown to interact with GM130 at the cis-Golgi however (discussed later), and while this interaction is not shown in the STRING network produced, it does show a possible mode of action (through activation) for ERK 2 in allowing SPARC and osteopontin to continue through the anterograde pathway. However, the image does show a direct interaction between ERK 2 (*MAPK1*) and clathrin light chain A and clathrin heavy chain 1 with a purple line indicating catalysis (phosphorylation) and the blue line representing binding. ERK 2 is indirectly linked to clathrin light chain B (*cltb*), but this indirect interaction may be functionally significant. Phosphorylation of clathrin light chain A or heavy chain 1 might be required for subsequent interaction or recruitment of other proteins to clathrin light chain B. Clearly there is a possibility by which activation of clathrin by ERK 2 might be required for CCV formation and subsequent forward trafficking. The implication of this will be discussed in the discussion (section 5.3.).

While potential binding partners for ERK 2 have been identified for promoting forward trafficking of SPARC and osteopontin, the most important factor or relationship between the proteins is that they must be able to interact with one another, and that includes the ability for MAPK to bind to a substrate and for that substrate to contain a sequence that the ERK kinases can phosphorylate.

The STRING software is very useful in that it also provides the amino acid sequence for all the proteins identified in a network. Using this sequence, software available online was used to identify consensus sequences that can be phosphorylated (by known kinases) as well binding partners for various known proteins including kinases. This software is called the PhosphoMotif finder and is available at http://www.hprd.org/PhosphoMotif_finder

Kinase motifs can be identified as well as specific serine/threonine phosphorylation sites or tyrosine phosphorylation sites.

5.2.3.1. SITES OF PHOSPHORYLATION AND BINDING FOR MAPK SUBSTRATES

To study phosphorylation sites in the proteins identified by STRING analysis, the serine/threonine phosphorylation option was selected as ERK 1 and 2 are serine/threonine kinases. The kinase sites for Trappc4 are highlighted in red (and bold underlined sequences are phosphorylation sequences for kinases other than ERK 1 and 2). I focused on proteins

that were indirectly linked to MAPK such as Trappc4 and GM130. Figures 5.5-5.8 show direct interactions with proteins as denoted by the blue line in the figure key indicating binding.

Trappc4 ERK phosphorylation motifs

MAIFSVYVVNKAGGLIYQW**DSYSP**RAEAE**KTFSYP**LDLLLKLHDERVLVAFGQRDGIRVGHAVLAINGM DVNG**KYT**ADGKEVLEYLG**NSANYP**V**SIR**FG**RPRLTSNE**KLML**ASMFHSL**FAIG**SQLSPE**QGSSGIEML**E TDTFKLHCFQTLTGI**KFVVLADPRQAGI**DSLLR**KIY**EIYS**DFALKNPF**YSLEMP**IRCELFDQNLKLALEVAE **KAGT**FGPGS

While potential phosphorylation sites that ERK could activate on Trappc4 exist, no binding motifs for the ERK kinases were identified in this amino acid sequence. The ERK kinases contain a DEF (Aspartic acid, Glutamic acid and Phenylalanine) motif to which substrates dock before they are phosphorylated. The docking site for ERK on substrates is 'FXFP' (Phenylalanine 'x' Phenylalanine, proline, where 'x' is any amino acid (Burkhard et al. 2011; Jacobs et al. 1999).

No ERK docking site was identified in the Trappc4 sequence (FXFP) and is therefore highly unlikely to be phosphorylated by the ERK kinases.

Golga2 (GM130)

Nine sequences were identified as possible targets for ERK kinase activity on GM130, however, no 'FXFP' motif was present in the entire amino acid sequence.

Cog3 (subunit of conserved oligomeric Golgi complex)

Potential sites of phosphorylation for the ERK kinases exist; however, no 'FXFP' motif is present in the amino acid sequence making this protein an unlikely target of the ERK kinases.

Uso1 (Vesicle docking protein)

The conserved 'FXFP' was also not present in the Uso1 amino acid sequence. However, a paper published recently showed that ERK 1 and 2 could bind with weaker affinity to the sequence 'FXSP' where 'S' is a serine. This sequence is present in Uso1, however the implications of this will be discussed later (Karpova et al. 2013).

In addition to the 'FXFP' binding motif, ERK substrates also bind to ERK through a D-domain. A D-domain is a conserved **(Arg/Lys)$_{2-3}$-(X)$_{1-6}$-Φ-X-Φ,** where 'X' is any amino acid and '**Φ'** is any hydrophobic amino acid. Consensus sequence above is taken directly from (Fernandes & Allbritton 2009).

We next used 'Motif Scan' a website available from the Massachusetts Institute of Technology (http://scansite.mit.edu/motifscan_seq.phtml) that could look specifically for consensus D-domains present in an amino acid sequence of interest. This would identify potential binding sites for ERK.

Since conserved 'FXFP' motifs were not found on Trappc4, Cog3, GM130 and Uso1, we looked for the presence of the D-domain in their amino acid sequences. The results here were far more interesting, and we found some proteins described above (but not all) that contained D-domains, helping us filter potential ERK 2 substrates. First, proteins that *did not* contain D-domains were Cog3, Trapcc4 and Uso1. We also looked for D-domain sequences present on Sec 13, 23b and 31 (COPII vesicle coat proteins) and found no presence of a D-domain.

Interestingly, a D-domain was present on GM130, Sec23a, Sec24, clathrin heavy chain 1 and Rab27 (D-domain sequences below). Sec proteins, clathrin and Rab27 were not included in the initial STRING analysis, but further consideration meant that there is a possibility these proteins might be involved in the forward trafficking of SPARC and osteopontin, as the entrapment observed (while apparently in the Golgi) could still be towards the trans-face of the Golgi hence clathrin and Rab27, or improper shedding of COPII coats might be taking place due to a lack of possible phosphorylation at the Cis-Golgi, so these proteins were included.

Protein	D-domain present	Sequence	FXFP motif
GM130	Yes	SRYQELAVALDSSYV	No
Rab27	Yes	GRGQRIHLQLWDTAG	No
Clathrin heavy chain 1	Yes	RKGQVLSVCVEEENI	No
Clathrin light chain A	No	-	No
Clathrin light chain B	No	-	No
Sec24a	Yes	RRIRVHTLCLPVVST	No
		QRSSAKEIHLTPSTD	
		KAKLPLGLLLHPFKD	
Sec23a	Yes	LRSSGVALSIAVGLL	No

TABLE 5.3, D-domain is found on various proteins that may be involved in the forward trafficking of osteopontin and SPARC. The D-domain represents an amino acid sequence that binds to ERK 1 and 2 and is therefore likely to be a substrate for the ERK kinases.

This section of the chapter concludes with binding partners for ERK 2. The implications of binding of these proteins to ERK 2 with respect to forward trafficking of SPARC and osteopontin will be discussed in the discussion section 5.3.

5.3. DISCUSSION

The experiments and results described in this chapter confirm the observations made on single cells with immunofluorescence, that is, that osteopontin and GFP-SPARC thus likely also endogenous SPARC are retained in a perinuclear compartment most likely the Golgi complex when the MAPK pathway is inhibited (specifically the ERK 2 kinase).

Subcellular fractionation was used to separate different cellular components on an Iodixanol density gradient and we observed that both SPARC and osteopontin were retained in a compartment that has characteristics of the Golgi apparatus. This was especially evident in the U0126 treated fractions. The SPARC antibody appeared to detect bands at the appropriate location on the membrane, however, the SPARC signal for the U0126 treated fraction in fraction 6 should be analysed with caution as it does not appear to show a band that might be typically observed in a western blot. It appears to show a 'mark' more representative of background staining. We could only detect SPARC once using the AON5031 antibody and this result has therefore been kept as a signal was detected at 43 kDa. Analysis of these bands will be made according to the observation on the western blot especially as the osteopontin antibody detects less total osteopontin in the untreated fraction 6 than it does in U0126 treated fraction 6.

GM130, a marker of the Cis-Golgi compartment was present in the same fraction as osteopontin and SPARC (fraction 6). ERp72 (ER marker) had a more wide distribution (fractions 7-9) whereas the lysosomal marker LAMP-1 was present in fraction 6 alone.

The results for ERp72 are not surprising because the endoplasmic reticulum is not simply a membrane surrounding the nucleus but extends outwards into the cytoplasm depending on how active the cells are. The ER occupies the majority of the cell's membrane component and strong staining of the ER was observed in ROS cells (chapter 3, section 3.3). It is therefore not surprising to see the ER marker ERp72 present in multiple fractions in the subcellular fractionation results. In fact, recent work has discovered that the ER has multiple contact sites (MCS) observed around mitochondria and endosomes, although whether these contact sites are functionally significant is not known (Phillips & Voeltz 2015). We did not observe any co-localisation of osteopontin or GFP-SPARC with the ER in immunofluorescence staining

(Concanavalin A) or ERp72, so although the presence of the ER marker does overlap with osteopontin and SPARC in fraction 6, this is most likely coincidental.

Similarly, we did not see any overlap between GFP-SPARC and osteopontin and a lysosomal marker in immunofluorescence staining, so we do not think retention is taking place here.

