The role of Tribbles 1 and Tribbles 3 in cartilage turnover

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Abstract

Arthritis is a term which encompasses a number of diseases characterised by cartilage degradation and joint destruction which represents an enormous and growing healthcare burden.

Matrix metalloproteinases (MMPs) are a family of enzymes involved in cleavage of extracellular matrix proteins. They have many roles in both development and normal tissue homeostasis. As well as this they have been shown to be important in a number of diseases, including arthritis. MMP-1 and -13 in particular have been shown to be important in arthritis, due to their ability to cleave type II collagen, a key component of cartilage. A greater understanding of the regulation of these MMPs could lead to the potential for new therapeutic arthritis treatments.

Tribbles (Trb) 1-3 are a group of proteins linked with diseases including diabetes, multiple sclerosis and cancer. Trb 1-3 are reported to play a role in regulating many cellular signalling pathways, such as mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase/Akt (PI3K/Akt) and nuclear factor kappa B (NFκB). These pathways are considered important in mediating gene expression changes, including MMPs.

Both Trb1 and Trb3 were shown to regulate MMPs in chondrocytes, with a greater effect being on MMP-13 regulation. Trb1 and Trb3 were both shown to regulate the major MMP transcription factor AP-1, as well as the ATF3 and NFκB transcription factors. Both Trb1 and Trb3 interacted with MAP2Ks MEK1, MKK4, MKK6 and MKK7, and in addition were shown to regulate MAPK activation, with Trb3 protein levels appearing to be affected by MAP2K levels. Trb3 also had the ability to affect both Akt and STAT activation.

These data demonstrate that Trb1 and Trb3 can regulate signalling pathways that have the ability to alter MMP expression and transcription factors within chondrocytes. This would suggest that Trb1 and Trb3 have the ability to affect cartilage degradation. This greater understanding of MMP regulation by Trb1 and Trb3 may help in the development of potential future therapeutic targets for arthritic disease.

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Declaration

This thesis is based on research performed in the Musculoskeletal Research Group (Rheumatology), Institute of Cellular Medicine, University of Newcastle, Newcastle upon Tyne, UK. Except for commonly held concepts, and where specific reference is made to other work, the content of the thesis is original. No part of this thesis has been submitted for the award of any other degree.

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Abbreviations

°C	degree Celsius
µg	microgram
µl	microlitre
ACPA	anti-citrullinated peptides antibody
ADAM	a disintegrin and a metalloproteinase
ADAMTS	a disintegrin and a metalloproteinase with thrombospondin motifs
ALK	activator receptor like kinase
AML	acute myeloid leukaemia
AP-1	activator protein-1
APS	ammonium peroxodisulphate
ATCC	American Type Culture Collection
ATF	activating transcription factor
bp	base pairs
Bcl	B-cell lymphoma
BSA	bovine serum albumin
CBP	CREB binding protein
cdc	cell division cycle
cDNA	complementary DNA
CEB	cytoplasmic extraction buffer
C/EBP	cytidine-cytidine-adenosine-adenosine-thymidine enhancer-binding protein
CER	cytoplasmic extraction reagent
CHOP	CCAAT/-enhancer binding protein homologous protein
CIA	collagen-induced arthritis
CIS	cytokine inducible SH2 protein
COP	constitutive photomorphogenic protein

CTLA	cytotoxic T lymphocyte antigen
dH_2O	distilled H_2O
DMARD	disease modifying anti-rheumatic drug
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
DUSP	dual specificity phosphatase
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular regulated kinase
Ets	E26 transformation specific sequence
Ezh	enhancer of zeste homolog
FCS	foetal calf serum
FGF	fibroblast growth factor
Fra	fos related antigen
g	relative centrifugal force
GAG	glycosaminoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HAC	Human Articular Chondrocyte
HEK	human embryonic kidney
HeLa	Henrietta Lack
HIF	hypoxia inducible factor
HLA-DR	human leukocyte antigen
HRP	horse-radish peroxidase
Htra	high temperature requirement A

Ig	immunoglobulin
IGF	insulin-like growth factor
Ihh	indian hedgehog
IκB	inhibitor of κ B
IKK	inhibitor of IκB kinase
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist
IL-1RAP	interleukin-1 receptor agonist accessory protein
IL-4R	interleukin-4 receptor
IRAK	interleukin-1 receptor associated kinase
JAK	janus kinase
JNK	c-Jun N-terminal kinase
kb	kilobase
kDa	kilodalton
LB	luria broth
LIFR	leukaemia inhibitory factor receptor
MAPK	mitogen-activated protein kinase
MAP2K	mitogen-activated protein kinase kinase
MAP3K	mitogen-activated protein kinase kinase kinase
MEB	membrane extraction buffer
mg	microgram
MMP	matrix metalloproteinase
mRNA	messenger RNA
miRNA	micro RNA
MMLV	Molony murine leukaemia virus
MT-MMP	membrane type MMP
MW	molecular weight
MYD	myeloid differentiation primary response protein

NEB	nuclear extraction buffer
NER	nuclear extraction reagent
NFκB	nuclear factor κB
NSAID	non-steroidal anti-inflammatory drug
OA	osteoarthritis
OSM	oncostatin M
OSMR-β	OSM receptor-β
PACE	paired basic amino acid cleaving enzyme
PAGE	polyacrylamide gel electrophoresis
PAR	protease activated receptor
PBS	phosphate buffered saline
PCA	protein complementation assay
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PEA	polyoma enhancer activator
PEB	cytoskeletal extraction buffer
PIAS	protein inhibitor of activated STATs
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphoinositol-4,5-biphosphate
PIP3	phosphoinositol-3,4,5-triphosphate
PTPN	protein tyrosine phosphatase non-receptor type
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor kappa B ligand
RF	rheumatoid factor
RFP	red fluorescence protein
RNA	ribonucleic acid
RNase	ribonuclease
RPTK	receptor protein tyrosine kinase

RT-PCR	reverse transcriptase PCR
RUNX	runt-related transcription factor
SDS	sodium dodecyl sulphate
SFM	serum-free medium
siRNA	small interfering RNA
SOC	Super Optimal Broth with Enhanced Catabolite Repression
SOCS	silencers of cytokine signalling
SOX	Sry high mobility group box
SHP	SH2 domain containing protein tyrosine phosphatase
ShRNA	short hairpin RNA
SIAH	seven in absentia homolog
STAT	signal transducer and activator of transcription
TEMED	N,N,N'N'-tetramethylethylene-diamine
TGF-β	transforming growth factor-β
TIMP	tissue inhibitor of metalloproteinases
TIP	tat interactive protein
TIR	toll/IL-1 receptor
TNF	tumour necrosis factor
TRAF	TNF receptor associated factor
Trb	Tribble
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	t-Octylphenoxypolyethoxyethanol
Tween 20	polyoxyethylene sorbitan monolaurate
TYK	tyrosine kinase
UTR	untranslated region
v/v	volume/volume
w/v	weight/volume

Chapter 1: Introduction

1.1 Articular cartilage

1.1.1 Structure and function

Articular cartilage is a type of hyaline cartilage, whose primary function is to cover the ends of bones to provide low friction movement between bones within the joint (Ateshian and Wang, 1995). Articular cartilage is both avascular and aneural (Buckwalter and Mankin, 1997), and contains a single cell type known as the chondrocyte. This cartilage has a high water content (70-80%) in order to withstand the compressive strains imposed on it (Wong and Carter, 2003). Cartilage resists large changes in shape when loaded, and this is partly due to the cartilage matrix having a low permeability (Mow *et al*, 1984).

Cartilage can be divided into 4 zones consisting of the superficial, transitional, middle and calcified zones, with each zone differing in terms of its matrix and biochemistry. Each of these 4 zones consists of 3 regions; the pericellular, the territorial and the interterritorial regions (Temenoff and Mikos, 2000). Each of these 3 regions consists of different matrix structure and components, with the interterritorial region being responsible for the mechanical properties of the cartilage, consisting of large diameter, parallel collagen fibrils (Buckwalter and Mankin, 1998). The superficial zone (zone1) is the thinnest of the 4 zones and consists of 2 layers; the top layer is acellular and consists of mainly collagen fibres, which cover the joint. The second layer consists of chondrocytes which are small and flattened lying parallel to the articular surface (Temenoff and Mikos, 2000; Goldring and Marcu, 2009). This layer has a high tensile strength due to the ratio of collagen to proteoglycans, as well as it containing fibronectin and water. The transitional zone (zone 2) is thicker than the superficial zone, and contains chondrocytes which are spherical, with the collagen fibrils of the interterritorial region being randomly arranged. The middle zone (zone 3) consists of the largest diameter collagen fibrils, and has the highest

proteoglycan content of the 4 zones. The middle zone chondrocytes are also spherical. The interterritorial fibres in this region are arranged perpendicular to the surface (Temenoff and Mikos, 2000). The calcified zone (zone 4) lies closest to the bone, providing the link between cartilage and bone. Due to this, this zone is exposed to high levels of shear stress (Cohen *et al*, 1998). The chondrocytes in this zone are smaller than the other zones, and as opposed to the cells of the middle zone which have high metabolic activity, these cells are thought to have low metabolic activity (Temenoff and Mikos, 2000).

Surrounding this articular cartilage is the synovial membrane which has a boundary layer consisting of lubricin and hyaluronic acid produced by chondrocytes and synovial cells. This layer provides lubrication which allows low friction gliding movement between the joints (Greene *et al*, 2011).

Beneath the articular cartilage lies the subchondral bone, and it is thought that this plays a role in providing support for the articular cartilage (Radin and Rose, 1986). Defects within the subchondral bone are a common feature of arthritis. This subchondral bone layer can be separated into two layers; a layer of mineralised cartilage, which lies next to the articular cartilage, and this can vary in thickness. Below this is woven or lamellar bone which is similar to normal trabecular bone (Madry *et al*, 2010). Unlike the articular cartilage, the subchondral bone is highly vascularised, and this has been shown to increase with greater loading (Lane *et al*, 1977). It is thought that one of the causes of cartilage degradation and its progression may be an increase in stiffness of this subchondral bone (Radin and Rose, 1986).

1.1.2 Chondrocyte

Chondrocytes are fairly low in number within cartilage, consisting of only approximately 2-5% of the cartilage (Goldring, 2006). Their role is to produce the cartilaginous extracellular matrix (ECM) which surrounds them (Temenoff and Mikos, 2000). However, once this ECM is formed there is low turnover by the chondrocytes, with collagen type II having a turnover of more than 100 years, and aggrecan having a turnover of 3-24 years (Goldring, 2006). This slow turnover is due to the fact that in normal cartilage these chondrocytes are

relatively quiescent (Loeser *et al*, 2012). Some chondrocytes have cilia protruding from them into the cartilage, this is thought to allow chondrocytes to detect changes within the environment due to loading of the cartilage. This in turn can cause the chondrocyte to modify its behaviour such as the production of matrix and growth factor expression (Wong and Carter, 2003; Buckwalter and Mankin 1998). The oxygen tension within the cartilage is low ranging from 10% at the surface to less than 1% in the deep zones; the chondrocytes have adapted to be able to work metabolically within this environment (Goldring, 2006).

1.1.3 Extracellular matrix (ECM)

1.1.3.1 Collagens

Collagens have been shown to be important in all connective tissue types. There are 26 known collagens (Sato *et al*, 2002), all of which are composed of trimeric triple helices, consisting of repetitions of Gly-X-Y (Gelse *et al*, 2003).The triple helix can either be composed of 3 identical chains, or 2 or more different chains (Gelse *et al*, 2003). Articular cartilage is composed of a number of different types of collagen, these are types II, VI, IX, X and XI, with the major one being type II, with type IX and XI also being important (Chen and Broom, 1998).Type II makes up approximately 90-95% of the collagen (Temenoff and Mikos, 2000). As mentioned above, the structure and composition of the collagen network varies throughout the depth of the cartilage, and compared with most connective tissues, the collagen network appears fairly randomly arranged (Chen and Broom, 1998).

Type II collagen provides the cartilage with tensile strength by forming a mesh-like structure made of collagen fibrils (Temenoff and Mikos, 2000). Type II collagen is composed of 3 identical chains within its triple helix, and consists of long triple helical domains approximately 300nm in length (Gelse *et al*, 2003). There are 2 splice variants of collagen type II, which are present in either mature cartilage or in embryonic cartilage (Sandell *et al*, 1991). Collagen IX

interacts with type II collagen fibrils, this is particularly evident around the chondrocyte (Hagg *et al*, 1998). Collagen XI is cross linked with itself, and is thought to prevent lateral growth of type II collagen (Blaschke *et al*, 2000).

1.1.3.2 Proteoglycans

The other major components of cartilage are proteoglycans. These proteoglycans consist of a central protein core, making up approximately 5% of the proteoglycan. Associated with this are one or more different glycosaminoglycans (GAGs), which consist of unbranched polysaccharides, which make up approximately 95% of the proteoglycan. The GAGs are negatively charged, due to sulphate and carboxylate groups, and this allows interaction with water (Temenoff and Mikos, 2000). This gives the cartilage its ability to resist compression during loading (Buckwalter and Mankin, 1998; Wong and Carter, 2003). There are a number of GAGs found in articular cartilage including hyaluronic acid, chondroitin sulphate and keratan sulphate (Roughley, 2006).

Cartilage proteoglycans can aggregate together and are known as aggrecans, consisting of monomers of keratan sulphate and chondroitin sulphate attached to the protein core (Roughley, 2006). Aggrecan can consist of up to 300 of these monomers linked to a central hyaluronic acid chain (Temenoff and Mikos, 2000). It is these aggrecan structures, along with type II collagen that are the primary components of cartilage (see Fig 1.1). The smaller proteoglycans are thought to play a role in the organisation of the collagen as well as cell function (Buckwalter and Mankin 1998).

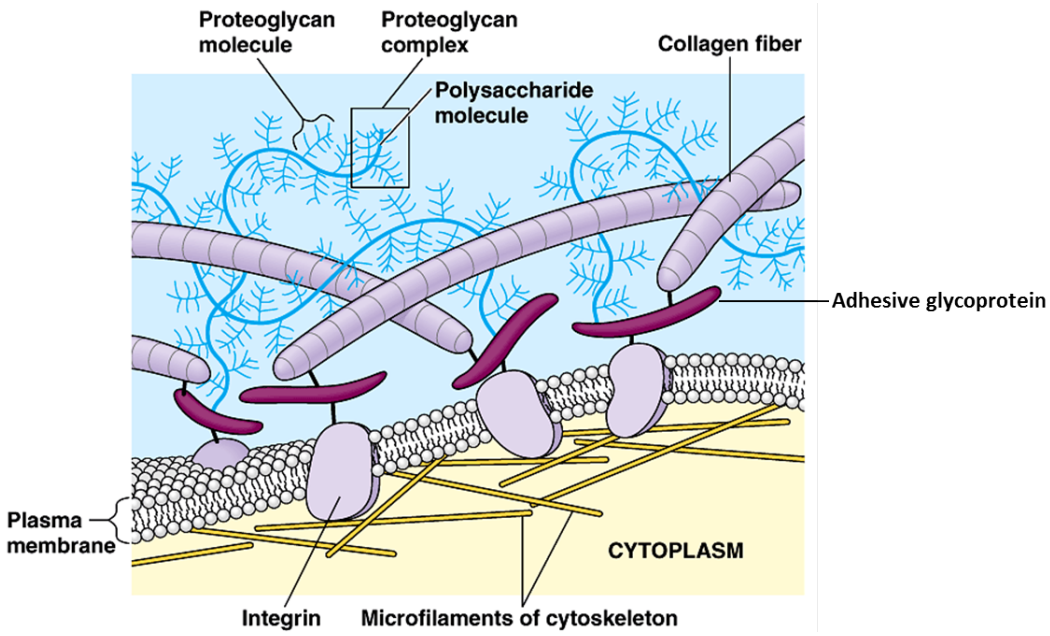


Figure 1.1. The components of cartilage.

1.1.3.3 Minor components of articular cartilage

As well as proteoglycans the cartilage also consists of non-collagenous proteins which include anchorin, fibronectin and tenascin. These proteins make up a relatively small component of the cartilage. However, they play a number of important roles including stabilisation of the ECM, and aiding in the interaction of chondrocytes with the surrounding matrix (Temenoff and Mikos, 2000; Buckwalter and Mankin, 1998).

1.2 Arthritis

Cartilage is a dynamic tissue, with the balance of synthesis and degradation of the matrix under tight control (Rowan *et al*, 2008). It is when this balance is interrupted that excessive cartilage degradation occurs and a disease state arises, with the two most predominant diseases being rheumatoid arthritis (RA) and osteoarthritis (OA). In particular, it is collagen degradation that is central to disease, since when aggrecan is lost this can be replaced but collagen degradation is irreversible (Jubb *et al*, 1980). The causal mechanisms of cartilage damage differ from disease to disease, indeed within a particular

disease, however, the end point is always characterised by excessive cartilage degradation and pain.

Cartilage degradation is driven primarily by 2 groups of enzymes, the A disintegrin and metalloprotease with thrombospondin motifs (ADAMTSs) and matrix metalloproteinases (MMPs), which cleave the two major components of the cartilage; the proteoglycans and collagen. This degradation process is driven by a large number of different cytokines including interleukin (IL) -1, -17, -6 and tumour necrosis factor-α (TNF-α) (Goldring *et al*, 2011; Boissier, 2010).

1.2.1 Rheumatoid arthritis (RA)

RA is a chronic autoimmune disease affecting between 0.5-1% of people in the industrialised world, occurring three times more frequently in women than men (Scott *et al*, 2010). It is an inflammatory disease, associated particularly with inflammation of the synovium. This persistent inflammation leads to damage of the cartilage, as well as bone and tendon (Scott, 2012). RA can in fact be thought of as a number of diseases with the same final outcome (van der Helm-van Mil and Huizinga, 2008). RA is mediated through the action of a number of cell types including B cells, T cells, macrophages, synovial-like fibroblasts, neutrophils and macrophage-like synoviocytes. Cytokine production such as IL-1, TNF, IL-6, IL-10, IL-17 and IL-8 is an important factor in driving RA. These different cytokines can be produced by a number of cell types and can lead to inflammation and joint destruction, as well as promoting autoimmunity (Feldman *et al*, 1996; Scott *et al*, 2010; McInnes and Schett, 2007). As well as this, osteoclasts also play a role in causing erosion of bone where receptor activator of nuclear factor-κB ligand (RANKL) plays an important part (McInnes and Schett, 2007). A large number of RA cases can be characterised by the presence of rheumatoid factor (RF) and anti-citrullinated peptides antibody (ACPA), with 50-80% of patients having one or both of these autoantibodies (Scott *et al*, 2010). Another important aspect of RA is the lack of inhibitory cytokines, such as IL-10 and IL-11, which are produced at lower levels in RA (Feldmann *et al*. 1996).

The initial cause of RA is still unknown, however it is thought that in many cases the disease starts many years before arthritis is apparent, as autoantibodies have been found in patients many years before cartilage damage (Schaeverbeke *et al* 2012). Infection may be one cause of RA, with a link between RA and periodontitis being suggested (Schaeverbeke *et al* 2012). There is also thought to be a genetic aspect to RA with the human leukocyte antigen (HLA)-DR genes, termed shared epitope alleles (in particular HLA-DR4 and DR1), thought to be the main risk factor. As well as this, polymorphisms in protein tyrosine phosphatase non-receptor type (PTPN) 22 and cytotoxic T lymphocyte antigen (CTLA)-4 (Andersson *et al*, 2008), are thought to be involved. There are also environmental factors contributing to RA, with a link between smoking and RA (Kallberg *et al*, 2011), and this is thought to be linked with shared epitope alleles and ACPA (Klareskog *et al*, 2006). In addition, factors such as child birth and stress have been linked to the onset of RA (Schaeverbeke *et al* 2012). RA is also associated with a number of co-morbidities, with the major one being cardiovascular disease; patients also have an increased risk of infection. The link between RA and many of these co-morbidities is unknown, however inflammation is thought to be a factor in cardiovascular disease (Levy *et al*, 2008).

1.2.2 Osteoarthritis (OA)

OA is a progressive disease of cartilage, affecting the majority of individuals over 65 (Goldring *et al*, 2007); with an ageing population this is a significant problem. OA was initially thought to be a disease caused simply by wear and tear, however, it is now thought to be much more complex involving a range of factors such as mechanical injury, obesity, joint instability and genetic factors. This is then perpetuated by inflammation and an imbalance in cellular signalling (Goldring *et al*, 2007). This leads to progressive degradation of the cartilage and bony growths termed osteophytes causing severe pain (Goldring *et al*, 2007), with the only real treatment at present being joint replacement.

The degree to which genetics plays a role in OA is still under debate, however twin studies have suggested this may be a factor in up to 70% of cases

(Goldring and Goldring, 2007), and there are a number of genes which may be involved.

In normal cartilage chondrocytes are relatively quiescent, however in OA the chondrocytes become more active, start to proliferate and produce matrix proteins. In addition to this, they produce matrix degrading enzymes, such as MMPs and aggrecanases, which cause cartilage degradation, and an imbalance between synthesis and degradation occurs (Goldring *et al*, 2009). The behaviour of the chondrocyte in this way is thought to represent a recapitulation of the chondrocyte in development during endochondral ossification (Goldring, 2012; Drissi *et al*, 2005; Tchetina *et al*, 2005).

As mentioned, age is the strongest correlating factor in OA, and it is thought that this may be due to the cartilage becoming more brittle and therefore more susceptible to damage. This may be due to an accumulation of glycation end products. In addition, increased cell death (Loeser *et al*, 2012), and changes in the composition of the cartilage matrix components, including proteoglycans and type II collagen, are thought to play a role (Goldring and Goldring, 2007).

OA was long thought not to have any inflammatory aspect to it, however recently it has come to be accepted that there is an inflammatory component affecting the synovial membrane of the joint. It has been shown that the synovium of OA patients contains increased TNFα, IL-1β and nuclear factor κB (NFκB) (Benito *et al*, 2005).

1.2.3 Arthritis treatment

1.2.3.1 Rheumatoid arthritis

Analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat the pain caused by RA, but do not treat the underlying cause of the disease (Scott *et al*, 2010). The two main treatments for RA are disease modifying anti-rheumatic drugs (DMARDs) and biological agents. The mechanism of action of DMARDs is still not fully understood (Scott *et al*, 2010), with the major DMARD for RA being Methotrexate, whilst Sulfasalazine and

Leflunomide are also used (Scott *et al*, 2010). DMARDs have also been shown to work effectively in combination (Choy *et al*, 2005).

There are a number of biological agents now being used therapeutically, with TNF inhibitors being the first to reach the clinic. Subsequent agents including Rituximab (Rituxan™ and MabThera™), a B cell targeting therapy (Edwards *et al*, 2004), Abatacept (Orencia™), which affects T cell signalling (Ruderman *et al*, 2005) and Tocilizumab (Actemra™ and RoActemra™), which affects IL-6 signalling (Scott *et al*, 2010; Mihara *et al*, 2005), are now being used in combination with DMARDs (Strangfeld *et al*, 2009). Glucocorticoids have also been used in the past, and they are still currently used for short term treatment (Scott *et al*, 2010), however they can have extreme side effects when used long term (Ravindran *et al*, 2009). In order to treat RA high doses of drugs are used initially, in order to reduce inflammation, and in some cases induce remission. It is in the early stages that a high amount of cartilage degradation occurs, therefore aggressive treatment is required early on which can subsequently be reduced to limit side effects (Schneider and Kruger, 2013). Due to the multi-factorial nature of RA, many patients still show poor responses to these treatments, and many adverse effects to these treatments have been reported (Salliot *et al*, 2009; Dixon *et al*, 2010). Furthermore, biological agents are very costly (Scott *et al*, 2009), therefore new approaches to RA treatments are required.

1.2.3.2 Osteoarthritis

There are few treatment options for OA, with a program of pain management and eventual joint replacement being the outcome in most cases. Tissue engineering treatments are currently being used, such as microfracture and autologous transplantation, however these treatments vary in success and may only delay ultimate joint replacement (Goldring and Goldring, 2007). Mesenchymal stem cells are currently being investigated to replace cartilage, however, much research is still required in this area with regard to their potential to form biologically active cartilage *in vivo*.

1.2.4 Mouse models

In order to study arthritis *in vivo* mouse models are used in order to represent the disease in humans. There are various mouse models used; collagen induced arthritis (CIA) involves injection of type II collagen emulsified in complete Freunds adjuvant into genetically succeptible mice (Brand *et al* 2007). This model mimics the pathological effects of RA, and is used extensively in order to study the viability of various drugs to treat RA. Destabilisation of the medial meniscus (DMM) involves sectioning of the medial meniscotibial ligament, which leads to destabilisation of the medial meniscus. This procedure induces OA, and is highly reproducible (Glasson *et al*, 2007). Injection with lipopolysacharide (LPS), a bacterial cell wall component, stimulates the immune inflammatory response (Capkova *et al*, 2012), and is used in order to study inflammatory diseases more broadly.

1.3 Cartilage degradation

1.3.1 Proteinases

There are 5 main classes of proteinases; these are aspartic proteinases, cysteine proteinases, threonine proteinases, serine proteinases and metalloproteinases (Cawston *et al*, 2006). Within the metalloproteinase class the major group of enzymes involved in cartilage degradation are those of the MMP family and the ADAMTS enzymes (Rowan *et al*, 2008). It is thought that for cartilage degradation to occur aggrecan must first be degraded by ADAMTSs which then allows collagen to be degraded via MMPs (Rowan *et al*, 2008). This is demonstrated by ADAMTS-5 gene depletion in a mouse model, which confers protection against OA (Glasson *et al*, 2005).

1.3.2 Matrix metalloproteinases

MMPs are a group of 23 enzymes which cleave ECM, and have been shown to be important in degrading cartilage ECM (Clark *et al*, 2008; Rowan *et al*, 2008). MMPs are divided into collagenases, gelatinases, stromelysins and membrane type MMPs (MT-MMPS). There are however MMPs which do not fit into these categories (MMP-7, MMP-12, MMP-19, MMP-20, MMP-21, MMP-23A, MMP-23B, MMP-26, MMP-27 and MMP-28) (Clark *et al*, 2008; Rowan *et al*, 2008). MMPs are generally regarded as being extracellular proteins, however, there are MMPs that can also work intracellularly (MMPs -1, -2 and -11) (Limb *et al*, 2005; Kwan *et al*, 2004; Luo *et al*, 2002). MMPs are important in terms of normal development and homeostasis (Cawston *et al*, 2005), but it is when their regulation is not under the correct control that excessive ECM degradation occurs and a disease state arises. As well as arthritis, MMPs have been linked with a number of other diseases such as cancer and cardiac disease (Nagase *et al*, 2006).

1.3.2.1 Structure of matrix metalloproteinases

MMPs share a common domain structure consisting of a signal peptide, a pro-peptide of approximately 80 amino acids, a catalytic domain of approximately 170 amino acids, a hinge region which can vary in length and a C-terminal haemopexin-like domain of approximately 200 amino acids (except MMP-7, MMP-23 and MMP-26) (Nagase *et al*, 2006; Clark *et al*, 2008; Rowan *et al*, 2008) (see Fig 1.2). As well as these common domains there are common structures within these domains including a zinc binding motif (HEXXHXXGXXH) in the catalytic domain, including a methionine eight residues after this forming a ‘met turn’, and a cysteine switch motif (PRCGXPD) in the pro-peptide, (except MMP-23) (Nagase *et al*, 2006; Rowan *et al*, 2008). These two motif regions are important in keeping pro-MMPs inactive (Nagase *et al*, 2006). There are however MMPs with a different structure, in particular the MT-MMPs, which include a membrane tethering region (Rowan *et al*, 2008).

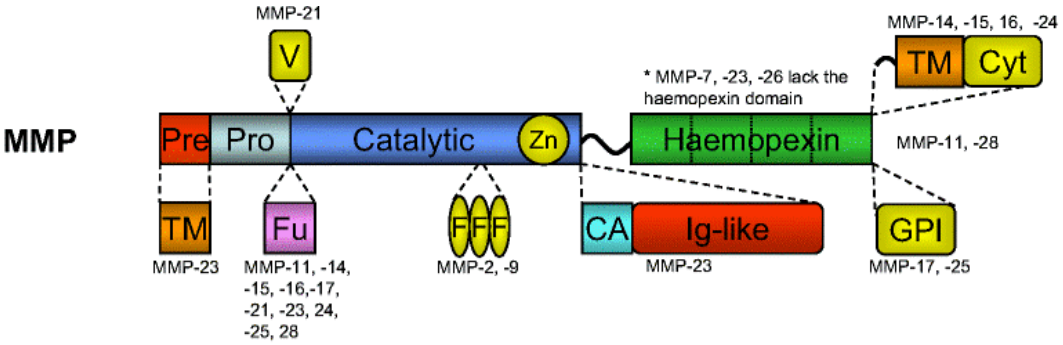


Figure 1.2 Domain structures of MMPs. Pre (pre domain); Pro (pro domain); Zn (zinc); Fu (furin recognition motif); F (fibronectin-like domains); V (vitronectin-like domain); TM (trans membrane domain); Cyt (cytoplasmic tail); GPI (glycosylphosphatidyl inositol); CA (cysteine array); Ig-like (immunoglobulin-like domain). Taken from Cawston and Young, 2010.

1.3.2.2 The collagenases

As mentioned, in terms of arthritis, the collagenases are perhaps the key enzymes, as they are known to degrade collagen type II, which is a key component of cartilage (Rowan *et al*, 2008), and when degraded cannot be replaced (Jubb *et al*, 1980). These collagenases include MMP-1, MMP-2, MMP-8, MMP-13 and MMP-14. MMP-1, MMP-8 and MMP-13 are the most potent in this collagenolytic action (Rowan *et al*, 2008), with MMP-13 being the most potent in terms of collagen type II cleavage (Knauper *et al*, 1996). Cleavage of collagen occurs at a specific peptide bond which causes the production of what is known as the one quarter and three quarter length fragments (Rowan *et al*, 2008). In terms of cartilage degradation in arthritis, it is thought that different collagenases have more predominant roles in OA and RA (Rowan *et al*, 2008). The fact that MMP-1 is produced by synovial fibroblasts as well as chondrocytes has led some to suggest that this may be the predominant collagenase in RA, and that MMP-13 maybe more predominant in OA which is thought to be a more chondrocyte-driven disease (Tetlow *et al*, 1998; Billinghurst *et al*, 1997).

1.3.2.3 The stromelysins

The stromelysins include MMP-3, MMP-10 and MMP-11. MMP-3 and -10 are similar in structure, and are involved in the activation of a number of proMMPs, including MMP-1 and -13 (Nagase *et al*, 2006; Suzuki *et al*, 1990; Amalinei *et al*, 2007). MMP-11 is known to cleave serpins (Pei *et al*, 1994). MMP-11 also contains a furin recognition motif which allows it to be activated intracellularly (Pei *et al*, 1995).

1.3.2.4 The gelatinases

There are two known gelatinases, MMP-2 and MMP-9. As well as their ability to cleave gelatin, they can also cleave type IV, type V, type XI collagen, laminin, aggrecan core proteins and elastin. In addition, MMP-2 also has the ability to activate proMMP-13 (Nagase *et al*, 2006; Cawston *et al*, 2006).

1.3.2.5 The matrilysins

The matrilysins include MMP-7 and MMP-26; these MMPs do not contain the haemopexin domain that most other MMPs contain (Nagase *et al*, 2006). MMP-7 can process a range of ECM components including fibronectin, laminin and proteoglycans (Amalinei *et al*, 2007). MMP-26 has the ability to undergo autocatalytic activation (Amalinei *et al*, 2007), and like MMP-7 can digest several different ECM molecules.

1.3.2.6 The membrane-type MMPs

The MT-MMPs include MMP-14 (MT-MMP-1), MMP-15 (MT-MMP-2), MMP-16 (MT-MMP-3), MMP-17 (MT-MMP-4) MMP-24 (MT-MMP-5) and MMP-25 (MT-MMP-6). These MT-MMPs are split into 2 groups; the type I transmembrane proteins (MMP-14, -15, -16 and -24) and the glycosylphophatidylinositol

anchored proteins (MMP-17 and -25) (Nagase *et al*, 2006). As with the stromelysin MMP-11, they contain a furin recognition motif allowing intracellular activation (Rowan *et al*, 2008). These MMPs (apart from MMP-17) have the ability to activate proMMP2 (Nagase *et al*, 2006; English *et al*, 2000). In addition, MMP-14 can activate proMMP-13 (Cawston *et al*, 2006), and also has collagenolytic activity, being able to cleave collagens type I, II and III yielding the characteristic one quarter and three quarter fragments (Ohuchi *et al*, 1997).

1.3.2.7 Regulation of matrix metalloproteinases

1.3.2.7.1 Synthesis

MMPs are regulated at several levels; an important method of pro-MMP regulation is at the transcriptional level. This occurs through a number of transcription factors, including activating protein-1 (AP-1), NFκB and runt-related transcription factor 2 (RUNX2) (Vincenti and Brinckerhoff, 2007), which will be discussed in detail later.

1.3.2.7.2 Activation of proenzymes

MMPs are regulated at the post-translational level through production as pro-MMPs, which require cleavage of the pro-peptide. This occurs through a number of proteinases including serine proteinases, such as matriptase (Milner *et al*, 2010), MMPs including MMP-2 and MMP-3, as well as autocatalysis (Bellayr *et al*, 2009; Hadler-Olsen *et al*, 2011). The majority of MMPs are secreted from the cells as pro-MMPs (apart from those with a furin recognition domain and MT-MMPs), and activation usually occurs at the cell surface (Cawston *et al*, 2006). The pro-peptide contains an invariant cysteine residue, which allows it to bind to the zinc ion within the active site, preventing binding to its substrate (Clark *et al*, 2008). The pro region contains within it what is termed a ‘bait’ region which is susceptible to, and cleaved by, proteinases. However, cleavage of the bait region does not activate the MMP, which requires further cleavage for activation (Nagase *et al*, 2006). In some cases, cleavage of the

pro-peptide is not required for activation, all that is required is a physical delocalisation of the pro-peptide from the active site. This may be achieved by cleavage or by removal of the pro-peptide away from the catalytic region through binding of a binding partner (Hadler-Olsen *et al*, 2011).

1.3.2.7.3 Inhibition

Tissue inhibitor of metalloproteinases (TIMPs) consist of TIMPs 1-4. TIMPs inhibit the action of MMPs by blocking their active site, by forming a wedge shape that allows them to interact with the active site (Rowan *et al*, 2008; Clark *et al*, 2008; Vincenti and Brinckerhoff, 2007). TIMPs bind and inhibit MMPs in a 1:1 ratio (Cawston *et al*, 2006). TIMPs consist of 2 domains, an N-terminal and C-terminal, with the N-terminal domain having the inhibitory activity (Rowan *et al*, 2008). The C-terminal domain may play a role in the rate of association between the TIMP and its MMP (Murphy *et al*, 2002). TIMPs have different modes of regulation, with TIMP-1 and -3 being inducible by factors such as transforming growth factor-β (TGF-β) and insulin like growth factor (IGF)-1 (Cawston *et al*, 2006). TIMP-4 exhibits restricted expression, whilst TIMP-2 is constitutively expressed (Rowan *et al*, 2008). TIMPs vary in their affinity for MMPs, however they all have a fairly broad specificity and collectively have the ability to inhibit all MMPs (Rowan *et al*, 2008; Nagase and Murphy, 2008). TIMP-3 is thought to have the broadest range, and as well as inhibiting MMPs it also has the ability to inhibit ADAMs and ADAMTSs, whereas the other TIMPs are fairly limited in their ability to inhibit these enzymes (Nagase and Murphy, 2008). TIMPs, although primarily inhibitors, can also in some cases play a role in activating MMPs. This occurs through co-localisation of different MMPs, through the ability of TIMPs to bind to more than one MMP at once. This is the case for the activation of MMP-2 by MT-MMP-1 through a ternary complex with TIMP-2 (Wang *et al*, 2000).

TIMPs may be important in terms of inhibiting cartilage degradation to prevent arthritic disease. TIMP-3 gene depletion was shown to exacerbate arthritis in an aged mouse model (Sahebjam *et al*, 2007). However TIMP-1 and -2 silencing have not been shown to have any major phenotype (Rowan *et al*, 2008).

As well as TIMPs, α2-macroglobulin is able to inhibit MMPs by binding to them and causing them to be cleared through endocytosis (Strickland *et al*, 1990).

1.3.3 A disintegrin and metalloprotease with thrombospondin motifs (ADAMTS)

The ADAMTS enzymes are members of the metalloproteinase family. As mentioned previously, along with MMPs the ADAMTSs are the most important proteinases in arthritis, as there is a subset which has the ability to degrade aggrecan, one of the major components of cartilage (Sandy *et al*, 1992). There are 19 different ADAMTSs, with the aggrecanases being composed of ADAMTS -1, -4, -5, -8, -9 and -15. The ADAMTSs consist of a signal peptide, a cysteine rich domain, a thrombospondin type I like repeat, a spacer region and C-terminal TS repeats (Kuno *et al*, 2000; Tang *et al* 2001; Apte *et al*, 2004) (see Fig 1.3). The ADAMTSs contain a furin recognition motif in their pro-domain, which as stated by Rowan *et al*. (2008) would suggest they are secreted from the cell as active forms of the enzyme, much like those MMPs containing furin recognition motifs, however this does not always appear to be the case, as some are not. ADAMTSs also have C-terminal cleavage in the spacer region, this cleavage can occur through the action of MMPs and alters the specificity of ADAMTSs to their substrate (Rodriquez-Manzaneque *et al*, 2000; Flannery *et al*, 2002). ADAMTSs are activated through members of the enzyme family termed pro-protein convertases, which include furin (as mentioned), pro-protein convertase 5/6, pro-protein convertase 7 and Paired Basic Amino Acid Cleaving Enzyme 4 (PACE4) (Troeberg and Nagase, 2012). As previously mentioned, ADAMTSs can also be regulated through the action of TIMPs, in particular TIMP-3. In addition to arthritis, ADAMTSs play roles in a number of other diseases, for instance ADAMTS-1 has been linked with coronary artery disease (Sabatine *et al*, 2008), due to its ability to cleave versican (Sandy *et al*, 2001), which plays a role in heart disease. ADAMTS-8 has been associated with cancer of the brain (Dunn *et al*, 2006), whereby ADAMTS-8 is down regulated in brain tumours, and due to its anti-angiogenic properties this may play a role in cancer progression.

ADAMTS-4 and -5 are the 2 most studied aggrecanases in terms of cartilage degradation (Tortorella and Malfait, 2008). ADAMTS-4 and -5 can cleave

aggrecan at 5 separate sites within the molecule (Lin and Liu, 2010). The most important cleavage site in terms of arthritic disease is Glu^{373}-Ala^{374}, since cleavage at this site leads to loss of the whole aggrecan molecule. Blocking cleavage at this site has shown to be protective against cartilage loss in a mouse model (Little *et al*, 2007). There is evidence that ADAMTS-5 is the most important aggrecanase of the two, as ADAMTS-4 deficient mice are not protected against aggrecan loss, however ADAMTS-5 deficient mice are (Stanton *et al*, 2005). Whether this is the case in human disease is still up for debate.

TIMP-3 is able to inhibit both ADAMTS-4 and -5 (Kashiwagi *et al*, 2001). As well as this the proteoglycan syndecan-4 is thought to be important in ADAMTS-5 regulation. Syndecan-4 deficient mice were seen to be protected against cartilage erosion, this was through a lack of ADAMTS-5 activity. Syndecan-4 acts through direct interaction with ADAMTS-5, as well as through regulation of MMP-3 which can itself regulate ADAMTS-5 (Echtermeyer *et al*, 2009).

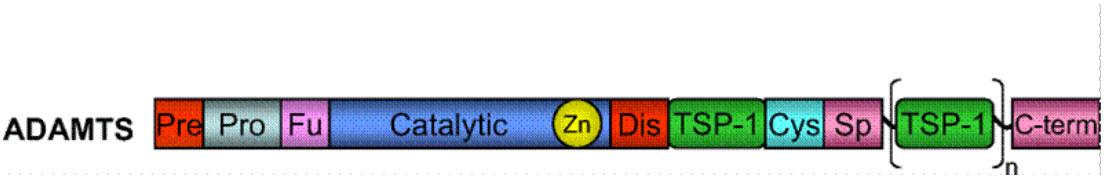


Figure 1.3 Domain structures of MMPs. Pre (pre domain); Pro (pro domain); Zn (zinc); Fu (furin recognition motif); Dis (disintegrin); TSP-1 (thrombospondin type-1); Cys (cysteine domain); Sp (signal peptide); C-term (C-terminus). Taken crom Cawston and Young, 2010.

1.3.4 Other proteinases involved in cartilage degradation

Although the collagenases and aggrecanases are clearly key enzymes in terms of cartilage degradation during arthritis, a number of other enzymes have been shown to be up regulated in disease and play important roles.

1.3.4.1 Serine proteinases

The serine proteinases are a group of enzymes, of which several are strongly implicated in arthritis. They are thought to play a major role in the activation of collagenases, and this activation may be a rate limiting step in arthritis development (Milner *et al*, 2001). Plasmin is thought to be important in MMP-1 and -13 activation (Zhang *et al*, 2007; Morgan and Hill, 2005). Matriptase has been shown to activate MMP-1 and -13 *in vitro*, as well as enhancing collagenolysis, this effect on MMPs is thought to occur through PAR-2 (discussed below) (Milner *et al*, 2010). Unlike those discussed so far, high temperature requirement A (Htra1) is thought to contribute to arthritis by direct action on the cartilage ECM, having been shown to act on a number of proteins including aggrecan, decorin and fibromodullin, as well as soluble type II collagen *in vitro* (Tsuchiya *et al*, 2005).

1.3.4.2 Cysteine proteinases

The cysteine proteinase cathepsin K is the only enzyme other than the MMPs which can cleave collagen type II (Kafienah *et al*, 1998). It is thought that cathepsin K may be important in arthritis, as overexpression in a mouse model demonstrated increased synovitis and cartilage degradation (Morko *et al*, 2005). Cathepsin K is also able to cleave aggrecan. This occurs at several sites within the aggrecan, and yields GAG fragments that cathepsin K must bind to in order to exhibit collagenolytic activity (Hou *et al*, 2003). Other cathepsins have been shown to be up regulated in arthritic cartilage. Cathepsins B, S and L have been implicated in cartilage degradation (Buttle and Saklatvala, 1992). However, the role of these enzymes in pathological cartilage degradation is still controversial (Hou *et al*, 2002; Bayliss and Ali, 1978; Sapolsky *et al*, 1973). As mentioned previously, an important feature of arthritic disease is chondrocyte death (Loeser *et al*, 2012). Caspases, members of the cysteine proteinase family, play a major role in cell apoptosis. It has been demonstrated that caspase-3 is up regulated in osteoarthritic chondrocytes, suggesting an important role in chondrocyte death and disease (Sharif *et al*, 2004).

1.4 Pro-inflammatory cytokines in arthritis

Inflammation has been shown to be a major contributing factor in arthritis. Whilst the inflammatory aspect of RA has been known for many years, and studied greatly (Mcinnes and Schett, 2007), it is only relatively recently been accepted that OA also has inflammatory characteristics, wiith OA patients being shown to have inflamed synovium (Benito *et al*, 2005). As mentioned previously, this inflammation is driven by a number of pro-inflammatory cytokines including IL-1, TNF-α, IL-17 and IL-6 (Benito *et al*, 2005; Mcinnes and Schett, 2007). These cytokines, as well as causing inflammation, drive the degradation of cartilage by stimulating the production of proteinases by the cells present including chondrocytes, macrophage-like synoviocytes and synovial fibroblasts (Mcinnes and Schett, 2007). Knowledge of these pro-inflammatory cytokines is therefore of great interest in arthritis.

1.4.1 Interleukin-1 (IL-1)

IL-1 is an extracellular signalling cytokine family that plays a multitude of roles in many cellular pathways contributing to inflammation, IL-1 therefore plays a role in immunity and a great deal of diseases. IL-1 has been vastly studied due to its important role in cellular signalling, and it was the first interleukin to be discovered. It was identified in the mid 1980s, however, it had been studied long before this under different guises such as haematopoetin 1 and leukocyte endogenous mediator. The IL-1 family consists of 11 members, which are IL-1α, IL-1β, Il-1 receptor antagonist (IL-1Ra), IL-18, IL-33, IL-1F5, IL-1F6, IL-1F7, IL-1F8, IL-1F9 and IL-1F10 (Sims and Smith 2010). IL-1 signals through specific receptors which contain extracellular immunoglobulin domains and a Toll/IL-1 receptor (TIR) domain in the cytoplasmic portion. IL-1 binds to the receptor subunit IL-1 receptor type 1 (IL-1R), this then leads to recruitment of a secondary subunit IL-1R accessory protein (IL-1RAP). The TIR domains in the cytoplasmic portion then recruit myeloid differentiation primary response protein 88 (MYD88), IL-1R associated kinase 4 (IRAK4), TNF receptor-associated factor 6 (TRAF6) and other signalling intermediates. This then leads to the

activation of a number of signalling pathways (O'Neill, 2008). All members of the IL-1 family are conserved at the genetic level (Taylor *et al*, 2002).

Two members of the IL-1 family termed IL-1α and IL-1β are potent pro-inflammatory cytokines and play a major role in arthritis. Both of these cytokines are thought to have identical biological activities, however they are spatially separated with IL-1β being secreted and IL-1α associated with the plasma membrane (Kurt-Jones *et al*, 1995; Dinarello, 1996). In addition, IL-1β requires cleavage of its pro-domain in order to be activated (Martinon *et al*, 2009). These 2 cytokines are also produced by different cells and are therefore differentially regulated (Dinarello, 1996; Rausch *et al*, 1994). IL-1α also has additional functions, as it has a nuclear localisation sequence allowing it to act within the nucleus, where it can play a role in epigenetic regulation, as it has been shown to interact with histone acetyltransferases (Buryskova *et al*, 2004).

IL-1α and IL-1β due to their importance are tightly regulated. These 2 cytokines are regulated at the transcriptional level, and also through mRNA stability through various cytokines and stimuli (Rausch *et al*, 1994). There are also IL-1 antagonists, which prevent IL-1 signalling. IL-1Ra can bind to the receptor subunit IL-1R to block IL-1 binding (Granowitz *et al* 1991). There is also a second IL-1R termed IL-1R2 which acts as a decoy receptor, IL-1R2 is able to bind IL-1, but does not transmit signalling (Colotta *et al*, 1993).

1.4.1.1 IL-1 and arthritis

As has been demonstrated many times, IL-1 is likely to play a major role in arthritis, since it is involved in the induction of various signalling cascades, such as mitogen activated protein kinase (MAPK) and NFκB. These signalling cascades lead to the regulation of MMPs and ADAMTSs and therefore cartilage degradation. In addition, IL-1 has been shown to reduce type II collagen production by chondrocytes *in vitro* (Goldring *et al*, 1994).

IL-1 has been shown to be important in RA as anti-IL-1α and β treatment of a mouse collagen induced arthritis (CIA) model demonstrated reduced disease (Joosten *et al*, 1999). In confirmation of its important role in RA, treatment of RA

patients with recombinant IL-1Ra (Anakinra), although not universally successful, has been shown to be effective in many patients (Martens and Singh, 2009).