Centrifugation of the nuclear and cytoplasmic fractions took place before the gradients were loaded and only the post nuclear supernatant was loaded onto the gradient. Despite this, it is clear that subcellular fractionation cannot separate all cellular compartments perfectly especially because some organelles naturally have the same density hence they overlap on density gradients. We tested several gradients before deciding on the one described in the results section and did not find better resolution of the different fractions with similar yield. This technique is thus useful for separating compartments at a large scale to verify results obtained with immunofluorescence and to carry out further experiments such as immunoprecipitation to see if proteins interact specifically at certain sites. As we are unsure of the protein interaction that is causing the retention of osteopontin and GFP-SPARC, further techniques such as immunoprecipitation followed by mass spectroscopy would help identify which proteins SPARC and osteopontin are interacting with in fraction 6. The ERK kinases have over 200 substrates, so carrying out mass spectroscopy of fraction 6 would help tremendously as the range of interacting partners would be reduced significantly and it is likely that we could identify the interacting protein that is responsible for the forward trafficking of osteopontin and SPARC.

The density gradient that we used did successfully isolate the Golgi apparatus to one fraction, fraction 6. Immunofluorescence staining did show osteopontin to overlap (and merge in some cases) with the HPA marker of the Golgi apparatus. Furthermore, osteopontin and SPARC levels were much higher in the treated fraction compared to the untreated fraction. While osteopontin and SPARC levels appear to be higher after equalising protein concentration across fractions, there does still appear to be less GM130 in the untreated fraction. This result should be taken into account as it may be indicating less total protein content and therefore, SPARC and osteopontin levels may be higher in untreated fractions. However, as we consistently observed less SPARC and osteopontin across the two independent experiments, the potential significance of this difference should be considered.

Could it be the case that these matricellular proteins were packaged appropriately for secretion but do not receive a signal to continue from this point? Rab27, a marker for secretory vesicles, has been shown to co-localise with the Golgi apparatus (at the trans-Golgi network, the point at which secretory vesicles are released towards the plasma membrane) (Ostrowski et al. 2010).

In our experiments, Rab27 was present across fractions 6 and 7 in the untreated cells and 6-9 in the treated cells. The lack of bands in untreated fractions 8 and 9 may be due to antibody detection problems, but it may also be significant. It is not known if the secretion of SPARC and osteopontin are regulated or if they are constitutively secreted (Brion et al. 1992). Rab27 has been shown to be important for the trafficking of procollagen in osteoblasts. Microarray analysis following addition of ascorbic acid in MC3T3 cells showed that Rab27 expression was upregulated. Subsequent experimentation by immunofluorescence staining showed that ascorbic acid addition leads to co-localisation of procollagen and Rab27. Addition of a mutant Rab27 plasmid showed dispersed trafficking of procollagen with less presence at the plasma membrane. Subsequently, researchers found the total amount of procollagen secreted in cells transfected with a mutant form of Rab27 was lower than that of control cells (Nabavi et al. 2012). Together these results implicate Rab27 as an important GTPase in exocytosis.

Two models for protein secretion have been defined, some proteins such as casein are secreted constitutively (without storage) upon synthesis in lactating mammary epithelial cells (Turner et al. 1992) while other proteins such as insulin (a peptide hormone) are stored in intracellular compartments and released upon receiving a signal in response to an increase in blood glucose levels (7mM). These signals induce the exocytosis of insulin in β-cells in the pancreas (Fu et al. 2013).

Again, it is not known whether SPARC and osteopontin undergo regulated or constitutive secretion, but Rab27 has been shown to be a regulatory GTPase on secretory granules that undergo regulated secretion in melanocytes (Izumi et al. 2003). There is evidence that Rab27 containing vesicles are involved in both regulated and constitutive secretion (Fukuda 2013). For this reason it would be difficult to say how SPARC and osteopontin secretion takes place (whether regulated or constitutive). The absence of Rab27 in fractions 8 and 9 of the untreated cells could indicate that SPARC and osteopontin are being secreted normally. It

could be the case that Rab27 containing vesicles which should contain SPARC and osteopontin might have been dispersed further in the cell and are either present in lighter fractions, or too few to be detected by the Rab27 antibody in fraction 8 and 9 because a normal rate of trafficking is taking place. Presence of Rab27 in fractions 6-9 in the treated samples could implicate these GTPases in the retention that we are seeing if secretion is compromised. In the first fractionation experiment carried out, osteopontin was present in fraction 6 of the untreated cell fractions. U0126 treated cell fractions however, showed osteopontin presence in fractions 6, 7 and 8 indicating a shift in osteopontin localisation after inhibitor treatment. Alternatively, if SPARC and osteopontin undergo regulated secretion, the presence of osteopontin in fraction 7 and 8 may indicate that the MAPK pathway is required to signal the release of these matricellular proteins from holding compartments that contain Rab27 GTPases for secretion.

A review on membrane trafficking in osteoblasts and osteocytes does not detail the specific type of secretion that takes place for various proteins, but rather describes problems associated with defects along the trafficking pathways. Since the specific mechanisms of protein secretion in osteoblasts have not been elucidated, whether proteins undergo constitutive or regulated secretion, and which proteins undergo these types of secretion in osteoblasts remains unclear (Zhao 2012).

We did not have a marker for clathrin coated vesicles, nor did we have one for COPII coated vesicles, so it would be interesting to see if there was co-localisation of osteopontin and SPARC with clathrin or COPII where the retention was taking place, and if there was a difference between treated and untreated cells. Co-localisation of osteopontin and SPARC with Rab27 should be considered as well (the Rab27 antibody used did not support immunofluorescence staining).

COPI coated vesicles are responsible for transporting ER resident proteins from the Golgi to the ER. We found a signal for COPI in fraction 9, a fraction that did not correspond with SPARC or osteopontin presence. This indicates that there is no backwards trafficking of osteopontin or SPARC into the ER.

COPI coated vesicles have also been reported to shuttle between different compartments of the Golgi apparatus, but again, we do not consider this significant in the retention of osteopontin and SPARC (Popoff et al. 2011).

ERGIC-53 is a lectin (carbohydrate binding) protein that is present in the ER-Golgi intermediate compartment (ERGIC) where COPII coated vesicles fuse to form larger bodies that are bound for the Cis-face of the Golgi apparatus. A defect in this compartment (or ERGIC-53) can disrupt glycoprotein transport and so we asked if the retention might be taking place here (Hauri et al. 2000).

We did not see the presence of ERGIC-53 in fractions 6-9 in untreated or treated cells. In conclusion, it is highly unlikely that SPARC or osteopontin are retained in the ERGIC.

The subcellular fractionation successfully isolated osteopontin and SPARC to one fraction, however, markers of compartments like the Golgi apparatus, the endoplasmic reticulum and lysosomes were present in the same fraction. While this technique did not perfectly separate all compartments, conclusions are made in conjunction with results obtained using immunofluorescence staining in chapters 3 and 4.

Taken together, the results of the subcellular fractionation support our hypothesis that retention or accumulation of osteopontin and SPARC is taking place in the Golgi apparatus, especially in light of immunofluorescence staining which discounts the ER and compartments between the ER and Golgi (ERGIC) as the sites for accumulation.

^{35}S labelling of methionine confirmed that the accumulation or retention effect taking place inside the cell was correlating with reduced total protein secretion, truly implicating the ERK 1 and 2 module of the MAPK pathway in the forward trafficking of secretory proteins. While it would be interesting to specifically see the reduction of osteopontin and SPARC secretion, it is interesting to see the effect of ERK 1 and 2 on protein secretion (total reduction of 34 and 30% for cells treated with the U0126 inhibitor and cells treated with ERK 2 siRNA respectively), indicating a global effect on protein secretion not restricted to matricellular proteins (Murphy-Ullrich & Sage 2014; Frantz et al. 2010).

It is possible to isolate specific proteins using radioactive labelling of amino acids. A study looking at the trafficking of MHCII (an antigen presenting peptide) detailed the 'pulse chase' assay and immunoprecipitation to pull down MHCII as well as SDS-PAGE to carry out western blotting before using scintillation fluid to measure radioactively labelled MHCII specifically (Hou et al. 2013).

The study was limited to the transport of MHCII inside the cell, so cell lysates were used in the study. In theory we could have carried out the same technique, but we were interested in the secretion of osteopontin and SPARC so would have needed to use an antibody for immunoprecipitation as western blotting did not work for detecting SPARC and osteopontin in media. Therefore, the total protein content should suffice, and it was also promising to see that ERK 2 was likely involved in the secretion of other proteins separate from SPARC and osteopontin hinting at a conserved mechanism and involvement in membrane trafficking.

We expected that the knockdown of ERK 1 would maintain total protein secreted similar to that of the control cells. Surprisingly we observed some results that were completely different. We observed an increase of total protein secreted in some experiments compared to the control cells. This was surprising at first, however coming back to the apparent redundant roles of ERK 1 and 2 (and the fact that they can compensate for each other) this may not be so surprising (Buscà et al. 2015; Frémin et al. 2015; Yao et al. 2003).

ERK 1 and 2 compensate for each other with respect to activation of transcription factors, and the theory for why such an increase of total protein content was observed in some instances when ERK 1 was knocked down centres around this principle. If ERK 1 is knocked down, it may induce the activation of ERK 2 more than it is activated normally at basal levels (chapter 4, section 4.3, figure 4.11). This could be a compensatory mechanism employed to ensure that all of the work load (activation of transcription factors) can be handled by ERK 2 since the kinases cannot share this work when ERK 1 is knocked down (this theory should be irrespective of whether transcription factors are activated by ERK 1 or ERK 2 alone, or both). If ERK 2 activation is increased in response to downregulation of ERK 1, it may be the case that the extra ERK 2 activation also increases secretion of matricellular proteins, not

necessarily specifically, but as a side effect for the extra activation in order to compensate for activating transcription factors.

The instances when ERK 2 phosphorylation was unchanged when ERK 1 is knocked down could explain why protein secretion was very similar to the control cell in some cases when we treated cells with ERK 1 siRNA. This variation could explain some of the differences in total protein content secreted in the radioactive assay between higher values, and lower values.

Finally, we employed STRING analysis to look at protein-protein interactions to try and identify potential candidates for ERK 2 that might be causing a retention or accumulation of osteopontin and SPARC. There are limitations to this method, namely that not every single known interaction may be recorded in the search results, meaning that a potential target, or the substrate for ERK 2 that is causing the observed effect is completely missing in this software. However, it is useful to have the sequence of each protein available after search results were obtained because it meant we could rule out any candidates simply by looking for the 'FXFP' or D-domain motifs to see if substrates could bind to ERK in the first place.

I created networks of interactions based on areas of the cell in which an effect was observed, namely, the Golgi apparatus and tethering factors around the Golgi. Before looking at interacting partners that connect ERK with potential substrates linked to the Golgi or a tethering factor, we sought to find out if ERK could directly interact with factors such as GM130 or Uso1. These were not identified to be directly linked to ERK 1 or 2 on STRING, so it would be interesting to see if there was a possible mode of interaction in the first place. No 'FXFP' binding motif was identified on any of these proteins, however, Uso1 contained an 'FXSP' motif where the 'S' is a serine. A study carried out in 2013 showed that ERK could interact with weak affinity with a protein called Jacob (a protein involved in synaptic signals) that contained a similar motif (Karpova et al. 2013). However, it is unlikely that this is the interaction that we are interested in. ERK 2 could potentially bind to and activate Uso1, a coiled-coil tethering factor involved in ER-Golgi transport. Activation of Uso1 might be required to initiate tethering of vesicles with the Cis-Golgi (Grabski et al. 2012).

The weak interaction discovered between ERK and Jacob is highly unlikely to cause such a pronounced effect as we saw in the immunofluorescence staining images, as well as in the reduction in total protein content in the supernatant.

Additionally, it has been shown that Uso1 works with GM130, another tethering factor at the Cis-Golgi by binding to it in order to provide a membrane docking site for vesicles that will interact with Uso1. Mitotic phosphorylation of GM130 showed that binding with Uso1 diminished, suggesting that interaction between Uso1 and GM130 is dedicated to the ER-Golgi trafficking pathway (Nakamura et al. 1997).

The effects observed when MAPK is inhibited appear to be highly specific simply by the high level of accumulation or retention of SPARC and osteopontin. This is unlikely to be the result of a weak interaction. For this reason, it is unlikely that ERK 2 interacts with Uso1 to facilitate fusion of COPII coated vesicles, and that this mechanism of action may be separate to a requirement of activation by MAPK. Uso1 also did not contain a D-domain.

There is no known interaction between any of the MAPKs and GM130. GM130 is a tethering factor that facilitates fusion of vesicles with the Cis-face of the Golgi. GM130 contains 6 coiled-coil regions and causes interaction of vesicles with SNAREs, although the binding of Uso1 (p115 in humans) to GM130 is thought to be essential to facilitate fusion. The extent to which GM130 acts alone is not fully understood (Nakamura 2010). GM130 is phosphorylated at serine 25 (by CDC2) in order to induce fragmentation of the Golgi in response to mitotic signals. But there is no evidence that GM130 requires phosphorylation (nor is there any reported evidence the ERK kinases phosphorylate GM130) for facilitating docking and fusion of COPII derived vesicles (Cargnello & Roux 2011; Lowe et al. 1998).

GM130 contains a D-domain sequence. A search for GM130 in NCBI Blast showed a very highly conserved amino acid sequence across species from rat and mice to humans. Not only was the sequence of GM130 highly conserved, but the D-domain sequence 'SRYQELAVALDSSYV' is highly conserved across species with the addition of one amino acid in the human sequence (although the essential components of the D-domain remain intact). *Why would a specific domain found on substrates that bind to ERK be conserved on GM130?* The highly conserved nature of this domain likely means that ERK binding needs to be

retained as it serves a functional purpose, and has therefore been evolutionarily conserved. It may very well be the case that ERK interacts with (and activates) GM130 in order to facilitate the fusion of osteopontin and SPARC containing vesicles, and that a lack of phosphorylation might prevent the forward trafficking of these proteins and also cause the accumulation of SPARC and osteopontin in the region we observed.

However, one observation that has not been mentioned is that western blot analysis of SPARC and osteopontin showed the presence of these two proteins in their modified form (i.e. osteopontin was observed at 75kDa and SPARC at 43 kDa). This suggests that modification in the Golgi apparatus is taking place and that the retention observed may be taking place inside the Golgi apparatus or in a post-Golgi compartment.

A D-domain was observed also in COPII components, Sec23a (LRSSGVALSIAVGLL) and sec 24a but not 24b (RRIRVHTLCLPVVST, QRSSAKEIHLTPSTD and KAKLPLGLLLHPFKD). Again the sequence homology across species is very high as well as the conserved D-domain hinting at a conserved mechanism involving ERK and the Sec 23 and 24a isoforms. As mentioned in the discussion for chapter 3 (section 3.7), COPII coat phosphorylation by Hrr25p is important in preventing backwards trafficking into the ER ensuring fusion with the Cis-Golgi. It seems the conserved D-domain might be important for ERK binding and phosphorylation to serve other functions that may include binding to tethering factors.

We implicate GM130 and COPII coated vesicle components sec23 and 24a as potential ERK binding partners as they contain conserved D-domains and can therefore bind to ERK and also contain sites than can be phosphorylated by ERK. We remain sceptical however, as total protein analysis by western blot did not show smaller versions of SPARC and osteopontin. Trafficking from the ER-Golgi should present SPARC and osteopontin in an immature, un-modified form, but such bands (at smaller molecular weights) were not identified. It could be possible; however, that the antibodies against SPARC and osteopontin did not detect smaller un-modified forms of these two matricellular proteins. With respect to tethering and transport to the ER, GM130 and COPII coat components are good candidates for ERK binding and entry into the forward trafficking pathway.

Rab27a, a GTPase present on secretory vesicles was shown also to have a highly conserved D-domain across species (GRGQRIHLQLWDTAG). This was interesting as it suggests that not only does Rab27a act through effectors to initiate exocytosis, but phosphorylation of Rab27 may regulate exocytosis of secretory vesicles. There are limitations to STRING analysis as it is a text based analysis where interactions identified are primarily predicted interactions as well as known interactions scanned only from abstracts of papers and never the full text. In order to verify that Rab27 (or any other protein of interest) is phosphorylated, a western blot or immunofluorescence staining could be carried out using antibodies that specifically recognise the phosphorylated form of the protein. Mass spectroscopy could also identify proteins that are phosphorylated. In order to verify if a protein can be phosphorylated specifically by ERK 1 or 2, a co-immunoprecipitation could be carried out to see if the two proteins interact and if there is ERK specific phosphorylation. Alternatively, an in-vitro phosphorylation assay could be carried out to see if ERK can phosphorylate a substrate which can be further confirmed by mutating the target phosphorylation sites to alanine.

There is no evidence in the literature that Rab27 is phosphorylated for any purpose. It has been shown that Rab27 effector proteins (synaptotagmin like protein) can be phosphorylated by AKT to change cellular distribution which can affect Rab27 binding (Johnson et al. 2005).

Similarly, there may be a mechanism whereby ERK 2 phosphorylates Rab27 in order to induce or promote secretion of Rab27 containing vesicles, assuming SPARC and osteopontin are packaged in such vesicles before entrapment takes place. A lack of phosphorylation due to inhibition or knockdown of ERK 2 may prevent a crucial signal of Rab27 containing vesicles to continue along the secretory pathway in osteoblasts, and since it has been shown that Rab27 can co-localise in the trans-Golgi as well as cluster in a perinuclear region, this could explain the retention of osteopontin and SPARC we are seeing in this region.

Finally, we identified clathrin as another potential candidate as improper packaging of SPARC and osteopontin into clathrin coated vesicles (CCVs) could prevent SPARC and osteopontin secretion and thus cause the retention we are seeing. The clathrin heavy chain contained a sequence (RKGQVLSVCVEEENI) that was identified as a D-domain for ERK binding (other components of clathrin coat such as the light chain did not contain such a sequence).

Phosphorylation of β-Arrestin has been shown to be important for clathrin recruitment to clathrin coated pits (sites of CCV formation) as well as endocytosis. ERK 1 and 2 phosphorylate β-Arrestin (a protein important in regulating G protein-coupled receptor signalling) on serine 412. The phosphorylation of β-Arrestin inhibits its endocytosis and its de-phosphorylation enhances its ability to bind to clathrin and therefore its internalisation implicating the ERK kinases as negative regulators of endocytosis (Delom & Fessart 2011; Meng et al. 2009; Lin et al. 1999).

Although ERK 1 and 2 do not phosphorylate clathrin directly in this example, the presence of a D-domain on clathrin heavy chain means that MAPKs may function as a positive regulator of clathrin mediated transport by means of promoting clathrin vesicle formation and thus initiating SPARC and osteopontin exit from the trans-Golgi network.