1.4.2 Oncostatin M (OSM)

OSM is a multifunctional cytokine, and is a member of the IL-6 family of cytokines (Heinrich *et al*, 2003). OSM signals through two different heterodimeric receptor complexes, both of which contain gp130, and either leukaemia inhibitory factor receptor (LIFR) or OSM receptor-β (OSMR-β) (Thoma *et al*, 1994). The binding of OSM to either of its receptor complexes then leads to the activation of various signalling pathways including janus kinase/signal transducers and activators of transcription (JAK/STAT) (Li *et al*, 2001), MAPK and phosphoinositide-3 kinase (PI3K) (Godoy-Tunidor *et al*, 2005).

1.4.2.1 OSM and arthritis

As mentioned, OSM can regulate a number of pathways involved in arthritis, it is therefore thought to play a role within arthritic disease. OSM has been shown to work synergistically with IL-1α to degrade bovine nasal cartilage *in vitro* (Cawston *et al*, 1995), as well as enhancing MMP-1 and -13 production in chondrocytes and human cartilage co-cultures (Cawston *et al*, 1998; Fearon *et al*, 2006). OSM has also been shown to work synergistically with TNF-α; it was shown to enhance bovine cartilage degradation as well as enhancing MMP-1, -3 and -13 expression. OSM also worked synergistically with TNF-α to enhance joint damage when injected into the joint of a mouse, and showed increased MMP-13 expression (Hui *et al*, 2003).

OSM has been found to be elevated in RA synovial fluid (Manicourt *et al*, 2000). As well as this, addition of OSM in mouse joints led to enhanced RA like symptoms including synovial cell proliferation and infiltration of mononuclear cells, in which the synovium took on characteristics of the pannus (Langdon *et*

al, 2000). In a mouse CIA model, treatment with anti OSM antibodies showed reduced RA (Plater-Zyberk *et al*, 2001). However, as much as OSM appears to be pro catabolic, it has also been shown to increase TIMP-1 expression in chondrocytes *in vitro* (Sanchez *et al*, 2004), possibly suggesting a role in protection of cartilage. Such contradictions may be dependent on the context of cytokine profile present.

1.4.3 Tumour necrosis factor-α (TNF-α)

TNF-α along with IL-1 has been shown to be extremely important in arthritis. It is a key regulator of pro-inflammatory stimuli, as mentioned above, as well as a number of signalling pathways, many of which are also regulated by IL-1, such as MAPK (Schett *et al*, 2000) and NFκB (Roman-Blas and Jimenez, 2006). Due to its importance in inflammation it is clearly important in RA, and this has been demonstrated most obviously by the success of anti TNF-α therapy in treating RA patients (Rankin *et al*, 1995). As well as this, blocking TNF-α in chondrocyte explant cultures demonstrated reduced cartilage degradation, as well as reduced MMP-1, -3 and -13 expression (Kobayashi *et al*, 2005). In addition, TNF-α addition also led to a decrease in collagen type II production by chondrocytes *in vitro* (Reginato *et al*, 1993).

1.5 Anti-inflammatory cytokines in arthritis

Anti-inflammatory cytokines are essential in normal homeostasis as a mechanism of dampening down the pro-inflammatory responses which occur. It is therefore thought that a major factor in arthritic disease, which has a very much pro-inflammatory feature, is the dysregulation of anti-inflammatory cytokine production (Boissier *et al*, 2008).

1.5.1 Interleukin-4 (IL-4)

IL-4 is a potent anti-inflammatory cytokine, involved in the suppression of many pro-inflammatory pathways in a wide variety of cells. IL-4 signals through the JAK/STAT signalling pathway. The IL-4 receptor consists of two sub units, the IL-4 receptor α (IL-4Rα) chain, (Galizzi *et al*, 1990) and the γ common (γc) chain (Russel *et al*, 1993). These 2 subunits are associated with JAK1 and JAK3. In addition to this IL-4 signals through the same receptor as IL-13, which is made up of the 2 subunits IL-4Rα and IL-13Rα1 (Zurawski *et al*, 1995; Miloux *et al*, 1997); this also signals through the JAK/STAT pathway (Takeda *et al*, 1996).

IL-4 has been shown to inhibit the production of a number of pro-inflammatory cytokines, including IL-1, IL-6, TNF-α and IL-8 (Miossec *et al*, 1992; Morita *et al*, 2001). IL-4 can inhibit IL-1 signalling by inducing IL-1R2 which, as mentioned, acts as a decoy receptor for IL-1 (Colotta *et al*, 1993).

The anti-inflammatory effect of IL-4 may occur through a number of pathways in order to affect arthritis. It has been shown to reduce OSM-induced MMP-13 and ADAMTS-4 levels in bovine chondrocytes. IL-4 has also been shown to reduce IL-1-stimulated MMP-1 expression in synovial fibroblasts (Borghaei *et al*, 1998). As well as this, it was shown to reduce TGF-β-induced c-Jun N-terminal kinase (JNK) phosphorylation, which is a member of the MAPK family. However, somewhat contraindicative, the same stimulus was also shown to reduce TIMP-3 (Mabrouk *et al*, 2008). In addition, adenoviral transfer of IL-4 into the joints of a mouse CIA model showed protection from cartilage destruction (Watanabe *et al*, 2000).

1.5.2 Interleukin-10 (IL-10)

IL-10 is a potent anti-inflammatory cytokine. IL-10 can work by blocking the expression of pro-inflammatory cytokine genes, as well as inducing other anti-inflammatory cytokines (Moore *et al*, 2001). IL-10 signals through the JAK/STAT pathway; JAK1 and TYK2 are associated with the IL-10 receptor subunits IL-

10R1 and IL-10R2 (Kotenko *et al*, 1997). On IL-10 binding the receptor, JAKs are then activated leading to activation of STAT1, 3 and 5 (Finbloom and Winestock, 1995; Wehinger *et al*, 1996).

IL-10 has been shown to inhibit production of a vast array of pro-inflammatory cytokines, including IL-1, IL-6, IL-18 and TNF (de Waal Malefyt *et al*, 1991), as well as inhibiting NFκB (Lentsch *et al*, 1997). IL-10 has been shown to reduce the severity of arthritis in a mouse CIA model (Tanaka *et al*, 1996). As well as this, it has been shown that IL-10 leads to an increase in TIMP-1, demonstrating its ability to regulate MMP activation (Lacraz *et al*, 1995). IL-10 has also been shown to synergistically enhance the anti-inflammatory properties of IL-4; the addition of IL-10 to IL-4 enhances the release of IL-1Ra, hence increasing IL-1 inhibition (Crepaldi *et al*, 2002). However, in contradiction to the anti-inflammatory properties of IL-10, it has also been demonstrated that IL-10 can lead to AP-1 and NFκB activation (Hurme *et al*, 1994).

1.5.3 Interleukin-13 (IL-13)

IL-13 has also been shown to be an anti-inflammatory cytokine. IL-13 is very homologous to IL-4 and shares many functions with the cytokine. IL-13 signals through the JAK/STAT pathway, in particular through STAT6 much in the same way that IL-4 does (Takeda *et al*, 1996). IL-4 and IL-13 share the IL-4Rα subunit (Zurawski *et al*, 1995), this subunit in combination with IL-13Rα1, forms the IL-13 receptor (Miloux *et al*, 1997). IL-13 is also able bind to another receptor IL-13Rα2, however this is thought to possibly be a decoy receptor, as no IL-13 signalling was seen when bound to IL-13Rα2 (Donaldson *et al*, 1998). In addition, IL-13Rα2 has been shown to inhibit IL-13 signalling (Kawakami *et al*, 2001).

IL-13 has been shown to reduce pro-inflammatory cytokine secretion including IL-1, TNF-α and IL-8 levels in synovial explant culture (Woods *et al*, 2000). IL-13 has also been shown to inhibit IL-1 by also increasing IL-1Ra (Chizzolini *et al*, 2009). In addition, IL-13 has also been shown to be anti-angiogenic in a rat model of RA, and angiogenesis has been shown to be important in the

development of RA and the synovial pannus (Haas *et al*, 2007). IL-13 has also been shown to supress NFκB activation through preservation of the NFκB inhibitor, inhibitor of kappa B (IκB)-α (Lentsch *et al*, 1997).

1.6 Signalling pathways involved in arthritis/MMP regulation

1.6.1 MAP kinases (MAPK)

The MAPK pathway plays a role in a vast number of cellular processes, and is critical for cell growth, differentiation and survival (Pouyssegur *et al*, 2003). The MAPKs are ser/thr kinases (Loeser *et al*, 2008) involved in three signalling pathways, namely extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. Each pathway consists of 3 distinct levels, wherein MAPKs are activated through phosphorylation by MAP kinase kinases (MAP2K), which are activated by MAP kinase kinase kinases (MAP3Ks) through the same process (Thalhamer *et al*, 2008) (see Fig 1.4). MAPKs are activated by specific MAP2Ks (see Fig 1.4), and this occurs at a specific motif, which consists of T-X-Y. Each of the three MAPKs contain a different amino acid at the X region, with ERK containing glutamic acid, JNK a proline residue and p38 a glycine residue. This motif along with surrounding amino acids can influence the specificity of the MAPK for its substrate (Thalhamer *et al*, 2008). The MAPKs' catalytic activity is enhanced over 5000 fold when activated through phosphorylation (Lew, 2003). Once activated the MAPKs can then subsequently translocate to the nucleus or remain in the cytoplasm and elicit a number of cellular events (Sacks, 2006).

MAPKs are regulated by negative feedback through dual specificity phosphatases (DUSPs), threonine phosphatases, and tyrosine phosphatases. MAPKs themselves are able to regulate some such phosphatase activities and therefore contribute to their own regulation (Owens and Keyse, 2007). The JNK and p38 pathways are primarily activated by cellular stress, whilst ERK is primarily mitogen activated (Thalhamer *et al*, 2008). The MAPK pathways can be activated in conjunction, with a particular stimulus activating more than one MAPK pathway (Kiss-Toth *et al*, 2004), as is the case for TNF-α, IL-6 and IL-1,

which have been shown to activate all these MAPK pathways (Schett *et al*. 2000).

The MAPK pathways are important in terms of cartilage degradation and arthritis. These pathways are key mediators of cytokine signalling, which subsequently leads to regulation of a number of different proteins and transcription factors involved in cartilage degradation. All three of the MAPKs have been shown to be active in tissue of RA patients, each of these MAPKs were localised to different areas; JNK activation was localised around and within mononuclear cell infiltrates, ERK around synovial micro vessels, and p38 in the synovial lining layer and synovial endothelial cells (Schett *et al*, 2000). Furthermore, all three MAPKs were increased in a dog OA model (Boileau *et al*, 2006).

The activation of the MAPKs has been shown to lead to MMP-1 and -13 up regulation in chondrocytes (Mengshol *et al*, 2000). ERK has been associated with OA, having been shown to effect progression in a rabbit OA model (Pelletier *et al*, 2003). ERK inhibition was shown to reduce MMP-1 and -13 induction, with the inhibition of JNK shown to lead to a reduction in MMP-1 (Han *et al*, 2001). The inhibition of p38 was shown to reduce cartilage degeneration in an OA model in rats (Brown *et al*, 2008), as well as reducing both bone and cartilage degeneration in a CIA model in mice (Medicherla *et al*, 2006). Also, p38 has been shown to be involved in TNF-α and IL-1 production (Campbell *et al*, 2004; Baldassare and Bellone, 1999), which as mentioned are important in both OA and RA. In addition, p38 inhibition leads to a reduction in IL-6 production through IL-1 and TNF in chondrocytes (Kumar *et al*, 2001). SAPK/p38 has been shown to contribute to MMP-1 and MMP-13 expression (Raymond *et al* 2006, Mengshol *et al* 2000), as well as stabilising MMP-1 mRNA (Reunanen *et al* 2002).

MAPKs can regulate MMPs through the regulation of transcription factors. A major transcription factor involved in the regulation of MMP-1 and -13 is AP-1. AP-1 can be a heterodimer consisting of c-Fos and c-Jun, Jun B or Jun D, as well as members of the fos-related antigen (Fra) and activating transcription factor (ATF) family (Eferl and Wagner, 2003). There is much evidence for the MAPK pathways playing a role in regulating MMPs through AP-1. The inhibition

of JNK has been shown to inhibit the phosphorylation of c-Jun, as well as inhibiting AP-1 activity, leading to a reduction in MMP-1 expression (Han *et al*, 2001). JNK phosphorylation has been shown to increase transcription, protein stability and DNA binding of c-Jun (Minden *et al*, 1997). The inhibition of ERK has been shown to inhibit AP-1 activation (Han *et al*, 2001), whilst phosphorylation of ERK was shown to increase c-Jun expression and phosphorylation (Leppa *et al*, 1998). As well as this, ERK has also been shown to regulate E26 transformation specific sequence (Ets), which has been shown to be involved in MMP transcription by enhancing AP-1 activity (O'Hagan *et al*, 1996). Mengshol *et al*. (2001) demonstrated evidence of p38 activating MMP-13 through recruitment of AP-1, as well as the MMP-regulatory transcription factor runt related transcription factor (RUNX) 2 in chondrocytes. As with ERK, p38 can enhance AP-1 activity by activating transcription factors such as Ets (Minden *et al*, 1997) and ATF2 (Davis *et al*, 2000). MAPKs may also regulate MMPs through regulating the production of ECM fragments, which as mentioned, can regulate enzyme activity. It was demonstrated that inhibition of the MAPKs led to inhibition of fibronectin fragment-mediated MMP-13 production (Loeser *et al*, 2003).

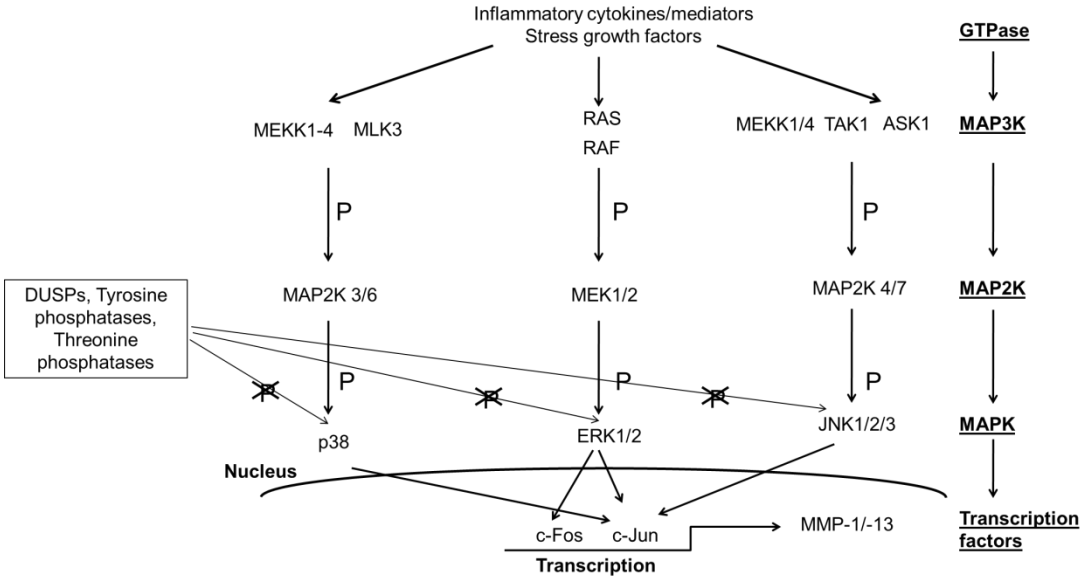


Figure 1.4. The different levels of the MAPK signalling pathway, demonstrating the specific regulation within each pathway. Adapted from Thalhamer *et al*, 2008. ASK, apoptosis signal-regulating kinase; ERK, extra-cellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MEK, MAPK kinase; MEKK, MEK kinase; MLK, mixed lineage kinase; MKK, MAPK kinase; TAK, TGFβ-activated kinase; RAF, rapidly accelerated fibrosarcoma; MMP matrix metalloproteinase; DUSP, Dual specificity phosphatase; P, phosphorylation.

1.6.2 Transforming Growth Factor-β/Sma and Mad related protein (TGF-β/Smad)

TGF-β is a family of growth factors involved in many different cellular pathways such as proliferation, differentiation and development (Van der Kraan *et al*, 2009). TGF-β commonly signals through Smad proteins, which mediate this signalling (Van der Kraan *et al*, 2009). Smads are activated through TGF-β pathway activation, whereby TGF-β binds to its receptor TGF-β type II, this then forms a complex recruiting TGF-β type I receptor, activator receptor like kinase (ALK) 5, which then leads to phosphorylation of Smad 2 or 3. Alternatively, TGF-β type I receptor ALK1 can be recruited leading to phosphorylation of Smad 1, 5, or 8. Once activated the Smads form a complex with Smad 4 which subsequently translocates to the nucleus to regulate gene expression (Finsson *et al*, 2008).

TGF-β may be important in OA as it has been shown to regulate chondrocyte hypertrophy (Blaney Davidson *et al*, 2007), which has been shown to be important in OA (Goldring, 2012). A paradox occurs with TGF-β as it has been shown to be both anabolic and catabolic. TGF-β has been shown to be chondroprotective (Blaney Davidson *et al*, 2005), and loss of TGF-β signalling in a mouse model resulted in enhanced cartilage degeneration (Serra *et al*, 1997), which was also the case for Smad 3 deficient mice (Yang *et al*, 2001). In addition, TGF-β has been shown to inhibit MMP-1 expression (Yuan and Varga, 2001). As well as this, TGF-β has been shown to induce TIMP-3 (Su *et al*, 1996). It has also been shown to both reduce the production of the pro-inflammatory cytokine IL-1, and increase its inhibitor IL-1Ra (Chizzolini *et al*, 2009). However, activation of the Smad 1/5/8 pathway has been shown to increase MMP-13 expression (Blaney Davison *et al*, 2009), and TGF-β itself has also been shown to promote MMP-13 gene expression (Uria *et al*, 1998). This apparent paradox may be due to different levels of TGF-β activity to give different effects, with high levels of TGF-β being catabolic and lower levels being anabolic. The ability of signalling pathways to be both catabolic and anabolic is common within signalling, and demonstrates the dynamism within signalling pathways. This allows both pro and anti-inflammatory cytokines to work through similar pathways giving different outcomes. This is often due to

the regulation of different transcription factors by these signalling pathways due to the specific cytokine stimulus. In addition, there is multiple cross talk between the different pathways. In this way these various pathways allow a diverse range of outcomes which in some cases can be pathological.

1.6.3 Janus kinase/signal transducers and activators of transcription (JAK/STAT)

The JAK/STAT pathway is a key pathway in many cellular events including proliferation, migration and apoptosis (Rawlings *et al*, 2004). The JAKs are a family of intracellular kinases consisting of 4 members; JAK 1, 2, 3 and tyrosine kinase 2 (TYK2). The STATs are a family of transcription factors consisting of 7 members; STAT 1, 2, 3, 4, 5A, 5B and 6 (Kisseleva *et al*, 2002). JAK activation occurs when a cytokine binds to its receptor leading to phosphorylation of the JAK, this then leads to recruitment and phosphorylation of STATs (Kisseleva *et al*, 2002). The activated STATs can then form dimers, both homo and heterodimers, and translocate from the cytoplasm to the nucleus, where they can then cause transcription of a number of genes (Kisseleva *et al* 2002; Korzus *et al*, 1997) (see Fig 1.5). In addition to being positive regulators of transcription, STATs can also be repressive, as is the case in the regulation of the immunoglobulin κ chain in B cells, where STAT5 can bind and recruit the histone methyltransferase enhancer of zeste homolog 2 (Ezh2), leading to repression of transcription at the epigenetic level (Mandal *et al*, 2011).

The JAK/STAT pathway is regulated through a number of different mechanisms. JAKs have been shown to be regulated through dephosphorylation by phosphatases, in particular SH2 domain containing protein tyrosine phosphatase (SHP) 1 and SHP2 (Kisseleva *et al*, 2002). As well as this, the JAK/STAT pathway has been shown to be regulated through negative feedback by cytokine inducible SH2 protein/suppressors of cytokine signalling (CIS/SOCS), of which there are 8 (Nicola *et al*, 1999). This CIS/SOCS group of proteins has the ability to inhibit both JAK and STAT activation (Seki *et al*, 2002; Matsumoto *et al*, 1999; Ungureanu *et al*, 2002; Ram and Waxman 1999). There are also a group of proteins termed protein inhibitor of activated

STATs (PIAS), which have been shown to bind STATs and prevent them from interacting with their target DNA (Liao *et al*, 2000).

A number of cytokines acting through the JAK/STAT pathway, such as interferons, IL-6, IL-10, IL-4 and IL-12 (Walker and Smith, 2005), are thought to be important in terms of arthritis regulation, and inflammatory pathways. The JAK/STAT pathway has been shown to regulate MMP expression. STATs can cause transcription of c-fos which as previously stated is a key component of the AP-1 transcription factor, and a key regulator of MMPs. STAT3 gene silencing has been shown to reduce c-fos, MMP-1 and -13 expression in human chondrocytes (Litherland *et al*, 2010). STATs have also been shown to directly regulate MMPs by binding to their promoters (Zugowski *et al*, 2011). In addition, it has been shown that JAK/STAT signalling is involved in OSM-mediated MMP expression (Li *et al*, 2001) and is a major regulatory route for MMP expression (Li *et al*, 2001, Mabrouk *et al*, 2007).

STAT1 was shown to be up regulated in synovial tissue from RA patients (van der Pouw Kraan *et al*, 2003). The silencing of STAT6 in a proteoglycan induced mouse model demonstrated a slower rate of cartilage erosion compared to wild type. In addition, STAT4 silencing in the same model led to protection against arthritis (Finnegan *et al*, 2002). As one would expect, the JAK proteins have also been shown to play a role in arthritis, with a strain of mouse which has a natural Tyk2 depletion being shown to be resistant to CIA (Ortmann *et al*, 2001; Shaw *et al*, 2003). Also, the use of a broad spectrum JAK inhibitor reduced the expression of inflammatory biomarkers in a CIA mouse model (Ghoreschi *et al*, 2011).

However, evidence for a role for STATs in RA is somewhat contradictory, as both STAT1 and STAT3 appear to be involved in both pro-inflammatory and anti-inflammatory pathways. STAT1 depletion in a zymosan-induced mouse model led to increased inflammation, suggesting STAT1 may be anti-inflammatory and protective in arthritis (Hooge *et al*, 2004). STAT3 may be important in the survival of synovial cells, as a depletion of STAT3 led to an increase in synovial fibroblast apoptosis *in vitro* (Krause *et al*, 2002).

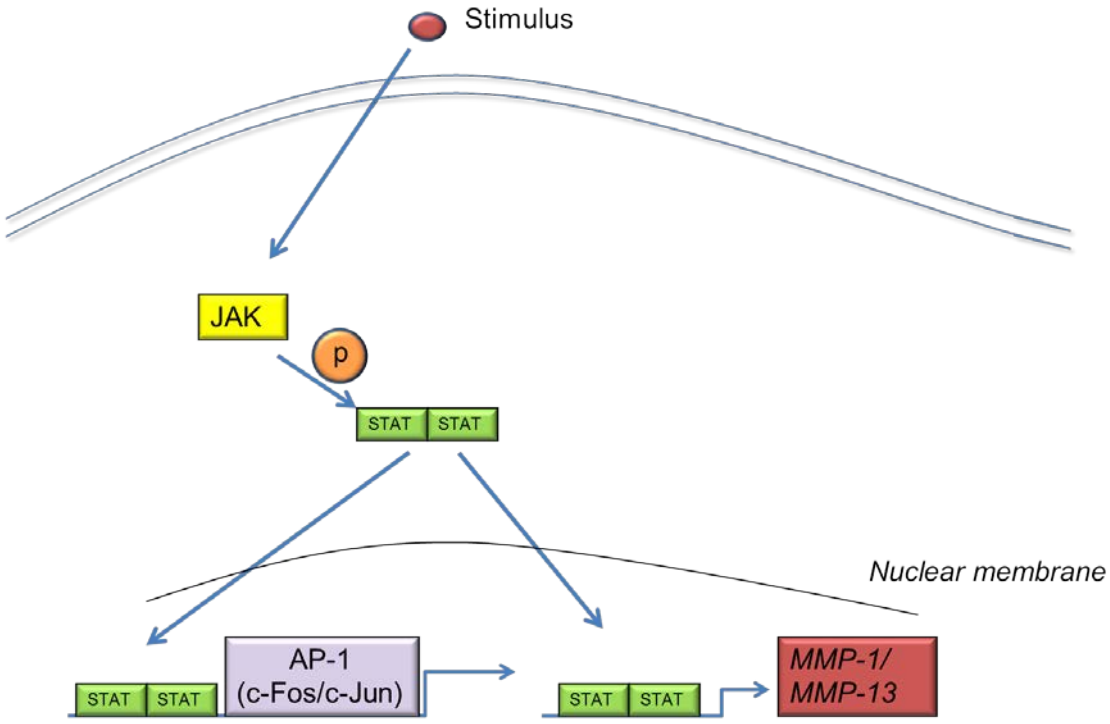


Figure 1.5. The JAK/STAT signalling pathway. JAK; Janus kinase, STAT; signal transducer and activator of transcription, MMP; matrix metalloproteinase; AP, activating protein.

1.6.4 Fibroblast growth factor (FGF)

The FGF family is a family of proteins known to play a large variety of roles in cells, such as growth, differentiation and migration (Ellman *et al*, 2008). These proteins bind heparin and heparin sulphate (Friedl *et al* 1997). In terms of cartilage homeostasis, FGF 2 is believed to be the most important. FGF 2 is thought to act as a mechanotransducer in cartilage. FGF 2 is bound to the heparin sulphate proteoglycan perlecan component of cartilage (Vincent *et al*, 2007). During loading conditions, FGF 2 is released and activates signalling (Vincent and Saklatyala, 2006). The role of FGF 2 within cartilage is under debate, as it has been shown to be either catabolic or anabolic. FGF 2 has been shown to be protective in terms of cartilage degradation and arthritis. This may be via affecting aggrecanases, as it has been shown that the addition of FGF 2 to chondrocytes *in vitro* led to a decrease in IL-1-induced ADAMTS-4 and -5 (Sawaji *et al*, 2008). In addition, FGF 2 deficient mice showed increased ADAMTS-5, and accelerated OA (Chia *et al*, 2009). In terms of catabolic effects, FGF 2 has been shown to increase MMP-13 expression in chondrocytes, and

this appears to occur through the MAPK and NFκB pathways (Muddasani *et al*, 2007). As well as this, FGF 2 was shown to be increased in synovial tissue of patients with RA (Qu *et al*, 1995).

1.6.5 Protease activated receptor-2 (PAR-2)

PAR-2 is a G protein coupled receptor (Rothmeier and Ruf, 2012). As the name suggests PAR-2 is activated through proteolytic cleavage, more specifically, cleavage of its extracellular domain, by serine proteases (Troeberg and Nagase, 2012), one of which is matriptase (Milner *et al*, 2010). PAR-2 is considered to be important in arthritis, as PAR-2 deficient mice are protected against OA (Ferrell *et al*, 2010), as well as being protective in a CIA mouse model, which also showed lower levels of IL-6, TNF-α and IL-1β (Crilly *et al*, 2012). PAR-2 has also been shown to lead to an increase in MMP-1 and -13 expression *in vitro* (Boileau *et al*, 2007).

1.6.6 Phosphoinositide 3-kinase (PI3K)/Akt

The PI3K/Akt pathway plays many central roles in cellular signalling including that of cell survival, cell differentiation and cell cycle progression (Fayard *et al*, 2005), as well as influencing the MAPK pathways (Ren *et al*, 2010). The PI3K/Akt pathway is initially activated through the binding of an extracellular factor to its membrane bound receptor, known as a receptor protein tyrosine kinase (RPTK), which subsequently results in autophosphorylation of tyrosine residues on the receptor. PI3K then translocates to the membrane and binds to the RPTK, which then leads to activation of PI3K and the conversion of phopshoinositol-4,5-biphosphate (PIP2) to phopshoinositol-3,4,5-triphosphate (PIP3). The production of PIP3 leads to recruitment of Akt and its subsequent activation. Once activated Akt then translocates to the nucleus and regulates various targets (Meier *et al* 2005) (see Fig 1.6).

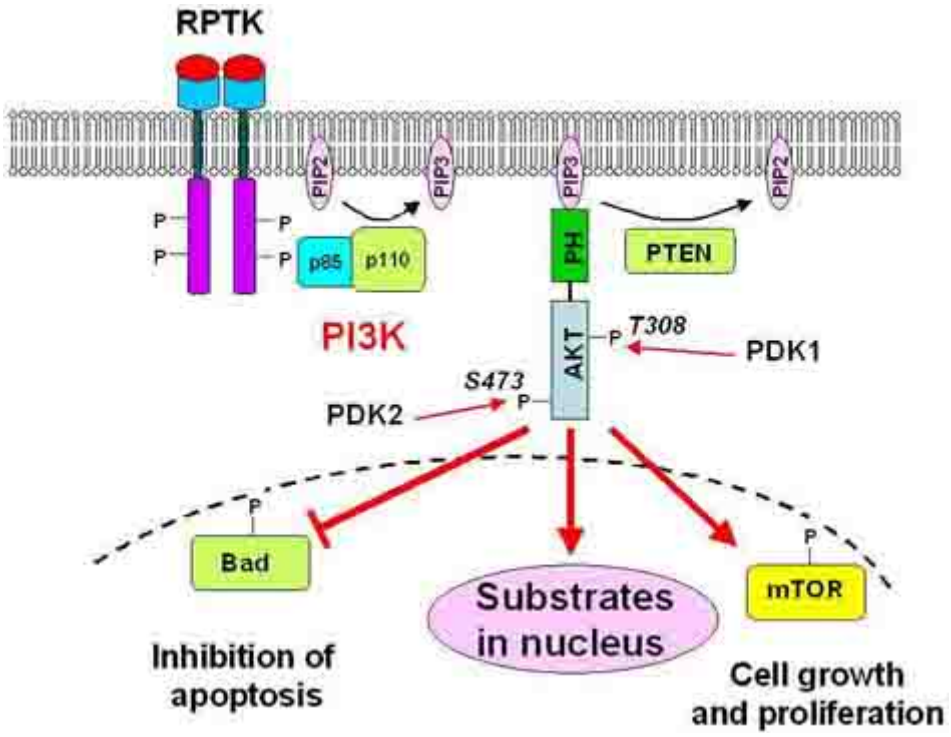


Figure 1.6. The PI3K/Akt pathway, demonstrating the various proteins involved in the signal transduction through the pathway. Taken from Meier *et al*. (2005). PI3K; phosphatidylinositol-3 kinase, RPTK; receptor protein tyrosine kinase, PIP2; phopshoinositol-4,5-biphosphate, PIP3; phopshoinositol-3,4,5-triphosphate, PH; pleckstrin homology, PTEN; phosphatase and tensin homologue deleted from chromosome 10, PDK; phosphoinositide-dependent kinase, mTOR; mammalian target of rapamycin.

The PI3K/Akt pathway has been shown to be up regulated by a number of pro-inflammatory cytokines involved in arthritis such as OSM, TGF-β and IL-6 (Godoy-Tundidor *et al*, 2005; Kim *et al*, 2002). As well as this, the PI3K/Akt pathway has been demonstrated to be involved in MMP-1 and -13 expression, as the inhibition of this pathway was shown to inhibit induction of these MMPs (Litherland *et al*, 2008). More specifically, it was demonstrated that the PI3K isoform p110α and the Akt isoform Akt1 were involved in MMP-1 and -13 induction, as well as Akt3, p110δ and PDK1 being involved in MMP-13 expression. In addition, inhibition of the PI3Kγ isoform was protective against cartilage degradation in an inflammatory mouse model (Hayer *et al*, 2009).

As stated previously, TIMPs are involved in the inhibition of MMP activity, and it has been shown that the PI3K/Akt pathway is required for the up regulation of TIMP-3 by TGF-β (Qureshi *et al*, 2007). As well as this the PI3K/Akt pathway has been shown to cooperate with other signalling pathways, such as the

MAPK pathway, which as previously demonstrated is important in arthritis signalling (Yart *et al*, 2002).

As has been shown with other signalling pathways discussed, the PI3K/Akt pathway may have somewhat opposing roles, being both anabolic and catabolic. Akt may be important in preventing chondrocyte apoptosis, as inhibition of Akt resulted in an increase in apoptosis in a chondrocytic cell line (Chrysis *et al*, 2005). In addition, it was demonstrated by Starkman *et al*. (2005) that IGF-1-stimulated proteoglycan synthesis by chondrocytes required PI3K.

1.7 Transcriptional control of MMPs

1.7.1 Activating protein (AP) 1

As mentioned earlier, AP-1 is a major transcription factor involved in the regulation of MMPs. The genes that encode the proteins of the AP-1 heterodimer are termed immediate early response genes, since they are induced within minutes of stimulation, independent of protein synthesis (Clark *et al*, 2008). The regulation of AP-1 occurs through a number of pathways including the MAPK pathway (Whitmarsh *et al*, 1996) (by IL-1), and the JAK/STAT pathway (Korzus *et al*, 1997) (through OSM as well as IL-1) (Catterall *et al*, 2001), with the MAP kinase pathway being studied most (see above for details). The importance of AP-1 in MMP regulation is highlighted by the fact that the majority of MMPs contain AP-1 binding sites in their promoters (Chunghong *et al*, 2007). A great deal of work has looked to study the regulation of the collagenolytic MMPs, including their regulation by AP-1 (Clark *et al*, 2008). In terms of MMP-1 and -13, the heterodimer of c-Fos and c-Jun has been shown to be the most important transcription factor in their regulation (Hu *et al* 1994; Saez *et al* 1995). However, other combinations have also been shown to be important, for instance a Fra-1 containing AP-1 complex has been shown to regulate MMP-1 (Hao *et al*, 2008). As well as this, the effect of AP-1 is abrogated by other transcription factors such as Ets and polyoma enhancer activator 3 (PEA3) which can work in conjunction with AP-1 (Sharrocks *et al* 1997; Benbow *et al* 1997). In many cases, AP-1 may be dependent on these

transcription factors for transcriptional activity, for instance an NFκB-like element was required for AP-1 to induce MMP-1 in rabbit fibroblasts during IL-1 stimulation (Vincenti and Brinckerhoff, 1998). As well as direct transcriptional activation, AP-1 has also been shown to regulate MMP-1 through binding to its promoter and preventing PPAR-γ from binding, which is a known inhibitor of MMP-1 (Francois *et al*, 2004). In addition, AP-1 has been shown to be increased in RA synovium (Asahara *et al*, 1997).

1.7.2 Activating transcription factor (ATF)

The family of transcription factors known as ATF are thought to form heterodimers with a number of other transcription factors, including their family members, AP-1 and C/EBP. These transcription factors are both repressors and activators of transcription (Hai and Hartman, 2001). ATF transcription factors have been shown to play a role in MMP expression. ATF4 is one of the proteins capable of forming the AP-1 heterodimer which regulates MMPs (Fung and Demple, 2007). ATF3 has been shown to suppress MMP-1 expression, and this was shown to be STAT1 dependent (Hao *et al*, 2008), and ATF2 has been associated with up regulation of MMP-1 (Westermarck *et al*, 2000). Also, ATF1 has been shown to increase expression of both MMP-2 and MT1-MMP (Melnikova *et al*, 2007). Although ATF3 is thought to be a transcriptional repressor, silencing of ATF3 was shown to suppress MMP-13 expression (Kwok *et al*, 2009), suggesting a greater complexity in the transcriptional control of MMP-13.

1.7.3 Hypoxia inducible factor (HIF)

The HIF transcription factors are heterodimeric, and mediate the response to altered oxygen levels (Lim *et al*, 2013). The HIF-α subunits (HIF-1α and HIF-2α) respond to oxygen levels, whilst the HIF-β subunit is constitutively expressed (Weisner *et al*, 2003). Hypoxia has been shown to be increased in OA (Yudoh *et al*, 2005), and in these hypoxic conditions HIF is no longer degraded, which

allows the heterodimerization of these subunits and transcriptional up regulation of their targets. In particular HIF-2α is thought to be important in OA. HIF-2α may play a role in arthritis through regulation of collagenase and aggrecanase expression, including MMP-1 and -13 and ADAMTS4, as well as other genes involved in cartilage destruction (Yang *et al*, 2010). In a mouse model it was demonstrated that HIF-2α contributed to cartilage destruction (Yang *et al*, 2010). As well as this, HIF-2α was shown to be increased in osteoarthritic tissue of both humans and mice (Saito *et al*, 2010).

1.7.4 Sry high mobility group box (SOX) 9

There are twenty SOX genes which have been identified, these are divided into eight groups, with SOX9 being a member of group E along with SOX8 and SOX10 (Akiyama, 2008). SOX9 is a major transcription factor important in cartilage development and chondrogenesis (Zhao *et al*, 1997). During this process it has been shown to be important in cartilage formation, including Col2a1 (Lefebvre *et al*, 1997) and aggrecan (Sekiya *et al*, 2000) expression. As well as this, it is thought that SOX9 is important in preventing chondrocyte hypertrophy during development (Bi *et al*, 2001). In addition, SOX9 depletion in mouse embryos demonstrated reduced chondrocyte proliferation, as well as reduced Indian hedgehog (Ihh) signalling (Akiyama *et al*, 2002). It is therefore thought that SOX9 may be important in normal cartilage homeostasis and preventing chondrocyte hypertrophy. Consistent with this, SOX9 has been shown to be down regulated in OA tissues (Brew *et al*, 2004). Chondrocyte hypertrophy occurs in development during the process of endochondral ossification, when cartilage which has been deposited is replaced by bone. During which the chondrocytes terminally differentiate and apoptose (Van der Kraan and Van den Berg, 2012). However, during arthritis chondrocytes have been shown to have characteristics of a hypertrophic phenotype, which include type X collagen (Nurminskya and Linsenmayer, 1996) and MMP-13 (Alvarez *et al*, 2000) expresssion. Proteins which regulate chondrocyte hypertrophy are therefore of great interest in arthritic disease.

1.7.5 Cytidine-Cytidine-Adenosine-Adenosine-Thymidine (CCAAT) enhanced-binding protein (C/EBP)

The C/EBP family are a group of transcription factors consisting of six proteins, C/EBP α, β, δ, ε, γ and ζ, which are involved in the differentiation of a number of tissues types (Yokoyama *et al*, 2011). C/EBPs are thought to play a role in arthritic disease, as they have been shown to be pro-inflammatory. C/EBP-β in particular has been shown to have binding motifs in the promoters of a number of pro-inflammatory cytokines including IL-6, IL-8 and TNF-α (Akira *et al*, 1990).

C/EBP can form a heterodimer with AP-1 proteins c-Fos and c-Jun (Friedman, 2007), as well as interacting with NFκB (Leclair *et al*, 1992). CEBP-β has been shown to regulate MMP-1, 3, 10 and 13 and ADAMTS-5 (Doyle *et al*, 1997; Armstrong *et al*, 2009; Hayashida *et al*, 2009; Tsushima *et al*, 2012). It has also been shown that C/EBP-β and the MAPKs combine within the same pathway to regulate MMPs. Raymond *et al*. (2006) demonstrated IL-1β induction of MMP-1 in chondrocytes occurred through ERK-dependent activation of C/EBP-β. C/EBP-β was also shown to work cooperatively with RUNX2 to regulate MMP-13 expression in chondrocytes, and this was thought to be induced by HIF-2α (Hirata *et al*, 2012). Whilst C/EBPδ depletion showed reduced arthritic damage in a mouse CIA model (Chang *et al*, 2012).

1.7.6 Nuclear factor kappa B (NFκB)

NFκB comprises a group of transcription factors, with five subunits identified consisting of NFκb1 (p50), NFκb2 (p52), Rel A (p65), Rel B and C Rel. They can form an array of homodimers and heterodimers, with the ability to regulate different genes. The most common dimer is p65:p50, which is involved in the canonical pathway (Chakraborty *et al*, 2010). NFκB is a major regulator in many cellular pathways, playing an important role in immune function and inflammation (Chakraborty *et al*, 2010). Due to its influence within the cell its activity is under tight control, most notably by IκB which binds and inhibits NFκB. Upon activation of the NFκB pathway, through cytokines such as IL-1β and TNF-α, inhibitor of IκB kinase (IKK) is activated, which subsequently

phosphorylates IκB, this leads to its proteasomal degradation and allows NFκB to translocate to the nucleus and have its effect (Roman-Blas and Jimenez, 2006; Chakraborty *et al*, 2010). NFκB is also regulated through other mechanisms, such as the phosphorylation of the protein subunits themselves (Perkins, 2006).

NFκB has been linked with arthritis as it is a key mediator of both IL-1 and TNF-α expression, both of which are key pro-inflammatory cytokines in arthritis, as previously mentioned (Kapoor *et al*, 2011). As well as this, NFκB has been shown to be increased in RA and OA (Han *et al*, 1998). In addition, activation of the NFκB pathway resulted in enhanced inflammation and RA-like symptoms in rats (Takk *et al*, 2001). NFκB has also been associated with MMP-1 and -13 expression (Vincenti and Brinckerhoff, 2002), and when NFκB is inhibited by constitutively expressing IκB, MMP-1 and -13 levels are reduced (Bondeson *et al*, 2000). It is also thought that NFκB can cooperate with AP-1 to regulate MMP-1 and -13 expression (Vincenti and Brinckerhoff, 2002). In addition, it has been demonstrated that NFκB can recruit B cell lymphoma-3 (Bcl-3), which serves to allow docking of transcriptional co-activators of MMPs – Tat interactive protein 60 (TIP60) and CREB binding protein (CBP)/p300 (Na *et al* 1998; Dechend *et al* 1999). Data by Elliott *et al*. (2002), would suggest that recruitment of Bcl-3 is important in MMP-1 regulation, as it was shown that Bcl-3 was required for MMP-1 gene expression in chondrocytes under IL-1β stimulation.

1.7.7 Runt related transcription factor (RUNX2)

RUNX2 is a transcription factor which has been shown to be important in development during endochondral ossification. It plays an important role in influencing chondrocyte hypertrophy (Takeda *et al*, 2001). It is therefore thought that RUNX2 may play a role in arthritis. RUNX2 is not expressed in normal cartilage, its expression is however enhanced in OA cartilage (Wang *et al*, 2004). In addition, RUNX2 has been shown to be important in the regulation of both MMP-13 and ADAMTS-5 *in vitro*, where it is shown to be catabolic

(Tetsunaga *et al*, 2011). RUNX2 has been shown to cooperate with AP-1 to regulate MMP expression, in particular MMP-13 (Selvamurugan *et al*, 1998).

1.8 Tribbles 1-3

Mammalian Tribbles (Trb) are a group of three proteins named Trb 1, 2 and 3, discovered in the late 1990s (Kiss-Toth *et al*, 2011). They were originally shown to regulate a protein named string, an orthologue of cell division cycle (cdc) 25 in *Drosophila* (Mata *et al* 2000). Trb1-3 have since been shown to play a role in many cellular pathways, including those involving diabetes, multiple sclerosis and cancer (Keeshan *et al*, 2006; Liu *et al*, 2010; Cavanillas *et al*, 2011). Trb1-3 proteins are highly conserved, especially between mouse and humans, and within the human Trb1-3 family there are many sequence similarities (Yokoyama and Nakamura, 2011).

1.8.1 Trb1-3 structure

Trb1-3 consist of 3 domains; an N-terminal domain, which has a high serine and proline content (Hegedus *et al*, 2007); this is indicative of a protein with a high turnover (Rechsteiner and Rodgers 1996). The N-terminal domain has also been suggested to be a region of high phosphorylation (Hegedus *et al*, 2007), and has been shown to be required for the nuclear localisation of Trb1 and Trb3 in Henrietta Lack (HeLa) cells (Kiss-Toth *et al*, 2006). Trb1-3 also consist of a central domain which contains a kinase-like domain (Hegedus *et al*, 2007). The kinase-like domain resembles a serine/threonine kinase and has many of the features of a kinase, however as yet no evidence has been shown that this domain functions as a kinase (Hegedus *et al*, 2007). This domain lacks certain common characteristics of a kinase, although lacking such characteristics does not necessarily make a kinase inactive (Hegedus *et al*, 2007). The kinase-like domain is highly conserved, suggesting that its function is important (Hegedus *et al*, 2007), and it has been shown to play a role in the binding of proteins (Eder *et al*, 2008). It has been suggested that Trb1-3 may act as decoy kinases

regulating other kinases (Hegedus *et al*, 2007). The C-terminus has also been shown to be involved in protein binding, including the MAP2K MEK1 (Yokoyama *et al*, 2010), and the E3 ubiquitin ligase constitutive photomorphogenic protein (COP) 1 (Qi *et al*, 2006).

1.8.2 Trb1-3 location

Trb1 and Trb3 have been shown to be predominantly present within the nucleus, with Trb2 localised to the cytoplasm (Kiss-Toth *et al*, 2006). Trb1-3 have been shown to be expressed in most human tissues, with the highest expression of Trb1-3 mRNA being found in the bone marrow, pancreas, peripheral blood leukocytes, thyroid gland and muscle (Kiss-Toth *et al*, 2004).

1.8.3 The regulation of Trb1-3

It is unknown how Trb1-3 are regulated; however there are a number of possibilities. Perhaps one way in which Trb1-3 are regulated is at the transcriptional level through mRNA degradation. It has been shown that Trb1 mRNA in particular has an extremely short half-life of less than 1 hour, with Trb2 and Trb3 having half-lives of 1.8 and 2.8 hours, respectively (Sharova *et al*, 2009). This study investigated the half-life of 19,977 genes and showed that less than 100 of these genes tested had a half-life of less than 1 hour, demonstrating the unusually rapid Trb1 mRNA turnover rate.

Trb3 has been shown to be regulated through micro (mi)RNA. In vascular smooth muscle cells, platelet derived growth factor-BB (PDGF-BB) has been shown to induce miRNA-24 which in turn down regulates Trb3 (Chan *et al*, 2010). In addition, miRNA-98 has been show to downregulate Trb2 (Xie *et al*, 2012). As well as this Ord *et al*. (2009) describe the existence of several human Trb3 isoforms which differ in their 5’ untranslated region (UTR), and it is thought that this may be a way of regulating Trb3 expression, by affecting factors such as mRNA stability (Hughes *et al*, 2006).

Trb3 protein levels appear to be controlled through proteasomal degradation. It has been shown that Trb3 interacts with an E3 ubiquitin ligase called seven in absentia homolog (SIAH) 1, which has been shown to cause polyubiquitination of Trb3 and direct it to the proteasome for degradation (Zhou *et al*, 2008). Due to the fact that, as mentioned, Trb3 has been shown to be a nuclear protein (Kiss-Toth *et al*, 2006), its degradation through the proteasomal pathway may at first appear unusual. However, it has been demonstrated that the ubiquitin proteasome system is also present and active within the nucleus (Rockel *et al*, 2005). Trb3 has also been shown to be regulated by PI3K during nutrient starvation in prostate cancer cells (Schwarzer *et al*, 2006). Inhibition of PI3K reduced Trb3 levels, and this was unique for Trb3 among the Trb1-3 proteins. A further level of complexity to the regulation of Trb1-3 is that they may respond to the same stimuli differently depending on the cell type. This has been shown through the regulation of Trb2 by IL-1; in THP-1 cells the addition of IL-1 leads to the up regulation of Trb2; in synoviocytes it is down regulated by the exact same stimulus (Sung *et al*, 2006). As well as these methods of Trb1-3 regulation, interactions with other proteins may also stabilise Trb1-3 proteins, as it has been shown that interaction of Trb1 and Trb3 with MAP2Ks increases the levels of these Tribbles (Kiss-Toth *et al*, 2004).