Having identified potential binding sites for the ERK kinases, we filtered out interactions that might have led to the entrapment of SPARC and osteopontin when ERK 1 and 2 activation was inhibited. We add into the equation, possible intermediate proteins (as identified by STRING analysis) that MAPK could activate, which may then serve as chaperones to matricellular proteins, whether it be required for packing of proteins into vesicles, or factors required to keep the proteins moving along the pathway.

It remains to be seen whether MAPK interacts with proteins such as Rab27, clathrin and GM130, as well as look at the localisation of these proteins when MAPK is inhibited.

Wrapping up this chapter, I propose the following mechanism for what may be happening when ERK 1 and 2 activation is inhibited.

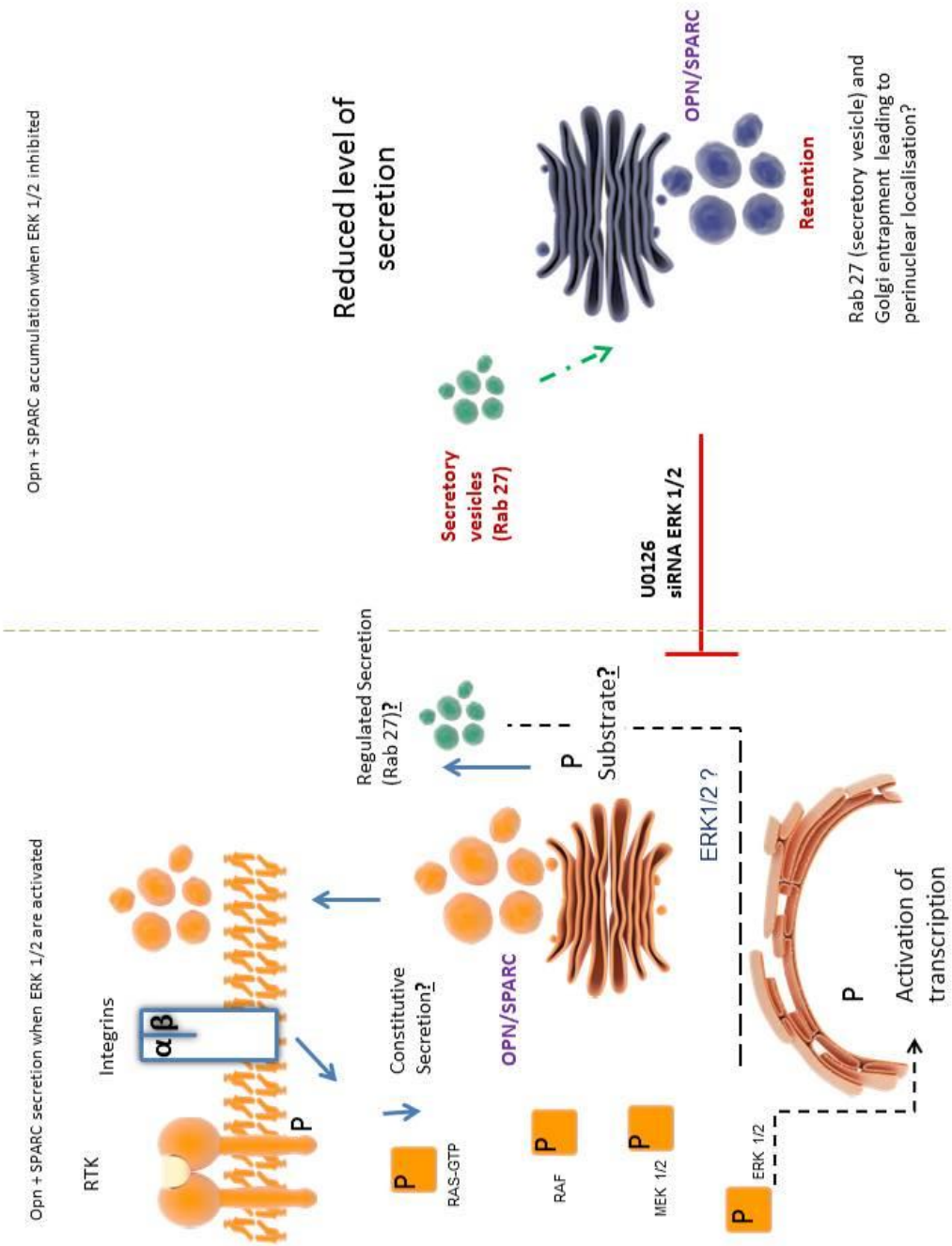


FIGURE 5.9, **Illustration of possible ERK interactions that could lead to retention of osteopontin and SPARC. Left,** An active ERK 1 and 2 kinase pathway leads to the activation of transcription factors but is also part of an early event whereby activation is likely activating a substrate/s that is allowing SPARC and osteopontin to continue along the secretory pathway. **Right**, **Inhibition** of the ERK 1 and 2 module of the MAPK pathway causes retention

of SPARC and osteopontin in a perinuclear region. The image shows different possible mechanisms by which ERK 2 may be causing the retention of SPARC and osteopontin including possible signals to secretory vesicles, improper packaging of clathrin coated vesicles at the trans-Golgi and a possible involvement in the fusion of COPII derived vesicle to the cis-Golgi.

6.0. GENERAL DISCUSSION

The extracellular matrix plays an important role in tissue homeostasis and is altered in diseases such as cancer. Expression and secretion of matricellular proteins such as SPARC and osteopontin have been shown to change in cancer and to affect the development of metastasis. We sought to elucidate the role of intracellular trafficking of these proteins in response to MAPK, a signalling pathway downstream of Ras activation which is frequently altered in cancer. ERK 1 and 2 have a vast array of cellular substrates localised to all cellular compartments and have been implicated in trafficking from the ER. We specifically concentrated on the changes in osteosarcoma and used a mature osteosarcoma derived cell line which constitutively expresses and secretes matricellular proteins, including SPARC and osteopontin.

We hypothesised that the MAPK pathway may be involved in the trafficking of matricellular proteins, specifically SPARC and osteopontin. To this end we monitored trafficking of GFP-SPARC and endogenous osteopontin through the secretory pathway in ROS cells by using fluorescence microscopy. Using siRNA knockdown of ERK 1 and 2 and inhibition of MEK 1 and 2 with the small molecule inhibitor U0126, the ERK MAPK pathway was effectively shut down with the latter, and partially with siRNA knockdown of ERK 1 or 2. There is no compelling evidence that clearly outlines individual roles for ERK 1 and 2. ERK 1 and 2 appear to be interchangeable, are co-expressed, are both phosphorylated by MEK and both contain similar substrate recognition sequences (Voisin et al. 2010).

For this reason, it would be hard to distinguish between the two kinases, and therefore we decided to knock both down using the inhibitor, and knock down ERK 1 or 2 using siRNA oligos specific for the respective target mRNA.

Our initial experiments were treating cells with the U0126 inhibitor for 30 minutes and 2 hours. U0126 treatments were effective within 30 minutes totally diminishing phosphorylated ERK bands in treated samples (western blot, chapter 3, section 3.2.1, figure 3.1).

Transfection of GFP-SPARC allowed us to see differences in localisation of GFP-SPARC containing vesicles after treatment with the U0126 inhibitor and retention of GFP-SPARC was observed in the perinuclear region. Treatments during a 6 hour time course (1, 2 and 6 hours)

showed bright green vesicles around the nucleus which appeared aggregated with very few GFP vesicles seen spread in the cytoplasm. This was unlike the untreated control cells where green punctate vesicles were more dispersed and seemed to traverse the Golgi complex on route to the plasma membrane. Unfortunately, due to problems with transfection efficiency we could not further investigate the localisation/entrapment of GFP-SPARC after treatment with U0126. Nevertheless, if the MAPK pathway is involved in vesicular trafficking of SPARC, *where precisely would this interaction take place and what effect would it have on the vesicle?* We repeated the 6 hour U0126 treatment and also looked at the localisation of endogenous osteopontin. We found the effect to be very similar to that observed with GFP-SPARC. This also gave us confidence in using GFP tagged SPARC as a model to look at trafficking. GFP tagged proteins appear to behave in a way similar to the native protein, thus making GFP tagged proteins a good model to study trafficking (Maloney et al. 2009); however, overproduction of GFP-SPARC might inadvertently lead to accumulation in multiple cellular compartments simply because the secretory pathway could be saturated by the overexpressed protein. GFP-SPARC is expressed under the CMV promoter, a promoter that is constitutively active and can yield high protein expression (Xia et al. 2006). However, we were unable to achieve a consistent rate of GFP-SPARC transfection. As far as I could search in the literature, there is no evidence of any involvement of MAPK on the CMV promoter, whether it be to repress or promote its function. Regardless of this, it appears that the trafficking of GFP-SPARC, even in the 6 hour sample where there is less transfection is still affected by the inhibitor. Another explanation for cells that do not express GFP-SPARC so well is that there may exist a mechanism by which there is negative feedback on the GFP-SPARC plasmid from endogenous SPARC expression. The result of this might cause less GFP-SPARC expression inside the cell due to repression of the promoter by promoter methylation. Alternatively, plasmid transfection in itself may be very low hence a lack of GFP-SPARC expression.