1.8.4 Trb1-3 regulation of signalling pathways

1.8.4.1 Trb1-3 and the MAPK pathway

Trb1-3 proteins have been shown to influence the MAP kinase pathways. They are thought to impact this pathway by binding to the MAP2Ks, as demonstrated by Kiss-Toth *et al*. (2004). Trb1 binds to MEK1 and MKK4, and it has been shown that the kinase-like domain is required for Trb1 interaction with MKK4 (Sung *et al*, 2007). Both Trb2 and Trb3 were demonstrated to bind MEK1 and MKK7 (Kiss-Toth *et al*, 2004). As with Trb1, it has been shown that the kinase-like domain is important in binding of Trb2 to these MAP2Ks (Eder *et al*, 2008).

It is thought that the ratio of Trb1-3 to MAP2Ks influences whether the MAPK pathway is up regulated or down regulated. It has been shown by Kiss-Toth *et*

al. (2004) that low levels of Trb3 can up regulate ERK and JNK but inhibit p38, whilst higher levels inhibit all 3 MAPKs. This, perhaps, is consistent with Trb1-3 acting as scaffolds, as it has previously been suggested by Sacks *et al*. (2006) that the ratio of the scaffold protein to its target proteins is essential in whether the pathway is activated or inhibited. Trb2 has been shown to inhibit both ERK and JNK activation via its interaction with MKK7 and MEK1 in monocytes (Eder *et al*, 2008). Interestingly, MAPKs may also play a role in Trb1-3 regulation, as it has recently been demonstrated that ERK could regulate Trb1 expression (Soubeyrand *et al*, 2013).

1.8.4.2 Trb3 and the TGF-β/Smad pathway

Trb3 has been shown to regulate TGF-β signalling and this may be through the stabilisation of Smad proteins (Park *et al*, 2009). It has been shown that Trb3 interacts with BMP receptor II; upon activation of BMP, Trb3 dissociates from the receptor. Trb3 can then subsequently interact with Smurf1 and Smurf2, which are E3 ubiquitin ligases involved in the degradation of various proteins within the BMP pathway, including Smads (Park *et al* 2009; Fang *et al*, 2011). Interaction of Trb3 with Smurf causes the ubiquitination of Smurf and its degradation through the proteasomal pathway, leading to an increase in Smad proteins, and an enhancement in BMP signalling (Park *et al* 2009). It has also been demonstrated that Trb3 interacts directly with Smad3 and this has been shown to increase nuclear localisation and therefore signalling of Smad3 (Hua *et al*, 2011).

In concordance with Trb3 regulating this pathway, depletion and overexpression of Trb3 resulted in a reduction and increase in TGF-β respectively (Chan, 2010; Chan *et al* 2007). Also, the overexpression of Smad3 and stimulation of TGF-β has been shown to increase Trb3 expression levels (Hua *et al*, 2011), which the authors suggest indicates Trb3 is involved in feedback control within this pathway. Interestingly, Trb3 may regulate TGF-β signalling through the JNK pathway, as previously mentioned, Trb3 can regulate the MAPKs, and JNK has been shown to regulate Smad4 (Giehl *et al*, 2007).

1.8.4.3 Trb2, Trb3 and the PI3K/Akt pathway

Trb2 and Trb3 have been shown to interact with Akt. Both Trb2 and Trb3 have been shown to bind Akt and prevent its phosphorylation (Du *et al*, 2003), inhibiting this pathway, and therefore being implicated in a number of diseases. There is a great deal of evidence suggesting that Trb3 may play a role in insulin signalling and diabetes, and this is thought to be through the regulation of Akt (Du *et al*, 2003). It has been suggested that this interaction of Trb3 with Akt may influence the IGF-1 signalling pathway (Cravero *et al*, 2009). Cravero *et al*. (2009) demonstrated that Trb3 was up regulated in OA cartilage, with overexpression of Trb3 leading to a reduction in Akt phosphorylation and an increase in chondrocyte death by approximately 40%. They proposed that the effect of Trb3 on Akt phosphorylation could be a factor in reduced response of chondrocytes to IGF-1 in OA. It is possible that Trb3 may influence MMP signalling by disruption of the insulin/IGF-1 signalling pathway, as it has previously been shown that addition of IGF-1 can lead to an increase in both MMP-1 and -13 in cartilage (Wheeler *et al*, 2009). Contradictory to this, IGF-1 was shown to reduce IL-1-induced MMP-1 and -13 expression in human articular chondrocytes (Hui *et al* 2001). It is also possible that the negative regulation of Akt by Trb2 and Trb3 may lead to regulation of MMPs by altering TIMP activity. As stated previously, TIMPs are involved in the inhibition of MMP activity, and it has been shown that the PI3K/Akt pathway is required for the up regulation of TIMP-3 by TGF-β (Qureshi *et al*, 2007). It is therefore possible that Trb2 and Trb3 prevent TIMP-3 induction by TGF-β. As well as this, Trb3 may regulate cartilage degradation through its interaction with Akt and disruption of insulin signalling (Du *et al*, 2003), as previous work has demonstrated that insulin prevents cartilage degradation in mice (Claassen *et al*, 2006). As mentioned previously, inhibition of PI3K reduced Trb3 levels, (Schwarzer *et al*, 2006) and Akt is regulated by PI3K (Fayard *et al*, 2005); this may suggest that Trb3 may be acting as a negative feedback regulatory mechanism to control PI3K induced Akt activation.

1.8.4.4 Trb1-3 and C/EBP

Trb1-3 have been shown to play a role in C/EBP regulation. Trb3 has been shown to interact with CCAAT/-enhancer-binding protein homologous protein (CHOP), which is a member of the C/EBP family (Ohoka *et al*, 2005). In addition, both CHOP and C/EBP-β bind to the Trb3 promoter, and are involved in Trb3 expression (Selim *et al*, 2007). As well as this, Trb1 and Trb2 have been shown to negatively regulate both C/EBP-α and C/EBP-β, by reducing their levels (Keeshan *et al*, 2006; Naiki *et al*, 2007; Yamamoto *et al*, 2007). It has been shown that Trb2 reduces the levels of C/EBP-α by increasing its proteasomal degradation, and binds directly to C/EBP-α (Keeshan *et al*, 2006).

1.8.4.5 Trb1, Trb3 and NFκB

Work by Kiss-Toth *et al*. (2006) suggested that Trb1 was not involved in NFκB signalling, however in contrast Ostertag *et al*. (2010) suggested that Trb1 may have a pro-inflammatory role in adipocytes involving NFκB. This study stated that Trb1 is induced via pro-inflammatory pathways and plays a role in this pro-inflammatory pathway through interaction with the p65 subunit of NFκB. Trb3 has also been shown to interact with p65, with overexpression of Trb3 inhibiting NFκB activation (Duggan *et al*, 2010), which would suggest a possible anti-inflammatory role for Trb3. In contrast to this, Kiss-Toth *et al*. (2004) demonstrated that both Trb3 overexpression and silencing showed no effect on NFκB. This work on Tribbles regulation of NFκB highlights an important feature of Tribbles, in that their effect on various signalling pathways, appears to be very cell type specific.

1.8.4.6 Trb1-3 and cytokines

Trb1-3 proteins have been shown to be involved in both pro-inflammatory and anti-inflammatory cytokine signalling. Trb2 has been shown to play a role in IL-8 signalling, negatively regulating this cytokine, with the silencing and

overexpression of Trb2 increasing and decreasing IL-8 levels, respectively (Eder *et al*, 2008). This therefore suggests an anti-inflammatory role for Trb2, since IL-8, as previously mentioned, is a pro-inflammatory mediator and has been shown to be up-regulated in arthritis (Sakao *et al*, 2009). In contrast to this, it is suggested that Trb2 may have a pro-inflammatory role, as it has been shown to reduce levels of (anti-inflammatory) IL-10 in macrophages (Deng *et al*, 2009; Woodell-May *et al*, 2011). Trb3 may have the ability to regulate a number of different cytokines, as Trb3 silencing in mast cells showed an increase in TNF-α, lL-4 and IL-6 (Kuo *et al*, 2012).

1.8.4.7 Trb1-3 and AP-1

It has previously been demonstrated that Trb1-3 proteins can regulate AP-1, therefore suggesting that they may play a role in MMP regulation through regulation of this transcription factor. It was shown in HeLa cells that Trb1, 2 and 3 were involved in the regulation of AP-1 (Kiss-Toth *et al*, 2004; Eder *et al*, 2008), where overexpression inhibited AP-1 activity. The kinase-like domain was required for this inhibition (Eder *et al*, 2008). Kiss-Toth *et al*. (2004) stated that both overexpression and silencing of Trb3 inhibited AP-1 activity. This possibly adds further evidence for Trb1-3 acting as scaffolds, as both overexpression and silencing of scaffolds has been shown to give similar effects (Morrison and Davis, 2003). It was also suggested that the regulation of AP-1 by Trb1-3 may be due to their regulation of the MAPK pathways, as the MAP3K MEKK1 mediated activation of AP-1 was inhibited by Trb1-3 (Kiss-Toth *et al*, 2004). However, in contrast to these findings, Hegedus *et al*. (2006) found that overexpression of Trb3 did not inhibit MEKK1 mediated activation of AP-1. This may demonstrate the cell type specificity of Trb1-3 action, as the work by Hegedus *et al*. (2006) was conducted in human embryonic kidney (HEK)-293 cells whereas the work by Kiss-Toth *et al*. (2004) involved HeLa cells.

1.8.4.8 Trb3 and ATF

It has been shown that Trb3 can interact with ATF4 (Bowers *et al*, 2003). Trb3 is involved in the feedback control of ATF4, inhibiting ATF4; ATF4 initially causes the up regulation of Trb3, then through negative feedback it is inhibited by Trb3 (Jousse *et al*, 2007). In addition, ATF3 levels are sustained by TGF-β1, which has been shown to be regulated by Trb3 (as discussed previously) (Kwok *et al*, 2009).

1.8.4.9 Trb3 and RUNX2

It was demonstrated by Park *et al*. (2009) that Trb3 silencing led to a reduction in RUNX2 during stimulation with the amiloride derivative phenamil, suggesting that Trb3 may regulate MMP-13 through this pathway.

1.8.4.10 Trb1-3 and disease

Recent studies of Trb1-3 have suggested that they may play an important role in cellular signalling, and it has been proposed that they are involved in disease states. They have been suggested to play a role in cancer, with Trb1 and Trb2 involved in acute myeloid leukaemia (AML). This role is thought to be due to the ability of Trb1 and Trb2 to influence C/EBPα (Keeshan *et al*, 2006; Gilby *et al*, 2010). Trb3 has been implicated in breast cancer, and this is thought to be through possible regulation of the MAPK and TGF-β pathways (Izrailit *et al*, 2013). In addition, Trb3 was shown to be up regulated in lung cancer, and depletion of Trb3 was shown to reduce the malignant behaviour of the cancer cells *in vitro* (Zhou *et al*, 2013).

As previously stated, there is a great deal of evidence implying that Trb3 may play a key role in insulin signalling and diabetes, possibly through the regulation of Akt (Liu *et al*, 2010). Trb3 in has been shown to be up regulated in a diabetic mouse model, and overexpression of Trb3 led to diabetes in mice (Du *et al*, 2003). Interestingly, it has been suggested that Trb2 may be an autoimmune

antigen; a genome wide association study has linked Trb2 with multiple sclerosis (Cavanillas *et al*, 2011), and anti Trb2 antibodies were seen in uveitis patients (Zhang *et al*, 2005), both of which are autoimmune diseases. This therefore possibly lends itself to Trb2 playing a role in rheumatoid arthritis by acting as an auto antigen.

As previously described, Trb3 has been implicated in OA (Cravero *et al*, 2009). Trb3 has also been shown to be up regulated in stress conditions such as ER stress and hypoxia (Ohoka *et al*, 2005; Bowers *et al*, 2003). Trb3 is possibly a mediator of ER stress, as depletion of Trb3 reversed the effect of ER stress on insulin signalling in cardiac myocytes (Avery *et al*, 2010). This is linked with arthritis as both hypoxia and ER stress have been implicated in arthritis (Yudoh *et al*, 2005; Takada *et al*, 2011).

1.9 Summary and aims of this study

Arthritic diseases, in particular osteoarthritis (OA) and rheumatoid arthritis (RA) are a major cause of disability throughout the world, having an enormous cost to healthcare organisations. Both OA and RA can be caused by a number of factors, and in both cases can be considered as a number of diseases under the same umbrella. Ultimately however, the final outcome is the same with excessive cartilage degradation by proteases.

Matrix metalloproteinases (MMPs) are a family of enzymes involved in cleavage of extracellular matrix proteins. They have many roles in both development and normal tissue homeostasis. As well as this they have been shown to be important in disease, one of which is arthritis. In terms of arthritis, the collagenases are perhaps the most important as they are known to degrade collagen type II, which is a key component of cartilage (Rowan *et al*, 2008), and when degraded cannot be replaced (Jubb *et al*, 1980). The two major collagenases are MMP-1 and -13. A greater understanding of the regulatory mechanisms of these MMPs could lead to the potential for new therapeutic arthritis treatments.

Trb1-3 are a group of proteins linked with diseases including diabetes, multiple sclerosis and cancer. Trb1-3 are reported to play a role in regulating many cellular signalling pathways, such as mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase/Akt (PI3K/Akt) and nuclear factor kappa B (NFκB). These pathways are considered important in mediating gene expression changes, including MMPs. In addition to this, Trb3 has also been shown to be up regulated in OA (Cravero *et al*, 2009). It is therefore hypothesised that Trb1-3 may regulate various signalling pathways within chondrocytes, in order to regulate MMPs, and control cartilage degradation.

1.9.1 Overall aim

- To assess whether Trb1 and Trb3 impact on cytokine-induced MMP expression in human chondrocytes.

1.9.2 Specific aims

- Gain a greater understanding of the regulation of Trb1 and Trb3 within the chondrocyte.
- Determine whether Trb1 and Trb3 regulate MMP-1 and MMP-13 within chondrocytes, and determine if this regulation was through known MMP transcription factors.
- Determine whether Trb1 and Trb3 interact with MAP2Ks, and in doing so regulate the MAPK pathway in chondrocytes.
- Determine whether Trb1 and Trb3 regulate other signalling pathways known to regulate MMPs, such as NFκB, PI3K/Akt and JAK/STAT in chondrocytes.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Antibodies

All antibodies are rabbit polyclonal, and were used at a concentration of 1:2000 in 5% BSA, unless otherwise stated. Phospho-Akt (Ser473) (#4060), phospho-STAT1 (ser727) (#8826), phospho-STAT1 (tyr701) (#9167), phospho-STAT3 (ser727) (#9134), phospho-STAT3 (tyr705) (#9145), phospho-ERK (thr202, tyr204) (#4370), phospho-p38 (thr180, tyr 182) (#4511), p65 (#8242), phospho-p65 (ser536) (#3033), c-Fos (#2250), Histone H3 (#9717B), Lamin A/C (#2032) and Caveolin 1 (#3267B) were from Cell Signalling Technology (Danvers, MA, USA). Trb1, made up in 5% milk, (#09-126) and mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody, (1:50000 dilution) (#MAB374) were from Millipore (MA, USA). Trb3 (#2488-1), c-Jun (#1254-1), phospho-JNK (Tyr 185, Tyr 223) (#2155-1) and MEK2 (#04-377) monoclonal antibodies and β-tubulin (#1799-1) were from Epitomics (Insight Biotech, Wembley). Mouse Ubiquitin antibody was from Enzo Life Sciences (Exeter, UK) Mouse c-Myc monoclonal antibody, (1:500 dilution), (#sc-40), phospho-c-JUN (thr93), (1:500 dilution) (#sc-101722) and ATF3, (1:500 dilution) (#sc-188) were from Santa Cruz (California, USA).

2.1.2 Cells

The human chondrosarcoma cell line, SW1353, and the human embryonic kidney cell line HEK 293T were from the American Type Culture Collection (ATCC) (Middlesex, UK). Mach1TM-T1® chemically competent *E.coli* were from Invitrogen (Paisley, UK).

2.1.3 Cell culture reagents

Dulbecco's Modified Eagle's Medium (DMEM), DMEM-F12, and foetal bovine serum (FBS) were from Invitrogen. Phosphate buffered saline (PBS) was from Lonza (Wokingham, UK). Penicillin-streptomycin solution (10,000 U/ml and 10 mg/ml, respectively), L-glutamine solution (200 mM), Nystatin suspension (10,000 U/ml), trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.5 g porcine trypsin and 0.2 g/L EDTA), hyaluronidase (from bovine testes, 439 U/mg), trypsin (from porcine pancreas, 1020 U/mg) and collagenase (from Clostridium histolyticum type I) were from Sigma-Aldrich (Poole, UK). Tryptone, yeast extract and bacto-agar were from Difco Laboratories (Detroit, MI, USA). Syringe filters (0.2 µm) were from Pall Life Sciences (Portsmouth, UK). Cell strainers (100 micron) were from Scientific Laboratory Supplies (Hessle, UK). Tissue culture plates and chamber slides were from Nunc, Fisher Scientific (Loughborough, UK).

2.1.4 Commercially available kits

Qiaprep Spin Midi/Maxi Kit was from Qiagen (Crawley, UK). Subcellular Protein Fractionation Kit, Nuclear Fractionation kit and c-Myc immunoprecipitation kit were from ThermoScientific (Loughborough, UK). Lenti-x-concentrator kit was from Clontech (Saint-Germain-en-Laye, France).

2.1.5 Molecular Biology Reagents

Dharmafect™ 1 lipid reagent and SMARTpool® small interfering RNA (siRNA) negative control (D-001810), Trb1 siRNA SMARTpool® (smartpool 1) (L-003633-00) and Trb3 siRNA SMARTpool® (L-003754-00) were from Dharmacon (Cramlington, UK), Trb1 # 1-6 (SI00138425, SI03032358, SI03054800, SI03100223, SI05066817, SI05066824) and AllStars negative control (#1027280) were from Qiagen. Trb1 siRNA smartpool 2 (SC-77704) was from Santa Cruz. TRIPZ inducible lentiviral shRNA negative control (RHS4743),

TRIPZ Trb1 shRNA # 1-3 (V2THS_137339, V2THS_137340, V2THS_137343), GIPZ non-silencing lentiviral shRNA control (RHS4346) and GIPZ Trb1 shRNA # A-D (V2LHS_405278, V2LHS_234767, V2LHS_137339, V2LHS_337823) were from ThermoScientific. JetPEI Transfection Reagent was from Polyplus transfection (Illkirch, France). Trb1 and Trb3 over-expression plasmids (see appendix A for plasmid maps) and GFP protein complementation assay (PCA) constructs Trb1V1, Trb1V2, Trb3V1, Trb3V2, NFκBV1, MEKV1, MKK4V1, MKK6V1 and MKK7V1 were a kind gift from Dr Endre Kiss-Toth (Sheffield University, UK). Sidestep™ Lysis and Stabilization Buffer was from Agilent Technologies, Stratagene Product Division (CA, USA) Cells-to-cDNA lysis buffer was from Ambion, (Huntington, UK). Real-time reverse transcriptase polymerase chain reaction (RT-PCR) primers were from Sigma-Aldrich. 18S rRNA, Trb1, Trb3, MMP-1 and MMP-13 probes were from Sigma-Aldrich. IL-8, c-jun, ATF3, lKKβ, MEK1, MKK4, MKK6 and MKK7 probes were from Roche (Burgess Hill, UK). c-fos gene expression assay on-demand was from Life Technologies (Paisley, UK). First strand buffer, Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase, Dithiothreitol (DTT) and RNaseOut Recombinant Ribonuclease inhibitor were from Invitrogen. VECTASHIELD mounting medium with DAPI was from Vector Labs (Peterborough, UK). DNase-free H_2O was from Sigma-Aldrich.

2.1.6 Immunoblotting reagents

Bradford Ultra was from Expendeon (Cambridgeshire, UK), Ammonium peroxodisulphate (APS) was obtained from BDH (Leicestershire, England). Bovine serum albumin (BSA), β-mercaptoethanol, N,N,N'N'-tetramethylenediamine (TEMED), 1,10-phenanthroline and polyoxyethylenesorbitan monolaurate (Tween 20) were obtained from Sigma-Aldrich. 40% (w/v) acrylamide/bis-acrylamide (37.5:1) solution was obtained from Severn Biotech (Worcestershire, UK). PageRuler™ prestained protein ladder was from ThermoScientific. Enhanced chemiluminescence (ECL) western blot detection reagents were from Amersham Biosciences (Little Chalfont, UK).

2.1.7 Inhibitors

UO126 (MEK inhibitor) was from Calbiochem (Nottingham, UK), MG132 (proteasome inhibitor) was from Sigma-Aldrich. All inhibitors were reconstituted in sterile filtered Dimethyl sulphoxide (DMSO) (Sigma-Aldrich).

2.1.8 Cytokines

Recombinant human IL-1α was a kind gift from GlaxoSmithKline (Stevenage, UK) and was stored at -20^{o}C prior to formulation at the appropriate concentration in the relevant culture medium. Recombinant OSM was prepared in-house (method described in Uddin *et al*, 2002) and stored at a stock concentration of 85 µg/ml at -80^{o}C prior to formulation at the appropriate concentration in the relevant culture medium.

All other standard laboratory chemicals and reagents, unless otherwise indicated, were commercially available from Sigma-Aldrich, Invitrogen or BDH Chemicals.

2.2 Methods

2.2.1 Isolation of Human Articular Chondrocytes (HACs)

Human cartilage from OA patients was observed macroscopically for normal cartilage and dissected. Cartilage pieces were then enzymatically digested, firstly in hyaluronidase (1 mg/ml, 4 ml/g of cartilage) at 37°C for 15 minutes, then trypsin (2.5 mg/ml, 4 ml/g) at 37°C for 30 minutes, and finally bacterial collagenase (2 mg/ml, 3 ml/g) overnight at 35°C. HAC were subsequently obtained by centrifugation of the digested material at 112 x *g* for 10 minutes after filtering debris through a 100 micron Nylon cell strainer. HAC were maintained in DMEM containing 10% (w/v) FBS, 400 IU penicillin, 400 µg/ml

streptomycin, 40 IU/ml nystatin and 2 mM L-glutamine. Consent was obtained from all patients, and the study was approved by the Newcastle and North Tyneside Joint Ethics Committee, REC reference 09/H00906/72.

Approximately one joint out of four per week was obtained which had sufficient normal cartilage for cell culture. An average of 8 million cells would be obtained from each viable joint, allowing for 2-3 HAC experiments per week.

2.2.2 Cell culture

Reagents:

- HAC were cultured in DMEM culture medium containing 10% (w/v) FBS, 2 mM L-glutamine, 400 IU/ml penicillin, 400 µg/ml streptomycin and 40 IU/ml Nystatin.
- SW1353 cells were cultured in DMEM in a 1:1 ratio with Ham's F-12 medium (DMEM:F12) containing 10% (w/v) FBS, 2 mM L-glutamine, 200 IU/ml penicillin and 200 µg/ml streptomycin.
- HEK293T cells were cultured in DMEM culture medium containing 10% (w/v) FBS, 2 mM L-glutamine, 200 IU/ml penicillin and 200 µg/ml streptomycin.

Method:

Cells were counted and seeded at a density of 20,000 cells/cm^2 for SW1353 cells and HEK293T cells and 40,000 cells/cm^2 for HAC into 96 well plates, chamber slides, 6-well plates, 6 cm dishes, 10 cm dishes or 500 cm^2 dishes. Cells were grown to the confluency required for the individual experiment, at 37°C in 5% (v/v) CO_2/humidified air.

2.2.3 Lentivirus production

HEK 293T cells were plated at the seeding density above into 500cm^2 dishes. Cells were grown to approximately 70-80% confluency. Cells were

subsequently transfected with the DNA plasmids; pCMV packaging plasmid, D2 envelope plasmid and lentivirus transfer vector plasmid, using jetPEI transfection reagent, 1:2 ratio (DNA:jetPEI) (see overexpression method for details). Medium was changed after 24 hours and then removed 72 hours after this. The medium was subsequently filtered through a 0.45 µm filter, aliquoted and frozen at -80°C. This was performed in medium containing heat inactivated serum, achieved through 56°C treatment for 30 minutes. Reproducibility of batches produced was assessed through comparison of transduction efficiency of each virus batch.

2.2.4 Lentivirus concentration

Lentiviral medium was removed and filtered as above. Lentivirus was then concentrated as per manufacturer's instructions. Briefly lentivirus medium was added to lentivirus-x-concentrator, 1:3 ratio (virus:concentrator). This was then incubated at 4°C for 30 minutes, and subsequently spun at 1500 x *g* for 45 minutes at 4°C. The lentivirus pellet was then resuspended in the desired volume of PBS, aliquoted and frozen at -80°C.

2.2.5 siRNA transfection of cells

Cells were seeded into 96 well plates, 6 well plates or 6 cm dishes at a seeding density above. Cells were grown to approximately 60% confluency. Medium was then removed and replaced with fresh medium (50 µl-96 well plate, 500 µl-6 well plate, 1.25 ml-6cm dish) then subsequently treated with siRNA diluted in serum-free medium to give a final concentration of 100 nM siRNA. siRNA had been mixed with Dharmafect^{TM} transfection reagent (0.26 µl/well for a 96 well plate, 3.25 µl/well for a 6 well plate and 8.125 µl/dish for a 6cm dish) for 20 minutes at room temperature prior to addition to cells for 48 hours. Cells were then serum-starved overnight before addition of IL-1α (0.5 ng/ml for SW1353 cells, 0.05 ng/ml for HAC) + OSM (10 ng/ml) or serum-free medium alone for a

designate time. Cells were then lysed or a nuclear fractionation performed for either immunoblotting or RT-PCR.

2.2.6 shRNA transfection of cells

Cells were seeded into 96 well plates or 6 well plates at a seeding density above. Cells were grown to approximately 60% confluency, then subsequently treated with shRNA at stated concentrations, in addition to 8 µg/ml polybrene in serum-free medium for 6 hours, then 20% (w/v) FBS medium added for 24 hours. Medium was then changed with 10% (w/v) FBS medium, and in the case of the inducible TRIPZ shRNA, 3 µg/ml doxycycline was also added. The cells were then incubated for 48 hours before visualisation by fluorescence microscopy. Cells were then lysed for either immunoblotting or RT-PCR.

Gene	Sequence
Trb1 smartpool 1	ACAACATAGACACTATGTA
	AGCGTGATTGCGAGAGCAC
	GAAACTCTTCTCCCTCCTG
	TACCGGCAAAGACTGAACA
Trb1 smartpool 2	ACACTTACTAATAACCGTT
	CGTTTGATGATTGTACGTA
	CGAGAGCACCAACGAGCTG
	TCCACCCTTAGGTCGGAGA
Trb1 #1	CATGACACATGAAACATGA
Trb1 #2	GACATAAACTCTTTACCGA
Trb1 #3	TAGTCATTGACAGAGAACC
Trb1 #4	GAAAACATCCACCCTTAGG
Trb1 #5	AGACAATCTATACAGGTCT
Trb1 #6	CGGAGACAATCAATACAGG
Trb3 smartpool	GATGCACCCTGGACTCTAT
	GTTGAATCTATGGCTCGCA
	CTTTGCTCGAGCTTCACCC
	CGTGACTCATATGGTCGTT

Table 2.1. siRNA sequences.

Gene	Sequence
Trb1 #1	AAGGGAAACAAATTTGCAG
Trb1 #2	TATTGGCCCAATTTGTCAC
Trb1 #3	TTCTCTACAAGTCAGAAAC
Trb1 #A	ATTACACACTTGACCACCG
Trb1 #B	TTATTACACACTTGACCAC
Trb1 #C	AAGGGAAACAAATTTGCAG
Trb1 #D	TTCTGAGTCGATGTACCCG

Table 2.2. shRNA sequences.

2.2.7 Addition of MG132 proteasomal inhibitor or UO126 MEK inhibitor to cells

Cells were seeded into 6 well plates at a seeding density above. Cells were grown to approximately 70-80% confluency. Cells were then serum-starved overnight before addition of 5 µM MG132 for 3 hours or 10µM UO126 for 1 hour. For UO126 treatment cells were then stimulated with IL-1α (0.05 ng/ml) + OSM (10 ng/ml) or serum-free medium for 20 minutes. Cells were then lysed for immunoblotting.

2.2.8 Overexpression plasmid transfection

Cells were seeded into 96 well plates, 6 well plates and chamber slides at a seeding density above. Cells were grown to 70-80% confluency. Plasmid DNA was then added (0.01 ng/cell for HAC and 0.005 ng/cell for SW1353 cells for standard overexpression and 0.01 ng/cell for protein complementation assay (PCA)). All plasmids were pcDNA3.1 vector. Control was pcDNA3.1 for standard overexpression, and venus 1 and venus 2 empty vectors for PCA. Cells were transfected using jetPEI transfection reagent in a 1:2 ratio (DNA:jetPEI) in 150mM NaCl in a volume of 10% of the final medium volume following the manufacturer's instructions. Briefly, jetPEI was vortexed and

added to 150mM NaCl, plasmid DNA was then added to an equal volume of NaCl. JetPEI NaCl solution was then vortexed and added to the DNA NaCl solution, vortexed and left for 20 minutes before addition to the cells. Medium was then changed after 6 hours. Cells were then incubated for 48 hours. Cells were then serum-starved overnight before addition of IL-1α (0.5 ng/ml for SW1353 cells, 0.05 ng/ml for HAC) + OSM (10 ng/ml) or serum-free medium alone. Cells were then lysed for either immunoblotting or RT-PCR.

For PCA; after 48 hours transfection, cells were starved for 6 hours, then washed twice in PBS and fixed using 4% (w/v) paraformaldehyde for 10 minutes. Cells were then washed a further 2 times in PBS and visualised using VECTASHIELD with DAPI mounting medium under a fluorescence microscope (Leica DMLB) (Leica, Milton Keynes UK), with SPOT ADVANCED software (Michigan, USA).

2.2.9 Preparation of plasmid DNA

Reagents:

- Luria broth (LB) containing 10 g NaCl, 10 g tryptone and 5 g yeast extract per litre dH_2O.
- LB Agar containing 10 g NaCl, 10 g tryptone, 5 g yeast extract and 15 g agar per litre dH_2O.
- Qiaprep Spin Midi/Maxi Kit.

Reagents were autoclaved prior to use and ampicillin added at a final concentration of 100 µg/ml in filter-sterilised water for selection. Standard aseptic technique was used throughout the procedure.

Mach1TM-T1® chemically competent *E.coli* (30 µl) and 0.5 µl plasmid (see appendix A for plasmid maps) was added to a 1.5 ml Eppendorf tube and

swirled to mix. The tube was then incubated on ice for 30 minutes and subjected to heat shock at 42°C for 30 seconds. The tube was incubated on ice for 2 minutes before the addition of 500 µl pre-warmed Super Optimal Broth with Catabolite Repression (SOC) medium (37°C). Tubes were incubated for 1 hour in a 37°C water bath. On pre-warmed agar plates containing 100 µg/ml ampicillin, 50 µl volumes were streaked out. Plates were allowed to dry for 5 minutes and then incubated at 37°C overnight. A single colony for each plasmid was picked and placed into a universal with 5 ml LB containing 100 µg/ml ampicillin. This was shaken at 37°C for 8 hours at 250 rpm before transferring the culture to a conical flask containing 250 ml LB containing 100 µg/ml ampicillin. The conical flasks were shaken at 37°C overnight at 250 rpm. Overnight cultures were centrifuged at 6000 x *g* for 15 minutes at 4°C to pellet cells. Plasmid DNA was then isolated using the Qiaprep Spin Midi/Maxi Kit as follows:-

The pellet was resuspended in 10 ml of buffer P1 containing RNase A. 10 ml of buffer P2 was then added, mixed by inverting 4-6 times and incubated at room temperature for 5 minutes. 10 ml of chilled buffer P3 was added, mixed by inverting 4-6 times and incubated on ice for 20 minutes. This was then centrifuged at 20,000 x *g* for 30 minutes at 4°C. The supernatant was then removed and centrifuged again at 20,000 x *g* for 15 minutes at 4°C. A QIAGEN-tip 500 was equilibrated by applying 10 ml buffer QBT and allowing the column to empty by gravity flow. The supernatant was then applied to the QIAGEN-tip, and allowed to enter through gravity flow. The QIAGEN-tip was then washed with 2 x 30 ml buffer QC. DNA was then eluted with 15 ml buffer QF, and the DNA precipitated with 10.5 ml room temperature isopropanol. This was then mixed and centrifuged at 15,000 x *g* for 30 minutes at 4°C. The supernatant was then removed and the DNA pellet washed with 5 ml room temperature 70% ethanol, and centrifuged at 15,000 x *g* for 10 minutes. The supernatant was removed, and the pellet air dried for 10 minutes, then re-dissolved in 250 µl dH_2O. DNA concentration was quantified by spectrophotometry (Nanodrop ND 1000 spectrophotometer, ThermoScientific) and stored at -20°C until required.

2.2.10 RT-PCR

Culture medium was removed and cells were washed once with ice-cold PBS. For HAC 20 µl SideStep™ Lysis and Stabilization Buffer was added to each well and the plate was then vortexed for 2 minutes to ensure complete cell lysis. 5 µl cell lysate was transferred to a new 96 well plate and diluted with 15 µl dH_20. 4 µl of the diluted cell lysate was transferred to a new 96 well plate for reverse transcription. To each well of the 96 well plate 1 µl random hexamers (0.2 µg/ml) 4µl of dH_20 and 3 µl dNTPs (2.5 mM) were added and the plate heated at 70°C for 5 minutes. For SW1353 cells 30 µl Cells-2-cDNA lysis buffer was added to each well, and the cells lysed by tip and transferred to a separate 96 well plate. The plate was then heated at 75°C for 10 minutes. 8 µl cell lysate was transferred to a new 96 well plate for reverse transcription. To each well of the 96 well plate 1 µl random hexamers (0.2 µg/ml) and 3 µl dNTPs (2.5 mM) were added and the plate heated at 70°C for 5 minutes. For both cell types the plate was immediately placed on ice where 4 µl 5x First Strand Synthesis Buffer, 2 µl DTT (0.1 M), 0.125 µl RNaseOut (40 U/µl), 1.375 µl dH_20 and 0.5 µl MMLV (200 U/µl) were added to each well. The plate was incubated at 37°C for 50 minutes, followed by 70°C for 15 minutes. 30 µl dH_2O was then added to each well prior to first use. SW1353 cDNA was diluted 1:100 and HAC cDNA was diluted 1:20 for housekeeping gene quantification. 5 µl cDNA was added to a 96 well PCR plate, along with 4.5 µl taqman gene expression master mix, 0.2 µl forward and reverse primers (30 µM) and 0.1 µl probe (15 µM). Relative quantification of genes was performed using the 7900HT system (Applied Biosystems, Foster City, CA). Cycling conditions were 10 minutes at 95°C, then 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C. The 18S ribosomal RNA gene was used as a housekeeping gene control to normalise for differences in RNA in each sample. Primers and probe sequences are shown in Table 1.

Gene	Forward primer	Reverse primer	Probe
18S rRNA	CGAATGGCTCATTAAATCAGTTATGG	TATTAGCTCTAGAATTACCACAGTTATCC	TCCTTTGGTCGCTCGCTCCTCTCCC
TRB1	CCCCAAAGCGAGGTGCCT	TACCCGGGTTCCAAGACG	CAGCCTCTTGAGAGGGGA
TRB3	CTGGCCGCTGTCTGGTTC	GGGCATCGGGTCCTGTCG	TGCCTCCTTCGTCGGGAG
MMP-1	AAGATGAAAGGTGGACCAACAATT	CCAAGAGAATGGCCGAGTTC	CAGAGAGTACAACTTACATCGTGTTGCGGCTC
MMP-13	AAATTATGGAGGAGATGCCCATT	TCCTTGGAGTGGTCAAGACCTAA	CTACAACTTGTTTCTTGTTGCTGCGCATGA
c-fos	ABI assay on demand #Hs00170630_m1		
c-jun	CCAAAGGATAGTGCGATGTTT	CTGTCCCTCTCCACTGCAAC	Universal probe library 19
ATF3	TTTGCCATCCAGAACAAGC	CATCTTCTTCAGGGGCTACCT	Universal probe library 53
IL-8	AGACAGCAGAGCACACACAAGC	AGGAAGGCTGCCAAGAGAG	Universal probe library 72
IKKβ	CTGAGCCAGCAAGAAGAGT	TGTGCCTCTTCTAGCAA	Universal probe library 83
MEK1	TCCAGCTTTCTTCAGGACTTG	TTCTACAGCGATGGCGAGAT	Universal probe library 58
MKK4	CAGCGATATCAATCGACATAC	GGCCAAAGTATAAAGAGCTTC	Universal probe library 33
MKK6	CCAGTTCCATTATAGGCTCCA	CAAGGCTTGCATTTCTATTGG	Universal probe library 8
MKK7	GGAGCTCTCTGAGGATGG	CGCAGGATCGACCTCAAC	Universal probe library 24

Table 2.3. Primers and probe sequences used for RT-PCR. TaqMan chemistry was used for analysis. Universal probe library probes were FAM labelled, all other probes were FAM-TAMRA labelled.

2.2.11 Western Blotting

Culture medium was removed, and cells washed with ice-cold PBS. Cells were then lysed with ice-cold lysis buffer (50 mM Tris-Cl, pH 7.5, 1.2 M glycerol, 1 mM EGTA, 1 mM EDTA, 1 mM Na_3VO_4, 10 mM 2-glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate, 1% (v/v) Triton X-100, 1 µM microcystin-LR; 0.1% (v/v) 2-mercaptoethanol, protease inhibitor cocktail. Cells were then scraped into the lysis buffer, vortexed thoroughly, incubated on ice for 20 minutes, before removal of particulate material by centrifugation at 13,000 x *g* for 5 minutes at 4°C. The supernatant was then removed and stored at -80ºC. For subcellular and nuclear fractionation, cells were isolated in PBS and pelleted at 500 x *g* for 5 minutes prior to the procedures described below.

Cell lysate samples had their protein content quantified through the Bradford assay, using BSA as a standard and Bradford Ultra reagent, which was subsequently measured using a Tecan Sunrise microplate reader (Tecan, Reading UK) at a wavelength of 495 nm to assess the colourimetric change. Samples were then made up in water to give equal amounts of protein per sample. 5 x Sample buffer (0.625 mM Tris HCl pH 6.8, 40% (v/v) glycerol, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol) was then added to 20 µl of each cell lysate sample and heated to 105°C for 5 minutes to denature the protein. Lysates/fractions were electrophoresed on 10% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE). Molecular weight markers were PageRuler pre-stained protein standards. Protein was transferred to polyvinilydene fluoride (PVDF) membranes using an iBlot gel transfer device (Invitrogen) according to manufacturer's instructions. Following transfer, the blot was blocked with TBS with 0.2% Tween 20 (TBS-T) containing 5% (w/v) non-fat dried milk (blocking buffer) for 1 hour at room temperature. The blot was then washed three times in 20 ml TBS-T for 5 minutes, and then incubated overnight at 4ºC with primary antibody diluted in TBS-T (containing 5% BSA or non-fat dried milk). The blot was then washed three times in 20 ml TBS-T for 10 minutes. The blot was incubated for 1 hour with either mouse or rabbit secondary antibody conjugated with horseradish peroxidase (HRP), and then washed three times in TBS-T for 10 minutes. Proteins were detected with enhanced chemiluminescent (ECL) reagent according to the manufacturer's

instructions, using the GeneGnome chemiluminescent detection machine (Syngene, Cambridge, UK).

2.2.12 Subcellular fractionation

Packed cell volume (µl)	**CEB (µl)**	**MEB (µl)**	**NEB (µl)**	**NEB (µl) + $CaCl_2$, MNase***	**PEB (µl)**
10	100	100	50	50	50
20	200	200	100	100	100
50	500	500	250	250	250

* MNase = Micrococcal nuclease

Table 2.4. Subcellular protein fractionation reagent volumes for different packed cell volumes.

Cytoplasmic extraction buffer (CEB), was added to the cell pellet, incubated on ice for 10 minutes with gentle mixing, then centrifuged at 500 x *g* for 5 minutes at 4°C. The supernatant was then removed and frozen at -80°C (cytoplasmic extract). Ice cold membrane extraction buffer (MEB) was added to the cell pellet, vortexed and incubated at 4°C for 10 minutes with gentle mixing, then centrifuged at 3000 x *g* for 5 minutes at 4°C. The supernatant was then removed and frozen at -80°C (membrane extract). Ice cold nuclear extraction buffer (NEB) was then added to the cell pellet, vortexed, and incubated at 4°C for 30 minutes with gentle mixing, then centrifuged at 5000 x *g* for 5 minutes at 4°C. The supernatant was then removed and frozen at -80°C (soluble nuclear extract). Chromatin bound extraction buffer was then prepared by adding 5 µl of 100 mM $CaCl_2$ and 3 µl of Micrococcal Nuclease per 100 µl NEB. Room temperature chromatin bound extraction buffer was then added to the pellet, vortexed, and incubated at room temperature for 15 minutes. After incubation the solution was then vortexed and centrifuged at 16,000 x *g* for 5 minutes at 4°C. The supernatant was then removed and frozen at -80°C (chromatin bound nuclear extract). Room temperature cytoskeletal extraction buffer (PEB) was then added to the cell pellet, vortexed and incubated for 10 minutes at room

temperature, then centrifuged at 16,000 x *g* for 5 minutes at 4°C. The supernatant was then removed and frozen at -80°C (cytoskeletal extract).

2.2.13 Nuclear fractionation

Packed cell volume (µl)	**CERI (µl)**	**CERII (µl)**	**NER (µl)**
10	100	5.5	50
20	200	11	100
50	500	27.5	250

Table 2.5. Nuclear protein fractionation reagent volumes for different packed cell volumes.

Cytoplasmic extraction reagent I (CERI) was added to the pellet, vortexed, and incubated on ice for 10 minutes. Cytoplasmic extraction reagent II (CERII) was then added, vortexed, incubated on ice for 1 minute, vortexed again, then centrifuged for 5 minutes at 16,000 x *g* at 4°C. The supernatant was then removed and frozen at -80°C (cytoplasmic extract). The cell pellet was then resuspended in nuclear extraction reagent (NER), vortexed every 10 minutes for 40 minutes, whilst on ice, then centrifuged for 10 minutes at 16,000 x *g* at 4°C. The supernatant was then removed and frozen at -80˚C (nuclear extract).

2.2.14 Immunoprecpitiation

Immunoprecipitation was carried out as per manufcaturers instructions. Briefly. cells were initially lysed. Lysates were then added to Pierce spin columns, followed by anit-c-Myc agarose slurry. Columns were then incubated with gentle end-over-end mixing overnight at 4ºC. Columns were then pulse centrifuged for 10 seconds and the flow through discarded. TBS-T wash solution was then added to each column and pulse centrifuged for 10 seconds, and the wash

discarded, this was repeated twice. Conditioning buffer was then added to the column and pulse centrifuged for 10 seconds and the flow through discarded. Non-reducing sample buffer was then added to each column, the column heated at 100ºC for 5 minutes, and pulse centrifuged for 10 seconds to obtain the sample.

2.3 Statistical analysis

Statistical analysis of data was performed by a Student's unpaired 2 sample t-test (Microsoft EXCEL) on standard error of the mean, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Chapter 3: RNA inhibition and overexpression optimisation

3.1 Introduction

In order to establish the effect that a specific protein has on various pathways it is common practice to alter these levels, whether *in vitro* or *in vivo*, and assess the effect of this change on the pathway. Therefore, in order to assess the effect of Trb1 and Trb3 on various signaling pathways it was first required to optimize the ability to both inhibit and overexpress Trb1 and Trb3 protein expression.

In order to inhibit Trb1 and Trb3 expression, two methods were assessed; this was small interfering RNA (siRNA) inhibition and short hairpin (shRNA) inhibition. These two methods essentially work on the same principle, which is inhibition of expression of specific genes through binding to RNA and preventing translation. The difference between siRNA and shRNA is that siRNA is simply transfected into a cell and is present only transiently. shRNA has an expression vector, often bacterial or viral, which integrates into the host genome, and the shRNA is constitutively expressed, meaning continued silencing of the gene. The shRNA is processed within the cell where one of the double strands is degraded. This shRNA then binds to a complementary sequence of mRNA. If there is complete complementarity then the mRNA is degraded, if there is only partial complementarity then mRNA translation is repressed.

Overexpression of Trb1 and Trb3 was achieved through transfection of plasmid DNA containing the sequence for the protein of interest into the cell. This plasmid DNA is then expressed using the cells expression machinery.

Throughout these results chapters only Trb1 and Trb3 were assessed. Trb2 was not assessed as it had previously been demonstrated within our lab that Trb2 had no effect on MMP-1 or -13 mRNA expression following pro-inflammatory stimulation (Duncan, 2010), therefore it was determined that Trb1 and Trb3 would be the sole focus of this thesis.

Both primary Human Articular Chondrocytes (HAC) and a human chondrosarcoma cell line (SW1353 cells) were used throughout this study. Both

cell types were used as it was not always possible to use primary cells, the reasons for which will be discussed later, therefore SW1353 cells were used as a model cell type for HAC.

The aims of this chapter were to:-

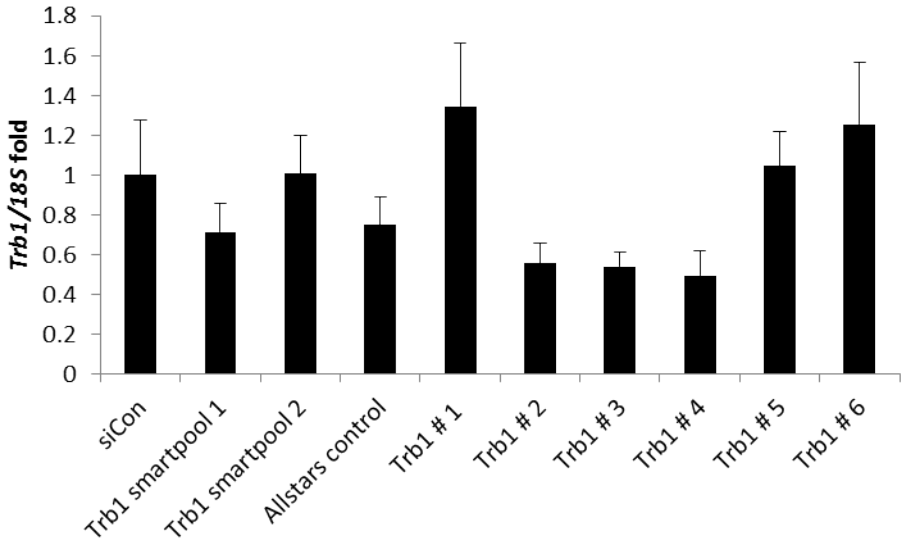
- Assess and optimise the ability to silence Trb1 and Trb3 using siRNA in SW1353 cells and HAC.
- Assess and optimise the ability to silence Trb1 using shRNA in SW1353 cells and HAC.
- Assess and optimise the ability to overexpress Trb1 and Trb3 in SW1353 cells and HAC.

3.2 Results

3.2.1 Trb1 gene silencing using siRNA in SW1353 cells and HAC

It was initially investigated whether it was possible to silence *Trb1* gene expression using siRNA. A number of different siRNAs were tested. This included pooled siRNA of 3 different siRNAs (Trb1 smartpool 1 and 2), as well as individual siRNA (Trb1 # 1-6). *Trb1* siRNA gene silencing was tested in both SW1353 cells and HAC. Trb1 smartpool 1 and 2 were compared to the siCon smartpool control, and Trb1 # 1-6 individual siRNAs were compared to the AllStars individual siRNA control. It was not possible to reproducibly silence *Trb1* to a sufficient level in either SW1353 cells (Fig 3.1A) or HAC (Fig 3.1B) using siRNA. Figures 3.1A and 3.1B demonstrate silencing after 24 hours, this was also attempted at a number of other time points (data not shown). 100 nmol siRNA was used as this was previously shown to be the optimum concentration to use for SW1353 cells and HAC without becoming toxic to the cells.

A



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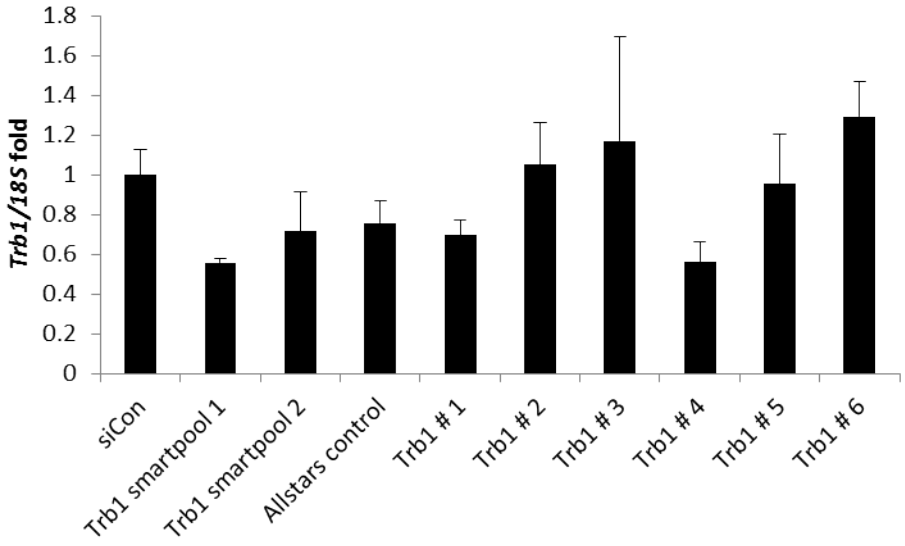


Figure 3.1. *Trb1* gene silencing optimisation using siRNA in SW1353 cells and HAC. mRNA expression analysis of *Trb1* after transfection with Trb1 siRNA of SW1353 cells (A) and HAC (B). Cells were transfected with siRNA in 96 well plates (100 nmol/well) for 24 hours and then serum-starved for 6 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *Trb1* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate (+/-standard error of the mean). Trb1 smartpool 1 and 2 refer to two different pools of three different Trb1 siRNA. Trb1 # 1-6 refer to 6 different individual Trb1 siRNA. Results are representative of 2 independent experiments, from 2 separate donors in regard to HAC. Ct values for siCon were 10.14 +/- 0.39 for 18S and 27.42 +/- 0.35 for *Trb1* for SW1353, and 17.38 +/- 0.19 for 18S and 32.77 +/- 0.10 for *Trb1* for HAC (+/- = standard error of the mean).

3.2.2 Trb3 gene silencing using siRNA in SW1353 cells and HAC

It was demonstrated that *Trb3* mRNA expression could be successfully silenced in SW1353 cells (Fig 3.2). 100 nmol siRNA was used, for the reason discussed above.

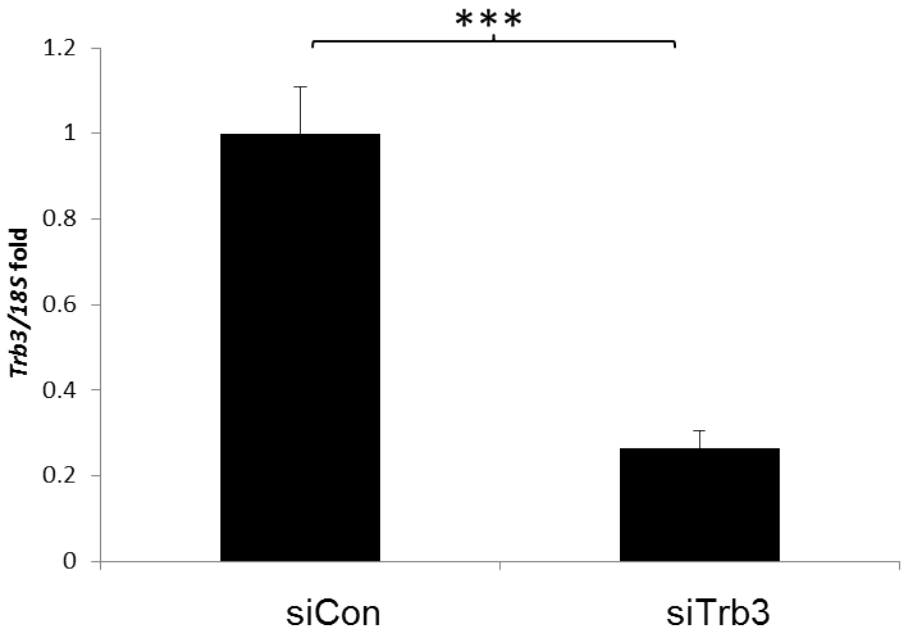
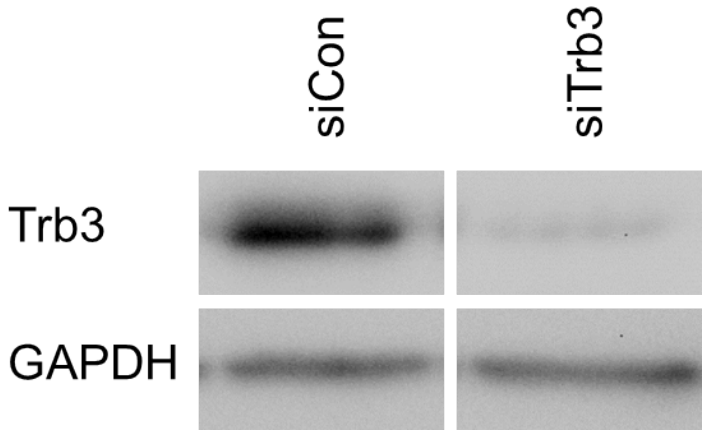


Figure 3.2. *Trb3* gene silencing using siRNA in SW1353 cells. mRNA expression analysis of *Trb3* after transfection with Trb3 siRNA in SW1353 cells. Cells were transfected with Trb3 siRNA in 96 well plates (100 nmol/well) for 24 hours and serum-starved for 6 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *Trb3* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean), ***p<0.001. Results are representative of 2 independent experiments. Ct values for siCon were 14.48 +/- 0.08 for 18S and 26.96 +/- 0.18 for *Trb3* (+/- = standard error of the mean).

Trb3 protein expression was also depleted when Trb3 was silenced using siRNA in SW1353 cells (Fig 3.3).

A



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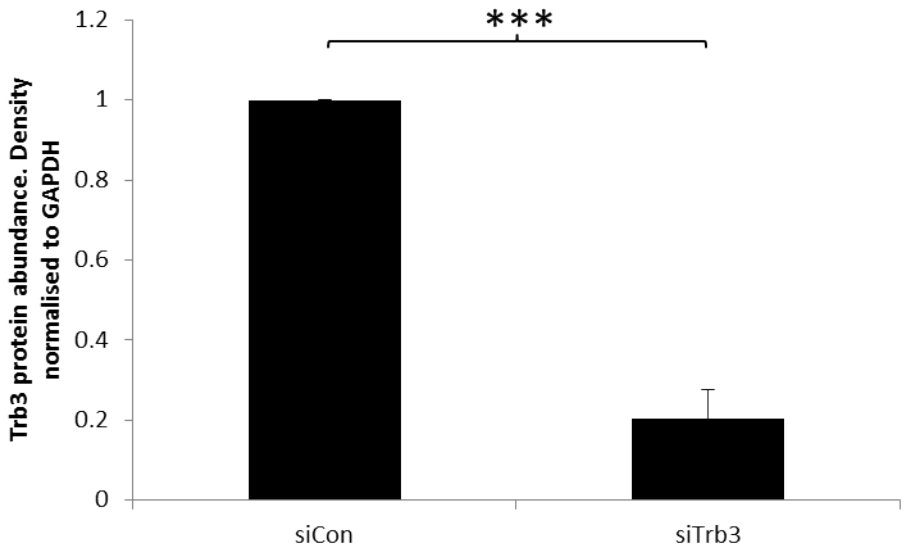


Figure 3.3. *Trb3* gene silencing using siRNA effect on Trb3 protein expression in SW1353 cells. A. Trb3 and GAPDH abundance after transfection with Trb3 siRNA in SW1353 cells. Cells were transfected with Trb3 siRNA in 6 well plates (100 nmol/well) for 48 hours and serum-starved for 6 hours. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Lanes were extracted from the same membrane. Results are representative of approximately 30 independent experiments. B. Combined densitometric scans from 4 separate blots representative of approximately 30 blots performed. Values were normalised to GAPDH. Results are +/-standard deviation of the mean, ***p<0.001.

It was then demonstrated that *Trb3* mRNA expression was successfully silenced using Trb3 siRNA in HAC (Fig 3.4). 100 nmol siRNA was again used.

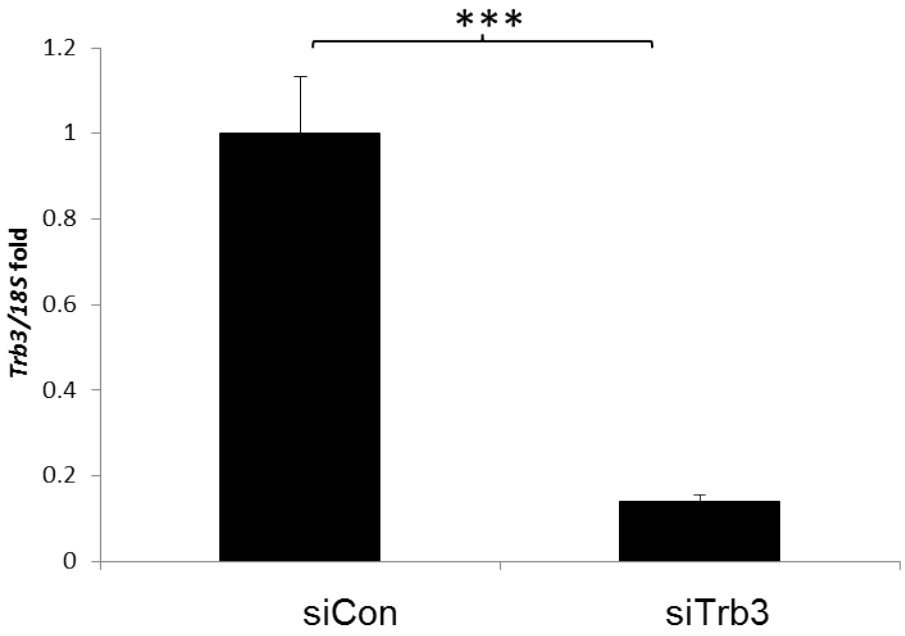
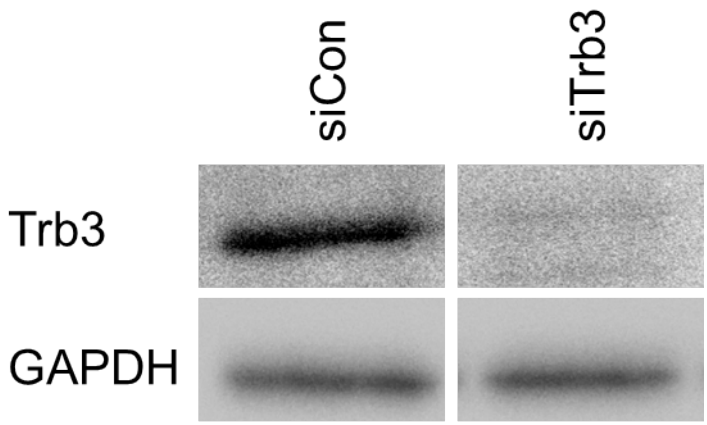


Figure 3.4. *Trb3* gene silencing using siRNA in HAC. mRNA expression analysis of *Trb3* after transfection with Trb3 siRNA in HAC. Cells were transfected with Trb3 siRNA in 96 well plates (100 nmol/well) for 24 hours and serum-starved for 6 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *Trb3* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). ***p<0.001. Results are representative of 2 independent experiments, from 2 separate donors. Ct values for siCon were 17.01 +/- 0.10 for 18S and 35.53 +/- 0.24 for *Trb3* (+/- = standard error of the mean).

Trb3 protein expression was also depleted when Trb3 was silenced using siRNA in HAC (Fig 3.5).

A



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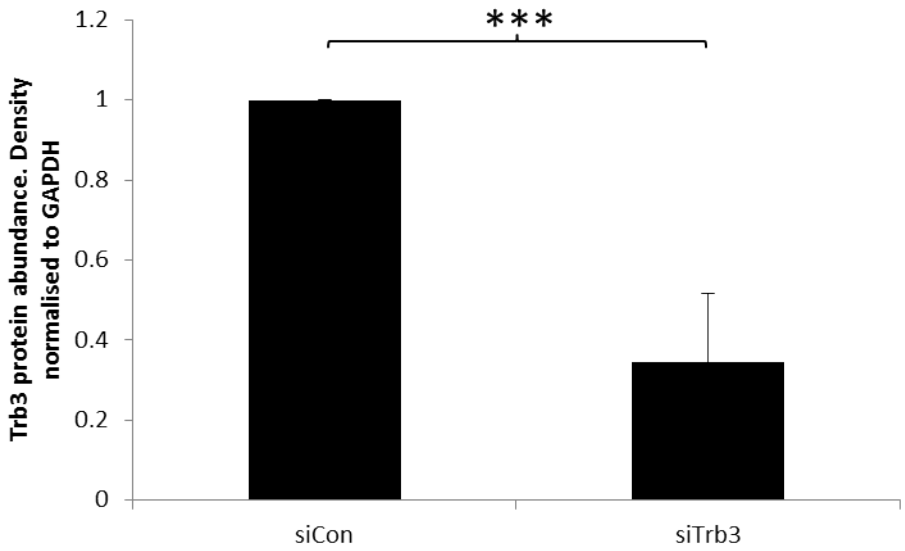


Figure 3.5. *Trb3* gene silencing using siRNA effect on Trb3 protein expression in HAC. A. Trb3 and GAPDH abundance after transfection with Trb3 siRNA in HAC. Cells were transfected with Trb3 siRNA in 6 well plates (100 nmol/well) for 48 hours and serum-starved for 6 hours. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Lanes were extracted from the same membrane. Results are representative of approximately 30 independent experiments, from 30 separate donors. B. Combined densitometric scans from 4 separate blots representative of approximately 30 blots performed. Values were normalised to GAPDH. Results are +/- standard deviation of the mean, ***p<0.001.

3.2.3 Trb1 overexpression optimisation in SW1353 cells and HAC

It was assessed whether Trb1 protein could be overexpressed in SW1353 cells. Trb1 was successfully overexpressed, as shown by the overexpressed Trb1 above the native Trb1 (Fig 3.6). It was decided not to go above 0.9 µg of plasmid DNA as this amount gave good overexpression, and the transfection reagent used can be toxic to the cells, therefore as low an amount as possible to get good overexpression was used. Overexpressed Trb1 gave a larger than expected band (55kDa) compared to the native Trb1 (40kDa).

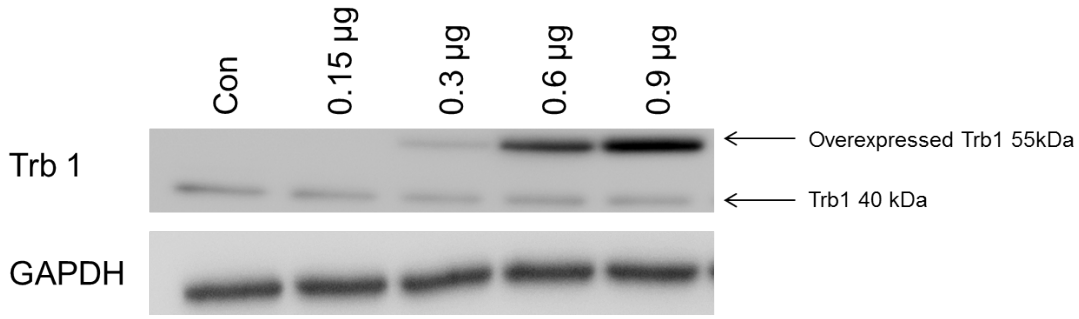


Figure 3.6. Trb1 overexpression optimisation in SW1353 cells. Trb1 and GAPDH abundance after transfection with Trb1 overexpression plasmid in SW1353 cells. Cells were transfected with Trb1 overexpression plasmid in 6 well plates, as indicated, for 48 hours and then serum-starved for 6 hours. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 2 independent experiments.

It was then assessed whether Trb1 could be overexpressed in HAC. Trb1 was successfully overexpressed, as can be seen by the overexpressed Trb1 protein above the native Trb1 (Fig 3.7). The amount of overexpressed protein increased with the amount of plasmid added up to 4 µg, whilst 6 µg did not generate any more overexpressed protein (Fig 3.7). As seen for SW1353 cells, overexpressed Trb1 gave a larger than expected band (55kDa) compared to the native Trb1 (40kDa).

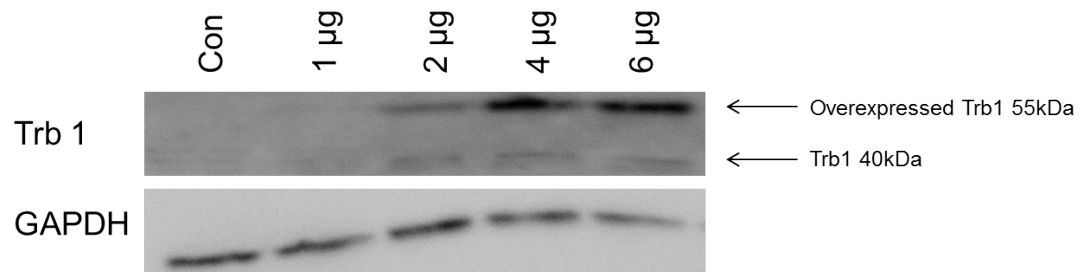


Figure 3.7. Trb1 overexpression optimisation in HAC. Trb1 and GAPDH abundance after transfection with Trb1 overexpression plasmid in HAC. Cells were transfected with Trb1 overexpression plasmid in 6 well plates, as indicated, for 48 hours and then serum-starved for 6 hours. Cells were then lysed and proteins were separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 2 independent experiments, from 2 separate donors..

3.2.4 Trb1 immunoprecipitation in SW1353 cells

Trb1 was also immunoprecipitated in SW1353 cells. Overexpressed Trb1 was immunoprecipitated through its c-Myc tag. Immunoprecipitated overexpressed Trb1 also gave a larger than expected band (55kDa) compared to the native Trb1 (40kDa) (Fig 3.8).

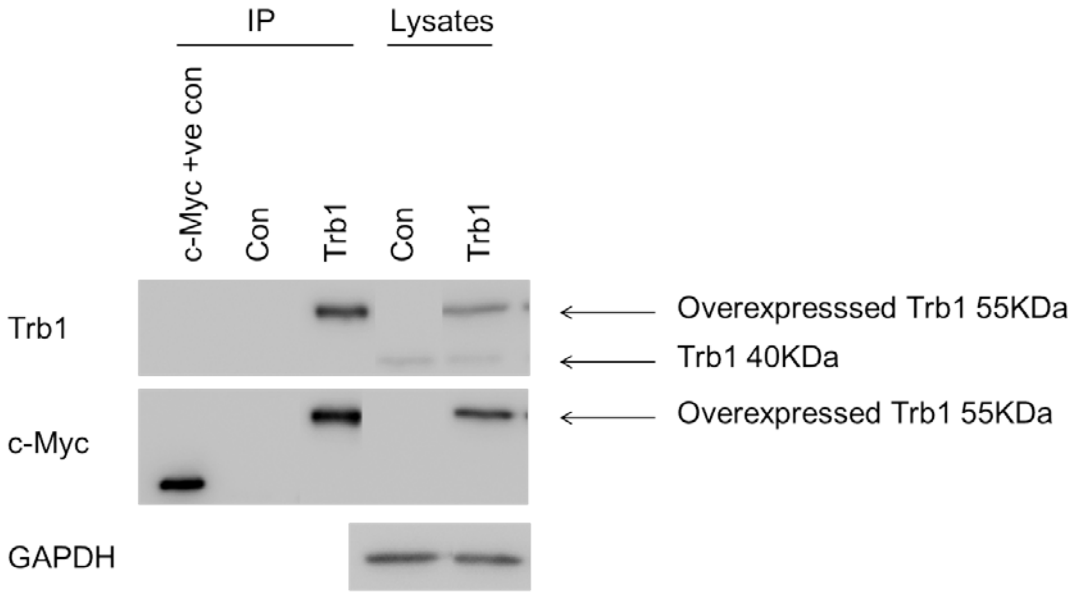


Figure 3.8. Trb1 immunoprecipitation in SW1353 cells. Trb1, c-Myc and GAPDH abundance after transfection with Trb1 overexpression plasmid in SW1353 cells. Cells were transfected with Trb1 overexpression plasmid in 6 well plates, as indicated, for 48 hours and then serum-starved for 6 hours. Cells were then lysed, and immunoprecipitation performed. and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 2 independent experiments.

3.2.5 Trb3 overexpression optimisation in SW1353 cells and HAC

It was assessed whether Trb3 could be overexpressed at the protein level in SW1353 cells. Trb3 was successfully overexpressed as observed by the c-Myc tag on the overexpression plasmid (Fig 3.9). For reasons unknown, overexpressed Trb3 was not detected by the Trb3 antibody in SW1353 cells. Both the c-Myc tagged Trb3 protein and the endogenous Trb3 were approximately the same size of 45 kDa, providing further evidence that the c-Myc antibody was in fact detecting the overexpressed Trb3. The amount of overexpressed protein increased with the amount of plasmid DNA used; 1.2 µg gave the highest overexpression (Fig 3.9). As before for Trb1 optimisation in SW1353 cells, it was decided not to go above 1.2 µg as this amount gave good overexpression, and the transfection reagent used can be toxic to the cells, therefore as low an amount as possible to get good overexpression was used.

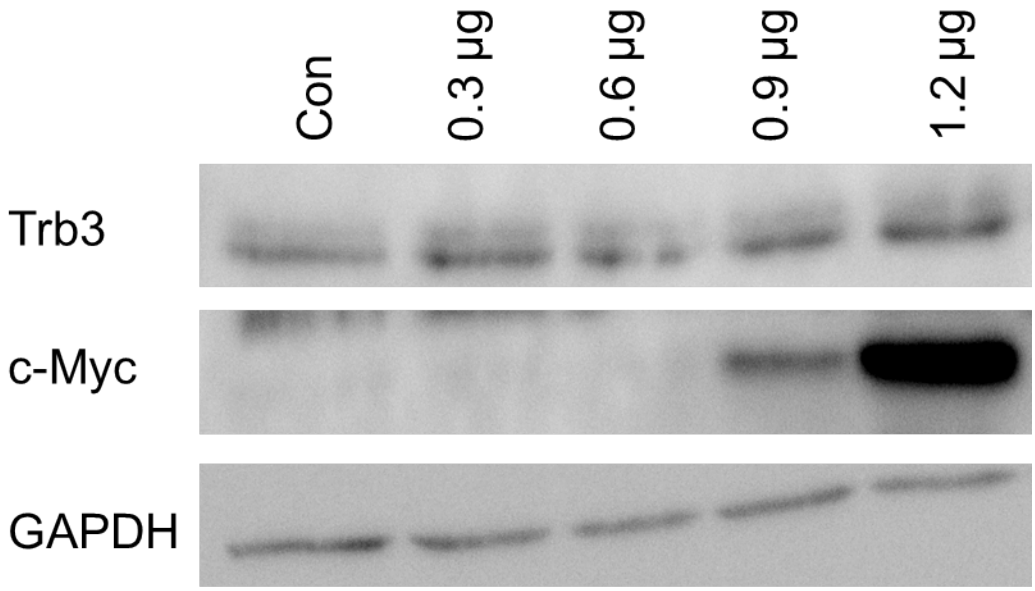


Figure 3.9. Trb3 overexpression optimisation in SW1353 cells. Trb3, c-Myc-tagged Trb3 and GAPDH abundance after transfection with Trb3 overexpression plasmid in SW1353 cells. Cells were transfected with Trb3 overexpression plasmid in 6 well plates, as indicated, for 48 hours and then serum-starved for 6 hours. Cells were then lysed and proteins were separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 2 independent experiments.

It was then assessed whether Trb3 could be overexpressed in HAC. Trb3 was successfully overexpressed, as can be seen by the c-Myc tag on the overexpression plasmid (Fig 3.10). Interestingly it appeared that the Trb3 antibody could also detect the overexpressed c-Myc-tagged Trb3 in HAC, which mimicked the increase in protein expression seen with the c-Myc antibody. The amount of overexpressed protein increased with the amount of plasmid added up to 4µg, whilst 6µg did not give any more overexpressed protein (Fig 3.10).

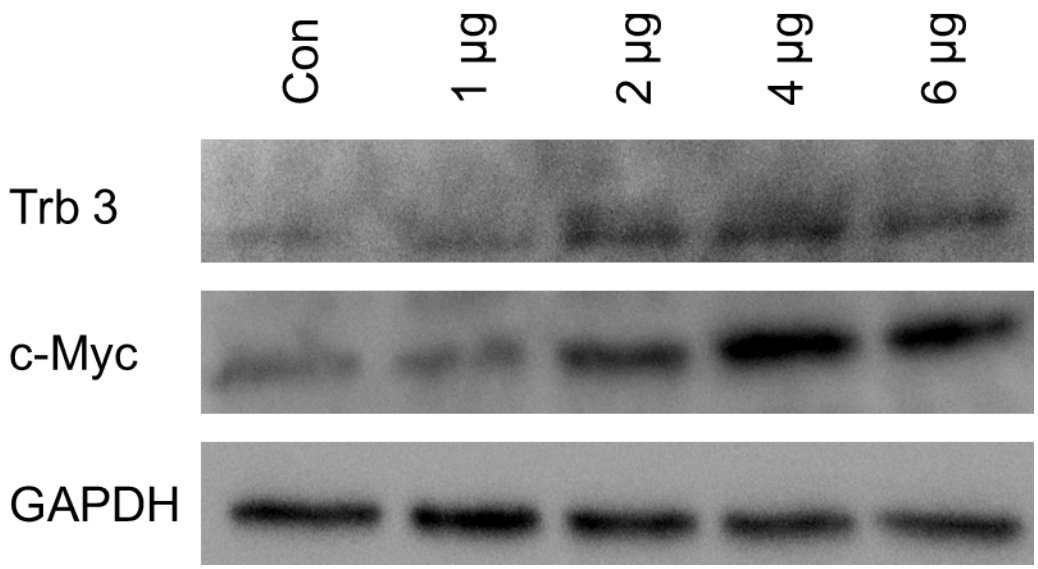


Figure 3.10. Trb3 overexpression optimisation in HAC. Trb3, c-Myc-tagged Trb3 and GAPDH abundance after transfection with Trb3 overexpression plasmid in HAC. Cells were transfected with Trb3 overexpression plasmid in 6 well plates, as indicated, for 48 hours and then serum-starved for 6 hours. Cells were then lysed and proteins were separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 2 independent experiments, from 2 separate donors.

3.2.6 Trb1 gene silencing optimisation using shRNA in SW1353 cells and HAC

3.2.6.1 Optimisation of GFP lentiviral shRNA in HAC and SW1353 cells

Due to the inability to silence *Trb1* using siRNA, silencing using shRNA was then attempted. In order to initially assess the feasibility of lentiviral transduction in both SW1353 cells and HAC, as well as optimising the technique, a lentiviral GFP was used. SW1353 cells were initially assessed due to ease of transduction. Specific volumes of virus were used, and transfection efficiency assessed. The volumes used were a percentage of the total volume, for instance 100% virus was pure virus-containing supernatant added to the cells, 50% virus was half virus-containing supernatant and half medium. It was demonstrated that virus production was successful, and the method of transduction was successful, as fluorescence from the GFP lentiviral empty

vector shRNA was detected within the cells (Fig 3.11). The best transduction efficiency was with 100% virus, which gave approximately 50% transduction, with transduction efficiency decreasing with lower concentrations (all transduction efficiencies were approximated by eye).

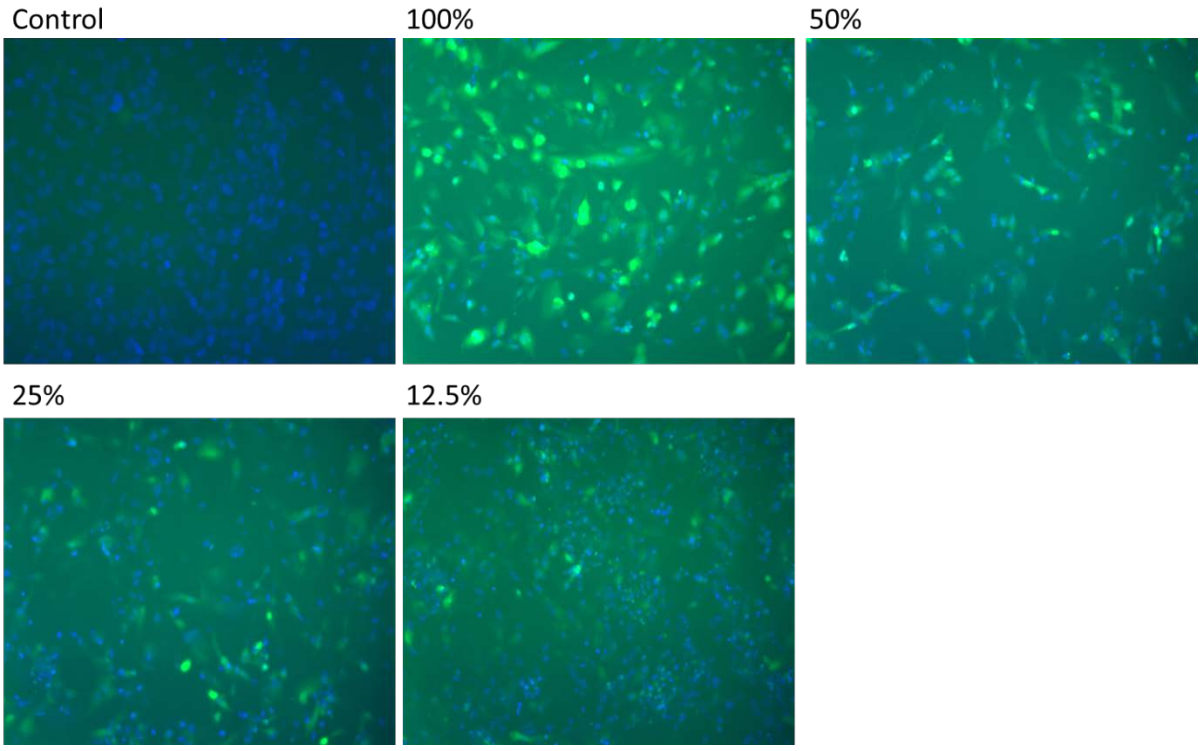


Figure 3.11. Optimisation of GFP lentiviral shRNA using different concentrations of shRNA in SW1353 cells. Fluorescent images of lentiviral GFP shRNA expression in SW1353 cells. Cells were transduced with lentivirus shRNA in 8 well chamber slides (12.5%, 25%, 50% and 100%) with 8 µg/ml polybrene for 24 hours, medium was then changed and the cells incubated for a further 48 hours, as detailed in the Materials and Methods, then fixed and visualised. Results are representative of a single experiment.

The same experiment as above was then performed on HAC, in order to test transduction efficiency. This was performed using live imaging, rather than fixing the cells, which was used from then on. It was observed that it was possible to transduce HAC with lentivirus. 100% lentivirus gave the best transduction efficiency with efficiency reducing with lower concentrations (Fig 3.12). Transduction efficiency was lower than that in SW1353 cells, with approximately 20% efficiency for 100% lentivirus.

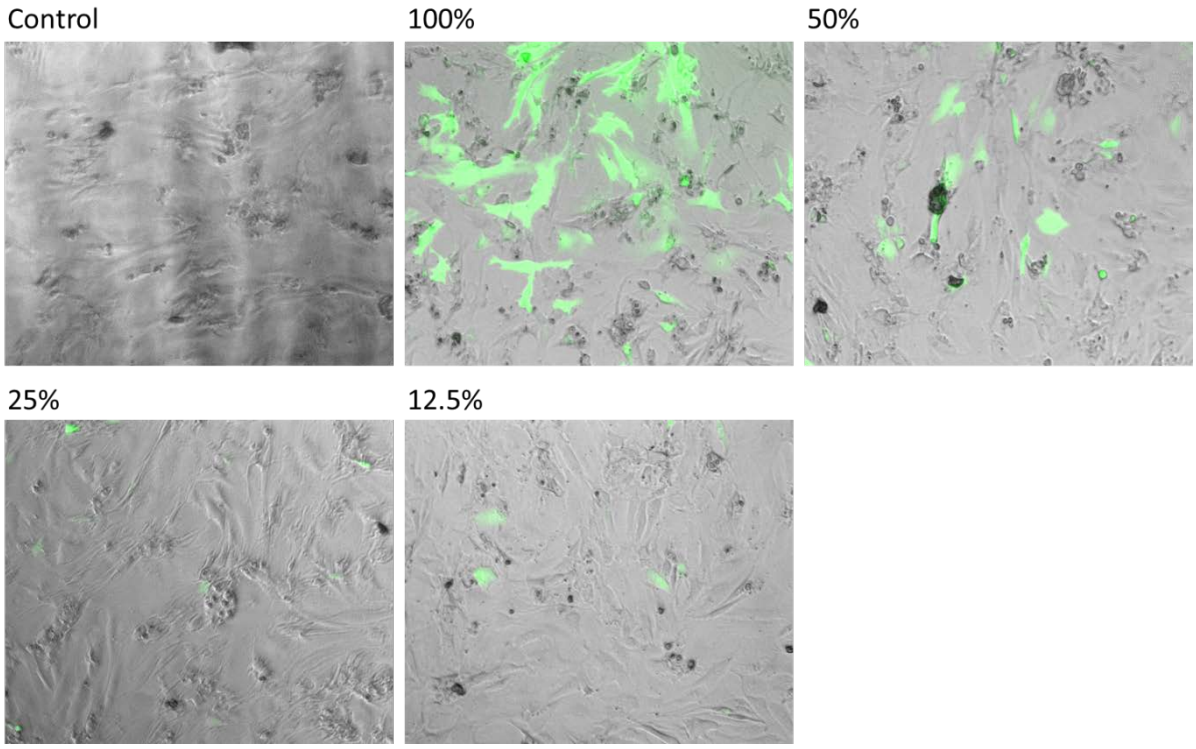


Figure 3.12. Optimisation of GFP lentiviral shRNA using different concentrations of shRNA in HAC. Fluorescent images of lentiviral GFP shRNA expression in HAC. Cells were transduced with lentivirus shRNA in 96 well plates (12.5%, 25%, 50% and 100%) with 8 µg/ml polybrene for 24 hours, medium was then changed and the cells incubated for a further 48 hours, as detailed in the Materials and Methods, then visualised. Results are representative of a single experiment.

3.2.6.2 Optimisation of polybrene concentration in HAC

Different concentrations of polybrene were tested in order to increase transduction efficiency in HAC. Polybrene is a cationic polymer which acts through “charge shielding” by neutralising the electrostatic repulsion forces between the lentivirus and sialic acid on the cell surface (Davis *et al*, 2004), therefore increasing transduction efficiency. The best concentration appeared to be 6-8 µg/ml of polybrene, concentrations above or below this range showed a decrease in transduction (Fig 3.13), therefore 8 µg/ml was continued to be used.

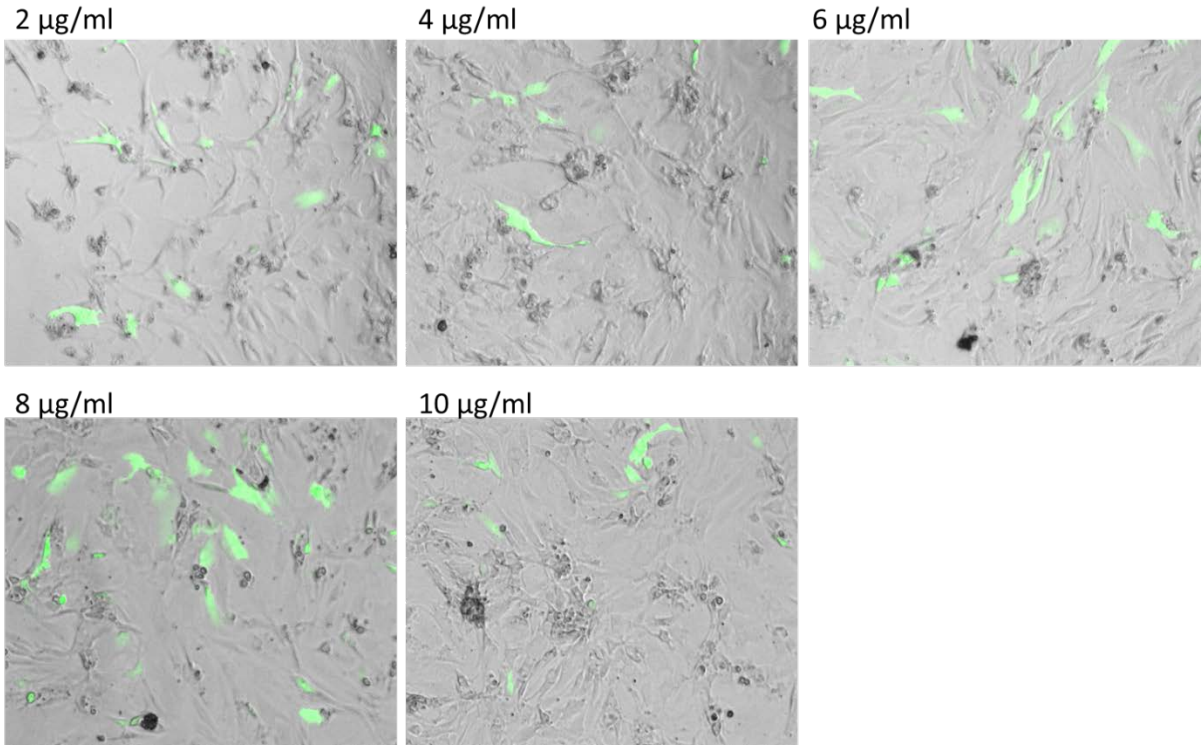


Figure 3.13. Optimisation of polybrene concentration in HAC. Fluorescent images of lentiviral GFP shRNA expression in HAC. Cells were transduced with lentivirus shRNA in 96 well plates with 100% virus and different concentrations of polybrene for 24 hours, medium was then changed and the cells incubated for a further 48 hours, as detailed in the Materials and Methods, then visualised. Results are representative of a single experiment.

3.2.6.3 Centrifugation of cells in order to increase transduction of shRNA in HAC

In order to further optimise transduction efficiency in HAC, centrifugation was performed after the lentivirus was added, and compared to non-centrifuged cells. This is thought to enhance transduction efficiency by enhancing the density of the virus at the cell surface. However, centrifugation did not enhance transduction efficiency, and appeared to give reduced transduction, compared to non-centrifuged cells, with non-centrifuged showing approximately 25% efficiency and centrifuged approximately 15% efficiency (Fig 3.14).

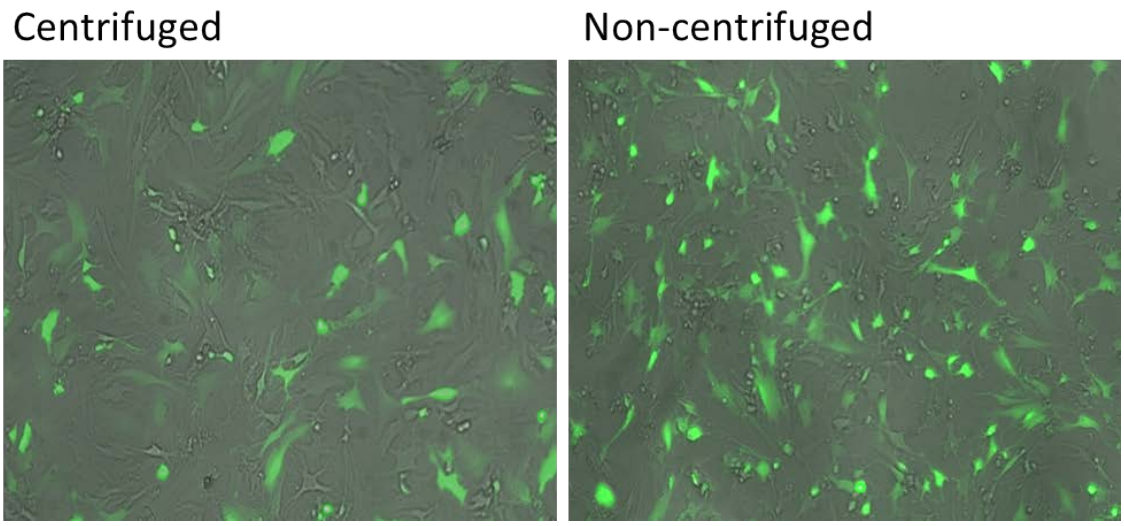


Figure 3.14. Centrifugation of cells in order to increase transduction of shRNA in HAC. Fluorescent images of lentiviral GFP shRNA expression in HAC. Cells were transduced with lentivirus shRNA in 96 well plates with 100% virus and 8 µg/ml polybrene, as detailed in the Materials and Methods, then spun at 1200 x g for 90 minutes at room temperature, and incubated for 24 hours, medium was then changed and the cells incubated for a further 48 hours, then visualised. Results are representative of a single experiment.

3.2.6.5 Optimisation of inducible TRIPZ Trb1 lentiviral shRNA in HAC and SW1353 cells

After it was confirmed lentiviral shRNA could successfully be produced and transduced into both SW1353 cells and HAC, Trb1 lentiviral shRNA was then tested. The Trb1 lentivirus used initially was TRIPZ inducible shRNA, whereby the expression of the shRNA was induced in the presence of doxycycline; this was through a tetracycline response element. In addition to this, the tetracycline response element also drives expression of the turbo red fluorescence protein (RFP) promoter which allows the fluorescence visualisation of transduction efficiency, and promoter activity.

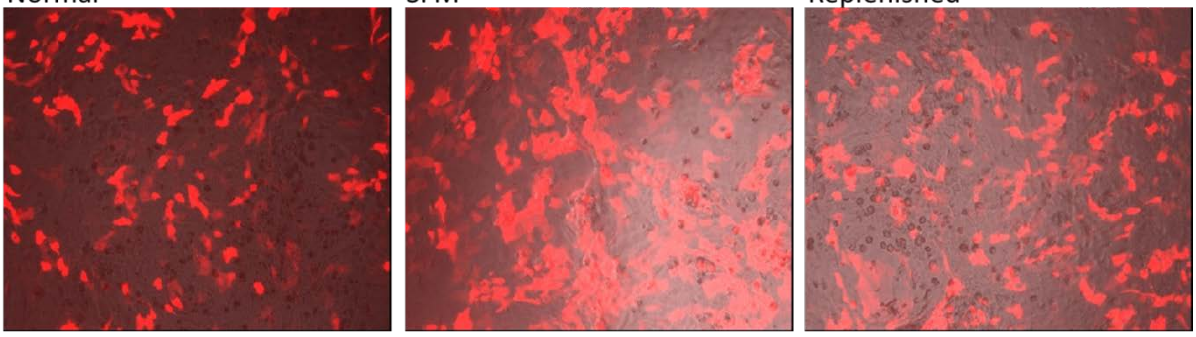
Three separate Trb1 shRNA (Trb1 # 1-3) were assessed, in order to give a better probability of identifying one which could silence Trb1. This was initially assessed in SW1353 cells. The lentivirus was concentrated in order to allow a greater amount of lentivirus to be added to maximise transduction efficiency. The lentivirus was concentrated through centrifugation, using a commercial kit, and concentrated 50-fold. In addition to this, transduction was also carried out three different ways: 1. As above; 2. Transducing in serum-free medium for 6

hours, then addition of 20% FBS medium; 3. Replenishing the doxycycline every day. The 300% virus gave the best transduction efficiency for all groups compared to the 200% (Fig 3.15). It also appeared that transduction in serum-free medium gave better transduction compared to the other methods (Fig 3.15), achieving almost 100% transduction for Trb1 # 1 and Trb1 # 3; Trb1 # 2 gave slightly lower transduction efficiency.

It was also assessed from these cells whether Trb1 shRNA led to gene silencing of *Trb1*. From the mRNA data Trb1 # 3 shRNA appeared to be the only shRNA which silenced Trb1 (Fig 3.16). Interestingly, this silencing occurred at 200% virus rather than the 300% which had given a higher transduction efficiency. Both serum-free (Fig 3.16B) and replenished (Fig 3.16C) conditions gave silencing of Trb1. However, *Trb3* mRNA expression was also assessed for the 200% virus in serum-free medium, and it was shown that the shRNA also silenced this gene (Fig 3.17), therefore it was not specific for *Trb1*.