Having observed an apparent retention or accumulation of GFP-SPARC and endogenous osteopontin, we looked at the specific distribution of endogenous osteopontin in the organelles of the vesicular pathway. For these experiments, MAPK inhibition was achieved by using both siRNA and U0126. siRNA achieved over 90% knockdown efficiency (JetPrime transfection reagent) for ERK 2 and more than 80% efficiency of knockdown for ERK 1. These

experiments yielded an interesting result. We stained control and treated cells with a Golgi marker (HPA) and used antibodies against osteopontin. One point to note is that extracellular matrix or matricellular proteins might be packaged together in the same vesicles as part of a system that defines a specific destination. An observation was made in the 1980's where human growth hormone (hGH) and norepinephrine were found to be packaged and released together upon stimulation of Carbachol. This study, although very old, suggests that secretory vesicles may be packaged specifically with proteins going to the same destination, although very little work has been carried out on this topic since (Hwang 2008; Schweitzer & Kelly 1985). Work carried out on matrix vesicles (extracellular membrane vesicles) in rat growth plates (epiphyseal plate) showed that these vesicles contained the matricellular proteins SPARC and osteopontin, as well as other proteins such as VEGF and bone morphogenetic protein (BMP) (Nahar et al. 2008). This is a very interesting observation and adds weight to the hypothesis that proteins bound for similar destinations such as the extracellular space would be contained in the same vesicle. We are still faced with the problem of what MAPK does to regulate the secretion of osteopontin and SPARC and we do not know if this regulation might take place when SPARC and osteopontin are contained in matrix vesicles or if the regulation takes place further down the secretory pathway.

The specific and collective packaging of matricellular proteins has also not yet been shown as far as I can find. However, this is why we think that MAPK is causing the retention of both osteopontin and SPARC, but precisely how this occurs is unknown.

Immunofluorescence staining showed that unlike untreated cells, ERK 2 knockdown meant that the majority of vesicles were localised around the Golgi. Intense red staining differentiates the vesicle from the Golgi in the treated cell whereas a merge was seen in untreated cells. It is generally accepted that a merge of two fluorophores of different colours indicates an occupation of a similar space between two probes, hence the conclusion we made that an overlap with the Golgi indicated an apparent co-localisation. At this point we could not make a conclusion whether (while the effect was definitely observed) osteopontin was or was not co-localising with the Golgi apparatus when ERK 2 was inhibited (Dunn et al. 2011). While retention of osteopontin was observed after siRNA knockdown of ERK 2, immunofluorescence staining did not show co-localisation between the Golgi marker and

osteopontin. However, osteopontin accumulated in the perinuclear region. We did not carry out further analysis such as staining of specific components of the Golgi apparatus such as the trans or cis-Golgi as well as other cellular compartments such as endosomes or CCVs that would be localised around the Golgi. Furthermore, we did not check co-localisation of osteopontin with Rab GTPases, which can specifically accumulate in the perinuclear region for post-Golgi associated trafficking (Ostrowski et al. 2010). Additionally, due to equipment limitations, we could not carry out confocal microscopy to see if osteopontin and the Golgi apparatus were occupying the same space using stacking analysis. Stacking analysis allows for the cross section of the cell to be imaged from top to bottom and a 3 dimensional picture can be generated. If osteopontin and the Golgi overlap in the same stack, then co-localisation could be confirmed.

ERK 1 siRNA results showed a pattern in the cell that appeared similar to control cells. This observation led us to the conclusion that the effect was ERK 2 specific.

U0126 treatment showed a very similar accumulation or retention of osteopontin when cells were stained for the Golgi apparatus, confirming the likely localisation of osteopontin and SPARC when MAPK is inhibited. Osteopontin and GFP-SPARC did not co-localise with the ER markers (Concanavalin A and ERp72 over 6-72 hours of treatment), helping narrow down the place of entrapment. Osteopontin did not co-localise with a lysosomal marker either (LAMP-1), so the entrapment effect was unlikely to be taking place in lysosomal compartments.

Taken together these results point to a role for MAPK in the regulation of matricellular protein trafficking. It seems that MAPK is causing retention during the trafficking steps following ER exit but not post-Golgi although secretory vesicle markers Rab27, endo/exosomal compartments would localise in a perinuclear region and co-localisation of osteopontin and GFP-SPARC must be checked with these compartments (Harris et al. 2013; Xiao & Samulski 2012; Ostrowski et al. 2010).

Improper folding of osteopontin and SPARC in the ER would mean that these proteins would be tagged for ubiquitination. Improperly folded proteins will be sensed by various chaperones, and they will attempt to refold them if an initial fold fails. A failure to refold the protein will cause a chaperone protein to unfold the newly synthesised protein even more,

in order to ensure that the protein is ubiquitinated. The proteins are then translocated into the cytoplasm where they are picked up by the 26S proteasome and degraded into smaller peptide fragments. This pathway is known as Endoplasmic Reticulum Associated Degradation (ERAD) (Vembar & Brodsky 2008). This should be considered, because if MAPK was having an involvement in folding of the matricellular proteins (and causing their retention in the ER), the enhanced rate of ubiquitination and subsequent degradation would be detected in the total protein levels (as well as smaller bands on western blot films due to less post translational modifications [approximately 32 and 33 kDa for osteopontin and SPARC respectively]). For this reason we consider the retention of SPARC and osteopontin almost certainly not to take place in the ER. The unfolded protein response (UPR) is a process that ensures ER function is maintained correctly and should there be a scenario where there is improper folding, increase expression of proteins involved in protein folding to rectify such problems. Irreversible stress in the ER can also activate the UPR to induce apoptosis (Hetz 2012). The UPR is important in osteoblasts, chondrocytes and fibroblasts due to the fact that they are secreting fibrillar proteins. Mutations in genes associated with the UPR have been shown to affect collagen I secretion in osteoblasts. The retainment of collagen I in the ER leads to a severely osteopenic phenotype in mice (Boot-Handford & Briggs 2010; Wei et al. 2008).

There is no evidence that the ERK 1 and 2 module of the MAPK plays a role in any stage of the protein folding mechanism, so it would be hard to see how this could take place, nevertheless, since osteopontin and GFP-SPARC vesicles were localised in this region, it was an important consideration.

MAPK and p38 (another module of the MAPK pathway) can have indirect effects on membrane trafficking as demonstrated by Sorokina, 2011 and Blagoveshchenskaya, 2008 (introduction, section 1.7.3 and chapter 3, discussion, section 3.7 respectively). These papers demonstrate that in addition to coat proteins and tethering factors the lipid composition of the membranes plays an important role in forward trafficking which is regulated by MAPK and p38. In our work, it may be likely that we are seeing something similar. A protein chaperone which might be activated by ERK 2 could be essential for the shuttling of vesicles out of the Golgi (or tethering to the Golgi apparatus).

COPII coated vesicles can shuttle directly to the Golgi, or, they can become part of the ER-Golgi intermediate compartment (ERGIC). The ERGIC is the result of fusion of COPII coated vesicles bound for the Golgi. COPII coated vesicles lose their coat and fuse together creating a larger compartment. This observation supports the cisternal maturation model for the Golgi, which states that the Golgi cisternae is physically composed of vesicles containing cargo bound for the Golgi. A protein known as ERGIC-53, a mannose specific lectin acts as a receptor for glycoproteins, so that they may be incorporated into the ERGIC (Appenzeller-Herzog & Hauri 2006; Hauri et al. 2000). The method by which cargo passes through the Golgi apparatus has been debated for decades. Different theories exist for how cargo passes through the trafficking pathway, and two theories that are debated are the cisternal maturation model and the transport between stable compartments model. Cisternal maturation described above describes a model of transport through the Golgi apparatus where there is constant turnover of each compartment of the Golgi as COPII derived vesicles fuse to form the cis-Golgi, progressively becoming the Golgi cisternae and then the trans-Golgi by backward transport of enzymes via COPI coated vesicles. Evidence for this model has been demonstrated by live cell imaging in yeast, where it has been shown that the cis-Golgi compartment progressively becomes trans-Golgi. If the stable compartment theory were correct, no such changes to the compartment would be detected. The time course for this progression was 2 minutes and only unidirectional (forward) changes were observed (Luini 2011; Glick & Luini 2011; Matsuura-Tokita et al. 2006).

We did not observe the presence of ERGIC-53 in the fractionation experiments leading us to believe that there was no involvement of MAPK in the trafficking of COPII coated vesicles and their interaction with the intermediary compartment.

The U0126 washout experiment carried out was crucial in determining the specificity of ERK 1 and 2 inhibition on the trafficking of osteopontin. Not only did it tell us that the effect observed was specific to ERK inhibition, but we observed a rapid effect upon removing the U0126 inhibitor on the re-initiation of osteopontin secretion, demonstrating the speed of the effect. The model we used to test our hypothesis was a rat osteosarcoma cell line and while we were able to quantify total ERK as well as phosphorylated ERK levels, the extent to which ERK activation is regulated is not known. It could be possible that the signal is

sustained as is the case in many cancers harbouring a Ras mutation that keeps the ERK 1 and 2 module of the MAPK pathway turned on constantly allowing for continued cancer cell growth (Samatar & Poulikakos 2014). The characteristics of the ROS 17/2.8 cell line have not been fully elucidated. It is not known if this cell line harbours a Ras mutation (or what the mutation is that caused this cell line to become cancerous in the first place). Interestingly, a study on the activation of ERK 1 and 2 in the ROS cell line in 1998 showed that ERK 1 activation was higher than ERK 2, the opposite to the activation profile we have observed. This raises an interesting question as to the involvement of ERK 2 in the trafficking of SPARC and osteopontin. Cell lines are bound to change over time and the more they are passaged and longer they are maintained they can acquire different mutations. Where ERK 1 activation was higher in the ROS cells of this lab (Chaudhary & Avioli 2008), it would have been interesting to see how ERK 2 knockdown would have affected the trafficking of SPARC and osteopontin. If functional redundancy exists between ERK 1 and 2, and the explanation for why ERK 2 knockdown shows an effect on protein secretion is because this kinase has more of the workload in the cell line we used in our lab, then the higher rate of activation of ERK1 in (Chaudhary's lab) should have also compensated appropriately for the trafficking of osteopontin and SPARC. Hypothetically, if this experiment were carried out and it did not, it would truly point to an ERK 2 dependent mechanism (Chaudhary & Avioli 2008).