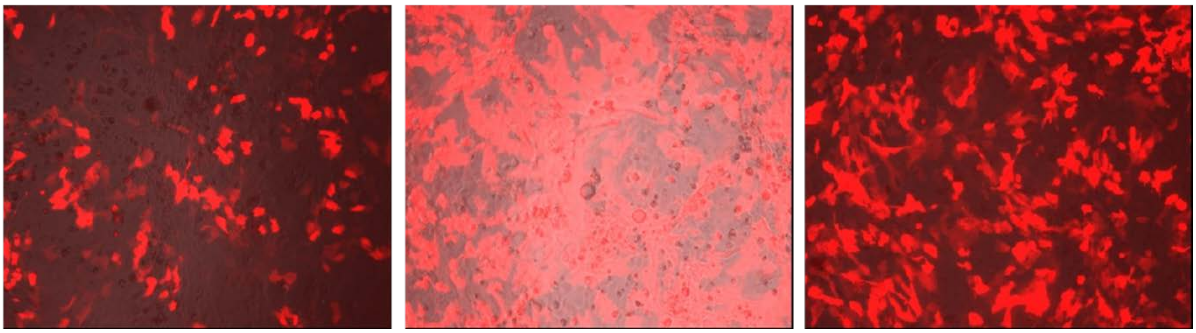
Trb1 # 1 200%

Normal SFM Replenished



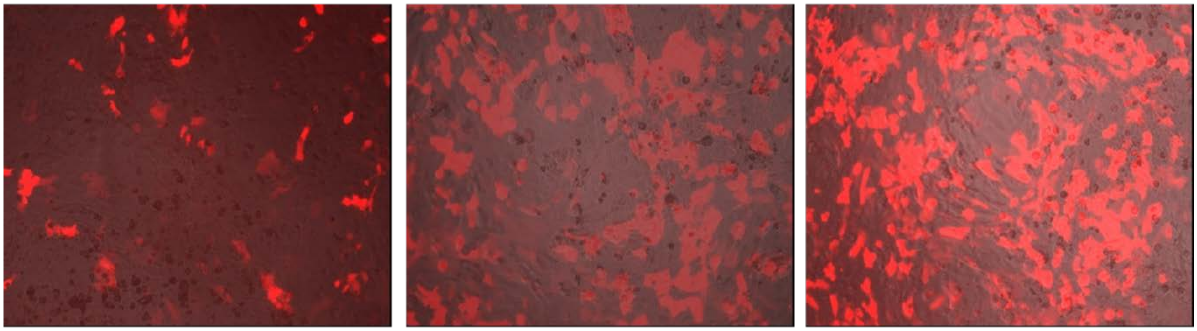
Trb1 # 1 300%

Normal SFM Replenished



Trb1 # 2 200%

Normal SFM Replenished



Trb1 # 2 300%

Normal SFM Replenished

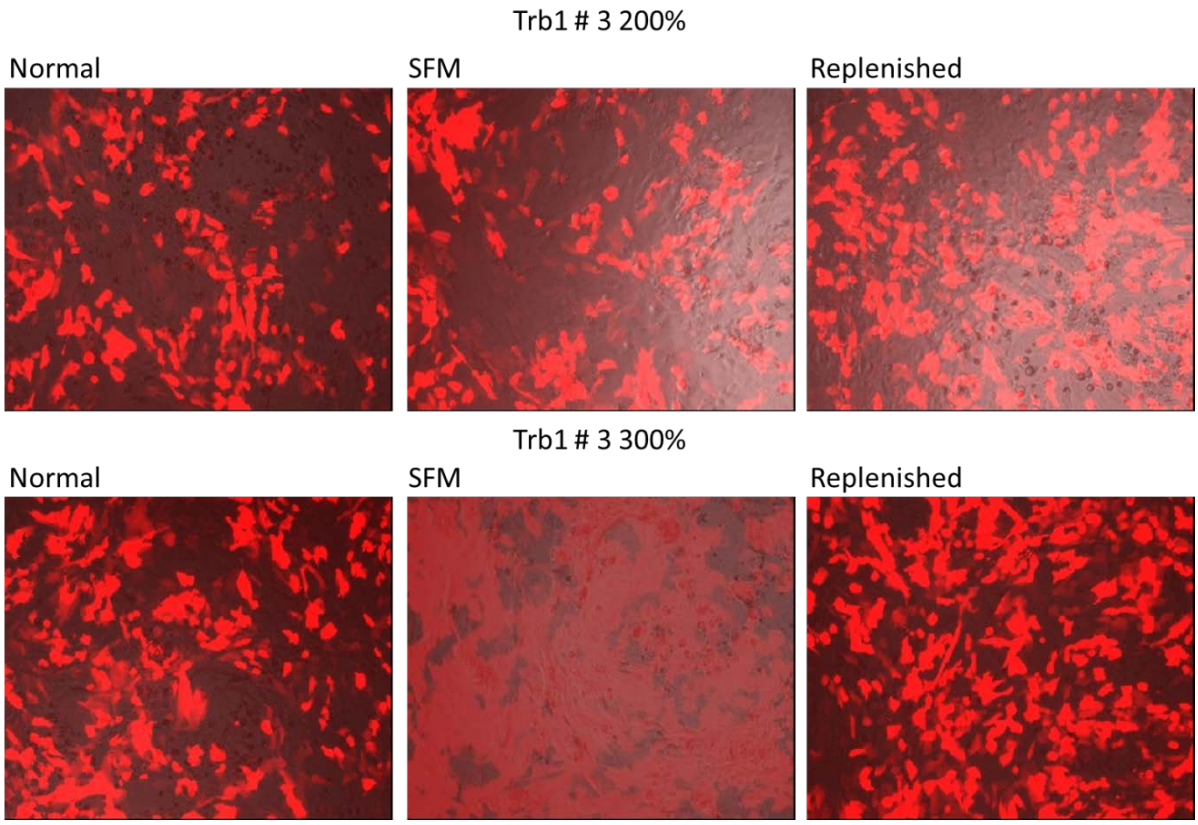


Figure 3.15. Optimisation of inducible TRIPZ Trb1 lentiviral shRNA through alteration of serum content and doxycycline in SW1353 cells. Fluorescent images of inducible lentiviral TRIPZ Trb1 shRNA expression in SW1353 cells. Cells were transduced with lentivirus shRNA in 96 well plates (200% or 300%) with 8 µg/ml polybrene in either 10% FBS medium or serum-free medium for 6 hours then 20% FBS medium added (SFM group). Medium was then changed after 24 hours and 3 µg/ml doxycycline was added, and the cells incubated for a further 48 hours, or medium changed and doxycycline added again after 24 hours (replenished group), as detailed in the Materials and Methods, then visualised. Three different Trb1 shRNA were tested (Trb1 # 1-3). Results are representative of 2 independent experiments.

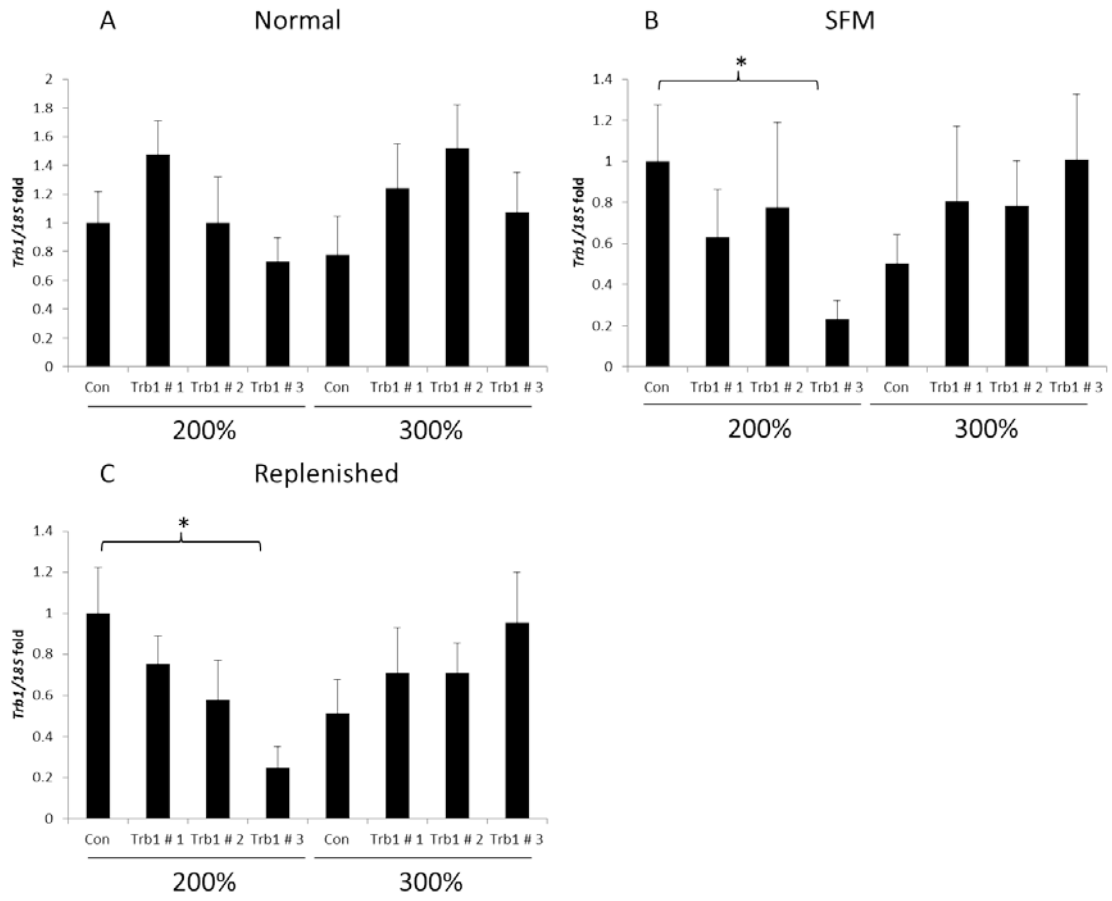


Figure 3.16. Optimisation of inducible TRIPZ Trb1 lentiviral shRNA through alteration of serum content and doxycycline in SW1353 cells: Assessment of Trb1 gene silencing. mRNA expression analysis of *Trb1* after transduction with inducible lentiviral TRIPZ Trb1 shRNA in SW1353 cells. Cells were transduced with lentivirus shRNA in 96 well plates (200% and 300%) with 8 µg/ml polybrene in either 10% FBS medium or serum-free medium for 6 hours then 20% FBS medium (B). Medium was then changed after 24 hours and 3 µg/ml doxycycline was added, and the cells incubated for a further 48 hours, or medium changed and doxycycline added again after 24 hours (C), as detailed in the Materials and Methods, before lysis. Total RNA was isolated, reverse transcribed to cDNA and *Trb1* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean values plotted. Results are measured in hextuplicate, (+/-standard error of the mean), *p<0.05. Results are representative of 2 independent experiments. Ct values for siCon werey 11.91 +/- 0.11 for 18S and 24.43 +/- 0.64 for *Trb1* for Normal,11.41 +/- 0.66 for 18S and 23.83 +/- 0.42 for *Trb1* for SFM, and 12.97 +/- 0.18 for 18S and 23.23 +/- 0.69 for *Trb1* for replenished (+/- = standard error of the mean).

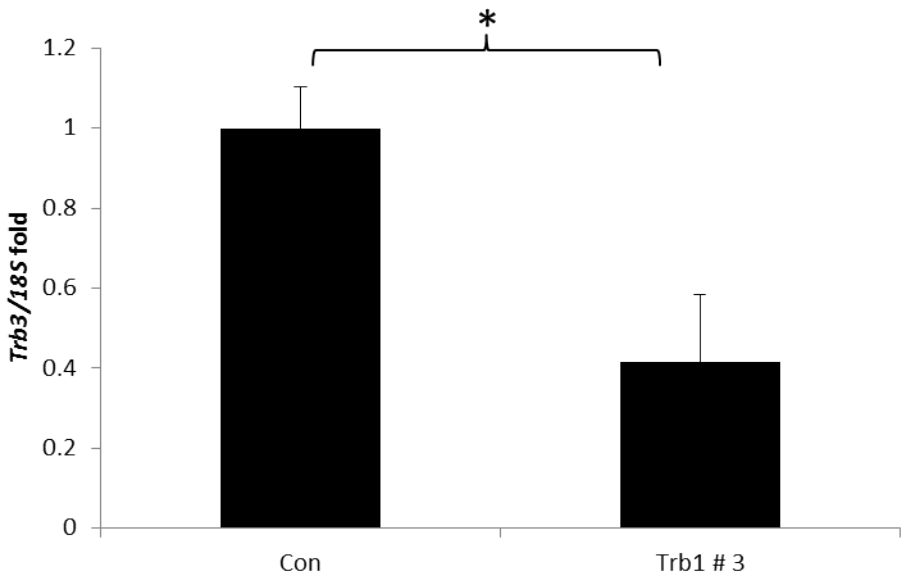


Figure 3.17. Assessment of *Trb3* gene silencing during TRIPZ Trb1 lentiviral shRNA transduction in SW1353 cells. mRNA expression analysis of *Trb3* after transduction with inducible lentiviral TRIPZ Trb1 shRNA in SW1353 cells. Cells were transduced with lentivirus shRNA in 96 well plates (200%) with 8 µg/ml polybrene in serum-free medium for 6 hours then 20% FBS medium. Medium was then changed after 24 hours and 3 µg/ml doxycycline was added, and the cells incubated for a further 48 hours, as detailed in the Materials and Methods, before lysis. Total RNA was isolated, reverse transcribed to cDNA and *Trb3* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/-standard error of the mean), *p<0.05. Results are representative of 2 independent experiments. Ct values for siCon were 12.23 +/- 0.25 for 18S and 25.39 +/- 0.48 for *Trb3* (+/- = standard error of the mean).

It was then assessed whether the TRIPZ Trb1 shRNA could be transduced into HAC. Taking into account the results from SW1353 experiments, the serum-free medium technique was used for all future experiments. In addition, due to the known difficulty in transducing HAC, as demonstrated by the GFP lentiviral shRNA data (see Fig 3.12, 3.13, 3.14), a higher concentration of virus was used. It was not possible to transduce HAC to a high efficiency using the Trib1 TRIPZ shRNA. It was only possible to transduce a few cells with 600% virus for the Trb1 # 3 shRNA (Fig 3.18).

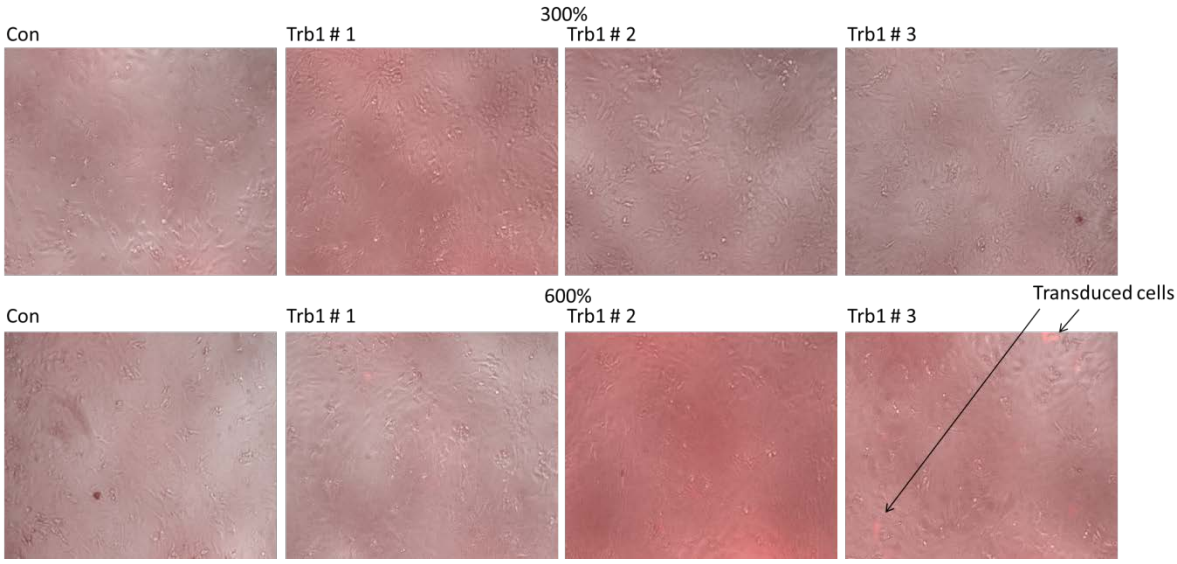


Figure 3.18. Optimisation of inducible TRIPZ Trb1 lentiviral shRNA using different concentrations of shRNA in HAC. Fluorescent images of inducible lentiviral TRIPZ Trb1 shRNA expression in HAC. Cells were transduced with lentivirus shRNA in 96 well plates (300% and 600%) with 8 µg/ml polybrene in serum-free medium for 6 hours then 20% FBS medium. Medium was then changed after 24 hours and 3 µg/ml doxycycline was added, and the cells incubated for a further 48 hours, as detailed in the Materials and Methods, then visualised. Three different Trb1 shRNA were tested (Trb1 # 1-3). Results are representative of 2 independent experiments, from 2 separate donors.

Due to the apparent lack of transduction of HAC, it was then tested whether it was the ability to induce the lentivirus which was the reason why there was such a low apparent transduction efficiency, rather than the ability to transduce the HAC per se. Therefore, non-inducible GIPZ and inducible TRIPZ controls were compared within the same HAC population. Three different concentrations of virus were compared; 250%, 500% and 750%. It was demonstrated that the GIPZ controls had a much higher percentage of fluorescent cells, compared to the TRIPZ controls (Fig 3.19), suggesting that the cells were being transduced but the doxycycline was not inducing the shRNA. It was therefore decided to use GIPZ Trb1 shRNA.

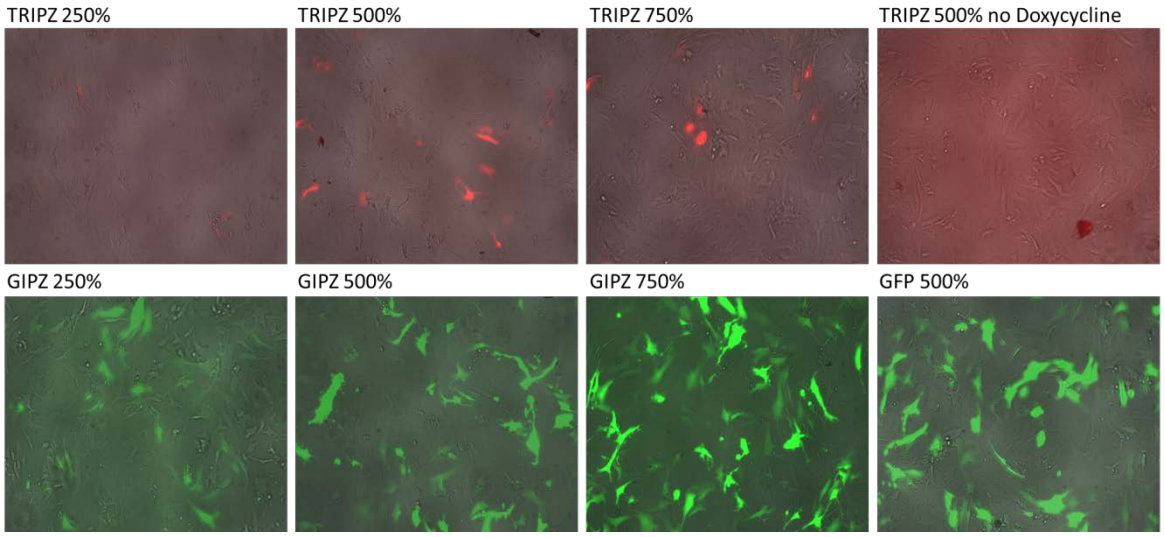


Figure 3.19. Assessment of the ability of doxycycline to induce lentivirus in HAC. Fluorescent images of inducible lentiviral TRIPZ control, non-inducible GIPZ control and GFP shRNA expression in HAC. Cells were transduced with lentivirus shRNA in 96 well plates (250%, 500% and 750%) with 8 µg/ml polybrene in serum-free medium for 6 hours then 20% FBS medium. Medium was then changed after 24 hours and 3 µg/ml doxycycline was added, and the cells incubated for a further 48 hours, as detailed in the materials and methods, then visualised. Results are representative of a single experiment.

3.2.6.6 Optimisation of non-inducible GIPZ Trb1 lentiviral shRNA in HAC

Four different Trb1 shRNA were tested (Trb1 # A-D) in order to increase the probability that one might silence Trb1. It was initially assessed if it was possible to transduce HAC with the GIPZ lentivirus, in addition to assessing the transduction efficiency. Through fluorescence microscopy, it was demonstrated that transduction of the virus was successful for all of the lentiviral constructs (Fig 3.20). Transduction efficiency appeared similar for all of the constructs at approximately 50-60%, with Trb1 # C being slightly lower. The increase in concentration from 750% to 1000% appeared to have little effect on transduction efficiency.

As transduction efficiency appeared sufficient, Trb1 silencing was measured at both the mRNA and protein levels. None of the four Trb1 shRNA had any apparent reproducible effect on either Trb1 mRNA or protein depletion (Fig 3.21 and 3.22). For measurement of protein depletion the 1000% concentration was used, as there appeared little difference in transduction efficiency between the

two different concentrations. Due to time constraints no further work was performed in order to silence Trb1 in either HAC or SW1353 cells.

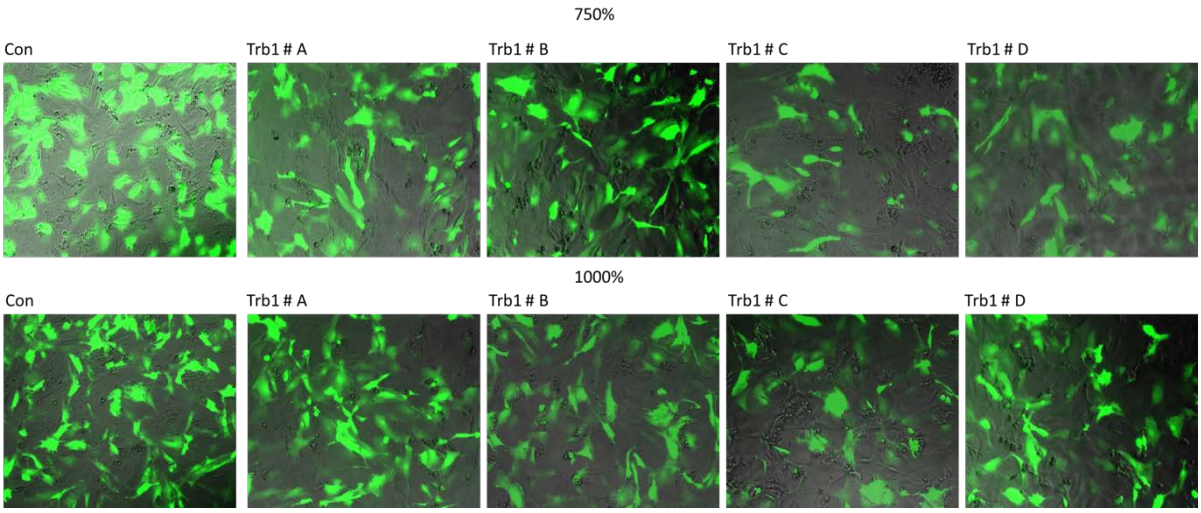


Figure 3.20. Optimisation of non-inducible GIPZ Trb1 lentiviral shRNA using different concentrations of shRNA in HAC. Fluorescent images of non-inducible lentiviral GIPZ Trb1 shRNA expression in HAC. Cells were transduced with lentivirus shRNA in 96 well plates (750% and 1000%) with 8 µg/ml polybrene in serum-free medium for 6 hours then 20% FBS medium added. Medium was then changed after 24 hours, and the cells incubated for a further 48 hours, as detailed in the Materials and Methods, then visualised. Four different Trb1 shRNA were tested (Trb1 # A-D). Results are representative of 2 independent experiments, from 2 separate donors.

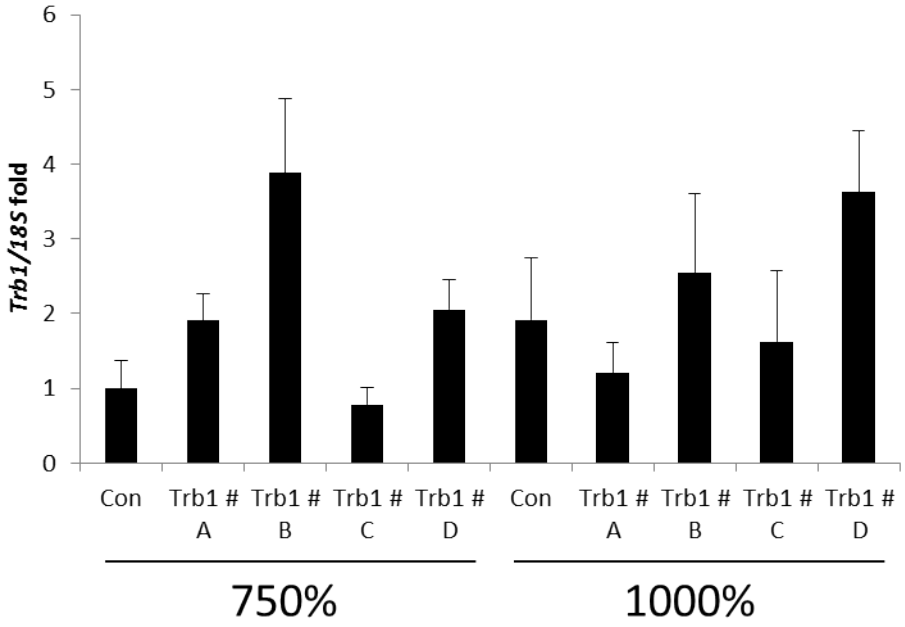


Figure 3.21. Optimisation of non-inducible GIPZ Trb1 lentiviral shRNA using different concentrations of shRNA in HAC: Assessment of *Trb1* gene silencing. mRNA expression analysis of *Trb1* after transduction with non-inducible lentiviral GIPZ Trb1 shRNA in HAC. Cells were transduced with lentivirus shRNA in 96 well plates (750% and 1000%) with 8 µg/ml polybrene in serum-free medium for 6 hours then 20% FBS medium added. Medium was then changed after 24 hours, and the cells incubated for a further 48 hours, as detailed in the Materials and Methods, before lysis. Total RNA was isolated, reverse transcribed to cDNA and *Trb1* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/-standard error of the mean). Four different Trb1 shRNA were tested (Trb1 # A-D). Results are representative of 2 independent experiments, from 2 separate donors. Ct values for siCon were 20.51 +/- 0.54 for 18S and 35.78 +/- 0.61 for *Trb1* (+/- = standard error of the mean).

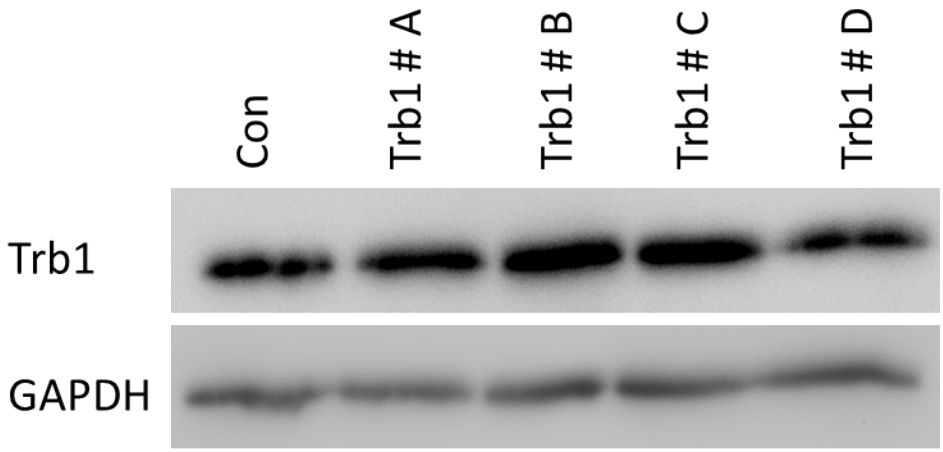


Figure 3.22. Optimisation of non-inducible GIPZ Trb1 lentiviral shRNA using different concentrations of shRNA in HAC: Assessment of Trb1 protein expression. Trb1 and GAPDH abundance after transfection with non-inducible lentiviral GIPZ Trb1 shRNA in HAC. Cells were transduced with lentivirus shRNA in 6 well plates (1000%) with 8 µg/ml polybrene in serum-free medium for 6 hours then 20% FBS medium added. Medium was then changed after 24 hours, and the cells incubated for a further 48 hours, as detailed in the Materials and Methods. Cells were then lysed and proteins were separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 2 independent experiments, from 2 separate donors.

3.3 Discussion

The ability to control and alter the expression of genes is critical in order to understand their roles within the cell. Therefore, initially it was essential to determine whether it was possible to control the genetic expression of the Trb1 and Trb3 in order to study them; by decreasing or increasing their expression. This was attempted through both siRNA and shRNA gene silencing, and plasmid overexpression.

It was initially investigated whether *Trb1* could be successfully depleted using siRNA. It was not possible to deplete *Trb1* in either HAC or SW1353 cells. *Trb1* mRNA has been shown to have a very short half-life of less than 1 hour (Sharova *et al*, 2009). This is fairly unique, as demonstrated by the study which tested 20,000 genes, and showed less than 100 had a half-life of less than 1 hour. Therefore, it may not have been possible to silence *Trb1* as the mRNA was being degraded too quickly and re-synthesised. This would prevent silencing of *Trb1*, as any of the mRNA that was being silenced would quickly be degraded along with the siRNA, and new functional mRNA synthesised before

an effect could be seen. This theory is consistent with *Trb3* having a longer half-life of 2.8 hours (Sharova *et al*, 2009) and successfully being silenced in SW1353 cells and HAC at both the mRNA and protein level. In order to demonstrate that *Trb1* mRNA levels were in fact being measured, and that what was being seen was not simply background, a negative control well of no template and water should have been performed. In addition, in order to verify that the correct amplicons were being produced a melt curve should have been performed.

Throughout this thesis 18S ribosomal RNA was used as the housekeeping gene. This is commonly used as a housekeeping gene for RT-PCR, as it is quite consistent in many cell types, however it does have limitations. 18S rRNA may not always represent the total cellular RNA. In addition, it has a high abundance, constituting 85-90% of total cellular RNA, meaning this may be problematic for medium and low expressing target genes.

Both Trb1 and Trb3 were successfully overexpressed in both HAC and SW1353 cells. It was not possible to visualise overexpressed Trb3 in SW1353 cells, however the c-Myc tag encoded within the plasmid was used to show that Trb3 was indeed being overexpressed. The reason for the lack of visualisation of overexpressed Trb3 was unknown. However, it was possible that the overexpressed Trb3 protein was not exposing the primary antibody binding site for the particular antibody used. Overexpressed Trb1 gave a larger than expected band (55kDa) compared to the native Trb1 (40kDa). The reason for this was unkown, however, there are a number of possible reason; the overexpressed Trb1 may be glycosylated, phosphorylated or sumoylated. Also, the overexpressed Trb1 may be polyubiquitinated, and an accumulation is seen due to the degradation machinery being saturated. This ability to overexpress both Trb1 and Trb3 therefore allows the effect of an increase in these proteins on the various signalling pathways to be investigated. In addition, it also allows a number of possible techniques to be performed, such as protein complementation assays (as seen in chapters 4, 6 and 7), and co-immunoprecipitation.

Due to the inability to silence *Trb1* using siRNA, shRNA was used in order to attempt to silence *Trb1*, as shRNA would provide constitutive silencing of the

gene. Initially, GFP was used in order test production of the lentivirus, scale up the lentiviral production, as well as optimising transduction efficiency of the lentivirus in both HAC and SW1353 cells. Production and transduction of the GFP lentivirus proved to be possible in both SW1353 cells and HAC. A number of different lentivirus concentrations were tested, as well as different polybrene concentrations.

The initial Trb1 shRNA tested was doxycycline inducible, which allowed the ability to induce expression of shRNA and therefore silence the gene of interest at the time point required. In addition, a red fluorescence protein promoter was also induced which allowed transduction efficiency to be assessed. Three different Trb1 shRNA were tested. In order to further improve transduction efficiency the lentivirus was concentrated through a centrifugation technique. This allowed a greater amount of virus to be added. Transduction in serum-free medium was tested as this has been shown to enhance transduction efficiency; as complement within the serum can inactivate the viral vector particles (Depolo *et al*, 2000). Replenishing of doxycycline was also tested as it has been shown that in culture conditions the half-life of doxycycline was 24 hours; and the cells were incubated for a further 48 hours after addition of doxycycline. It was demonstrated that concentrating the lentivirus was effective with the best transduction efficiency in SW1353 cells being 300% virus. In addition serum-free medium was also effective as this showed increased transduction in SW1353 cells. Replenishing doxycycline also proved to give greater induction of the shRNA compared to standard induction. This demonstrated that many factors can control the level of shRNA expression; and complete optimisation of expression can be complex. The depletion of *Trb1* was also tested. One shRNA, Trb1 # 3, showed *Trb1* depletion at the mRNA level, however, this was not specific and showed depletion of *Trb3* also. This was not entirely surprising, as it has been shown that Trb1 and Trb3 share common sequences (Yokoyama and Nakamura, 2011). Interestingly, however, the silencing at the mRNA level appeared to be greatest at 200% virus which showed lower transduction efficiency, the reason for this was unknown.

I was unable to observe apparent transduction of HAC to a sufficient level using the TRIPZ Trb1 lentivirus. It is thought that this was due to the inability of the doxycycline to either penetrate the HAC or have an effect once inside the cells.

The reason for this assumption was that when comparing the percentage of fluorescent cells of both the GIPZ and TRIPZ control, GIPZ gave a much higher percentage. This suggested that it was the induction of the shRNA that was not working, rather than the transduction.

Due to the inability to induce TRIPZ, GIPZ Trb1 was then tested. Four different Trb1 shRNA were used, which had different sequences to the TRIPZ shRNA. Transduction efficiency in HAC was good at approximately 50-60%, suggesting that it was in fact induction and not transduction of the TRIPZ shRNA which was the problem. However, mRNA and protein expression analysis showed no substantial depletion of Trb1 with any of the shRNA. The reason for this may possibly be the same reason why Trb1 siRNA did not work, in that *Trb1* mRNA is being turned over too quickly within the HAC, therefore the lentiviral shRNA is not being generated rapidly enough in order to bind sufficient *Trb1* mRNA to deplete it. Alternatively, it may be that the four sequences tested were not specific enough to bind and silence the mRNA.

3.4 Synopsis

It was not possible to specifically silence Trb1 using either siRNA or shRNA. Two methods of shRNA were used, both inducible and non-inducible. Inducible shRNA was unable to be induced in HAC due to the inducible factor doxycycline having little effect in these cells. However, non-inducible shRNA gave high transduction efficiency in HAC, but as mentioned Trb1 was not silenced. It is possible Trb1 could not be silenced in HAC due to the high degradation rate of this gene. *Trb1* was silenced in SW1353 using inducible shRNA, however this was not specific and also led to the silencing of *Trb3*. Trb1 was successfully overexpressed using plasmid overexpression, therefore its function within the cell can still be investigated. However, this result was not ideal, as in order to truly investigate the effects of a protein on signalling pathways both overexpression and silencing is required. In addition, silencing is more biologically relevant than overexpression, as overexpression produces expression levels not physiologically seen within the cell, and can therefore produce cellular anomalies. It was possible to silence Trb3 using siRNA and

overexpress Trb3 using plasmid overexpression, therefore its role within the cell can be more fully investigated.

3.5 Summary

- *Trb1* gene expression could not be silenced using siRNA in either SW1353 cells or HAC.

- *Trb3* gene expression was silenced using siRNA in both SW1353 cells and HAC.

- *Trb1* gene expression was silenced using shRNA in SW1353 cells; however it was not specific for *Trb1*, and also silenced *Trb3*.

- *Trb1* gene expression could not be silenced using shRNA in HAC.

- Trb1 and Trb3 were successfully overexpressed and optimised in both SW1353 cells and HAC using plasmid overexpression.

Chapter 4: The regulation of Trb1 and Trb3

4.1 Introduction

The method of regulation of Trb1 and Trb3 proteins is largely unknown. They may be regulated through a number of pathways, such as mRNA stability (Sharova *et al*, 2009), whilst Trb3 has been shown to be regulated through proteasomal degradation by SIAH1 (Zhou *et al*, 2008). Trb1 and Trb3 have been shown to be upregulated by IL-1β in a number of different cell types (Sung *et al*, 2006). In addition, Trb3 has been shown to be downregulated through miRNA, in particular miRNA 24 (Chan *et al*, 2010). Both Trb1 and Trb3 have been demonstrated to be predominantly nuclear (Kiss-Toth *et al*, 2006), and this subcellular distribution may give indications of their modes of regulation. Trb1 and Trb3 have also been shown to respond to stress conditions, being up regulated during endoplasmic reticulum (ER) stress and hypoxia (Ohoka *et al*, 2005; Bowers *et al*, 2003). An added level of complexity of Trb1 and Trb3 is that they appear to be somewhat cell type specific in many cases, whereby they appear to have an effect on a pathway in one cell type but not in another (Duggan *et al*, 2010, Kiss-Toth et al, 2004 Hegedus *et al*, 2006).

Trb1 and Trb3 have been shown to regulate many pathways involved in arthritis and MMP regulation including the MAPK (Kiss-Toth *et al*, 2004), PI3K/Akt (Du *et al*, 2003) and NFκB (Duggan *et al*, 2010) pathways. In addition, Trb3 has been shown to be up-regulated in OA and it has been suggested that it may play a role in OA progression (Cravero *et al*, 2009).

It is therefore hypothesised that Trb1 and Trb3 may be regulated in both HAC and SW1353 cells in similar manners to which they have previously been demonstrated within the literature. This regulation includes through cellular stress, cytokine stimulation and proteasomal degradation, as well as through their cellular distribution. A greater understanding in the way in which Trb1 and Trb3 are regulated may provide insights into how they may be involved in the signalling pathways involved in arthritis.

Cytokines play a major role in arthritic disease, with IL-1 playing a predominant role (Feldman *et al*, 1996; Scott *et al*, 2010; McInnes and Schett, 2007; Goldring

et al, 1994). In addition, OSM has also been shown to have a role in arthritis (Plater-Zyberk *et al*, 2001). IL-1 and OSM have been shown to work synergistically in terms of cartilage degradation *in vitro* (Cawston *et al*, 1995). OSM has also been shown to synergistically enhance IL-1 mediated MMP expression (Cawston *et al*, 1998; Rowan *et al* 2003; Fearon *et al*, 2006), confirming that IL-1 and OSM in combination is a potent pro-inflammatory cytokine stimulus. Therefore, throughout this thesis in order to mimic a pro-inflammatory environment *in vitro*, IL-1+OSM stimulation will be used.

The aims of this chapter were to:-

- Determine the subcellular distribution of Trb1 and Trb3, and investigate whether IL-1+OSM stimulation alters this distribution.
- Determine the effect of cellular stress on Trb1 and Trb3 over time, through a serum-starvation time course.
- Determine the effect of IL-1+OSM stimulation on Trb1 and Trb3 over time.
- Determine whether Trb1 and Trb3 are degraded through the proteasome. This was performed through proteasomal inhibition and determining Trb1 and Trb3 protein abundance.
- Investigate if Trb1 and Trb3 interact together, and therefore possibly regulate one another through this interaction.

4.2 Results

4.2.1 Trb1 and Trb3 subcellular localisation and the effect of IL-1+OSM stimulation in SW1353 cells

Whilst it has previously been demonstrated that Trb1 and Trb3 are predominantly present within the nucleus, this study was conducted in HeLa cells and not in a chondrocyte context (Kiss-Toth *et al*, 2006). In addition, the

effect of pro-inflammatory stimulation on Trb1 and Trb3 cellular localisation has not been studied previously. In order to assess subcellular distribution, experiments were performed in SW1353 cells, as due to the number of cells required, the amount of cartilage needed in order to perform the experiment was not available.

Trb1 protein was present within the cytoplasmic, membrane and soluble nuclear fractions, with the highest amounts appearing to be in the cytoplasm and nucleus (Fig 4.1). However, the fractionation was not completely successful as demonstrated by the fractionation markers (Caveolin, Lamin A/C, and MEK2). In particular the cytoplasmic fractionation marker MEK2 demonstrated similar abundance between the cytoplasm and membrane as that for Trb1 protein abundance.

IL-1+OSM stimulation appeared to have no effect on membrane bound and nuclear Trb1, however, it did appear to increase abundance of cytoplasmic Trb1 (Fig 4.1), stimulation did not appear to affect localization of the protein though.

Trb3 protein expression appeared to be mainly within the cytoplasm and soluble nuclear fractions (Fig 4.1). There was, however, protein present in the other fractions (membrane, chromatin nuclear and cytoskeletal). It is possible that this expression may be due to an inefficient fractionation process, as shown by the fractionation markers. As with Trb1; IL-1+OSM stimulation appeared to increase abundance of cytoplasmic Trb3 (Fig 4.1), but did not affect its localisation.

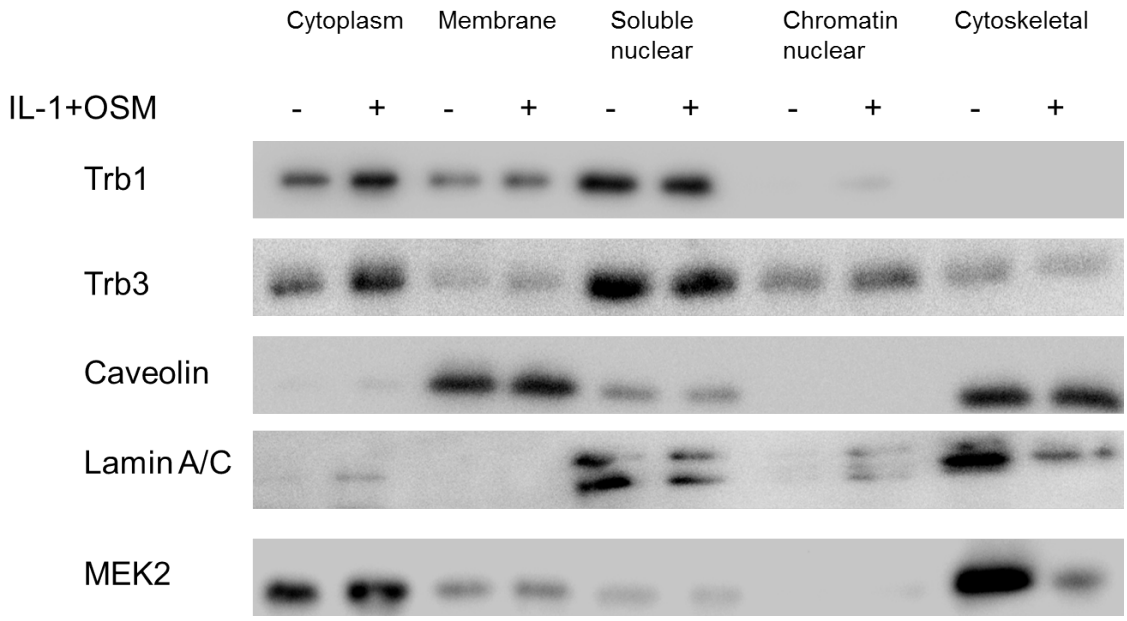


Figure 4.1. Trb1 and Trb3 localise predominantly within the nucleus and cytoplasm, as well as increasing in protein abundance within the cytoplasm during IL-1+OSM stimulation in SW1353 cells. Trb1, Trb3, Caveolin, Lamin A/C and MEK2 abundance within different subcellular compartments +/- IL-1+OSM stimulation in SW1353 cells. Cells were serum-starved overnight, then stimulated with IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 20 minutes. Cells were subjected to subcellular fractionation after stimulation. Proteins were separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Caveolin, Lamin A/C and MEK2 providing controls for Membrane, Nuclear and Cytoplasmic extracts, respectively. Results are representative of 2 independent experiments.

4.2.2 Trb1 and Trb3 protein abundance during 24 hour serum-starvation in HAC

Cellular stress is thought to be important in terms of arthritis (Yudoh *et al*, 2005; Takada *et al*, 2011), as it has been shown to activate a number of pro-inflammatory pathways, such as the MAPK pathway (Thalhamer *et al*, 2008). It was therefore investigated whether Trb1 and Trb3 may be up regulated during stress conditions.

HAC were serum-starved over time points up to 24 hours, in order to determine Trb1 and Trb3 protein abundance over this time period during cellular stress. Trb1 protein abundance appeared to increase slightly after 2 hours of starvation, and continued to increase up to 6 hours (Fig 4.2). By 12 hours Trb1 protein levels had reduced below the basal level which continued up to 24 hours.

Trb3 protein abundance gradually increased over time during serum-starvation (Fig 4.2). Protein abundance was shown to be increased by 2 hours, which then continued up to 12 hours. By 24 hours Trb3 protein levels had reduced slightly, but were still above basal level.

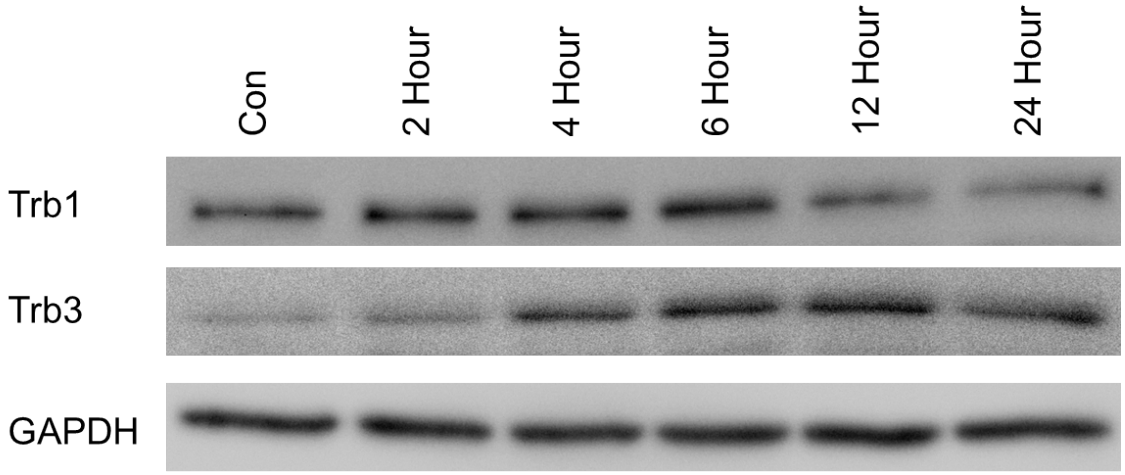


Figure 4.2. Trb1 and Trb3 respond to serum-starvation in HAC. Trb1, Trb3 and GAPDH abundance after serum-starvation treatment of HAC. Cells were serum-starved for the indicated time points. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 2 individual experiments, from 2 separate donors.

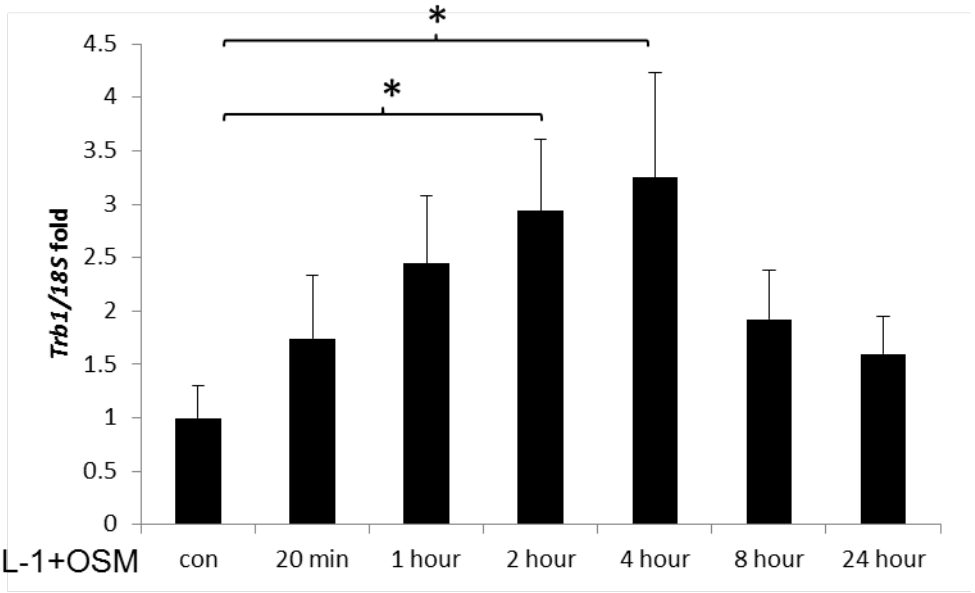
4.2.3 Trb1 and Trb3 mRNA expression and protein abundance during IL-1+OSM stimulation in HAC

In order to determine whether Trb1 and Trb3 were up regulated within a pro-inflammatory environment, HAC were stimulated with the potent pro-inflammatory cytokine combination of IL-1+OSM over a period of 24 hours.

IL-1+OSM stimulation led to a gradual increase in *Trb1* mRNA expression up to 4 hours which proved to be significant. An increase in *Trb1* mRNA expression appeared as early as 20 minutes, however this was not statistically significant (Fig 4.3 A). By 8 hours the mRNA expression had reduced and continued at this level at 24 hours.

IL-1+OSM stimulation showed a trend of a gradual increase in *Trb3* mRNA expression up to 24 hours (Fig 4.3 B), however, this was not significant.

A



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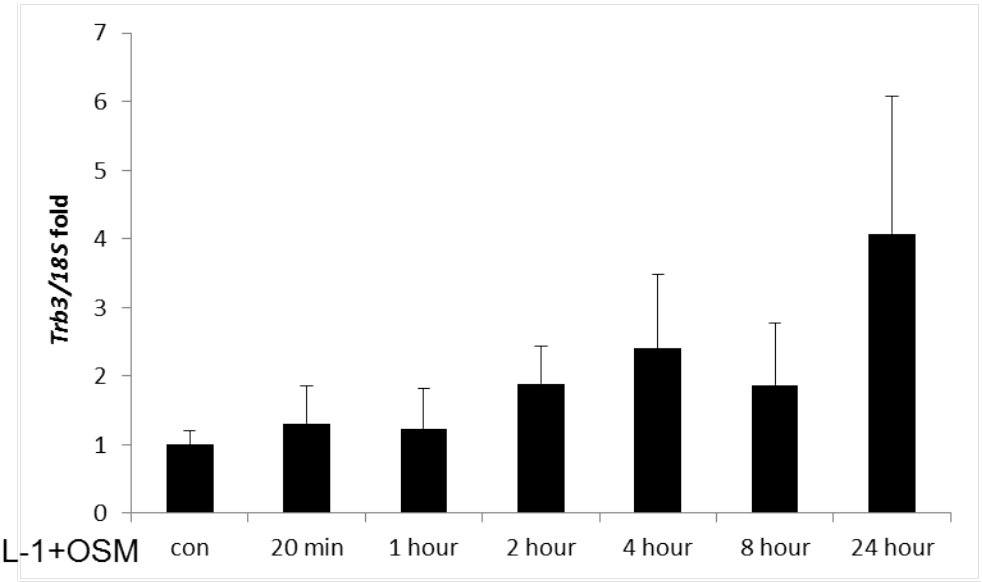

Figure 4.3. IL-1+OSM stimulation leads to an increase in *Trb1* and *Trb3* mRNA expression in HAC. mRNA expression analysis of *Trb1* (A) and *Trb3* (B) after IL-1+OSM stimulation of HAC. Cells were serum-starved overnight before stimulation with IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for the indicated time points before lysis. Total RNA was isolated, reverse transcribed to cDNA and *Trb1* and *Trb3* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/-standard error of the mean). Results are combined data from 3 separate donors. *p<0.05. Ct values for siCon were 18.68 +/- 0.19 for 18S and 34.50 +/- 0.64 for *Trb1* and 35.21 +/- 0.21 for *Trb3* (+/- = standard error of the mean).

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In addition to mRNA expression, protein abundance was also investigated during IL-1+OSM stimulation, over the same time course. Trb1 protein abundance appeared to increase at 1 hour, and subsequently decrease by 2 hours and remain constant (Fig 4.4). This increase then subsequent decrease was similar to that for Trb1 mRNA, however the increase in protein abundance was much less prolonged.

Trb3 protein abundance showed an increase with IL-1+OSM stimulation, increasing by 4 hours and continuing up to 8 hours, then subsequently decreasing by 24 hours back to basal (Fig 4.4). This was slightly different from the mRNA data which showed a trend for a continued increase up to 24 hours. The increase then decrease in protein abundance with IL-1+OSM stimulation was similar to that seen with Trb1, however this occurred more rapidly with Trb1.

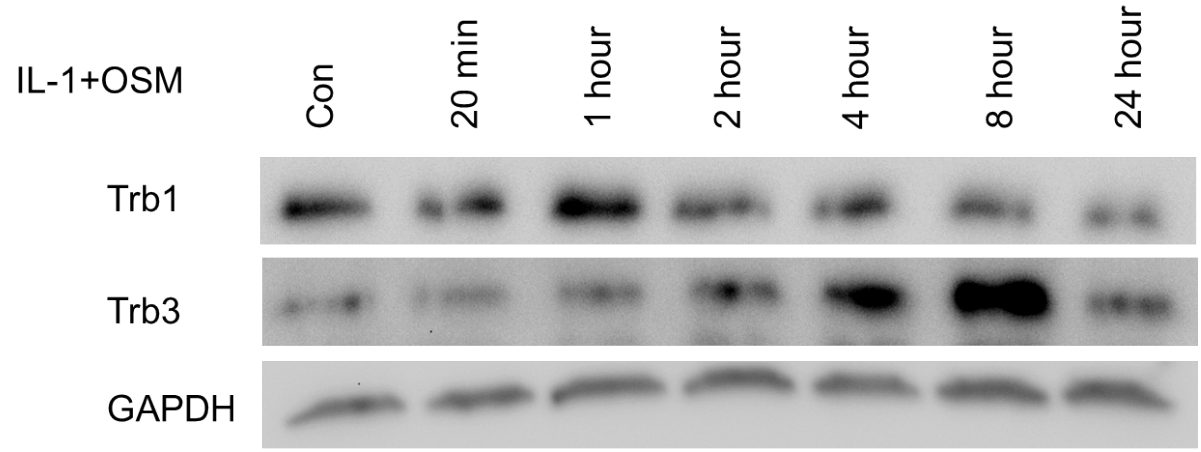


Figure 4.4. IL-1+OSM stimulation increases Trb1 and Trb3 protein abundance in HAC. Trb1, Trb3 and GAPDH abundance after IL-1+OSM stimulation of HAC. Cells were serum-starved overnight before stimulation with IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for the indicated time points before lysis. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments, from 3 separate donors.

4.2.4 Assessment of proteasomal degradation of Trb1 and Trb3 in HAC

Since Trb3 has been shown to be degraded through the proteasomal pathway in HEK 293T cells (Zhou *et al*, 2008), it was hypothesised that Trb1 may also be regulated in a similar manner, as this is a common cellular method of controlling levels of protein expression. Proteasomal degradation of Trb1 and Trb3 in HAC was therefore investigated using MG132. MG132 inhibits 20S proteasome activity by binding to the active site of the β subunits, and blocks the proteolytic activity of the 26S proteasome complex (Guo and Peng, 2013).

It was initially demonstrated that MG132 was inhibiting the proteasome by analyzing ubiquitinated protein levels. Proteins are first ubiquitinated in order to be recognized and degraded by the proteasome (Chen *et al*, 2008). Therefore, inhibition of the proteasome should show an increase in ubiquitinated proteins. It was observed that MG132 caused an increase in ubiquitinated proteins (Fig 4.5). The addition of MG132 had no effect on Trb1 protein abundance (Fig 4.6), suggesting that it was not degraded through the proteasome.

The addition of MG132 led to a considerable increase in Trb3 protein abundance (Fig 4.6). This suggested that Trb3 protein levels may be regulated through proteasomal degradation in HAC.

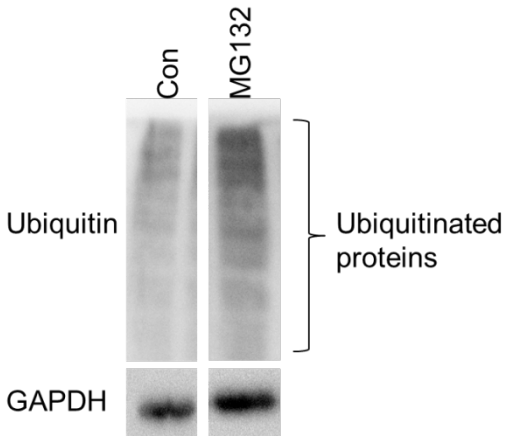
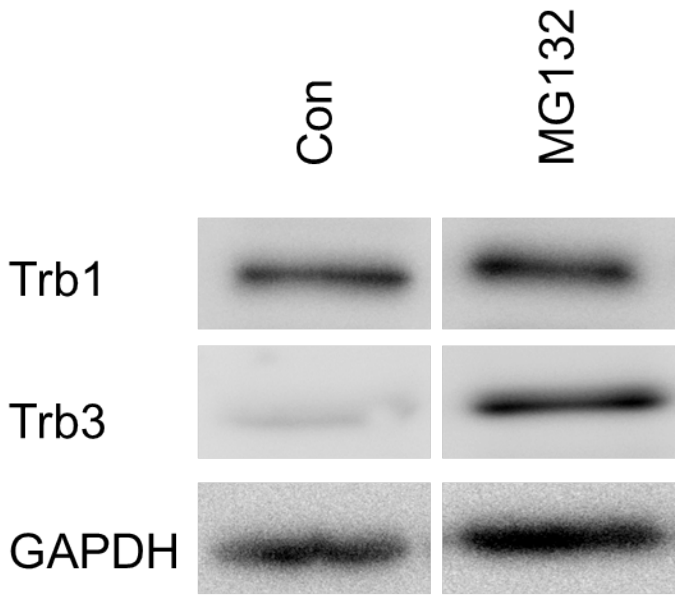


Figure 4.5. Ubiquitinated protein levels increase with the addition of MG132 in HAC. Ubiquitin and GAPDH abundance after MG132 treatment of HAC. Cells were serum-starved overnight, then treated with MG132 (5μM for 3 hours). Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Lanes were extracted from the same membrane.

A



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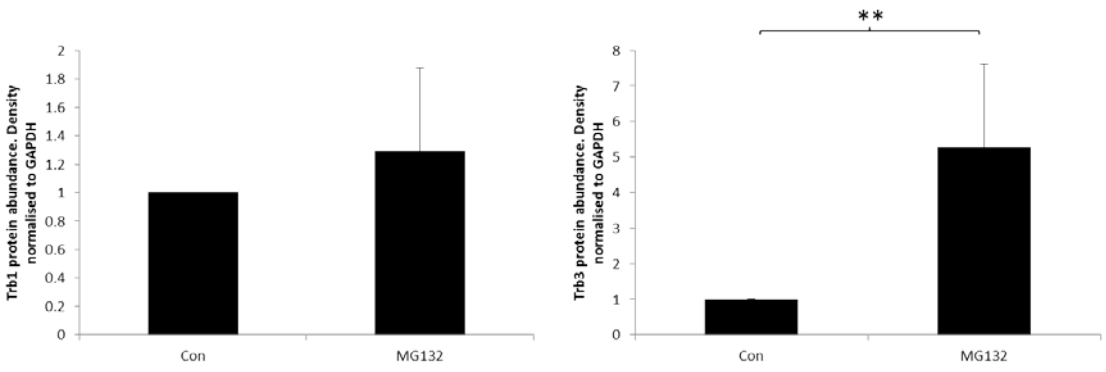

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Figure 4.6. The addition of MG132 leads to an increase in Trb3 protein abundance but has no effect on Trb1 in HAC. A. Trb1, Trb3 and GAPDH abundance after MG132 treatment in HAC. Cells were serum-starved overnight, then treated with MG132 (5μM for 3 hours). Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Lanes were extracted from the same membrane. Results are representative of 3 independent experiments, from 3 separate donors. B. Combined densitometric scans from 3 separate blots, from 3 separate donors for Trb1 (i) and Trb3 (ii). Values were normalised to GAPDH. Results are +/-standard deviation of the mean, **p<0.01.

4.2.5 Trb1 and Trb3 interaction

The potential for interaction between Trb1 and Trb3 was investigated as it was hypothesised that they may form heterodimers in order to regulate signaling pathways. This interaction was investigated using a technique called protein complementation assay (PCA). In this assay, two complementary Venus reporter fragments, V1 and V2, are fused to the protein(s) of interest. Upon direct interaction of the fusion proteins, the Venus fluorescent reporter activity is reconstituted and generates fluorescence.

The interaction was investigated in SW1353 cells, as the technique required transfection of two plasmid constructs into the same cell, which has a low efficiency. Since HAC are difficult to transfect, it was not possible to transfect two plasmid constructs at a high enough efficiency.

Trb1 and Trb3 did not appear to interact in SW1353 cells, as no fluorescence was observed when microscopically visualised (Fig 4.7), both Trb1V1-Trb3V2 and Trb1V2-Trb3V1 were tested (Trb1V2-Trb3V1 data not shown). Trb1-MEK1 was included as a positive control.

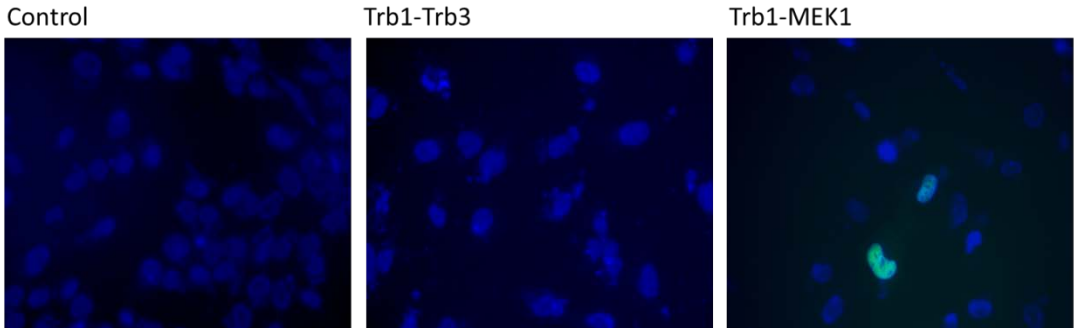

Figure 4.7. Fluorescent images of Trb1 (V1) and Trb3 (V2), as well as MEK1 (V1) and Trb1 (V2) overexpression constructs in SW1353 cells. Cells were transfected with each construct in 8 well chamber slides (0.2 µg per construct) for 48 hours, as detailed in the Materials and Methods, then serum starved for 6 hours, fixed and visualised. Results are representative of 2 independent experiments.

4.3 Discussion

In order to gain a better understanding of the context of their role within arthritis, a greater knowledge of the Trb1 and Trb3 proteins, and ways in which they may be regulated was sought. It was initially determined where Trb1 and Trb3 proteins were localised in SW1353 cells, and also whether this localisation changed following a pro-inflammatory stimulus.

Pro-inflammatory stimuli were used throughout in order to mimic the inflammatory environment in arthritis. RA in particular has been shown to be very much an inflammatory disease, with a number of pro-inflammatory cytokines involved including, IL-1, TNF-α, IL-6, OSM and IL-8 (Feldman *et al*, 1996; Scott *et al*, 2010; McInnes and Schett, 2007; Manicourt *et al* 2000). OA has also now been accepted to have an inflammatory aspect to its progression, with synovial inflammation being a characteristic, and IL-1 and TNF-α shown to be up-regulated in OA (Benito *et al*, 2005). In addition, it has been shown that MMPs are up-regulated by pro-inflammatory stimulation (Clark *et al*, 2008). Therefore in order to attempt to mimic this pro-inflammatory catabolic environment, the stimulus of IL-1+OSM was used.

In order to study cellular localisation, SW1353 cells were used instead of HAC, as discussed. SW1353 cells are a chondrocytic cell line that are often used as a model for primary human chondrocytes. However, they are just a model and as such there are differences within signalling pathways between primary human chondrocytes and SW1353 cells (Borden *et al*, 1996; Mengshol *et al*, 2000) which must be taken into account when interpreting results.

Trb1 and Trb3 were shown to be present predominantly in the cytoplasm and nucleus of SW1353 cells, suggesting that they have the ability to move between different cellular compartments. This is somewhat contradictory to Kiss-Toth *et al*. (2006), who showed Trb1 and Trb3 to be predominantly present in the nucleus. This may be due to cell type specificity, as that study was in HeLa cells. Both Trb1 and Trb3 contain a nuclear localisation sequence in their N-terminus (Kiss-Toth *et al*, 2006), and therefore their ability to move between the cytoplasm and nucleus was not unexpected. The presence of Trb1 and Trb3 both within the nucleus and cytoplasm suggests that they may be able to

regulate the transport of proteins to and from the nucleus, possibly regulating the ability of proteins to control transcription. The movement of proteins to and from the nucleus in order to regulate signalling pathways is a common occurrence within cellular signalling, including many proteins shown to be involved in arthritis such as NFκB, Akt and the MAPKs (Roman-Blas and Jimenez, 2006; Meier *et al,* 2005 and Sacks, 2006).

Stimulation with IL-1+OSM did appear to alter the cellular abundance of Trb1 and Trb3. Trb1 and Trb3 abundance increased within the cytoplasm during stimulation, suggesting that they were responsive to pro-inflammatory stimuli. It is unknown whether this response to IL-1+OSM stimulation is in a pro-inflammatory or anti-inflammatory context, as they may be increasing to propagate the pro-inflammatory response, or to dampen the response. However, stimulation did not appear to affect localisation of either Trb1 or Trb3. These similarities between Trb1 and Trb3 in terms of subcellular localisation, and response to IL-1+OSM, possibly suggests that Trb1 and Trb3 may work in a similar manner within a pro-inflammatory environment.

The effect of cellular stress on Trb1 and Trb3 expression was assessed in HAC; this was performed through serum-starvation. Trb1 increased during serum-starvation, suggesting that it was responsive to stress stimuli. This would be in concordance with data from Ostertag *et al*. (2010) who stated that Trb1 was increased during stress conditions in adipocytes. Trb1 was increased by 2 hours of starvation suggesting that its up regulation is fairly rapid, and the expression of Trb1 is under tight control, due to this rapid increase. By 12 hours serum-starvation Trb1 protein expression had reduced, suggesting that Trb1 may be involved in the initial response of HAC to stress conditions.

Trb3 was shown to be up regulated during serum-starvation. This was expected, as Trb3 has been shown to be a stress response protein, being up-regulated during both ER stress and hypoxia (Ohoka *et al*, 2005, Bowers *et al*, 2003). As with Trb1, Trb3 appeared to have a rapid response to cellular stress, and this response continued up to the final 24 hour time point, suggesting that Trb3 was involved in continued stress responses within the cell. A longer time course would be required to fully investigate the effects of starvation stress on Trb3.

The effect of the pro-inflammatory stimuli of IL-1+OSM on Trb1 and Trb3 was further investigated with a timecourse study. Trb1 appeared to respond to pro-inflammatory stimulation showing an increase in both mRNA and protein expression. The mRNA levels showed a more prolonged response than the protein levels, which were much more transient showing a rapid increase and subsequent decrease. This suggested that Trb1 protein levels are not directly controlled through transcriptional activation, and there is a greater complexity to their regulation such as degradative regulation. The fact that protein abundance is reduced in such a rapid fashion possibly indicates that Trb1 is not regulated through activation such as phosphorylation, as if this were the case one may not expect the level of the protein to be so tightly regulated, however, this is not always true. Trb1 has previously been shown to be up-regulated during inflammation; as previously stated it has been shown to be involved in pro-inflammatory signalling in adipocytes (Ostertag *et al*, 2010). Trb1 was also shown to be up regulated by the pro-inflammatory stimulus TNF-α, and was also shown to be up regulated during chronic antibody-mediated allograft failure (Ashton-Chess *et al*, 2008).

As with Trb1, Trb3 responded to a pro-inflammatory stimulus, showing an increase. The mRNA increase was not significant, however it showed a trend of a continuous increase up to 24 hours, suggesting a more prolonged response than Trb1. The increase in protein abundance was also more prolonged, however the initial response was less rapid. In addition, as with *Trb1*, the *Trb3* mRNA levels showed a more prolonged response than the protein levels. Suggesting that Trb3 protein levels are also not directly controlled through transcriptional activation, and there is a greater complexity to their regulation such as degradative regulation. Trb3 has previously been suggested to be involved in inflammatory signalling, as it has been shown to be involved in the survival and activity of mast cells which release pro-inflammatory mediators when activated (Ord *et al*, 2012). This role of Trb3 is thought to be through the action of IL-3 which regulates Trb3 mRNA expression. In addition, Trb3 silencing has also been shown to reduce inflammation in diabetic mice, showing a decrease in IL-6 and TNF-α (Ti *et al*, 2011). These studies along with the results above suggest Trb3 may be involved in pro-inflammatory signalling.

The regulation of Trb1 and Trb3 through the proteasomal degradation pathway was assessed using the proteasome inhibitor MG132. MG132 had no effect on Trb1 protein abundance. It is possible that no effect was seen due to full inhibition of the proteasomal pathway not being achieved. The level of ubiquitinated proteins present during MG132 treatment did not appear to be elevated to the extent one would expect from full proteasomal inhibition. Alternatively, it is possible that Trb1 protein levels are regulated through a different pathway, such as degradation through the lysosomal pathway.

Trb3 protein abundance was shown to increase with addition of MG132, suggesting that it was indeed degraded through the proteasomal pathway in HAC, and would suggest that Trb1 and Trb3 may be regulated differently. Proteasomal degradation would be a way of tightly regulating Trb3 protein levels within the cell. This result would concur with the IL-1+OSM timecourse data where mRNA and protein expression did not correlate. This regulation of Trb3 through proteasomal degradation has been reported previously, as it has been shown that Trb3 interacts with an E3 ubiquitin ligase called SIAH1. SIAH1 has been shown to cause polyubiquitination of Trb3 and direct it to the proteasome for degradation (Zhou *et al*, 2008). Due to the effect of cytokine stimulation on both Trb1 and Trb3 protein abundance, it is possible that cytokine stimulation may also have an effect on the proteasomal degradation of both Trb1 and Trb3, and should be investigated.

As mentioned, both Trb1 and Trb3 appeared to be present within the same cellular compartments, and in addition to this during IL-1+OSM stimulation they both appeared to increase within the cytoplasm. One possible explanation for this would be that Trb1 and Trb3 can in fact form a heterodimer within the cell. However, the protein complementation assay showed no signs of Trb1 and Trb3 interacting together in SW1353 cells. This study was performed in non-stimulatory conditions, therefore it is possible that they could in fact interact during stimulation, which should be investigated. In addition, it is possible that they do not interact but co-localise in order to modulate the consequences of signalling pathways following inflammatory stimuli.

4.4 Synopsis

Both Trb1 and Trb3 appear to be regulated similarly in some respects. They have similar localisation within SW1353 cells, and their translocation during pro-inflammatory stimulation is similar. In addition, they both appear to be up regulated under stress conditions and during pro-inflammatory stimulation in HAC. This suggests that they both may play a role within arthritic disease; as mentioned both cellular stress and inflammation are key characteristics of arthritis. They do however vary in the duration of this up regulation, suggesting that they may play differing roles. In addition to this, it appears that they are down regulated differently, further suggesting that Trb1 and Trb3 may play varying roles within the cell.

4.5 Summary

- Both Trb1 and Trb3 appeared to be predominantly nuclear and cytoplasmic in SW1353 cells, and IL-1+OSM stimulation led to an increase in both Trb1 and Trb3 within the cytoplasm.

- Serum starvation of HAC led to a rapid but transient increase in Trb1 protein expression, with an increase up to 6 hours, followed by a decrease. Trb3 also showed a rapid increase, but had a more prolonged increase in protein expression.

- IL-1+OSM stimulation showed a rapid and transient increase in Trb1 mRNA and protein expression, with protein abundance reducing earlier than mRNA expression. Trb3 showed a trend in the increase in mRNA expression, which was not significant, and protein abundance showed an increase which was less rapid and transient compared to Trb1.

- Proteasomal inhibition by MG132 had no effect on Trb1 but showed an increase in Trb3 protein abundance.

- Trb1 and Trb3 did not interact in SW1353 cells.

Chapter 5: The effect of Trb1 and Trb3 on MMPs and transcription factors which regulate these MMPs

5.1 Introduction

MMPs are a group of enzymes which cleave ECM components, and have been shown to be important in degrading cartilage ECM (Clark *et al*, 2008, Rowan *et al*, 2008). MMPs are important in terms of normal development and homeostasis (Cawston *et al*, 2005), but it is when their regulation is not under control that excessive ECM degradation occurs and a disease state arises.

In terms of arthritis, the collagenases are perhaps the most important proteinases as they are known to degrade collagen type II, which is a key component of cartilage (Rowan *et al*, 2008), and when degraded cannot be replaced (Jubb *et al*, 1980). MMP-1, MMP-8 and MMP-13 are the most potent in this collagenolytic action (Rowan *et al*, 2008), with MMP-13 being the most potent in terms of collagen type II cleavage (Knauper *et al*, 1996). There is therefore a great deal of research underway, in order to understand MMP regulation.

As mentioned, Trb1 and Trb3 are reported to play a role in regulating many cellular signalling pathways, such as MAPK (Kiss-Toth *et al*, 2004), PI3K/Akt (Du *et al*, 2003) and NFκB (Duggan *et al*, 2010). These pathways are considered important in mediating gene expression changes, including MMPs. In addition, it has been demonstrated that Trb3 is upregulated in OA (Cravero *et al,* 2009), and that Trb1 and Trb3 are induced during cellular stress (Ostertag *et al,* 2010; Ohoka *et al*, 2005; Bowers *et al*, 2003), which is a characterisitic of arthritis and MMP production. Trb1 and Trb3 have also been shown to regulate transcription factors known to regulate MMPs such as AP-1 (Kiss-Toth *et al*, 2004) and C/EBP (Ohoka *et al*, 2005; Gilby *et al*, 2010). It is therefore hypothesised that Trb1 and Trb3 may work through one or more of the pathways discussed, in order to regulate various transcription factors to regulate MMPs. It was initially assessed whether Trb1 and Trb3 could indeed regulate collagenolytic MMPs within a chondrocytic pro-inflammatory environment, and if this was through regulation at the transcriptional level.

The aims of this chapter were:-

- Determine whether Trb1 and Trb3 regulate *MMP-1* and *MMP-13* expression.
- Determine whether Trb1 and Trb3 regulate the expression and activation of the individual subunits of the transcription factor AP-1; c-Fos and c-Jun.
- Determine whether Trb1 and Trb3 regulate the transcription factor ATF3.

5.2 Results

5.2.1 Effect of Trb1 overexpression on MMP-1 and MMP-13 mRNA expression during IL-1+OSM stimulation in HAC and SW1353 cells

It was initially assessed whether Trb1 could regulate the collagenases MMP-1 and -13 within a pro-inflammatory chondrocytic environment. MMP-1 and -13 were chosen as they are thought to be the major collagenases in OA and RA (Tetlow *et al*, 1998; Billinghurst *et al*, 1997).

As demonstrated in Chapter 3, it was not possible to silence Trb1. Trb1 was therefore overexpressed in SW1353 cells, and the mRNA expression of *MMP-1* and *-13* investigated, following stimulation with the pro-inflammatory stimulus IL-1+OSM. Trb1 overexpression resulted in a significant decrease in both *MMP-1* and *-13* mRNA expression during stimulation with IL-1+OSM (Fig 5.1).

A B

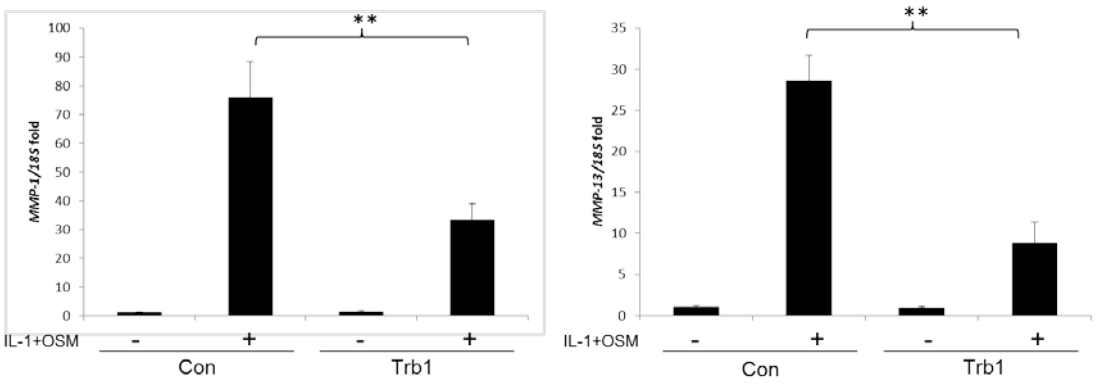

Figure 5.1. Trb1 overexpression leads to a reduction in *MMP-1* and *MMP-13* mRNA expression during IL-1+OSM stimulation in SW1353 cells. mRNA expression analysis of *MMP-1* (A) and *MMP-13* (B) after transfection with Trb1 overexpression plasmid +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb1 overexpression plasmid in 96 well plates (0.04 µg DNA/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 6 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *MMP-1* and *MMP-13* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate (+/-standard error of the mean). Results are combined data from 3 separate experiments, **p<0.01. Ct values for siCon were 13.99 +/- 0.27 for 18S, 29.73 +/- 0.29 for *MMP-1* and 31.03 +/- 0.08 for *MMP-13*. (+/- = standard error of the mean).

Overexpression of Trb1 in HAC also led to a significant reduction in *MMP-13* mRNA expression during IL-1+OSM stimulation, and the expression was reduced to non-stimulated basal levels (Fig 5.2B). However, overexpression of Trb1 had no significant effect on *MMP-1* expression (Fig 5.2A), unlike in SW1353 cells.

A B

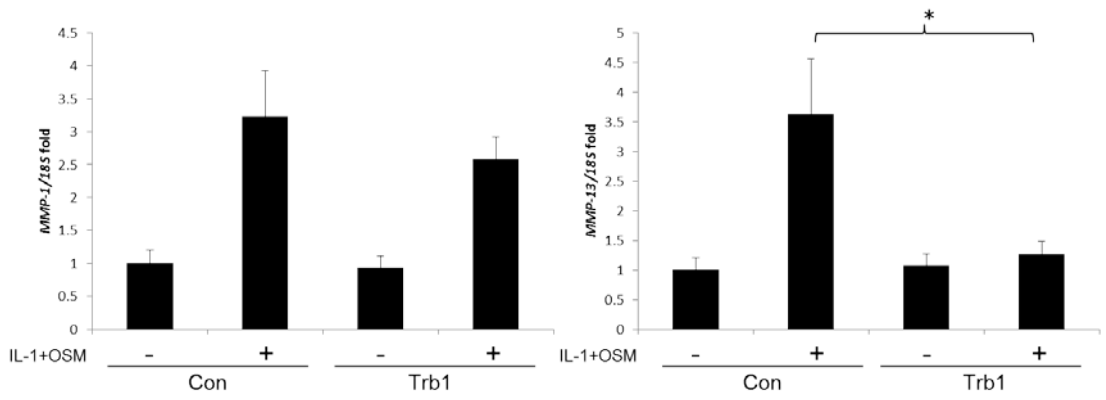

Figure 5.2. Trb1 overexpression leads to a reduction in *MMP-13* mRNA expression but has no effect on *MMP-1* during IL-1+OSM stimulation in HAC. mRNA expression analysis of *MMP-1* (A) and *MMP-13* (B) after transfection with Trb1 overexpression plasmid +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb1 overexpression plasmid in 96 well plates (0.2 µg DNA/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 24 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *MMP-1* and *MMP-13* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 3 separate donors, *p<0.05. Ct values for siCon were 17.48 +/- 0.33 for 18S, 25.49 +/- 0.49 for *MMP-1* and 30.59 +/- 0.37 for *MMP-13* (+/- = standard error of the mean).

5.2.2 Effect of Trb1 overexpression on c-fos and c-jun mRNA expression during IL-1+OSM stimulation in HAC

Due to the apparent effect of Trb1 on *MMP-13* mRNA expression, it was investigated whether Trb1 could regulate the AP-1 heterodimer subunits c-fos and c-jun. AP-1, as described previously, has been shown to be a major transcription factor involved in MMP regulation (Clark *et al*, 2008). The heterodimer of c-Fos and c-Jun has been shown to be the most important transcription factor in terms of MMP-1 and -13 regulation (Hu *et al* 1994, Saez *et al* 1995). In addition Litherland *et al*, (2010) demonstrated that silencing of c-fos and c-jun caused a reduction in IL-1+OSM induced *MMP-1* and *-13* expression. It has also been shown that Trb1 and Trb3 can regulate AP-1 transcription factor activation (Kiss-Toth *et al*, 2004).

The effect of Trb1 on *c-fos* and *c-jun* mRNA expression was not assessed in SW1353 cells, as work performed previously within the lab did not detect

expression of either of these 2 genes in this cell type, with the methods available. Trb1 overexpression led to a significant reduction in both *c-fos* and *c-jun* mRNA expression during IL-1+OSM stimulation in HAC (Fig 5.3). In addition, in non-stimulated conditions Trb1 overexpression showed a trend towards a reduction in *c-jun* expression (p=0.08), however this was not significant.

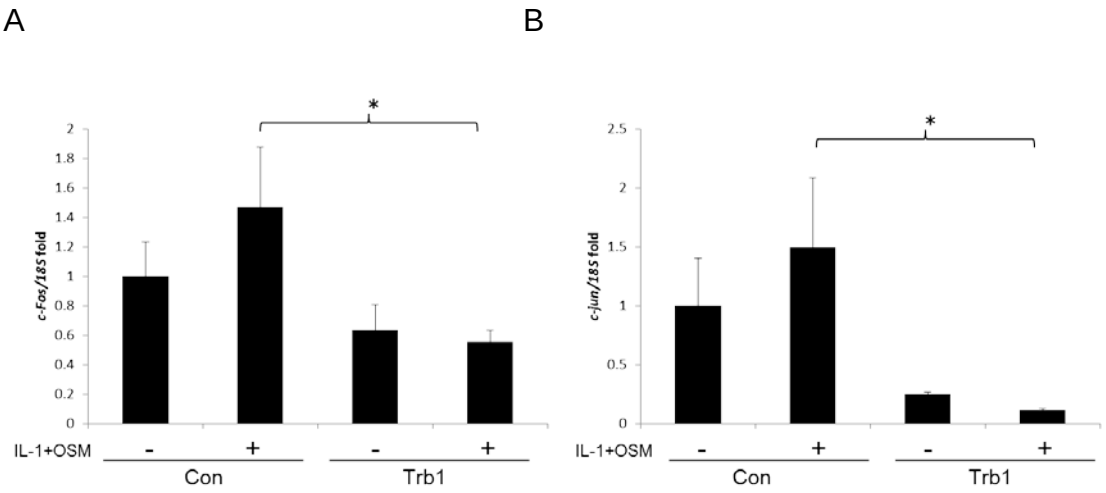

Figure 5.3. Trb1 overexpression leads to a reduction in *c-fos* and *c-jun* mRNA expression during IL-1+OSM stimulation in HAC. mRNA expression analysis of *c-fos* (A) and *c-jun* (B) after transfection with Trb1 overexpression plasmid +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb1 overexpression plasmid in 96 well plates (0.2 µg DNA/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 1 hour before lysis. Total RNA was isolated, reverse transcribed to cDNA and *c-jun* and *c-fos* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 3 separate donors, *p<0.05. Ct values for siCon were 20.68 +/- 0.81 for 18S, 33.53 +/- 1.20 for *c-jun* and 34.39 +/- 0.68 for *c-fos* (+/- = standard error of the mean).

5.2.3 Effect of Trb1 overexpression on ATF3 mRNA expression during IL-1+OSM stimulation in HAC

In addition to investigating the effect of Trb1 on AP-1, the effect of Trb1 overexpression on *ATF3* mRNA expression was investigated in HAC. ATF are a family of transcription factors that have been shown to regulate MMPs (Kwok *et al*, 2009, Melnikova *et al*, 2007, Fung and Demple, 2007). In addition, it had previously been demonstrated within our lab that ATF3 silencing led to a

reduction in *MMP-13* mRNA expression (Macdonald, 2013). As well as this, Trb1 and Trb3 may regulate the ATF pathway, as it was shown that Trb3 can regulate ATF4 (Jousse *et al*, 2007). It was therefore hypothesised that Trb1 may regulate ATF3 and therefore regulate MMP-13. Trb1 overexpression led to a significant reduction in *ATF3* mRNA expression in the presence of IL-1+OSM stimulation (Fig 5.4).

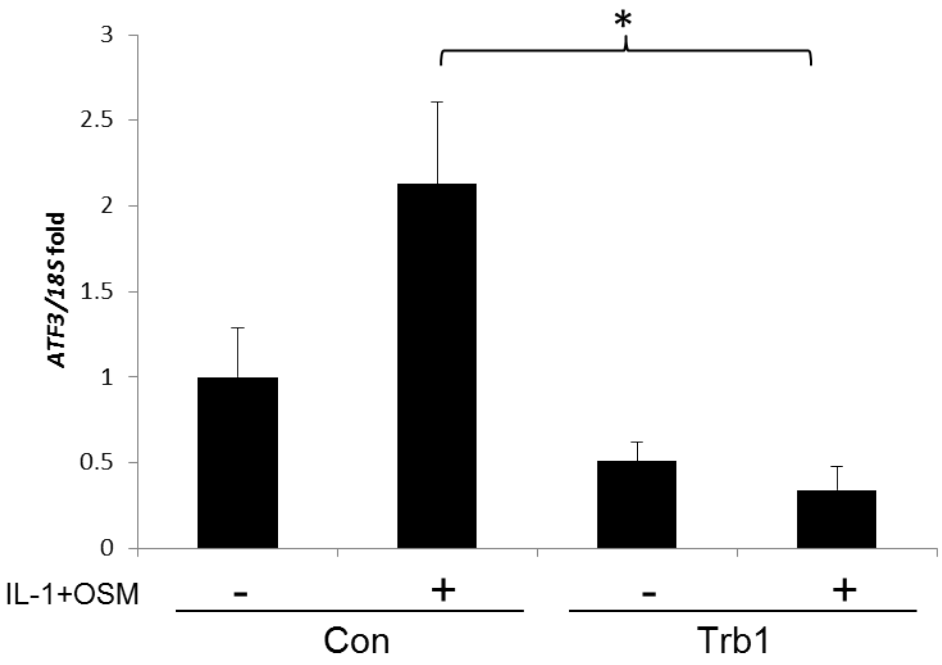

Figure 5.4. Trb1 overexpression leads to a reduction in *ATF3* mRNA expression during IL-1+OSM stimulation in HAC. mRNA expression analysis of *ATF3* after transfection with Trb1 overexpression plasmid +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb1 overexpression plasmid in 96 well plates (0.2 µg DNA/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 80 minutes before lysis. Total RNA was isolated, reverse transcribed to cDNA and *ATF3* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 3 separate donors, *p<0.05. Ct values for siCon were 19.85 +/- 0.75 for 18S and 36.31 +/- 0.40 for *ATF3* (+/- = standard error of the mean).

5.2.4 Effect of Trb3 siRNA gene silencing on MMP-1 and MMP-13 mRNA expression during IL-1+OSM stimulation in HAC and SW1353 cells

It was then assessed whether Trb3 could regulate the collagenases MMP-1 and -13 within a pro-inflammatory chondrocytic environment. The effect of Trb3

silencing on *MMP-1* and *-13* was initially investigated in SW1353 cells. Trb3 silencing showed a significant reduction in both *MMP-1* and *-13* in the presence of IL-1+OSM stimulation (Fig 5.5).

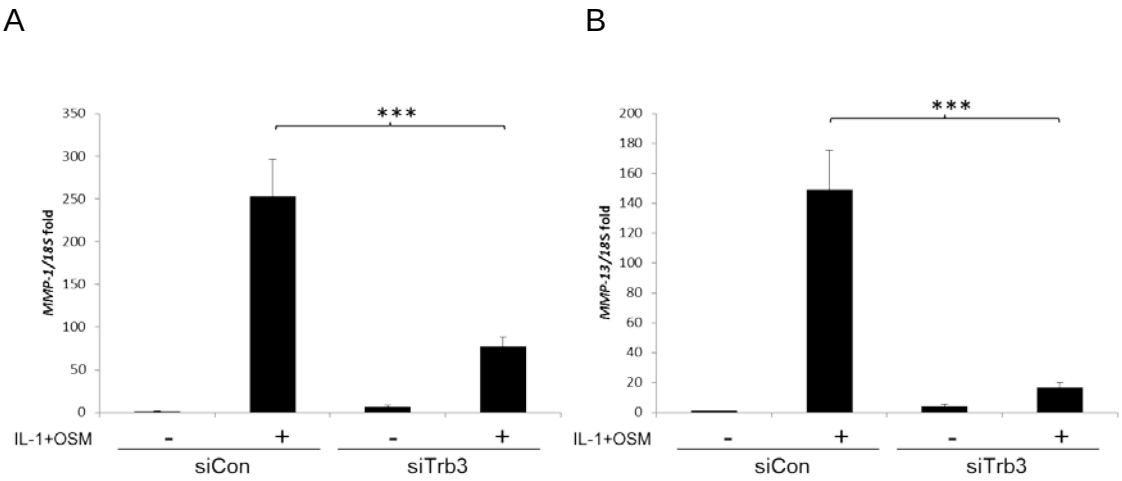

Figure 5.5. Trb3 gene silencing leads to a reduction in *MMP-1* and *MMP-13* mRNA expression during IL-1+OSM stimulation in SW1353 cells. mRNA expression analysis of *MMP-1* (A) and *MMP-13* (B) after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb3 siRNA in 96 well plates (100 nmol/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 6 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *MMP-1* and *MMP-13* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 2 separate experiments, ***p<0.001. Ct values for siCon were 14.39 +/- 0.11 for 18S, 29.20 +/- 0.49 for *MMP-1* and 28.47 +/- 0.19 for *MMP-13* (+/- = standard error of the mean).

Trb3 silencing in HAC led to a significant reduction in *MMP-13* mRNA expression during IL-1+OSM stimulation (Fig 5.6B). Silencing however, had no effect on *MMP-1* mRNA expression (Fig 5.6A). This varied from that seen in SW1353 cells where both *MMP-1* and *-13* appeared to be regulated by Trb3. This is in concordance with Trb1 which also demonstrated the same cell type specific differences.

A B

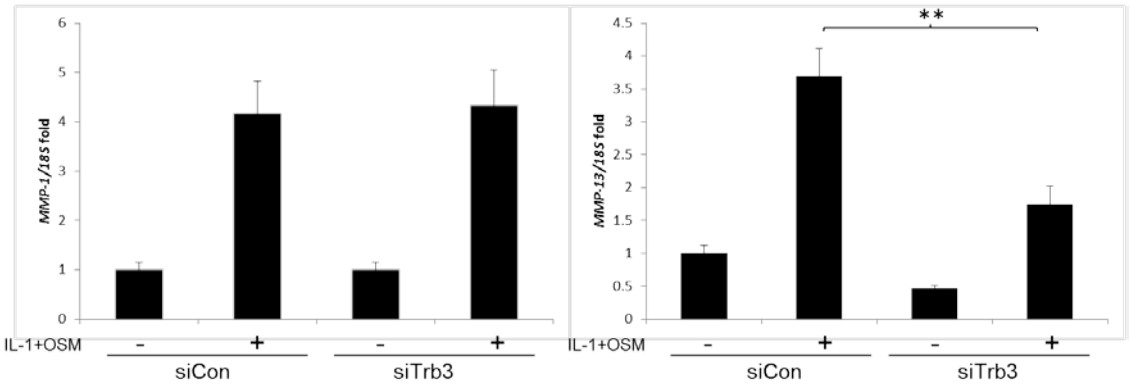

Figure 5.6. Trb3 gene silencing leads to a reduction in *MMP-13* mRNA expression but has no effect on *MMP-1* during IL-1+OSM stimulation in HAC. mRNA expression analysis of *MMP-1* (A) and *MMP-13* (B) after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 siRNA in 96 well plates (100 nmol/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 24 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *MMP-1* and *MMP-13* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 3 separate donors, **p<0.01. Ct values for siCon were 17.79 +/- 0.05 for 18S, 23.22 +- 0.07 for *MMP-1,* and 27.25 +/- 0.12 for *MMP-13* (+/- = standard error of the mean).

5.2.5 Effect of Trb3 overexpression on MMP-1 and MMP-13 mRNA expression during IL-1+OSM stimulation in HAC and SW1353 cells

Due to Trb3 silencing appearing to affect MMP mRNA expression, the effect of Trb3 overexpression on *MMP-1* and *-13* mRNA expression was also investigated. This was initially studied in SW1353 cells. Overexpression of Trb3 led to a significant reduction in *MMP-13* (Fig 5.7A), but had no effect on *MMP-1* (Fig 5.7B) following IL-1+OSM stimulation. This differed to the observed effects of Trb3 silencing, where Trb3 appeared to reduce both *MMP-1* and *-13* expression.

Both silencing and overexpression of Trb3 led to a reduction in *MMP-13*. This would appear to be somewhat counterintuitive, as one would generally expect opposing effects for silencing and overexpression.

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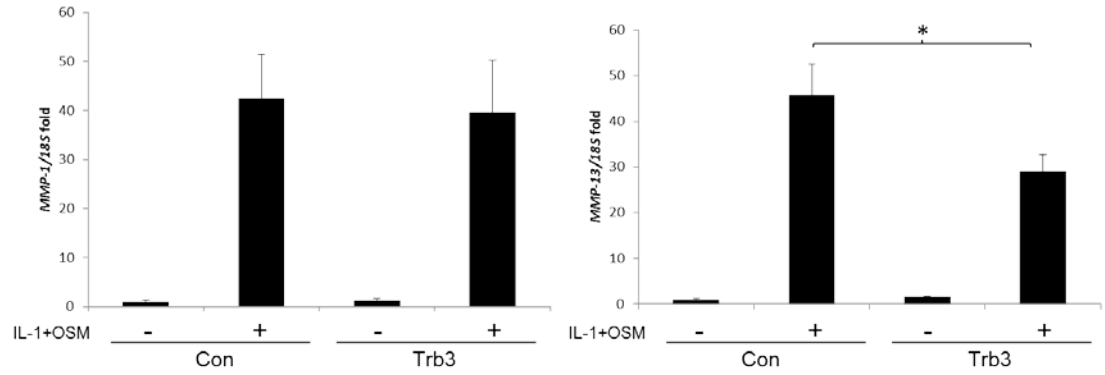

Figure 5.7. Trb3 overexpression leads to a reduction in *MMP-13* mRNA expression, but has no effect on *MMP-1* during IL-1+OSM stimulation in SW1353 cells. mRNA expression analysis of *MMP-1* (A) and *MMP-13* (B) after transfection with Trb3 overexpression plasmid +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb3 overexpression plasmid in 96 well plates (0.04 µg DNA/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 6 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *MMP-1* and *MMP-13* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 2 separate experiments, *p<0.05. Ct values for siCon were 13.06 +/- 0.22 for 18S, 31.59 +/- 0.30 for *MMP-1* and 31.65 +/- 0.41 for *MMP-13* (+/- = standard error of the mean).

Trb3 overexpression in HAC showed a significant reduction in *MMP-13* during IL-1+OSM stimulation (Fig 8B), but had no effect on *MMP-1* mRNA expression (Fig 8A). This result was similar to that seen with Trb3 silencing in HAC, whereby Trb3 had no effect on *MMP-1* but led to a reduction in *MMP-13*.

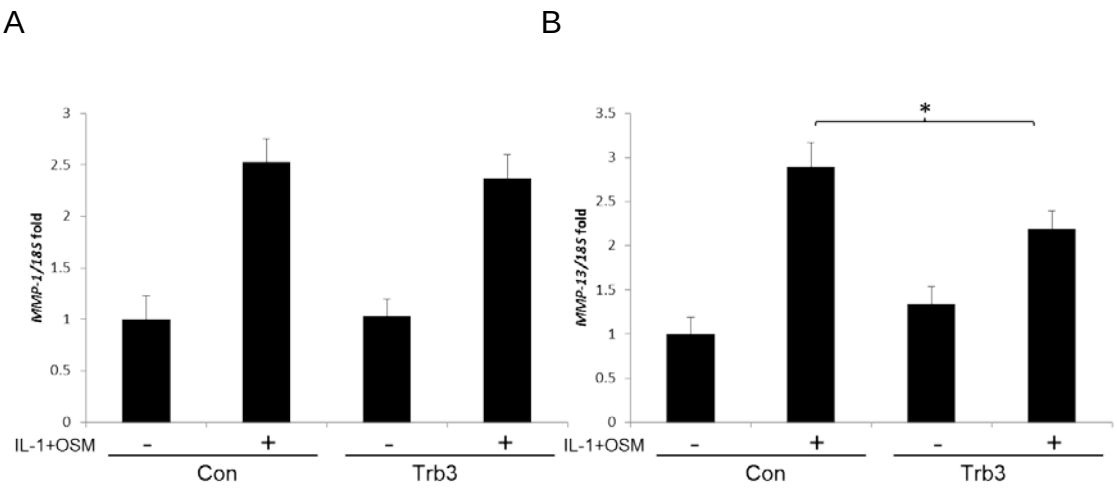

Figure 5.8. Trb3 overexpression leads to a reduction in *MMP-13* mRNA expression, but has no effect on *MMP-1* during IL-1+OSM stimulation in HAC. mRNA expression analysis of *MMP-1* (A) and *MMP-13* (B) after transfection with Trb3 overexpression plasmid +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 overexpression plasmid in 96 well plates (0.2 µg DNA/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 24 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *MMP-1* and *MMP-13* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 3 separate donors, *p<0.05. Ct values for siCon were 13 +/- 0.36 for 18S, 31.66 +/- 0.38 for *MMP-1* and 32.57 +/- 0.06 for *MMP-13* (+/- = standard error of the mean).