Overexpression of ERK 1 via plasmid delivery and lower ERK 2 levels by low concentrations of siRNA should be carried out to test this hypothesis, and this would help answer the question of specificity of ERK 2 versus a higher workload due to higher activation levels of this kinase.

A continued signal leading to persistent ERK activation may have a desired effect for the cancer cell, that is, one that not just maintains ERK activation for cancer cell proliferation, but one that maintains ERK activation to exploit ERK for its other activities (and we show trafficking to be a likely event influenced by ERK 2). We therefore implicate possible roles for cancer cells in utilising ERK activation to initiate/maintain secretion of proteins such as SPARC and osteopontin (in higher levels) that have been shown to be upregulated in many sarcomas as well as carcinomas. By the very nature of these two matricellular proteins, it is not surprising that their expression and secretion are upregulated as they carry out functions

essential to ECM maintenance and homeostasis that can help cancer cells metastasise (Delany 2010).

^{35}S labelling of ROS cells confirmed the observations made using immunofluorescence staining. The entrapment or accumulation of SPARC and osteopontin observed in the immunofluorescence experiments correlated with a reduction in protein secretion. While ^{35}S labelling measures total protein content secreted, it is likely that SPARC and osteopontin are part of this reduced secretion. The reduction in secretion of total protein applies to both U0126 treated cells and ERK 2 siRNA treated cells, with a similar reduction in both sets of treatments. This confirmed a role of the MAPK pathway in trafficking, and the specificity of the ERK 2 kinase in the effects observed as it could replicate the reduction in secretion seen with U0126 treatment.

The total protein secreted after ERK 1 knockdown by siRNA yielded a completely unexpected result with an increase in protein secretion recorded in some experiments. This increase could be a side effect of ERK 1 knockdown with an increase in phosphorylation of ERK 2 to compensate for the loss of ERK 1. Interestingly, the same cannot be said for ERK 1 phosphorylation when ERK 2 is knocked down and why this lack of compensation exists when it does apparently exist for ERK 1 knockdown is not clear. One possible explanation that should be considered is that ERK 1 acts as a limiting factor with respect to ERK 2 activation mediated by Ras (upstream of ERK 1 and 2). A study carried out in 2006 looking at the effects of ERK 1 and 2 activity in mouse fibroblasts (NIH3T3) found that ERK 1 could antagonize ERK 2 activity. Ectopic expression of ERK 1 and an oncogenic form of Ras could reduce cell growth in NIH3T3 cells and in vivo in nude mice (tumours induced by oncogenic Ras), with ERK 2 activation being reduced in ERK 1 overexpressing cells. Ectopic expression of ERK 2 did not affect tumour growth leading to the idea that ERK 1 was able to regulate ERK 2 signalling by an unknown mechanism (Vantaggiato et al. 2006).

With this in mind, it is possible that ERK 1 knockdown might lead to hyper activation of ERK 2 both as a compensatory mechanism but also due to a lack of ERK 1 regulation of ERK 2 phosphorylation if this sort of regulation also exists in osteoblasts. This could explain the increase in total protein secreted. This still does not explain why ERK 1 activation is not increased when ERK 2 is knocked down as we also observed a decrease in total cell number

when ERK 2 was knocked down using siRNA. While ERK 1 and 2 may be able to compensate for each other, it appears that either the cells have no contingency plan for ERK 2 knockdown, or that ERK 2 is affecting cell survival via a mechanism separate to the activation of transcription factors.

In ROS cells, ERK 2 is phosphorylated 1.8 times more than ERK 1. When ERK 2 is knocked down, the cell should be able to sense this loss and increase ERK 1 activation to compensate but doesn't. It may be the case that ERK 1 activation levels are sufficient for activation of transcription factors but not for the activation of cytoplasmic substrates, a deficiency possibly not sensed by the cell (or entirely specific to ERK 2), therefore affecting cell survival via deficiencies in cytoplasmic activities such as membrane trafficking.

The observation that the effects observed were ERK 2 specific was very interesting as it is a role that might be considered unorthodox for the MAPK proteins. Our work details cytoplasmic roles for the ERK kinases. While these roles are likely to be phosphorylations of target substrates to allow the trafficking process to take place, it is a role that is entirely separate to signalling in the traditional sense that the activation of downstream transcription factors leads to the production of proteins that help the cell survive. We observed an involvement (and a specific involvement for ERK 2 as oppose to ERK 1) in early cytoplasmic events (within 1 hour) in the forward trafficking of matricellular proteins in osteoblasts. This is a role most likely entirely separate from transcription of target genes as we demonstrated through western blot analysis (chapter 3, section 3.6, figures 3.19 and 3.25).

Considering that much of the evidence for the individual roles of ERK 1 and 2 point to a redundant system, we propose a theory that might explain the presence of two ERK kinases based on the observations we have made in this study, and a role that might give ERK 1 and 2 individual responsibilities within the cell. ERK 2 was able to cause the clustering and accumulation (as observed in fractionations) of SPARC and osteopontin and reduce the overall protein content secreted in osteoblasts. ERK 1 did not show the same effect and we think that redundancy likely exists within the ERK kinase system with respect to the activation of transcription factors. It is unlikely that functional redundancy exists with respect to the activities that ERK 1 and 2 carry out in the cytoplasm. The observation that forward trafficking is physically affected by ERK 2 and not ERK 1 implicates ERK 2 as a likely and

essential regulator of cytoplasmic activity in osteoblasts, with trafficking being an example of this. However, this must be shown to be the case in order for this theory to be plausible.

Studies looking at individual roles for ERK 1 and 2 in cell migration during zebrafish development showed that cell migration was affected when ERK 2 was knocked down but not when ERK 1 was knocked down (with very mild effects compared to ERK 2 knockdown) (Krens et al. 2008).

We used software to identify the presence of possible D-domains (ERK binding domains) in proteins that might be substrates for ERK 2. Using the 'Motif Scan 'feature from the Massachusetts Institute of Technology website, D-domain motifs were identified to be present on several proteins we searched for. Not only were these motifs present on the amino acid sequences we used for the rat species, Rattus Norvegicus, but the motifs were highly conserved amongst species. While these sequences are highly conserved, there were no apparent studies that I could find that implicate these proteins (such as Rab27 and GM130) and activity with the ERK kinases, hinting at a conserved unknown mechanism and interaction between ERK and these substrates. Having said this, they may or may not be linked to trafficking, but these proteins also do not activate transcription factors, again implicating ERK in activities separate from the activation of transcription. It is the collective evidence of conserved domains as well as the effects we see on trafficking when ERK 1 and 2 are inhibited that lead us to believe redundancy does not exist between ERK 1 and 2 in cytoplasmic activities carried out by the kinases.

Membrane trafficking is critical, and improper trafficking or retention can lead to disease. Therefore, a full understanding of the factors that affect trafficking is essential. In addition to this, our work revealed some interesting characteristics about how the ERK kinases function and raised questions as to what their full functional roles are within the cell. *What is the long term implication of our work?* Vesicular transport as a process is very well understood. The logical progression from the ER to a target membrane as well as endocytosis of cargo and subsequent recycling or degradation of this cargo has been observed and is well defined. However, the specific process, selection of cargo and factors that affect cargo transport are not well defined. Research implicating SAC1 as a mediator of vesicle transport shows that there are many more factors that determine when vesicles will begin to form and

travel (Blagoveshchenskaya et al. 2008). The MAPK pathway has been implicated in this process more than once, and while the evidence is not abundant, it still serves as a good candidate to contribute to the trafficking process and our studies so far agree with this.

We show a function for the ERK kinases that doesn't lead to the transcription of target genes. The field of cell signalling (and MAPK signalling) in particular has been saturated since it was known how important the function of the MAPK signalling pathways were to survival of a cell/organism, and arguably more so than other signalling pathways. Add in the fact that the ERK 1 and 2 module of the MAPK pathway are essential to the survival of a cancer cells and this has also become an attractive target for curing cancer. Unfortunately, it has been difficult to show precisely what the ERK kinases do, and the link of these kinases to over 200 substrates has made the task much more difficult. Much of the evidence points to a redundant system where ERK 1 and 2 co-exist and can compensate for each other where necessary. Work has revealed that there are individual roles for ERK 1 and 2 in some instances. For example, ERK 1 deficient mice appear to be healthy but are more susceptible to brain inflammation (Agrawal et al. 2006).