5.2.6 Effect of Trb3 siRNA gene silencing on c-fos and c-jun mRNA expression and protein abundance, as well as c-Jun activation during IL-1+OSM stimulation in HAC

Due to the observed effect of Trb3 on MMP-13 mRNA expression, it was investigated whether Trb3 could regulate the MMP transcription factor AP-1, specifically its subunits c-fos and c-jun, the importance of which on MMP transcription is described above.

Trb3 silencing did not appear to have any effect on *c-fos* or *c-jun* mRNA expression in HAC, either at the basal level, or during IL-1+OSM stimulation (Fig 5.9). This suggested that Trb3 does not regulate the AP-1 transcription factor at the mRNA level.

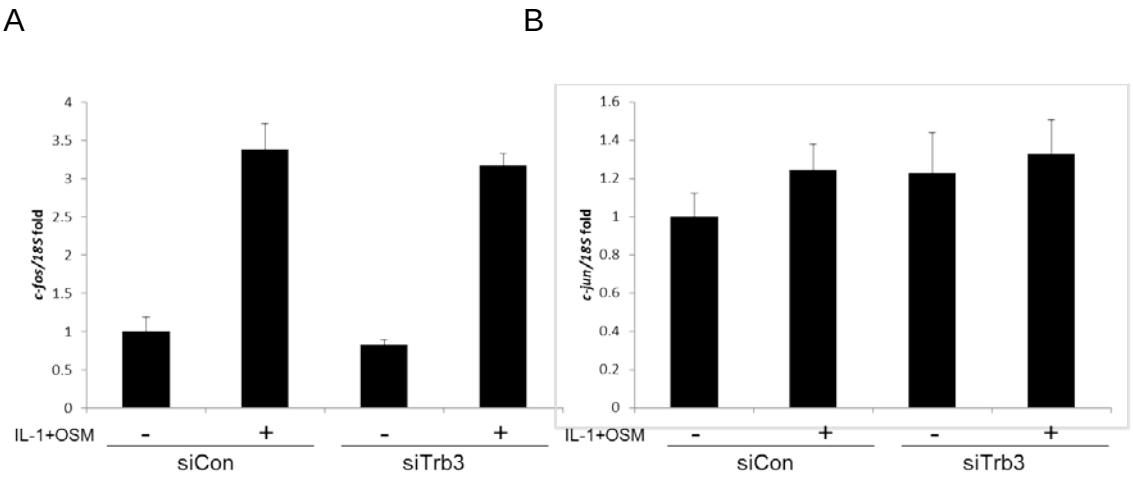

Figure 5.9. Trb3 siRNA gene silencing has no effect on *c-fos* and *c-jun* mRNA expression during IL-1+OSM stimulation in HAC. mRNA expression analysis of *c-fos* (A) and *c-jun* (B) after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 siRNA in 96 well plates (100 nmol/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 1 hour before lysis. Total RNA was isolated, reverse transcribed to cDNA and *c-fos* and *c-jun* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 3 separate donors. Ct values for siCon were 19.33 +/- 0.07 for 18S, 31.28 +/- 0.19 for *c-jun* and 29.87 +/- 0.24 for *c-fos* (+/- = standard error of the mean).

The effect of Trb3 silencing on c-Fos and c-Jun protein abundance as well as c-Jun activation was also investigated. It was hypothesised that although Trb3 silencing did not appear to regulate c-fos and c-jun at the mRNA level it may be involved in regulation at the protein level, as has been shown previously for Trb1 and Trb3 with other proteins.

In order to detect c-Fos and c-Jun, nuclear fractions were analysed, as they have a relatively low overall abundance within the cell. It was initially demonstrated that Trb3 was silenced at the protein level. Trb3 silencing led to a reduction in c-Fos protein abundance within the nucleus during IL-1+OSM stimulation. In addition, Trb3 silencing also led to a reduction in c-Jun activation (Fig 5.10). This suggested that Trb3 regulates the AP-1 transcription factor by regulating both c-Fos and c-Jun; c-Fos through its protein abundance within the nucleus, and c-Jun through activation by phosphorylation. There was no effect on native c-Jun expression during Trb3 silencing, both basally or in the presence of IL-1+OSM, c-Jun was therefore used as a loading control.

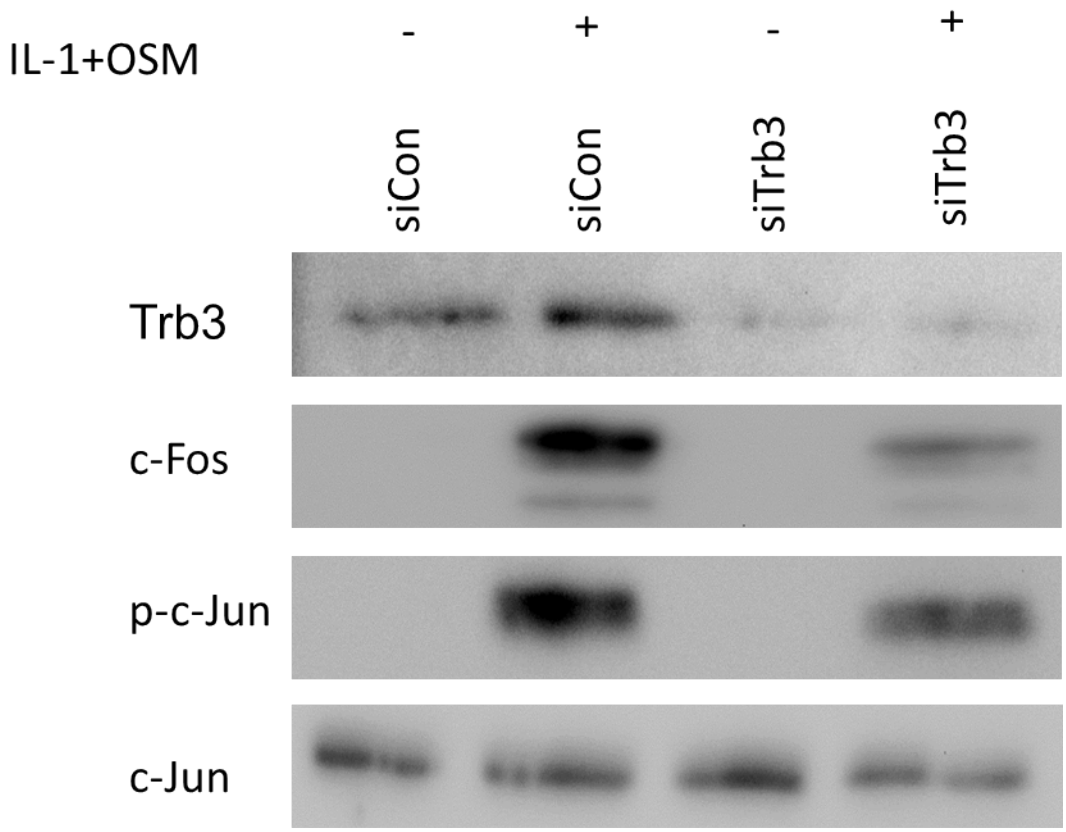

Figure 5.10. Trb3 siRNA gene silencing regulates both c-Jun and c-Fos at the protein level during IL-1+OSM stimulation in HAC. Trb3, c-Fos, p-c-Jun and c-Jun protein abundance after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 siRNA in 6 cm dishes (100 nmol/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 1 hour. Nuclear fractionation was then performed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments, from 3 separate donors.

5.2.7 Effect of Trb3 siRNA gene silencing on ATF3 mRNA expression and protein abundance during IL-1+OSM stimulation in HAC

As previously mentioned, it had been demonstrated within the lab that ATF3 silencing led to a reduction in *MMP-13* mRNA expression, but had no effect on *MMP-1* (Macdondald, 2013), much in the same way that Trb3 silencing in HAC was specific for *MMP-13*. It was therefore thought that Trb3 may regulate ATF3 in order to regulate MMP-13. Trb3 silencing led to a significant reduction in

ATF3 mRNA expression in the presence of IL-1+OSM stimulation in HAC (Fig 5.11).

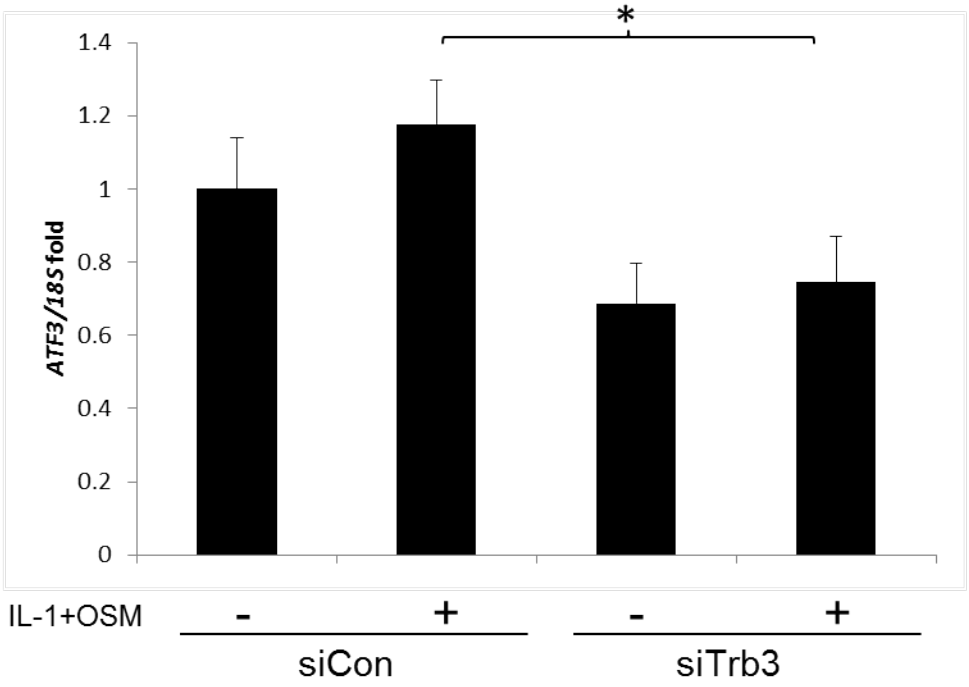

Figure 5.11. Trb3 siRNA gene silencing leads to a reduction in *ATF3* mRNA expression during IL-1+OSM stimulation in HAC. mRNA expression analysis of *ATF3* after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 siRNA in 96 well plates (100 nmol/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 80 minutes before lysis. Total RNA was isolated, reverse transcribed to cDNA and *ATF3* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 3 separate donors, *p<0.05. Ct values for siCon were 19.83 +/- 0.05 for 18S and 34.95 +/- 0.37 for *ATF3* (+/- = standard error of the mean).

Due to the effect of Trb3 on *ATF3* mRNA expression in HAC, the effect of Trb3 silencing on ATF3 nuclear protein abundance was also investigated. Trb3 protein expression was successfully silenced. Trb3 silencing showed a reduction in ATF3 protein abundance within the nucleus during IL-1+OSM stimulation (Fig 5.12). This would correlate with that seen at the mRNA level, where Trb3 silencing showed a reduction in *ATF3* mRNA expression.

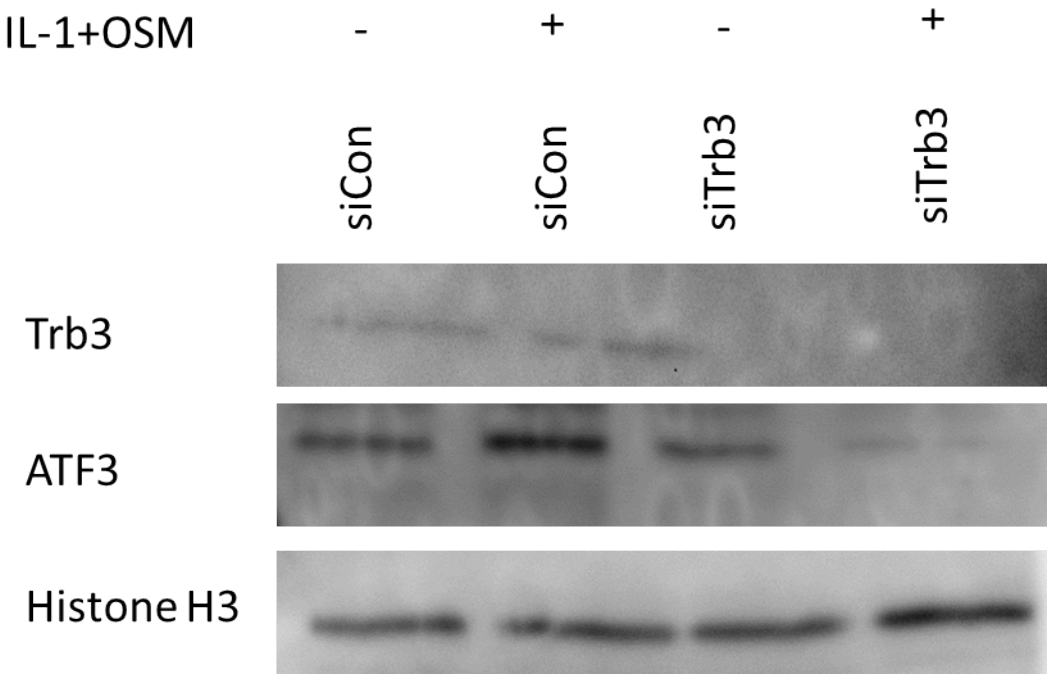

Figure 5.12. Trb3 siRNA gene silencing leads to a reduction in ATF3 nuclear protein abundance during IL-1+OSM stimulation in HAC. Trb3, ATF3 and Histone H3 protein abundance after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 siRNA in 6 cm dishes (100 nmol/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 80 minutes. Nuclear fractionation was then performed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments, from 3 separate donors.

5.2.8 Effect of Trb3 overexpression on ATF3 mRNA expression during IL-1+OSM stimulation in HAC

Due to Trb3 silencing appearing to effect *ATF3* mRNA expression, Trb3 was also overexpressed and *ATF3* mRNA expression investigated in HAC. As with Trb3 silencing overexpression of Trb3 also showed a significant reduction in *ATF3* during IL-1+OSM stimulation (Fig 5.13). This result is in agreement with that seen for Trb3 regulation of *MMP-13* in HAC, where both silencing and overexpression demonstrated a reduction in *MMP-13*.

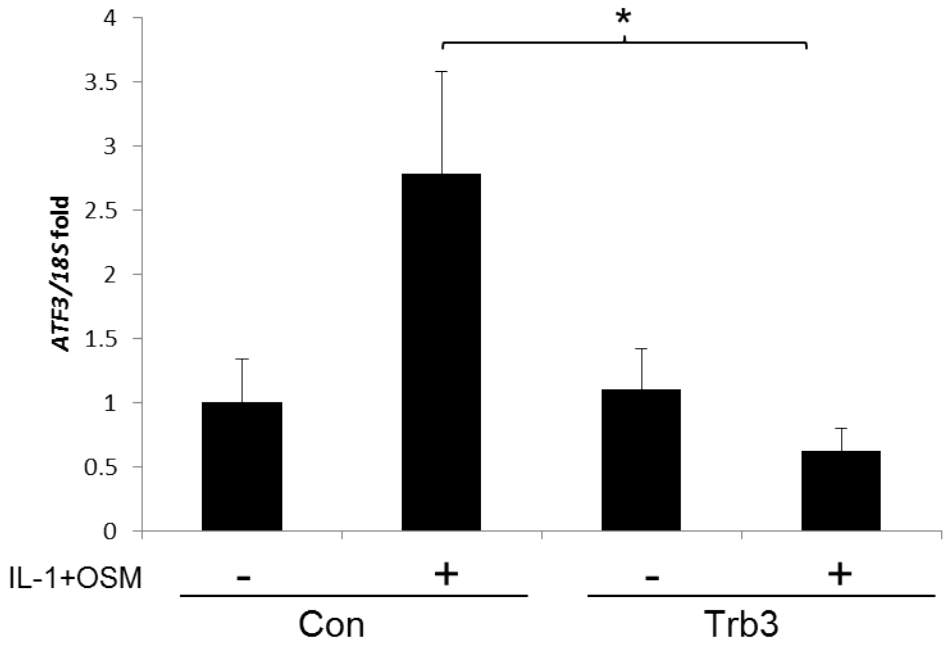

Figure 5.13. Trb3 overexpression leads to a reduction in *ATF3* mRNA expression during IL-1+OSM stimulation in HAC. mRNA expression analysis of *ATF3* after transfection with Trb3 overexpression plasmid +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 overexpression plasmid in 96 well plates (0.2 µg DNA/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 80 minutes before lysis. Total RNA was isolated, reverse transcribed to cDNA and *ATF3* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 3 separate donors. *p<0.05. Ct values for siCon were approximately 22 +/- 0.23 for 18S and 38.1 +/- 0.08 for *ATF3* (+/- = standard error of the mean).

5.3 Discussion

MMPs have been shown to be extremely important in terms of cartilage degradation. Understanding the proteins and pathways involved in their regulation is therefore of great interest. *MMP-1* and *-13* mRNA expression was investigated in both HAC and SW1353 cells, as in some experiments in other chapters it was not always possible to investigate the signaling in HAC for various reasons. Therefore, it was necessary to establish whether Trb1 and Trb3 could indeed regulate these MMPs in both cell types, and also investigate the differences between HAC and SW1353 cells in the way in which Trb1 and Trb3 regulate *MMP-1* and *-13* expression. As mentioned previously, it has been shown that there are differences in MMP signalling pathways between primary

human chondrocytes and SW1353 cells (Borden *et al*, 1996, Mengshol *et al*, 2000).

Trb1 overexpression led to a reduction in both *MMP-1* and *-13* mRNA expression during IL-1+OSM stimulation in SW1353 cells, however in HAC only *MMP-13* was affected. This may suggest that in SW1353 cells Trb1 regulates pathways which affect both MMP-1 and -13, whereas in HAC Trb1 regulates pathways more specific to MMP-13, suggesting cell type specificity. In addition, in HAC Trb1 overexpression reduced *MMP-13* expression levels down to the basal control levels, suggesting that it completely abolished the IL-1+OSM stimulatory affect.

This result, in both cell types, suggests that Trb1 may be involved in anti-inflammatory pathways which reduce pro-inflammatory stimuli and catabolic signaling including MMP expression and cartilage degradation. In agreement with this, previous work within our lab has suggested that Trb1 may be involved in the IL-4 pathway (Duncan, 2010), which is a known anti-inflammatory cytokine pathway that has been shown to inhibit IL-1 signaling (Colotta *et al*, 1993). IL-4 has also been shown to reduce OSM induced MMP-13 levels and IL-1 stimulated MMP-1 expression (Borghaei *et al*, 1998). Trb1 may therefore work through this pathway in order to reduce MMP expression. However, without the ability to silence Trb1 expression this cannot be confirmed.

Trb1 overexpression also resulted in a reduction in both *c-fos* and *c-jun* mRNA expression during IL-1+OSM stimulation in HAC. c-Fos and c-Jun are the two subunits which form the major AP-1 heterodimer involved in the transcriptional regulation of both MMP-1 and -13 (Hu *et al* 1994, Saez *et al* 1995). It has previously been demonstrated that Trb1 overexpression led to a reduction in AP-1 in HeLa cells (Kiss-Toth *et al*, 2004). However, this study did not state the specific AP-1 subunits which Trb1 regulated as regulation was demonstrated through an AP-1 luciferase construct. Regulation was only investigated in HAC as previous work within our lab could not detect *c-fos* or *c-jun* mRNA expression in SW1353 cells, highlighting a further difference between HAC and SW1353 cells in terms of MMP regulation. However, it has been previously demonstrated that c-fos and c-jun are expressed in SW1353 cells (Lim *et al*, 2011). The AP-1 result is consistent with that seen for the MMP data, suggesting that Trb1 may

be anti-inflammatory and protective to cartilage degradation. This may be through inhibition of AP-1 which subsequently leads to an inhibition of MMP expression. Also, in non-stimulated conditions Trb1 overexpression showed a strong trend towards a reduction in *c-jun* expression. This suggests that Trb1 may also regulate AP-1 in a non-inflammatory environment, perhaps implying that Trb1 may have a greater impact in OA rather than RA, which is more inflammation driven. The fact that Trb1 appears to regulate AP-1 would suggest it has the ability to regulate both MMP-1 and -13. The difference seen between *MMP-1* and *-13* expression during Trb1 overexpression in HAC may be due to additional transcription factors which Trb1 can regulate. It has been shown that the effect of AP-1 is abrogated by other transcription factors (Sharrocks *et al* 1997, Benbow *et al* 1997), and the modulation of AP-1 through these various transcription factors is only just being elucidated. These specific transcription factors may underlie the observation that Trb1 has a greater impact on *MMP-13* than *MMP-1*.

ATF3 is one of those transcription factors mentioned which may abrogate AP-1 transcriptional activation of MMPs. As mentioned, work within our lab had shown ATF3 to regulate *MMP-13* but not *MMP-1*. In addition, AP-1 was shown to regulate IL-1+OSM mediated *ATF3* expression (Macdonald, 2013). It has also been previously demonstrated that silencing of ATF3 suppresses *MMP-13* expression (Kwok *et al*, 2009). ATF3 has also been shown to work synergistically with c-jun in order to increase its activity (Pearson *et al*, 2003). Trb1 overexpression showed a reduction in *ATF3* mRNA expression during IL-1+OSM stimulation in HAC. This result would therefore correlate with the previous data for both MMP-13 and AP-1 suggesting that Trb1 is anti-inflammatory, whereby Trb1 can inhibit both AP-1 and ATF3 which leads to an inhibition in MMP expression. The effect of Trb1 on the AP-1 subunits c-jun and c-fos may subsequently lead to a reduction in *ATF3* mRNA expression; alternatively Trb1 may act directly on ATF3.

As with Trb1, the effect of Trb3 on MMPs appeared to be cell type specific, differing between HAC and SW1353 cells. Trb3 silencing in SW1353 cells showed a reduction in both *MMP-1* and *-13* mRNA expression, whilst only affecting *MMP-13* in HAC. This result suggested that Trb3 regulates pathways in HAC which ultimately lead to specifically regulating transcription factors

involved in MMP-13 expression, whereas in SW1353 cells Trb3 regulates pathways which lead to a broader effect on MMP transcription. From these results alone one could hypothesise that Trb3 was in fact a pro-inflammatory protein, its expression leading to an increase in MMP expression and cartilage degradation. However, the Trb3 overexpression data also showed a reduction in *MMP* expression. It is hypothesised that within the cellular environment, for normal homeostasis and signalling to occur, a specific level of Trb3 is required within the cell. When this level is altered, either increased or decreased, there is dysregulation within the pathways, leading to a reduction in signalling. This may suggest that Trb3 is acting as a scaffold protein, as both overexpression and silencing of scaffolds has been shown to lead to inhibition of signalling (Morrison and Davis, 2003). The reason behind this is that low levels of the scaffold protein do not allow the proteins within the pathway to combine, and high levels could segregate the components of this pathway, preventing them interacting with other proteins within a particular pathway.

In both SW1353 cells and HAC, Trb3 overexpression showed a reduction in *MMP-13* only. This correlates with the data observed in HAC, as only *MMP-13* was affected, further underlining the notion that Trb3 specifically regulates MMP-13 in HAC. However, in SW1353 cells the overexpression and silencing data are somewhat at odds, as silencing of Trb3 had an effect on both *MMP-1* and *-13*. It is possible that the siRNA transfection efficiency was greater than that of the overexpression plasmid transfection efficiency. A certain level of transfection of Trb3 may be required in SW1353 cells in order to alter *MMP-1* levels, which was not attained during overexpression. As mentioned, the level of Trb3 within the cell may be of critical importance to whether signalling occurs or is dysregulated, and the level of Trb3 required may differ for different pathways. This may suggest that MMP-13 is more responsive to changes in Trb3 than MMP-1 in SW1353 cells.

Trb3 silencing had no effect on *c-fos* or *c-jun* mRNA expression, suggesting that it was not directly involved in transcriptional regulation of these 2 proteins, unlike Trb1. However, Trb3 was shown to regulate both c-Fos protein abundance and c-Jun activation, demonstrating that Trb3 was involved in pathways that regulate AP-1. Trb3 may increase c-Fos protein abundance possibly through binding of c-Fos and stabilising it, although there is no

evidence of this within the literature. Alternatively, Trb3 may prevent c-Fos from being degraded; Trb3 has been shown to perform this role with other proteins, such as the Smad proteins (Park *et al* 2009). Trb3 may regulate c-Jun phosphorylation through regulation of the MAPK pathway, as this has been shown to regulate c-Jun phosphorylation (Han *et al*, 2001, Leppa *et al*, 1998), more of which will be discussed later. These results would be in agreement with that seen with the MMP-13 data, that Trb3 silencing caused a dysregulation in signalling. This dysregulation led to a reduction in c-Fos protein abundance and c-Jun phosphorylation, subsequently leading to a reduction in *MMP-13* mRNA expression. Kiss-Toth *et al*. (2004) had previously demonstrated that Trb3 had the ability to regulate AP-1, demonstrating that both silencing and overexpression of Trb3 led to a reduction in AP-1 luciferase expression. This would be consistent with the MMP data, showing both silencing and overexpression of Trb3 led to a reduction in *MMP-13* expression.

As mentioned previously, the fact that Trb3 only appears to regulate MMP-13 in HAC, but also regulates AP-1; a transcription factor known to regulate both MMP-1 and -13, suggests that there is a greater level of complexity occurring in regards Trb3 regulation of MMP-13. This additional complexity may be in the form of other transcription factors in which Trb3 regulates which can combine with AP-1 in order to specifically regulate MMP-13. Therefore, ATF3 was investigated as a possible transcription factor that could abrogate AP-1 activity, the reason for this being discussed earlier. Both overexpression and silencing of Trb3 led to a reduction in *ATF3* mRNA expression, which would be in agreement with that seen for the regulation of *MMP-13* by Trb3. In addition, Trb3 silencing was also shown to reduce ATF3 protein abundance, which would be expected, as it effects mRNA expression. These results suggest that Trb3 may regulate both AP-1 and ATF3, which can lead to a specific regulation of MMP-13, as discussed for Trb1. It is also possible that Trb3 regulates a number of other transcription factors which can abrogate AP-1 activity to specifically regulate MMP-13. Further research is therefore required in order to identify if other transcription factors are involved, and how they influence this transcriptional regulation.

5.4 Synopsis

Trb1 and Trb3 appear to regulate MMPs in both SW1353 cells and HAC. This regulation may indeed be cell type specific. Within HAC there appeared to be regulation of *MMP-13* but not *MMP-1* for both Trb1 and Trb3, whereas in SW1353 cells both Trb1 and Trb3 appeared to regulate both *MMP-1* and *-13*. This regulation of *MMP-13* within HAC may be through regulation of the 2 transcription factors AP-1 and ATF3. Both Trb1 and Trb3 appeared to regulate these 2 transcription factors. Trb1 and Trb3 both regulated the two AP-1 sub units c-Fos and c-Jun. However, this regulation appeared to be different between the Trb1 and Trb3 proteins, with Trb1 appearing to regulate AP-1 at the transcriptional level, whilst Trb3 appeared to regulate AP-1 at the protein level. Both silencing and overexpression of Trb3 had similar effects on the signalling pathways, suggesting that a specific level of Trb3 is required for signalling, and deviation from this level results in dysregulation of signalling.

5.5 Summary

- Trb1 overexpression led to a reduction in *MMP-1* and *-13* mRNA expression in SW1353 cells but only *MMP-13* in HAC.

- Trb1 overexpression led to a reduction in *c-fos* and *c-jun* mRNA expression in HAC, as well as *ATF3*.

- Trb3 silencing led to a reduction in *MMP-1* and *-13* mRNA expression in SW1353 cells. Whilst Trb3 overexpression led to a reduction in *MMP-13* only.

- Trb3 silencing and overexpression led to a reduction in *MMP-13* mRNA expression, but had no effect on *MMP-1* in HAC.

- Trb3 silencing had no effect on *c-fos* or *c-jun* mRNA expression but reduced both c-Fos protein abundance and c-Jun activation in HAC.

- Trb3 silencing and overexpression reduced *ATF3* mRNA expression, and Trb3 silencing also reduced ATF3 protein abundance.

Chapter 6: The effect of Trb1 and Trb3 on the MAP kinase pathway

6.1 Introduction

The MAP kinase pathway, consisting of ERK, JNK and p38, plays a role in a vast number of cellular pathways, and is critical for cell growth, differentiation and survival (Pouyssegur *et al*, 2003). The MAP kinase pathway has been shown to play a major role in arthritic disease. MAPK activation has been shown to be increased in arthritis (Schett *et al*, 2000, Boileau *et al*, 2006), and it is involved in the progression of arthritis in animal models (Pelletier *et al*, 2003, Brown *et al*, 2008). As well as this, MAPK activation has been shown to be enhanced by pro-inflammatory stimuli including IL-1 (Schett *et al*, 2000), and is an important regulator of MMPs, including MMP-1 and -13 (Mengshol *et al*, 2000, Han *et al*, 2001, Raymond *et al* 2006). In addition, one of the ways in which MAPKs are thought to regulate MMPs is through the AP-1 transcription factor (Han *et al*, 2001; Menghsol *et al* 2001), which as discussed previously is an important transcription factor in terms of MMP regulation (Clark *et al*, 2008).

Trb1 and Trb3 have previously been shown to regulate expression of a number of proteins important in cellular signaling, including TGF-β (Chan, 2010; Chan *et al* 2007), ATF4 (Bowers *et al*, 2003) and C/EBP-α (Gilby *et al*, 2010). In addition, Trb1 and Trb3 have been previously shown to regulate the MAPK pathway (Kiss-Toth *et al*, 2004), and they have also been shown to bind MAP2Ks (Sung *et al*, 2007, Kiss-Toth *et al*, 2004). Work in the previous chapter demonstrated that both Trb1 and Trb3 have the ability to regulate the AP-1 transcription factor. It was therefore hypothesised that Trb1 and Trb3 may regulate MMPs, and therefore cartilage turnover, through the MAPK pathway.

The aims of this chapter were:-

- Determine what MAP2Ks Trb1 and Trb3 interact with.
- Investigate the effect of Trb1 and Trb3 on MAPK activation.

- Investigate the effect of MAP2K gene depletion and inhibition on Trb1 and Trb3.

6.2 Results

6.2.1 MAPK activation during IL-1+OSM stimulation in SW1353 cells and HAC

The role of Trb1 and Trb3 in the MAPK pathway were studied in both SW1353 cells and HAC, as due to certain constraints which will be discussed it was not always possible to study this role solely in HAC. Therefore, it was initially investigated whether MAPKs had a similar activation profile during IL-1+OSM stimulation in both HAC and SW1353 cells, and thus SW1353 cells could be treated as a model for HAC in this context.

The activation profiles for all MAPKs were very similar between HAC and SW1353 cells. They become activated by 20 minutes IL-1+OSM stimulation and then progressively decrease after this (Fig 6.1).

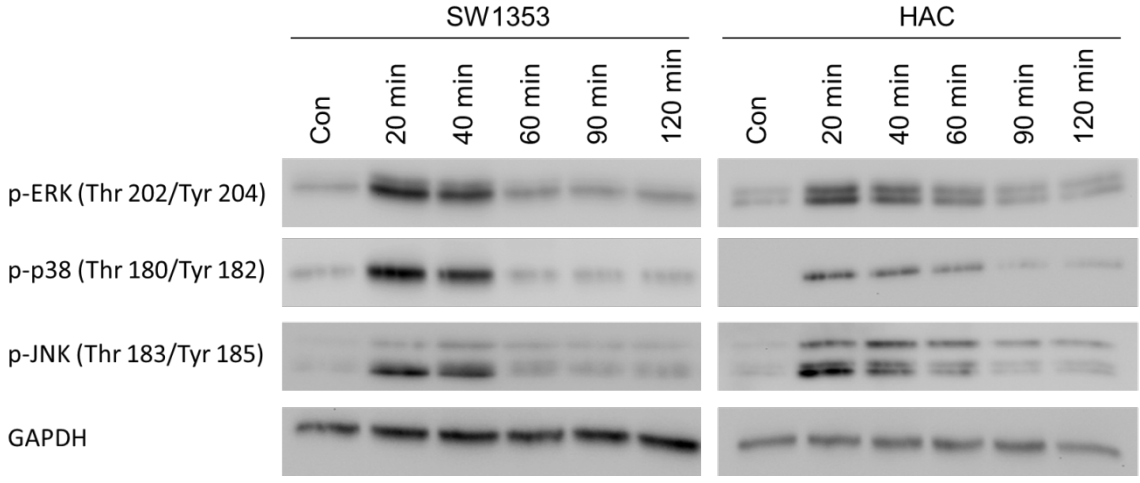

Figure 6.1. MAPK activation during IL-1+OSM stimulation in SW1353 cells and HAC. p-ERK, p-p38, p-JNK and GAPDH abundance after stimulation with IL-1+OSM in SW1353 cells and HAC. Cells were serum-starved overnight before stimulation with IL-1 (0.5 ng/ml SW1353, 0.05 ng/ml HAC) + OSM (10 ng/ml) for the indicated time points in 6 well plates. Cells were then lysed and proteins were separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 2 independent experiments, and from 2 separate donors with regard to HAC.

6.2.2 The interaction of Trb1 with MAP2Ks

It was previously demonstrated by Kiss-Toth *et al*, (2004) that Trb1 had the ability to bind to MEK1 and MKK4 in HeLa cells. However, due to the apparent cell type specificity of Trb1 and Trb3, it was initially investigated which MAP2Ks Trb1 had the ability to bind to in the context of chondrocytes. The interaction of Trb1 and MEK1, MKK4, MKK6 and MKK7 was investigated using the protein complementation assay, as described previously (see chapter 4). This was investigated in SW1353 cells due to the low transfection efficiency of HAC, as discussed in chapter 4.

Microscopic visualisation of fluorescence demonstrated that Trb1 interacted with all four of the MAP2Ks tested (MEK1, MKK4, MKK6 and MKK7) (Fig 6.2). In addition, depending on the MAP2K which Trb1 interacted with, the pattern of staining was different. Trb1-MEK1 demonstrated diffuse staining within the nucleus, as well as faint cytoplasmic staining. This was also similar for Trb1-MKK4. Trb1-MKK6 demonstrated distinct punctate staining within the nucleus, whilst Trb1-MKK7 also demonstrated diffuse nuclear staining, but did not appear to show any cytoplasmic staining. Transfection efficiency was low; this was due to the fact that 2 plasmids were required to enter the cell, as previously discussed.

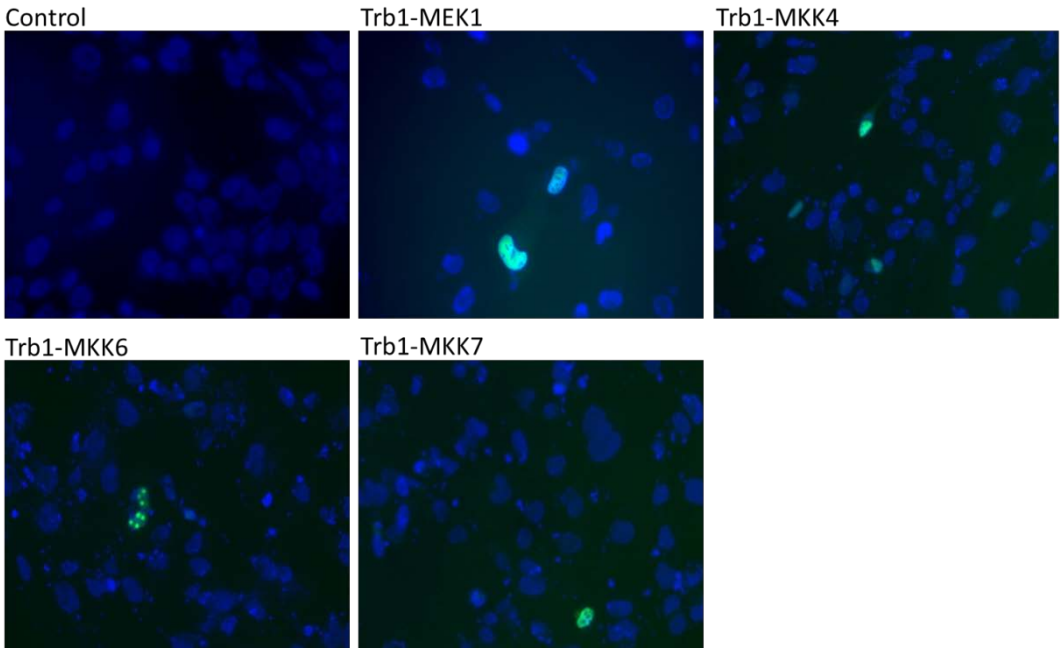

Figure 6.2. Trb1 interacts with MAP2Ks in SW1353 cells. Fluorescent images of MAP2K (V1) and Trb1 (V2) overexpression constructs in SW1353 cells. Cells were transfected with each construct in 8 well chamber slides (0.2 µg per construct) for 48 hours, as detailed in the Materials and Methods, then serum-starved for 6 hours, fixed and visualised. Results are representative of 2 independent experiments.

6.2.3 The effect of Trb1 overexpression on MAPK activation during IL-1+OSM stimulation in SW1353 cells

As it was demonstrated that Trb1 could indeed interact with MAP2Ks in SW1353 cells, the effect of Trb1 on the activation of all 3 MAPKs (ERK, JNK and p38) in SW1353 cells was investigated. This was performed in SW1353 cells rather than HAC, as unstimulated basal levels of protein phosphorylation in HAC were as high as stimulated levels when overexpression was performed with control plasmid, therefore it was not possible to determine if the HAC were performing as they should, i.e. increasing in phosphorylation with stimulation. Therefore throughout this thesis the effect of Trb1 and Trb3 on protein abundance during IL-1+OSM stimulation was only investigated in SW1353 cells. Trb1 was initially shown to be overexpressed. Trb1 overexpression showed a reduction in both p38 and JNK activation, but had no effect on ERK activation during IL-1+OSM stimulation (Fig 6.3).

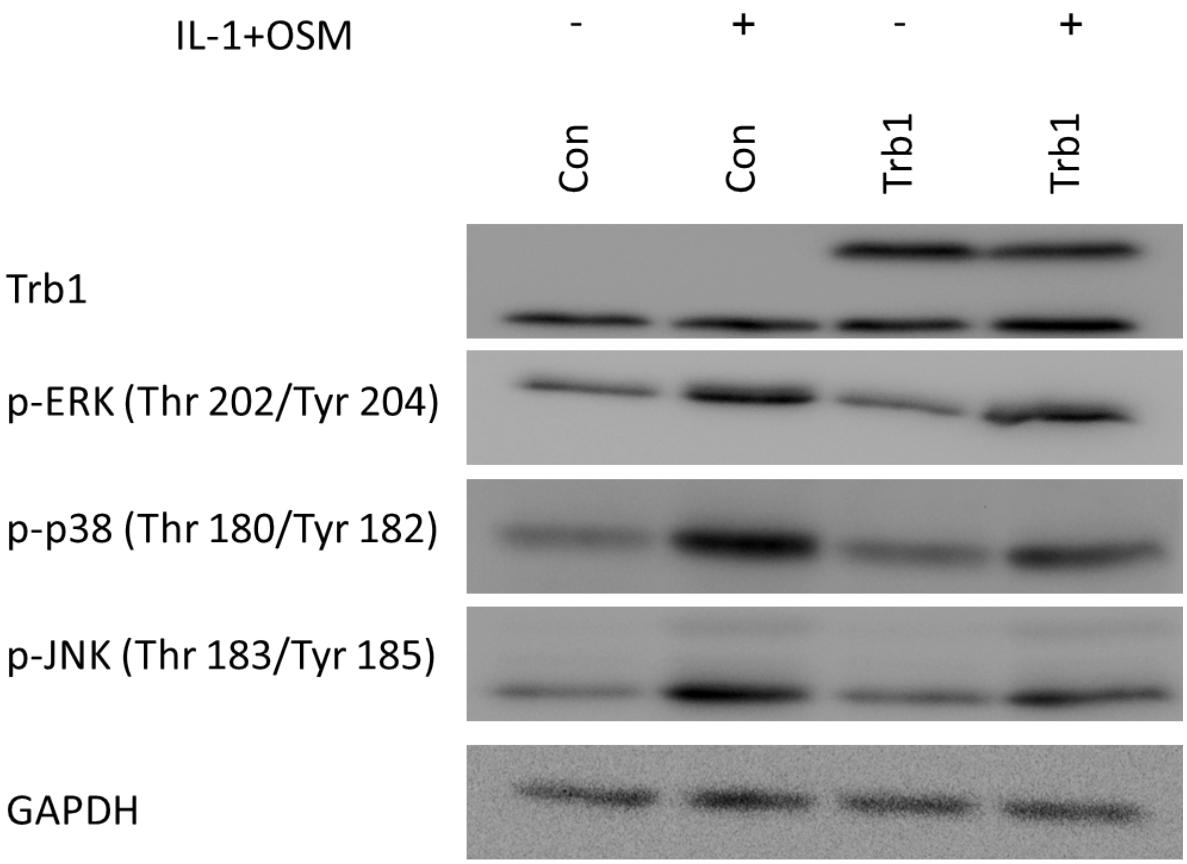

Figure 6.3. Trb1 overexpression regulates MAPK activation during IL-1+OSM stimulation in SW1353 cells. Trb1, p-ERK, p-p38, p-JNK and GAPDH abundance after transfection with Trb1 overexpression plasmid +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb1 overexpression plasmid in 6 well plates (0.9 µg DNA/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 20 minutes. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments.

6.2.4 The effect of combined siRNA gene silencing of the MAP2Ks on Trb1 protein abundance in HAC

Due to the ability of Trb1 to bind to the MAP2Ks and also its apparent effect on MAPK activation it was hypothesised that the level of MAP2Ks within the cell may influence the levels of Trb1. In addition, it was also demonstrated by Kiss-Toth *et al*. (2004) in HeLa cells that interaction of Trb1 with its MAP2K binding partners could stabilise Trb1. A combined silencing was performed as individual MAP2K silencing had no effect on Trb1 and Trb3 protein abundance (data not

shown). MEK1, MKK4, MKK6 and MKK7 were silenced as these were previously shown to bind Trb1 in the protein complementation assay (see Fig 6.2).

The combined silencing of the MAP2Ks had no effect on Trb1 protein abundance in HAC (Fig 6.5). The real time data demonstrated that the MAP2Ks were silenced at the mRNA level (Fig 6.4), however it could not be assessed whether the MAP2Ks were in fact silenced at the protein level due to poor results when immunoblotting chondrocyte lysates.

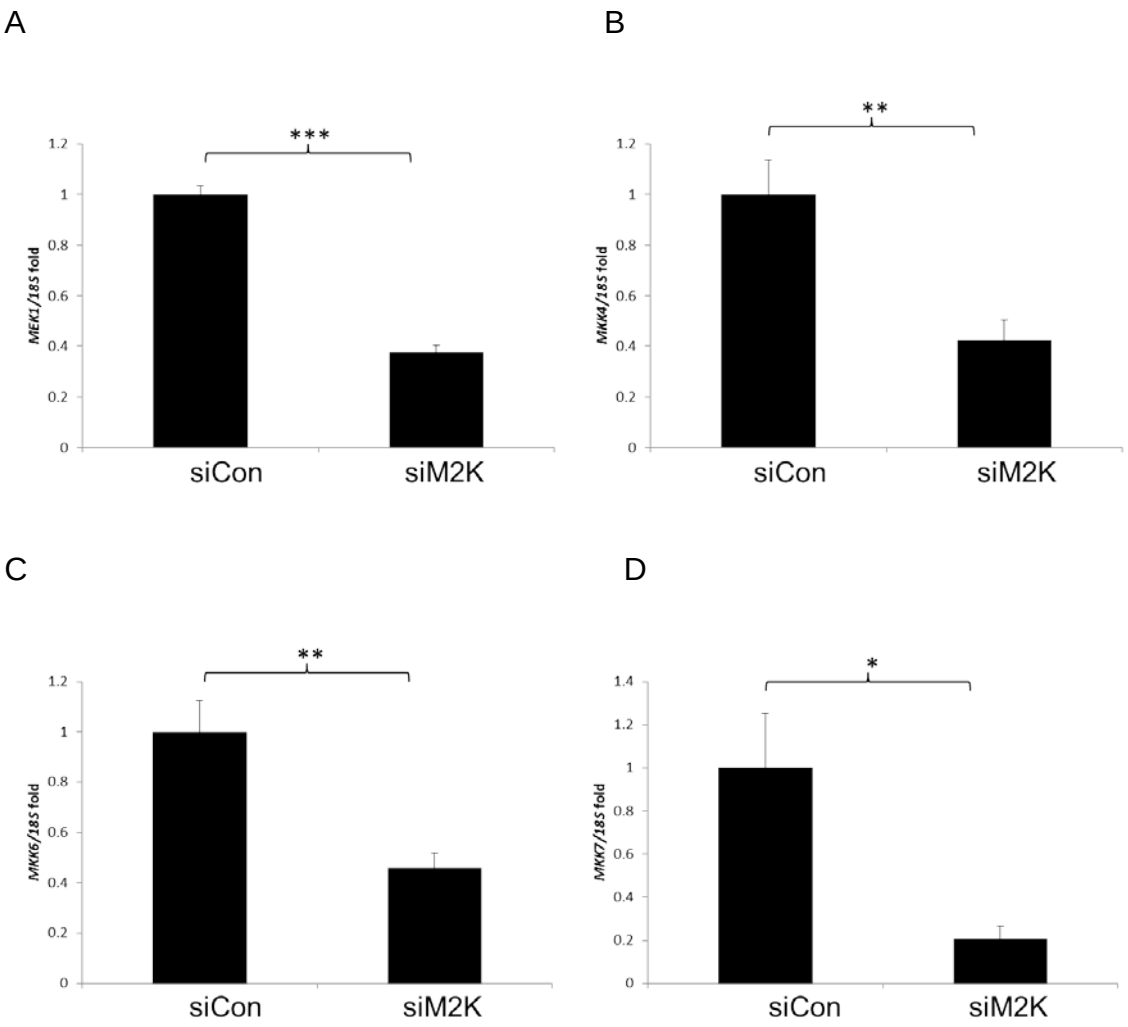

Figure 6.4. siRNA gene silencing of the MAP2Ks in HAC. mRNA expression analysis of *MEK1* (A), *MKK4* (B), *MKK6* (C) and *MKK7* (D) after transfection with the corresponding siRNA in HAC. Cells were transfected with siRNA in 96 well plates (25 nmol/siRNA/well) for 48 hours and serum-starved overnight before lysis. Total RNA was isolated, reverse transcribed to cDNA and *MEK1*, *MKK4*, *MKK6* and *MKK7* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). *p<0.05, **p<0.01, ***p<0.001. Ct values were approximately 18.72 +/- 0.16 for 18S, 32.34 +/- 0.35 for *MEK1,* 42.07 +/- 0.52 for *MKK4,* 34.74 +/- 0.25 for *MKK6,* and 37.94 +/- 0.47 for *MKK7* (+/- = standard error of the mean).