ERK 2 knockout in mice is lethal, and other papers discussed above describe how cell survival is affected giving ERK 2 a slight edge in the importance of the 2 kinases. This is extremely important; however, it is still not known exactly what ERK 1 or ERK 2 do in order to contribute to an effect, even in the case of ERK 1s protective effects against brain inflammation. In this respect, much more work is needed to really understand how these kinases specifically exert their effects, positive or negative.

We hope that our contribution to the field of signalling has helped understand how ERK 2 appears to carry out a physical role in the release of matricellular proteins from osteoblasts. We identified potential substrates for ERK 2. These substrates contain highly conserved domains that interact with ERK 2 and reveal yet unknown interactions (and functions) and in this respect, the role of the ERK kinases with respect to cytoplasmic activities is underestimated.

With respect to cancer, I believe that we have evidence to show that cancer cells utilise another mechanism in metastatic disease, that is, increased MAPK signalling to push proteins

such as SPARC and osteopontin out of the cell so that they can facilitate the invasion of a primary tumour. SPARC is implicated in metastasis (Tai & Tang 2008). SPARC plays a role in some of the major characteristics of metastasis. SPARC can induce the expression of MMPs which are capable of cleaving ECM components (Verma & Hansch 2007). Furthermore, SPARC itself can be cleaved. Cleavage of SPARC releases peptides containing lysine, glycine, histidine and lysine (HGHK) which are potent stimulators of angiogenesis. This is an essential process in metastasis, as tumours need blood vessels in order to survive and migrate to secondary sites. In vitro studies have shown that MMPs can cleave SPARC and release a peptide which can stimulate angiogenesis (Sage et al. 2003) but there isn't any evidence that links a protease specifically to SPARC in the invasion of cancer. In 2009, Podgorski et al, found that cathepsin K, a cysteine protease which cleaves collagen can also cleave other factors such as SPARC, and they found that SPARC upregulation in bone cancer was concurrent with cathepsin K expression, and cleavage of SPARC occurred. This recent evidence shows that SPARC may be a substrate for proteases in the ECM which can release peptides that facilitate tumour progression (Podgorski et al. 2009). The evidence that SPARC is upregulated is constantly being recognised in a large number of cancers. A clinical study of patients with oropharyngeal carcinoma showed that SPARC expression was found to be high in tumour samples and correlated with poor survival of the patients (Yoshida et al. 2015).

In addition, SPARC has been shown to contribute to the induction of epithelial mesenchymal transition (EMT), a process thought to be essential in metastasis. Tumours that migrate to secondary sites need to acquire a phenotype that allows easy manoeuvrability. Epithelial cells are polarised, form focal adhesions to the extracellular matrix and also form cell-cell junctions with adjacent cells. These characteristics have to be lost in order for cells to invade during metastasis. The biggest player in this transition is E-cadherin. E-cadherin forms cell-cell junctions, and the most notable characteristic of an epithelial cell becoming mesenchymal in phenotype is the downregulation of E-cadherin. Downregulation of E-cadherin is caused by the upregulation of another factor called SNAIL, a transcriptional repressor of E-cadherin (Thiery 2002).

SPARC has been shown to contribute to the downregulation of E-cadherin by inducing SNAIL expression but precisely how SPARC does this is not known (Hotchin et al. 2012; Robert et al. 2006).

With the evidence of SPARC inducing EMT, I would like to discuss very briefly the topic of circulating tumour cells (CTCs). CTCs are cells that have left a primary tumour and are circulating in the vascular system. 1-10 cancer cells can be found in about 10ml of blood in an average patient with 5 cells per 7.5ml considered a poorer prognostic marker (Yu et al. 2011).

However, the challenges we face in using CTCs as diagnostic and prognostic markers is the identification of CTCs that will successfully establish secondary sites, since not all CTCs will form secondary metastasis. Furthermore, while we can identify CTCs in the blood, *have tumours already been established at distant sites? Can a patient relapse as a result of CTCs post-treatment?*

The molecular characteristics of CTCs are important in answering these questions. Since cells undergo EMT, the molecular markers of the EMT are of interest in CTCs since it is thought that these cells would have had to undergo this process in order to metastasise. A study in breast cancer in mice showed that cells that were clustered could metastasise more effectively, and of all the circulating tumour cells, 50% of those that formed secondary tumours were found to be clustered. 3% of total CTCs were clustered, implicating the clustering of tumours in circulation as an important contributing factor to cells that can survive the passage through the blood stream (Bottos & Hynes 2014).

Increasingly recognised in the molecular profile of CTCs are the markers of EMT. It has been shown that more EMT markers exist in CTCs of late stage breast, prostate, lung and head and neck tumours, among other cancers. Markers of EMT include down regulation of E-cadherin and upregulation of proteins such as fibronectin and N-cadherin, which could explain and account for clustering of some tumour cells. It implicates also EMT as an important process in metastasis, and a maintained mesenchymal phenotype appears to be essential until mesenchymal epithelial transition (MET) can take place at a metastatic site (Mitra et al. 2015).

Consistent with markers of EMT are the expression of the matricellular proteins. SPARC has been found to be highly expressed in 100% of pancreatic cancer CTCs (in a mouse model of pancreatic ductal adenocarcinoma). It could be the case that SPARC is important for inducing metastasis through its roles in cell migration, detachment and inducing EMT, but also maintaining the integrity of cells that have acquired a mesenchymal phenotype while they circulate as CTCs (Ting et al. 2014).

Osteopontin may be important in this process as well. The upregulation of osteopontin has also been implicated in metastatic disease due to its role in the extracellular environment. A study in 2011 looking at non-small cell lung carcinoma (NSCLC) found that elevated levels of osteopontin were present in the plasma of patients after they had surgery for their primary tumour. No detectable metastasis had been seen pre-operation via various medical imaging techniques such as magnetic resonance imaging (MRI), ultrasound and bone scintigraphy. Tumour tissue samples (from the lung) showed that there was an elevated level of osteopontin (mRNA levels) in cancer patients versus control biopsies, and circulating osteopontin was detected to be higher in patients that had metastasis. The mRNA expression profile showed that in the lung tissue (cancer or control), 64.5% of lung cancer tissues had elevated levels of osteopontin, while 27.9% of control cells had elevated osteopontin mRNA, indicating a potential role for the use of osteopontin in facilitating metastatic disease. The fact that elevated osteopontin levels were found to be circulating in the tumour means that their upregulation must correlate with an invasive tumour showing its role in facilitating this process. It would have been interesting to know if there were elevated levels of SPARC in circulation also (Liang et al. 2011).

With regards to metastatic disease, understanding the mechanisms by which SPARC and osteopontin are transported out of the cell, we may be able to find a potential therapeutic target that might prevent these matricellular proteins from reaching the extracellular space. By doing this, tumours characterised by an increase in SPARC and osteopontin levels may struggle to invade their primary surrounding (and possibly distal sites in CTCs if their expression is important to maintain CTC integrity), and we are left having to deal with one tumour site to treat. Over 90% of cancer deaths are caused by metastatic disease. This makes metastasis an attractive target for treatment in order to reduce the amount of deaths that

occur from cancer. With SPARC and osteopontin being increasingly recognised as markers for poor prognosis where they are upregulated in cancer, it has become an attractive target for treatment of aggressive tumours, and we may have discovered an essential regulatory mechanism (via the ERK 2 kinase) whose substrate/s could be targeted in order to reduce the release of matricellular proteins in cancers and thus possibly reduce the potential for a tumour to invade.

These CTCs may secrete SPARC and osteopontin via exosomes. Exosomes are small lipid vesicles that range from 40-100nm in diameter. Unlike clathrin coated vesicles, exosomes form from multivesicular bodies (endosomal compartments) or directly from the plasma membrane. Exosome release normally transfers material to neighbouring cells or directly into the blood for circulation (Mulcahy et al. 2014; Li et al. 2014).

It is not fully understood how exosomes select cargo, but proteins involved in selection of cargo into endosomes may also be mediating sorting into exosomes (Stoorvogel 2015).

As I have discussed, it is not fully known how SPARC and osteopontin are secreted into the extracellular space, however, these proteins may be internalised into exosomes in metastatic cells where their release might be important for establishing the metastatic niche at a secondary site. Exosomes do not only contain protein cargo but have also been shown to carry RNA strands as well including functional miRNAs. In order to accompany the release of proteins such as SPARC and osteopontin which might be important for metastatic cell survival/entry into the secondary site, functional miRNAs may be important in silencing genes that could potentially prevent this invasion (Mulcahy et al. 2014).

Exosomes that contain integrins have been shown to be important for establishment of secondary tumour sites at specific organs. Integrin α6β1 and α6β4 have been shown to be important for lung metastasis while integrin αvβ5 has been linked with liver metastasis. The combination of specific integrins in exosomes can therefore be used to predict where metastatic cells might go, but also, this presents potential therapeutic targets (Hoshino et al. 2015).

There is no published work that shows proteins such as SPARC and osteopontin in exosomes. However, their involvement in the initiation of metastatic disease as well as their importance

(in the case of SPARC) in preparing the metastatic niche for metastatic tumour cells may very well suggest that these proteins might be present in exosomes. Timing and regulation of the secretion of SPARC and osteopontin containing exosomes at the appropriate moments, i.e., at the secondary sites may be an important factor for the successful establishment of a secondary tumour, and in this respect, the activation of the MAPK pathway may also be essential.

6.1. LIMITATIONS OF THE PROJECT

While this project produced promising data, there were limitations to the work. Unfortunately, the biggest limitation was the lack of a normal control. We did not have a 'normal' (even if immortalised) model of rat osteoblasts. The ROS cell line has not been fully characterised, and papers speak of specific expression/signalling patterns in the same way we characterised ERK activation but there is nothing more extensive than this. We also do not know specifically, the anatomical site from which the ROS cell line is derived. It would have been useful to know the activation of ERK in a 'normal' cell line (or primary osteoblast cells from the rat model), how this differs to ERK activation in the ROS cell line (if it differs at all) and *does this difference or similarity reflect in the trafficking of matricellular proteins, namely osteopontin and SPARC? Is ERK 2 involved in the anterograde trafficking pathway in the same way it is in the ROS cell line? If yes, is the effect as pronounced as it is in this osteosarcoma model? Does the extent of ERK activation dictate the level of matricellular protein secretion?* We would want to know how decreased levels of kinase activity also affect trafficking in the ROS cell line.

These are questions that must be answered. It may have been useful to reduce the concentration of the U0126 inhibitor (via a titration to determine levels of MEK inhibition) in order to look at the effects of untreated control cells versus treated cells that have less and less activation up to the point of total MEK inhibition. We tested our hypothesis using the latter, where we looked at untreated control cells versus cells that had complete inhibition of ERK activation at different time points.

We did have a pre-osteoblastic mouse fibroblast cell line derived from the skull cap of mice (MC3T3). Unfortunately our anti-osteopontin antibody did not work for immunofluorescence staining and we had non-specific binding with our secondary antibodies, giving us false positive results, that is, any staining did not necessarily represent osteopontin containing vesicles. Although this is a mouse model of osteoblasts, this lack of specific binding was unfortunate. Although we could not directly compare results from the MC3T3s to the ROS cell line, it would have been nice to know if inhibition of MEK also resulted in a retention of osteopontin and SPARC, and if this also meant there was less secretion out of the cell via ^{35}S labelling. This would help determine (although not conclusively) whether the effects observed in the ROS cell line are a mechanism utilised by the ERK 2 kinases in other cell lines, truly demonstrating a new role for the ERK 2 kinases in a cellular function previously not fully understood. It goes without saying that the trafficking of SPARC and osteopontin in late stage carcinomas should be investigated as well. *Does inhibition of ERK 2 produce the same result? If so, is there less invasion by that tumour?* This would also help determine the extent of SPARC and osteopontin involvement in the metastatic process.

Another limitation to the project was the absence of some essential equipment. We are convinced that we have solid data that tells us the likely accumulation of SPARC and osteopontin when MAPK is inhibited. It may be in or just prior to fusion with the Golgi apparatus. Fractionation and immunofluorescence made this observation likely. However, in order to truly understand the extent of co-localisation (or lack of) of osteopontin/SPARC with the Golgi apparatus, confocal and electron microscopy would be required. At the time of writing this has remained a limitation, however, much of the focus is now on obtaining data via confocal microscopy to confirm where specifically SPARC and osteopontin lie in relation to the Golgi apparatus. This is of particular interest with the treated cells as we can see further into where the entrapment is taking place, helping us narrow down the possible substrates for ERK 2 that could be causing this entrapment.

6.2. FUTURE PERSPECTIVES

There is a lot of potential in the role of MAPKs and cell signalling as we showed in our study. This study contributed to otherwise unknown functions for the MAPK pathway and we hope

that this work will be used to springboard the research of MAPK in the role of early events and how these contribute/to what extent they contribute to biological functions versus the canonical role of MAPK signalling in activating transcription factors.

Further work needs to be carried out in order to further confirm our study as well as consider the translational potential of our project (i.e. what practical use could come from this in terms of treatment of diseases such as cancer, or where SPARC might be utilised to facilitate too much collagen deposition as might be the case in fibrosis).

To what extent is ERK 2 utilised for the secretion of matricellular proteins in osteo/fibroblasts and epithelial cells in cancer? This is the next challenge. As mentioned in limitations, the trafficking of SPARC and osteopontin needs to be studied in different cell types, again to look at the extent or universality of ERK 2 in trafficking. If it turns out to be the case that MAPK is important in early cytoplasmic events, and substrates are identified, then new targets have been found for therapy and the efficacy of treatment of disease can be explored further.

With respect to the molecular events that take place in the trafficking influenced by ERK 2, more work needs to be carried out. Another interesting avenue of research is the mode of activation of the substrate that causes the forward trafficking of SPARC and osteopontin. Dimerisation of ERK 1 and 2 have been shown to be important, not just for their entry into the nucleus, but for the activation of their target substrates. *Could it be the case that dimerisation of ERK 2 is required before activating its substrate to help SPARC and osteopontin through the secretory pathway? Are scaffolding proteins involved?* Scaffolding proteins bring together the components of the MAPK pathways to allow for substrate activation (i.e. Ras-Raf-MEK-ERK). Different scaffolds exist at different sites in the cell and it is MAPK signalling at these sites that confer specificity to target substrate activation for different biological activities. *What scaffolds are required for ERK substrate activation in the cytoplasm for forward trafficking? Are these substrates recruited to these scaffolds for activation by ERK 2 at certain times of expression of matricellular proteins?*

These are essential investigations that need to be carried out in the future. By answering these questions, we will further understand the complexities of MAPK signalling. We are not sure also if the effects we observed are limited to ERK 2. Since 7 modules of the MAPK

pathway exist, it would be interesting if other MAPKs can produce similar effects. It may be the case that different MAPKs can activate substrates involved in the trafficking pathway, but that their activation might be dependent on a cell that has undergone stress as might be the case with p38, JNK and the other ERK kinases as they are primarily involved in the stress response. Maybe the activation of target substrates is dependent not on a specific kinase but the state of a cell. This could apply to other pathways such as the TGF-β SMAD pathway as well as the PI3-AKT-mTOR axis. *Is there lateral communication?* At this point we do not think that any of the kinase burden is shifted towards kinases in other signalling pathways as we observe the effect with fully activated phospho-s6 kinase as well as full TGF-β activation. Nevertheless, it should be considered, and knockdown of essential kinases must be carried out in order to verify there is no involvement in the secretion of SPARC or osteopontin, or that they do not produce a different phenotype in the secretory vesicle pattern. Their inhibition might result in changes further downstream of the trafficking pathway.

While we showed that overall protein secretion was reduced when ERK 2 activation was halted, it would be useful to know specifically how much SPARC and osteopontin secretion is reduced, as well as other proteins that are affected by ERK 2 inhibition whether they are matri- or extracellular proteins. This will help understand the mechanisms that dictate secretion of specific proteins. We attempted to run a western blot by concentrating the secreted content in the media of ROS cells, but were unable to observe osteopontin or SPARC in the samples representing the secreted content. For this reason we carried out the ^{35}S labelling as we did not think another technique such as an ELISA would be as sensitive as the western blot, however, it is worth trying an ELISA to look at protein secreted to see if any binding of supernatant SPARC or osteopontin might bind to antibodies on an ELISA plate. Alternatively, a very sensitive assay known as radioimmunoassay (RIA) could be employed. This is a technique that uses radioactively labelled SPARC/osteopontin which is then presented to the appropriate antibody for binding. A sample of the media would be added to this mixture of radioactively labelled SPARC/osteopontin bound to antibody. The non-radioactively labelled SPARC/osteopontin contained in the media will compete for binding of the antibody to which the radioactively labelled protein is bound. This competition would displace the radioactive protein and a measure of the remaining radioactive protein-

antibody bound would be taken. This is a very sensitive technique and can measure very low quantities of protein present.

Finally, whilst we did not notice any decrease in the expression of SPARC and osteopontin over a prolonged time course, a Real-Time PCR should be run alongside this time course, to verify that mRNA transcripts also are not affected by inhibition of ERK 1 and 2 activation.

We identified various proteins that contained conserved ERK binding sequences. We would seek to further isolate a substrate for ERK that was specifically causing the effect that we were seeing. However, we would also encourage work on the interaction of the ERK kinases with these substrates to identify what such interactions result in with respect to biological functions, as the presence of these domains represent as yet unknown molecular interactions.

What might result in targeting the substrate that causes the forward trafficking of SPARC and osteopontin? Once the questions posed above have been answered, the next step would be to target a substrate, prevent the secretion of these matricellular proteins in cancer cells and carry out techniques such as invasion assays to see if these cancer cells that express SPARC and osteopontin result in less of a metastatic potential. Side by side, the expression of proteins such as E-Cadherin, N-cadherin, fibronectin, SNAIL and other markers of the EMT should be looked at to see if the invasion potential is physically prevented because SPARC and osteopontin cannot induce or contribute to a greater extent to such phenotypes in cancer cells that might have the potential to invade. As SPARC has been shown to induce cell rounding and migration, *does less SPARC secretion result in more stabilised and rigid actin networks? Does the intracellular distribution of focal adhesion proteins such as vimentin change? Is the expression of MMPs affected?*

These are all essential questions that need to be answered, as these factors could change when SPARC and osteopontin secretion is reduced and thus prevent metastasis. We therefore identify a new method by which the MAPK pathway may be utilised by cancer cells in order to induce the metastatic process and it is important to find out specifically what ERK 2 is doing in osteoblasts to cause the forward trafficking of these matricellular proteins as they might present an attractive target for cancer treatment.

7.0. References

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“I have yet to see any problem, however complicated, which, when you looked at it in the right way, did not become still more complicated.”

-Poul William Anderson