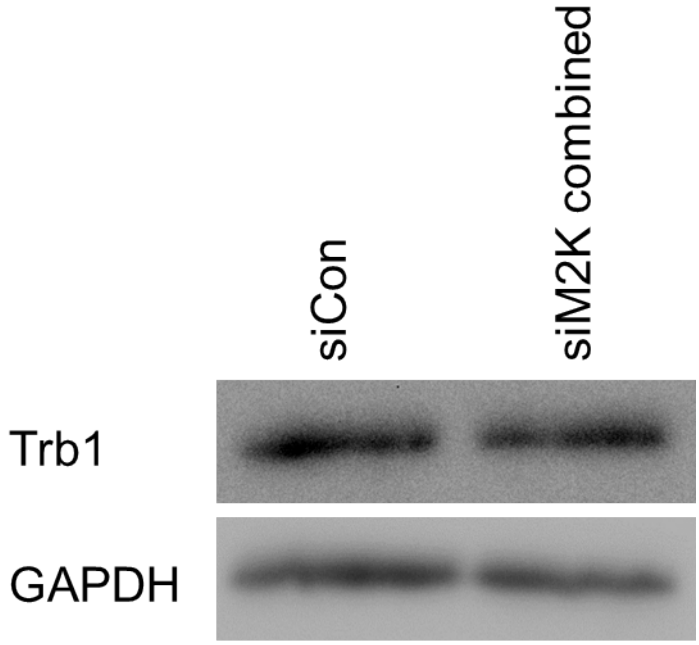

Figure 6.5. The combined silencing of MAP2Ks has no effect on Trb1 protein abundance in HAC. Trb1 and GAPDH abundance after transfection with MEK1 MKK4, MKK6 and MKK7 siRNA in HAC. Cells were transfected with MEK1 MKK4, MKK6 and MKK7 siRNA in 96 well plates (25 nmol/siRNA/well) for 48 hours and serum-starved overnight. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 4 independent experiments, from 4 separate donors.

6.2.5 The interaction of Trb3 with MAP2Ks in SW1353 cells

It had previously been demonstrated by Kiss-Toth *et al*. (2004) that Trb3 has the ability to bind to MEK1 and MKK7 in HeLa cells. Therefore the interaction of Trb3 with the four MAP2Ks MEK1, MKK4, MKK6 and MKK7 was assessed in SW1353 cells using the protein complementation assay. This was performed in SW1353 cells due to the low transfection efficiency of HAC, as discussed in chapter 4.

Microscopic visualisation demonstrated that Trb3 interacted with all four of the MAP2Ks tested (MEK1, MKK4, MKK6 and MKK7) (Fig 6.6), as was the case with Trb1. In addition, as with Trb1, the staining pattern varied depending on which MAP2K Trb3 was bound to. Trb3-MEK1 interaction showed diffuse staining within the nucleus, as well as cytoplasmic staining, which was similar to Trb1-MEK1. Trb3-MKK4 interaction appeared to vary between cells; in some cells there appeared to be punctate staining, in others the staining appeared to

be diffuse. Trb3-MKK6 showed discrete punctate staining within the nucleus, as was also seen for Trb1-MKK6, as well as faint cytoplasmic staining. Trb3-MKK7 also showed diffuse nuclear staining similar to Trb1-MKK7. As with Trb1-MAP2Ks, the transfection efficiency was low.

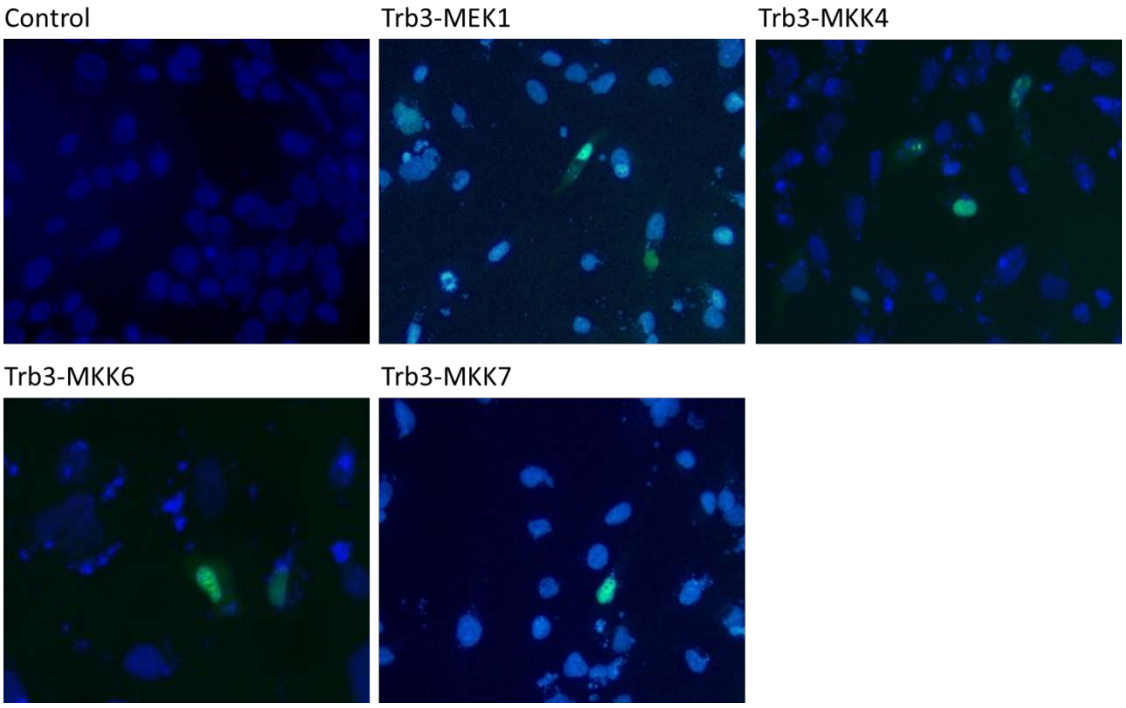

Figure 6.6. Trb3 interacts with MAP2Ks in SW1353 cells. Fluorescent images of MAP2K (V1) and Trb3 (V2) overexpression constructs in SW1353 cells. Cells were transfected with each construct in 8 well chamber slides (0.2 µg per construct) for 48 hours, as detailed in the Materials and Methods, then serum-starved for 6 hours, fixed and visualised. Results are representative of 2 independent experiments.

6.2.6 The effect of Trb3 siRNA gene silencing on MAPK activation during IL-1+OSM stimulation in SW1353 cells and HAC

It has previously been demonstrated that Trb3 can influence MAPK activation in HeLa cells, with different levels of Trb3 having differing effects (Kiss-Toth *et al*, 2004). In addition, it was shown in Figure 6.6 that Trb3 could interact with MAP2Ks. The effect of Trb3 on activation of all three MAPKs was therefore investigated. Trb3 was initially silenced, and the effect on MAPK activation during IL-1+OSM stimulation was explored in SW1353 cells. It was initially demonstrated that Trb3 was silenced at the protein level. Trb3 silencing showed a reduction in p38 activation during IL-1+OSM stimulation (Fig 6.7), no change

was seen for ERK or JNK activation. In addition Trb3 protein abundance appeared to increase with stimulation.

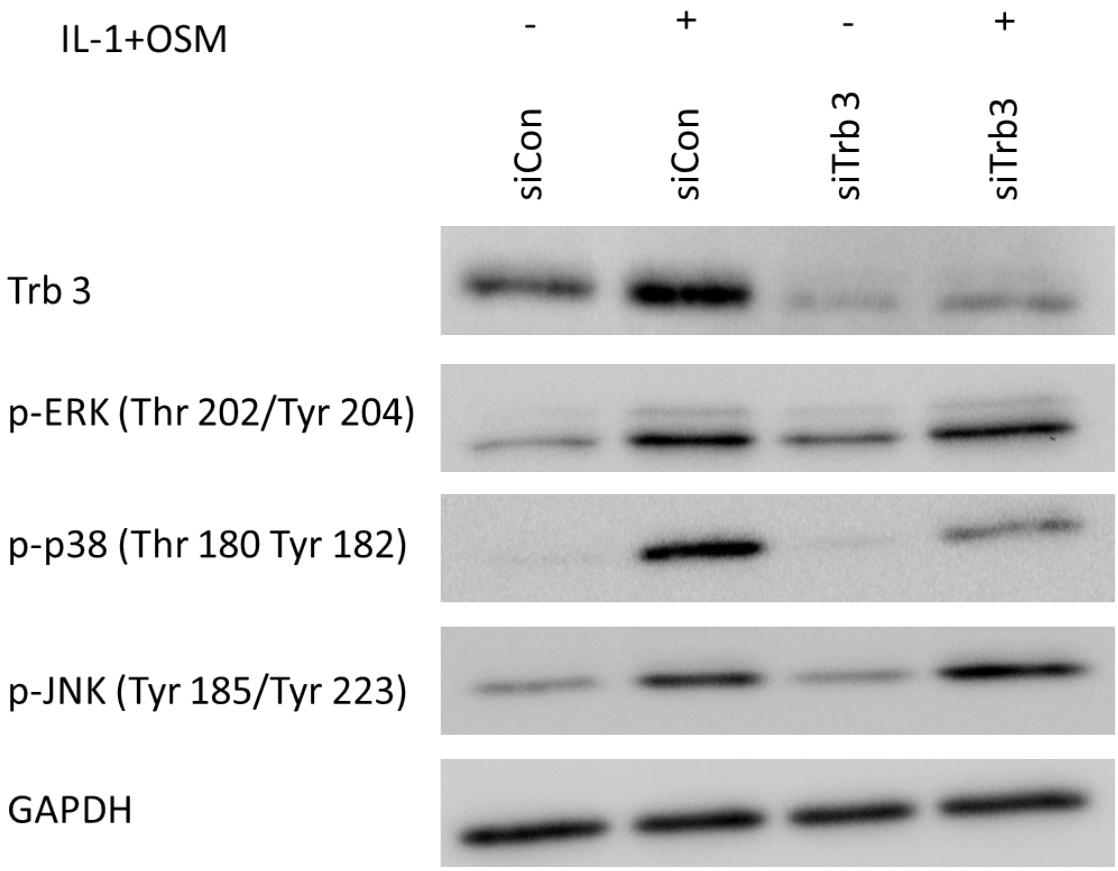

Figure 6.7. Trb3 siRNA gene silencing regulates MAPK activation during IL-1+OSM stimulation in SW1353 cells. Trb3, p-ERK, p-p38, p-JNK and GAPDH abundance after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb3 siRNA in 6 well plates (100 nmol/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 20 minutes. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments.

The effect of Trb3 silencing on MAPK activation in HAC was also investigated. It was initially demonstrated that Trb3 was successfully silenced. Trb3 silencing led to a reduction in activation of all 3 MAPKs during IL-1+OSM stimulation (Fig 6.8). This was unlike that seen in SW1353 cells, whereby only p38 activation was affected.

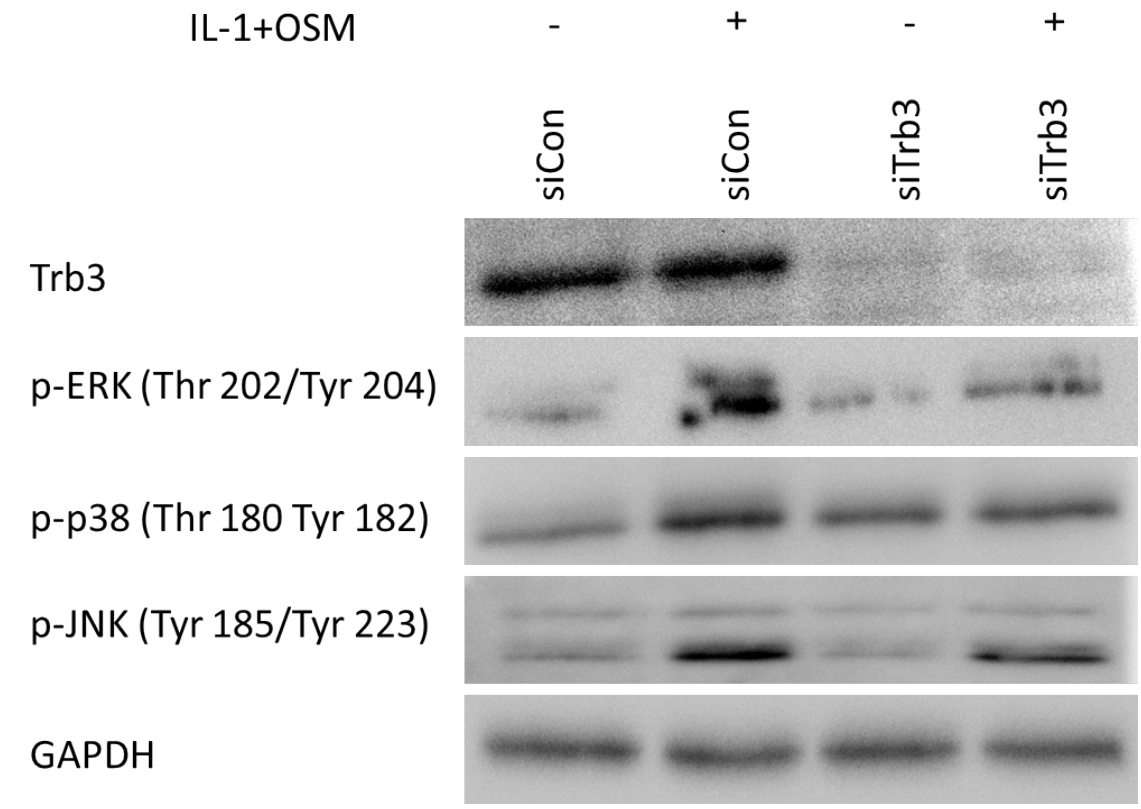

Figure 6.8. Trb3 siRNA gene silencing regulates MAPK activation during IL-1+OSM stimulation in HAC. Trb3, p-ERK, p-p38, p-JNK and GAPDH abundance after transfection with Trb3 siRNA +/- IL-1+OSM stimulation in HAC. Cells were transfected with Trb3 siRNA in 6 well plates (100 nmol/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 20 minutes. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments, from 3 separate donors.

6.2.7 The effect of Trb3 overexpression on MAPK activation during IL-1+OSM stimulation in SW1353 cells

Due to the effect of Trb3 silencing on MAPK activation, in both HAC and SW1353 cells, Trb3 was overexpressed and MAPK activation observed during IL-1+OSM stimulation in SW1353 cells. It was initially demonstrated that Trb3 was successfully overexpressed at the protein level; this was shown through the c-Myc tag on the Trb3 overexpression plasmid. Trb3 overexpression led to a reduction in activation in all 3 of the MAPKs during IL-1+OSM stimulation (Fig

6.9). This was in contrast to that observed when Trb3 was silenced in SW1353 cells, as only p38 activation was affected.

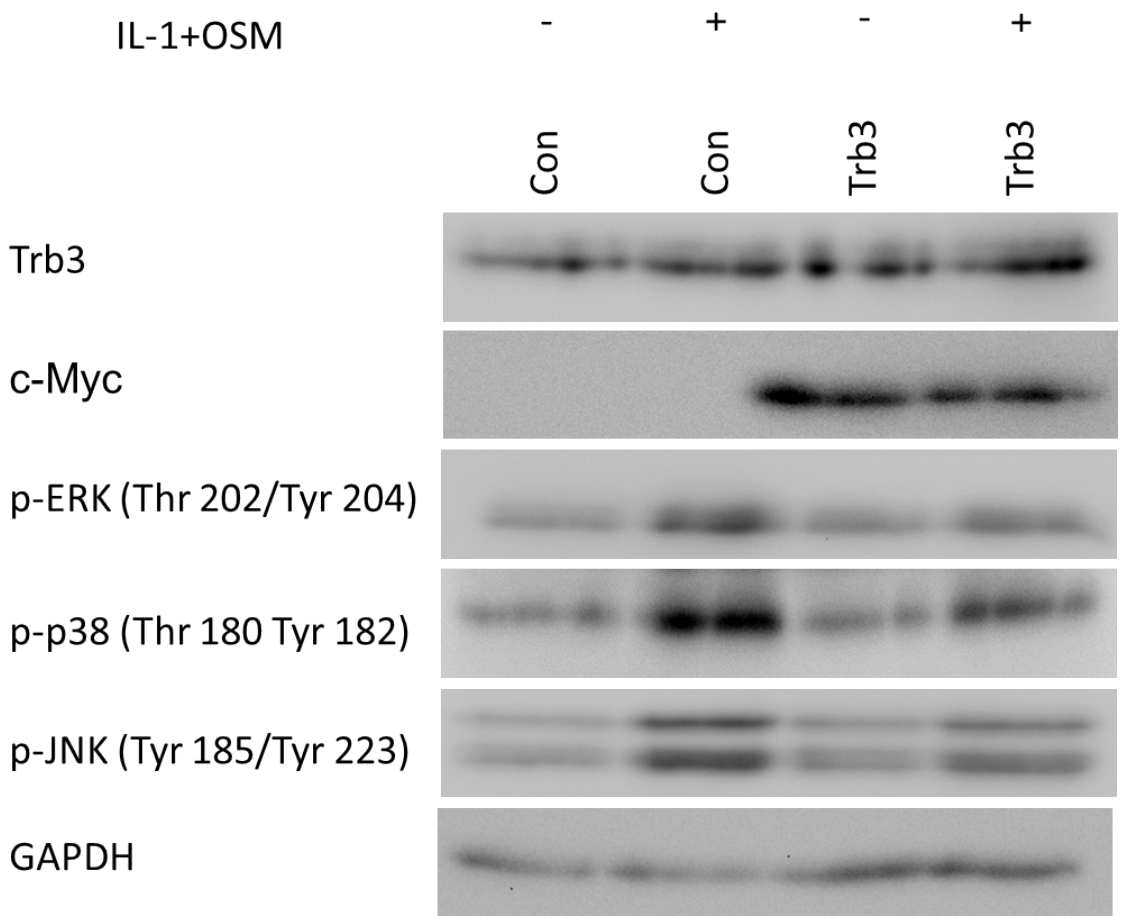

Figure 6.9. Trb3 overexpression regulates MAPK activation during IL-1+OSM stimulation in SW1353 cells. Trb3, p-ERK, p-p38, p-JNK and GAPDH abundance after transfection with Trb3 overexpression plasmid +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb3 overexpression plasmid (1.2 µg DNA/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 20 minutes. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments.

6.2.8 The effect of combined siRNA gene silencing of the MAP2Ks on Trb3 protein abundance in HAC

It has been shown previously that the binding of Trb3 to its MAP2Ks can stabilise Trb3 in HeLa cells (Kiss-Toth *et al*, 2004). In addition to the data above

demonstrating the ability of Trb3 to bind to MAP2Ks, and also regulate MAPK activation, it was thought that the binding of Trb3 to MAP2Ks may also stabilise Trb3 in chondrocytes. The effect of combined silencing of MAP2Ks on Trb3 was therefore investigated in HAC.

MEK1, MKK4, MKK6 and MKK7 were silenced as they were shown to bind Trb3 in the protein complementation assay (see Fig 6.6). As was discussed for Trb1, combined silencing was performed, as individual MAP2K silencing was attempted, however, this had no effect on Trb1 and Trb3 protein expression (data not shown).

The combined silencing of the MAP2Ks showed a reduction in Trb3 protein abundance (Fig 6.10). However, as discussed previously the real time data demonstrated that the MAP2Ks were silenced at the mRNA level (Fig 6.4). However, it could not be assessed whether the MAP2Ks were in fact silenced at the protein level due to poor results when immunoblotting chondrocyte lysates. Therefore, it could not be confirmed whether all of these MAP2Ks were silenced at the protein level, and therefore having an effect on Trb3 protein expression.

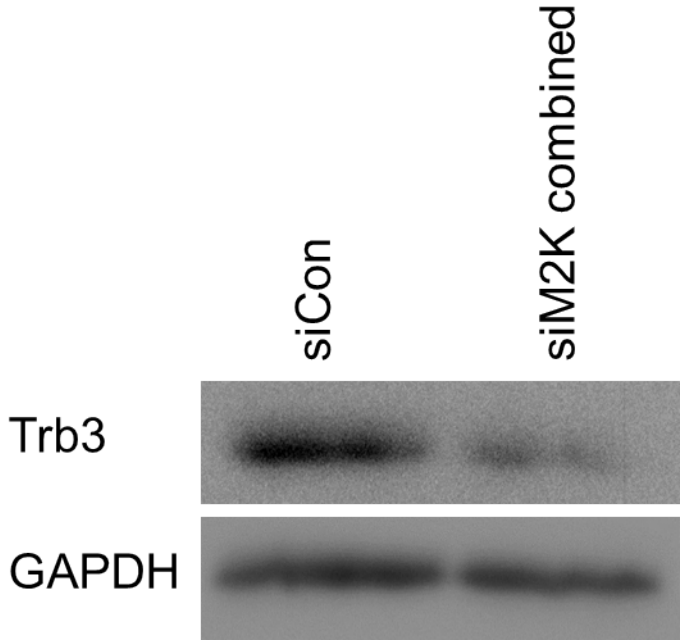

Figure 6.10. The combined silencing of MAP2Ks leads to a reduction in Trb3 protein abundance in HAC. Trb3 and GAPDH abundance after transfection with MAP2Ks MEK1, MKK4, MKK6 and MKK7 siRNA in HAC. Cells were transfected with MAP2Ks MEK1, MKK4, MKK6 and MKK7 siRNA in 6 well plates (25 nmol/siRNA/well) for 48 hours and serum-starved overnight. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 4 independent experiments, from 4 separate donors.

6.2.9 The effect of the MEK inhibitor UO126 on Trb3 protein abundance during IL-1+OSM stimulation in HAC

Due to MAP2K combined silencing influencing Trb3 protein abundance, the impact of inhibiting a specific set of MAP2Ks involved in a specific MAPK pathway, namely MEK1 and MEK2 and the ERK pathway was investigated. UO126 was used to inhibit the ERK pathway. UO126 exerts its biological effect by inhibiting MEK1 and MEK2, thereby inhibiting the ERK pathway (Favata *et al*, 1998).

UO126 inhibition of the ERK pathway showed a reduction in Trb3 protein abundance at all concentratons. Stimulation with IL-1+OSM had no impact on this result (Fig 6.11). However, UO126 did not appear to be a specific inhibitor for the ERK pathway, inhibiting both p38 and JNK activation, even at low concentrations of UO126; however, this was to a lesser extent than ERK

inhibition. This would perhaps suggest that UO126 is affecting the activation of a number of MAP2Ks other than MEK1/2. Therefore it cannot be determined whether inhibition of a specific MAPK pathway, namely ERK, has an influence on Trb3 protein abundance. The conclusion from this result can only be that an overall inhibition of the MAPK pathway results in a reduction in Trb3 protein abundance.

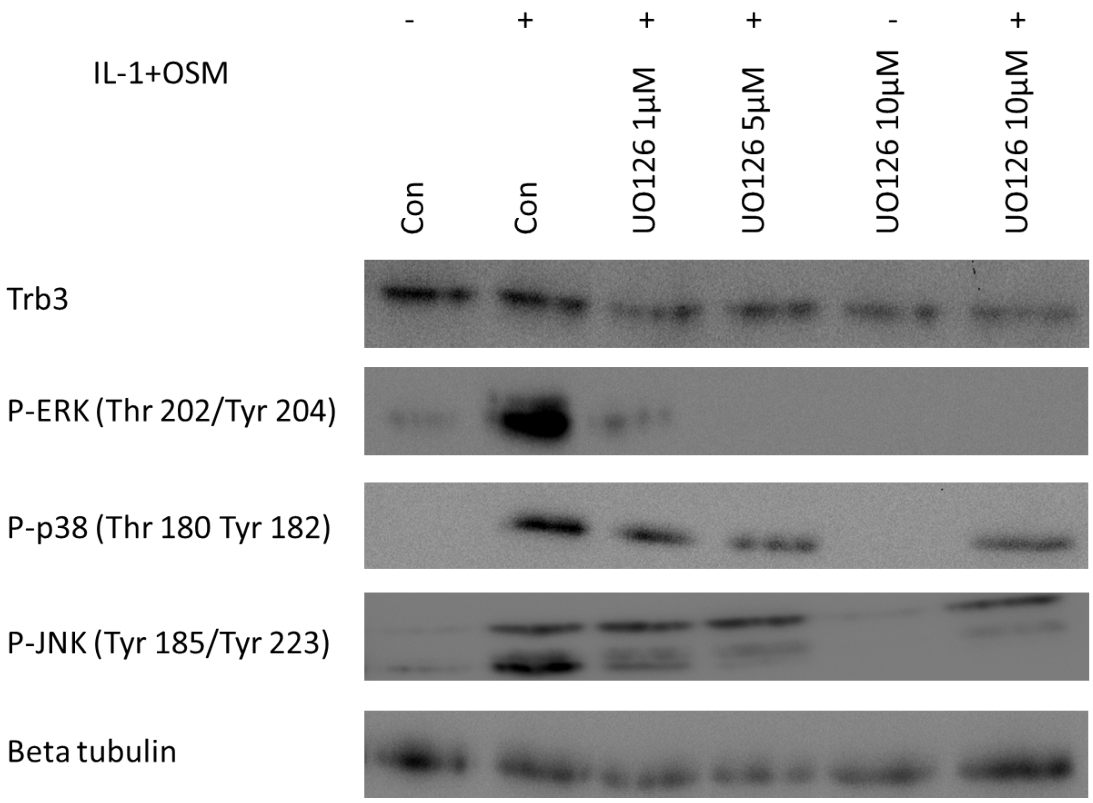

Figure 6.11. The MEK inhibitor UO126 leads to a reduction in Trb3 protein abundance in HAC. Trb3, p-ERK, p-p38, p-JNK and β tubulin abundance after addition of UO126 +/- IL-1+OSM stimulation in HAC. Cells were serum-starved overnight before addition of 1μM, 5μM or10μM UO126 for 1 hour before stimulation with IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 20 minutes. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 2 independent experiments, from 2 separate donors.

6.3 Discussion

Due to the effect of Trb1 and Trb3 on both AP-1 and ATF3 to regulate MMPs, a logical pathway to investigate was the MAP kinase pathway. This pathway is a key regulator of MMP-1 and -13 (Mengshol *et al*, 2000, Han *et al*, 2001, Raymond *et al* 2006), as well as being a regulator of AP-1 (Han *et al*, 2001, Leppa *et al*, 1998, Menghsol *et al* 2001). In addition, the MAPK pathway has also been implicated in ATF3 regulation, with p38 (Lu *et al*, 2007) and JNK (Hamdi *et al*, 2008) shown to regulate ATF3.

Trb1 was shown to interact with all of the MAP2Ks tested (MEK1, MKK4, MKK6 and MKK7) in SW1353 cells. This was contrary to previous data in a different cell line which demonstrated that Trb1 only interacted with MEK1 and MKK4 (Kiss-Toth *et al*, 2004). This discrepancy may simply be due to cell type specificity, which is common for Trb1 and Trb3. However, the method of analysis of interaction was also different. Kiss-Toth *et al*. (2004), studied interaction by co-immunoprecipitation, whereas the method used here was the PCA method, which involved overexpressing both proteins of interest and observing their interaction through fluorescence. The PCA method is open to false results as overexpression of both proteins produces a somewhat artificial physiological environment within the cell, and may cause an interaction which may not occur physiologically. Therefore, it is possible that Trb1 has the ability to interact with all of these MAP2Ks, but under physiological conditions it preferentially binds to specific MAP2Ks, or possibly none. However, the co-immunoprecipitation method is also open to error, as due to the mechanical and chemical stresses that occur through co-immunoprecipitation, proteins that in fact do interact physiologically may not co-immunoprecipitate. This may be why Trb1 was not shown to interact with the other MAP2Ks in this method. In order to help confirm interaction of Trb1 with these MAP2Ks in SW1353 cells, co-immunoprecipitation could also be used, however as stated, a negative result would not necessarily indicate the proteins do not interact physiologically. In addition, mutation of amino acid sites known to be involved in interaction, and a subsequent lack of interaction, would also help to confirm the result.

Interestingly, the fluorescent staining pattern varied depending on which MAP2K Trb1 interacted with. All showed strong nuclear staining, however some

also showed cytoplasmic staining (MEK1 and MKK4), and the nuclear staining pattern also differed from diffuse staining (MEK1, MKK4, MKK7) to punctate staining (MKK6). This may possibly provide evidence that the interactions which are occurring are in fact real, as if this was not the case, one may expect the staining pattern to be similar. In addition, this possibly demonstrates that Trb1 may play individual specific roles for each MAP2K, rather than a common role for all of them. The punctate staining for MKK6 would suggest that Trb1:MKK6 interaction may be occurring within specific organelles within the nucleus. Interestingly, as mentioned, all MAP2Ks show strong staining within the nucleus. MAP2Ks are not known to be nuclear proteins. This is possibly suggesting that as well as their role in MAPK activation, Trb1 may also be shuttling MAP2Ks into the nucleus in order to play an additional role within the cell, not yet identified. However, higher than normal physiological levels of these proteins may cause them to move into the nucleus. This has been demonstrated previously, whereby overexpression of NFκB has seen nuclear translocation without activation, which would not be expected (Carlotti *et al*, 1999)

Trb1 overexpression demonstrated a reduction in both p38 and JNK activation, but no effect on ERK in SW1353 cells. From the PCA results one would expect Trb1 to affect p38 activation as MKK6 regulates p38 activation. In addition Trb1 interacting with MKK4 and MKK7 also supports Trb1 affecting JNK activation, as these are the 2 MAP2Ks which regulate JNK. However, ERK is regulated by the MAP2Ks MEK1 and MEK2, therefore one may expect Trb1 to also influence ERK activation, as it has been shown to bind MEK1. It is possible that MEK1 is in fact affected by Trb1 overexpression, but MEK2 is able to compensate for this, although it has not been investigated whether Trb1 also interacts with MEK2. It was previously demonstrated that Trb1 negatively regulates JNK activation, as well as ERK (Sung *et al*, 2007), however this may be cell type specific, and this was also in the context of LPS activation.

These data would suggest that Trb1 is involved within the anti-inflammatory pathway, which had also been suggested by the MMP, AP-1 and ATF3 results. Overexpression of Trb1 may lead to a reduction in MAPK activation through interaction with MAP2Ks, which reduces both AP-1 and ATF3 expression, subsequently leading to a reduction in MMP expression. However, as

mentioned, without the ability to silence Trb1 the role of Trb1 cannot be verified, particularly in light of the way in which Trb3 appears to function, whereby both silencing and overexpression have similar effects. This effect on MAPK activation may suggest that Trb1 is binding to the MAP2Ks and inhibiting their activity. It is possible that Trb1 may be shuttling the MAP2Ks into the nucleus, as suggested by the interaction data, and this may be inhibiting their ability to interact with and phosphorylate the MAPKs within the cytoplasm.

It would appear that the interaction of Trb1 with MAP2Ks does not stabilise Trb1, as a combined silencing of the MAP2Ks which Trb1 was shown to interact with (MEK1, MKK4, MKK6 and MKK7) showed no effect on Trb1 protein expression in HAC. This was based on the assumption that the MAPK pathways behave in a similar fashion in both HAC and SW1353 cells, as their activation profiles with IL-1+OSM stimulation were similar. This result was contradictory to that shown by Kiss-Toth *et al*. (2004), as they demonstrated that interaction of Trb1 with the MAP2Ks MEK1 and MKK4 led to an increase in Trb1 protein abundance. This may simply be due to cell type specificity. Alternatively, only a small percentage of Trb1 may bind MAP2K in HAC, therefore when MAP2KS are silenced this reduction in Trb1 cannot be detected. It is also possible in HAC, when MAP2Ks are not present, Trb1 has the ability to bind another protein, and become stable, or it simply does not require interaction for stability. However, the optimum amount of total siRNA used was 100 nmol, meaning each MAP2K siRNA was at 25 nmol, rather than the normal 100 nmol for a single siRNA. Silencing of these individual MAP2Ks may therefore not have been at a sufficient level to have an effect, although mRNA silencing data for the MAP2Ks showed silencing. Individual silencing of the MAP2Ks was also attempted, however no effect was seen. It is thought that this could possibly be due to redundancy, as the silencing of a single MAP2K may simply allow another MAP2K to bind to Trb1 and therefore stabilise it. Overexpression of the MAP2Ks, and analysis of Trb1 protein abundance, which was performed by Kiss-Toth *et al*. (2004), may provide an improved way to analyse the ability of MAP2Ks to stabilise Trb1, as redundancy for individual MAP2Ks would not be an issue in this case.

Trb3, as with Trb1, also interacted with all 4 of the MAP2Ks tested (MEK1, MKK4, MKK6 and MKK7). This was not consistent with previous work by Kiss-

Toth *et al*. (2004), which demonstrated through co-immunoprecipitation that Trb3 only interacted with MEK1 and MKK7. The possible reasons for this difference are discussed above. In addition, as with Trb1, depending on the MAP2K in which Trb3 was interacting with, the pattern of staining was different. Interestingly, this staining pattern was very similar for both Trb1 and Trb3. Due to the similarities seen within subcellular distribution between Trb1 and Trb3, this was not entirely unexpected. Trb3-MKK4 interaction, appeared to vary between cells, in some cells there appeared to be punctate staining, in others the staining appeared to be diffuse. This variation could possibly be due to the cells being in a different state within the cell cycle, although cells were serum starved prior to fixation. This ability of Trb1 and Trb3 to bind MAP2Ks within the nucleus would also be consistent with the ability of the MAPKs themselves to move from the cytoplasm to the nucleus (Sacks, 2006).

There was a difference in the way in which Trb3 regulated MAPK activation between SW1353 cells and HAC. Trb3 silencing in HAC reduced activation of all 3 MAPKs, whereas in SW1353 cells only p38 was affected. This suggested that there may be cell type specific differences between HAC and SW1353 cells in the way in which Trb3 regulates MAPKs. The HAC data would actually be in concordance with the MAP2K-Trb3 interaction data in SW1353 cells, as all of the MAP2Ks that Trb3 was shown to interact with play a role in one of the MAPK pathways (p38 – MKK6, ERK – MEK1, JNK – MKK4/7). As mentioned previously, in SW1353 cells it was possible that ERK was not affected due to redundancy from MEK2. However, Trb3 was shown to bind both MKK4 and MKK7, therefore if this interaction was a true reflection on the effect of Trb3 on MAPKs one would expect JNK activation to be affected. This cell type specificity for MAPK regulation by Trb3 has been suggested previously by Sung *et al*. (2006), where they demonstrated that Trb3 overexpression had differing effects on the JNK-AP-1 pathway depending on the cell type. This cell type specificity for the MAPKs may explain the differences seen for Trb3 regulation of MMP-1 and -13 between HAC and SW1353 cells seen in chapter 5. In addition, in SW1353 cells, stimulation appeared to increase Trb3 protein abundance suggesting stimulation may perhaps be preventing degradation of Trb3, which may be influencing signalling within the nucleus.

Trb3 overexpression in SW1353 cells showed a reduction in activation of all 3 MAPKs. This result would be in agreement with the PCA interaction results, however, interestingly, it does not concur with the Trb3 silencing data in SW1353 cells. A possible reason for this may be that, as previously mentioned, the levels of Trb1 and Trb3 within the cell may be vital in the regulation of various pathways, and this may be specific for individual pathways. It is therefore possible that the overexpression levels of Trb3 were sufficient to affect all 3 MAPK pathways, however silencing of Trb3 was not at a sufficient level to influence all 3 MAPK pathways in SW1353 cells. This also suggested that p38 is more susceptible to changes in Trb3 levels than ERK and JNK in SW1353 cells. Kiss-Toth *et al*. (2004) demonstrated a similar occurrence, whereby alterations in Trb3 levels had differing effects on the MAPK pathway; low levels of Trb3 up-regulated ERK and JNK activation but inhibited p38, whilst higher levels inhibited all 3 MAPKs. Taking this reasoning into account, the MAPK data would concur with previous data, seen in chapter 5, for MMP-13, and ATF3, whereby both overexpression and silencing of Trb3 led to a dysregulation in signalling. This may therefore suggest that Trb3 can regulate MAPK activation through interaction with MAP2Ks, which can then regulate AP-1 and ATF3, which subsequently leads to transcriptional regulation of MMP-13.

The role that Trb3 plays in MAPK regulation may also be influenced by the MAP2Ks themselves. It was demonstrated that the combined silencing of the MAP2Ks in which Trb3 was shown to interact with (MEK1, MKK4, MKK6 and MKK7) led to a reduction in Trb3 protein abundance. This therefore suggested that MAP2Ks can bind Trb3 and influence its protein level. This could possibly be through stabilisation of the Trb3 protein, or by preventing Trb3 proteasomal degradation. This would be in concordance with that demonstrated by Kiss-Toth *et al*. (2004), which showed that binding of Trb3 to MAP2Ks increased protein abundance, which they suggested was through stabilisation. As mentioned, individual MAP2K silencing was attempted however no effect was seen possibly due to redundancy of other MAP2Ks.

In addition to the silencing of MAP2Ks, inhibition of MAP2Ks also appeared to impact Trb3 protein abundance. The MEK inhibitor UO126 led to a reduction in Trb3 protein abundance. Interestingly however, the MEK inhibitor did not appear to be specific for MEK, as one would only expect ERK activation to be

affected, however both JNK and p38 activation were also affected. This would suggest that UO126 may be inhibiting a number of MAP2Ks involved in all 3 pathways. UO126 inhibits MEK through direct interaction, it is therefore possible that in binding and inhibiting these different MAP2Ks, UO126 prevents Trb3 binding. The prevention of Trb3 binding may affect Trb3 protein abundance much in the same way as combined silencing of the MAP2Ks would. In order to assess if specific inhibition of the MEK pathway impacts Trb3 protein abundance one would require silencing of both MEK1 and MEK2, and the protein level assessed.

Throughout this chapter the effect of Trb1 and Trb3 on the MAPKs was only investigated at the phospho level, and total protein abundance of the MAPKs was not investigated. Whilst this data indicates that Trb1 and Trb3 affect the abundance of the phopsho-MAPKs, it is entirely possible that this is due to Trb1 and Trb3 affecting the total protein abundance, and should be investigated. However, the fact that Trb1 and Trb3 appear to interact with the MAP2Ks may suggest that they do influence activation of MAPKs rather than their abundance.

6.4 Synopsis

Both Trb1 and Trb3 bind to MAP2Ks and regulate the activation of MAPKs. Trb1 and Trb3 appeared to differ in their ability to regulate the 3 MAPK pathways. In addition, they may also be cell type specific, with differences between both HAC and SW1335 cells. One commonality from the data between Trb1 and Trb3 and both HAC and SW1353 cells was that Trb1 and Trb3 did not have a positive effect on MAPK activation. However, Trb1 was not silenced and MAPK activation assessed within these experiments. There may be differences in the way in which Trb1 and Trb3 are regulated within this pathway, as Trb3 protein abundance was affected by MAP2K silencing whilst Trb1 was not.

6.5 Summary

- The activation profile of the MAPKs during IL-1+OSM stimulation was very similar between both HAC and SW1353 cells.

- Both Trb1 and Trb3 interacted with all of the MAP2Ks tested; MEK1, MKK4, MKK6 and MKK7 in SW1353 cells. In addition, depending on the MAP2K, the fluorescent staining pattern varied.

- Trb1 overexpression led to a reduction in both p38 and JNK activation, but did not affect ERK in SW1353 cells.

- Combined silencing of MEK1, MKK4, MKK6 and MKK7 had no effect on Trb1 but reduced Trb3 protein abundance in HAC.

- Trb3 silencing led to a reduction in p38 activation but did not affect ERK or JNK in SW1353 cells. In HAC, Trb3 silencing reduced the activation of all 3 MAPKs.

- Trb3 overexpression led to a reduction in activation of all 3 MAPKs in SW1353 cells.

- The non-specific MEK inhibitor UO126 led to a reduction in Trb3 protein abundance in HAC.

Chapter 7: The effect of Trb1 and Trb3 on other signaling pathways known to regulate MMPs

7.1 Introduction

In addition to the MAPK pathways MMP regulation has been shown to involve a variety of other signaling pathways. The aim of the following chapter is to investigate some of those signaling pathways in relation to their regulation by Trb1 and Trb3.

7.1.1 NFκB

NFκB is a major transcription factor influencing many cellular pathways. NFκB is important in arthritis as it is a key mediator of pro-inflammatory cytokine signaling including IL-1 (Kapoor *et al*, 2011), which as discussed previously can regulate MMP expression (Goldring *et al*, 1994). In addition, NFκB has also been linked with arthritis progression (Takk *et al*, 2001). One of the roles of NFκB in arthritis is to regulate MMPs, as it has been shown to be involved in regulation of MMP-1 and -13 (Vincenti and Brinckerhoff 2002, Bondeson *et al*, 2000).

Trb1 and Trb3 have been shown to bind to NFκB through the p65 subunit and regulate this transcription factor (Ostertag *et al*, 2010, Duggan *et al*, 2010). However, this regulation does appear to be cell type specific (Kiss-Toth *et al*, 2004). It was therefore hypothesised that Trb1 and Trb3 may regulate MMPs through the NFκB pathway in order to regulate cartilage degradation in arthritis.

7.1.2 Akt

The PI3K/Akt pathway plays many central roles in cellular signalling including that of cell survival, cell differentiation and cell cycle progression (Fayard *et al*, 2005), as well as influencing the MAP kinase pathway (Ren *et al*, 2010). The

PI3K/Akt pathway has been associated with arthritis as it has been shown to be activated through pro-inflammatory stimuli involved in arthritis, including OSM (Godoy-Tundidor *et al*, 2005, Kim *et al*, 2002). In addition, the PI3K/Akt pathway has been shown to be involved in cartilage degradation (Hayer *et al*, 2009), as well as regulation of MMP-1 and -13 (Litherland *et al*, 2008). It has also been suggested that the PI3K/Akt pathway may have a protective role in arthritis as it has been shown to be involved in the maintenance of chondrocytes (Chrysis *et al*, 2005) and proteoglycan synthesis (Starkman *et al*, 2005).

Trb3 has been suggested to be involved in the regulation of the PI3K/Akt pathway, as Trb3 has been shown to inhibit this pathway through preventing Akt phosphorylation (Du *et al*, 2003). In addition, it has been suggested that this influence of Trb3 on Akt may be a contributing factor to OA progression (Cravero *et al*, 2009), as Trb3 overexpression led to a reduction in Akt phosphorylation and an increase in chondrocyte cell death. It has also been demonstrated that there is cross talk between the PI3K/Akt and the MAPK pathways (Hong *et al,* 2008), of which Trb1 and Trb3 were shown to regulate in chapter 6. It was therefore hypothesised that Trb1 and Trb3 may regulate MMPs and cartilage turnover through the PI3K/Akt pathway.

7.1.3 JAK/STAT

The JAK/STAT pathway is a key pathway in many cellular events including proliferation, migration and apoptosis (Rawlings *et al*, 2004). JAKs regulate STATs which then cause transcription of a number of genes (Kisseleva *et al* 2002, Korzus *et al*, 1997).

The JAK/STAT pathway is thought to be important in arthritis, as it has been suggested that this pathway contributes to cartilage degradation *in vivo* (Finnegan *et al*, 2002, Ortmann *et al*, 2001, Shaw *et al*, 2003). This pathway has also been shown to regulate MMP-1 and -13 expression (Litherland *et al*, 2010, Li *et al*, 2001, Mabrouk *et al*, 2007).

It has previously been demonstrated that there is cross talk between the JAK/STAT and the MAPK pathways, in which the MAPK pathways can regulate

STAT phosphorylation (Rawlings *et al,* 2004). Due to the apparent ability of Trb1 and Trb3 to regulate a number of pathways involved in MMP regulation, as well as their ability to regulate the MAPK pathways, it was hypothesised that they may also regulate this pathway in order to regulate MMPs and cartilage turnover.

The aims of this chapter were to:-

- Determine whether Trb1 and Trb3 interact with the NFκB subunit p65.
- Investigate the effect of Trb1 and Trb3 on the NFκB pathway.
- Investigate the effect of Trb1 and Trb3 on Akt activation.
- Investigate the effect of Trb3 on STAT activation.

7.2 Results

7.2.1 The NFκB pathway

7.2.1.1 The interaction of Trb1 and the NFκB subunit p65 in SW1353 cells

As mentioned, it has previously been demonstrated that Trb1 can bind the p65 subunit of NFκB in adipocytes (Ostertag *et al*, 2010). It was therefore initially investigated whether Trb1 could interact with the NFκB subunit p65 in SW1353 cells. SW1353 cells were used rather than HAC due to low transfection efficiency in HAC, as discussed previously. This interaction was observed by the PCA method detailed in chapter 4. Microscopic analysis demonstrated that both Trb1 and p65 interacted (Fig 7.1). This interaction was nuclear, with diffuse staining present within the nucleus. There appeared to be no cytoplasmic interaction between Trb1 and p65.

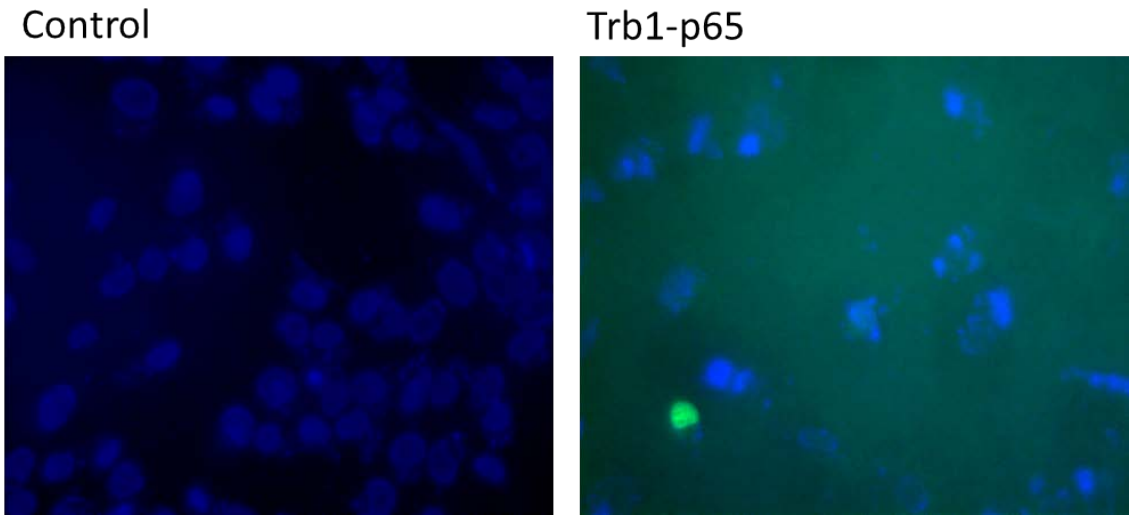

Figure 7.1. Trb1 interacts with the NFκB subunit p65. Fluorescent images of p65 (V1) and Trb1 (V2) overexpression constructs in SW1353 cells. Cells were transfected with each construct in 8 well chamber slides (0.2 µg per construct) for 48 hours, as detailed in the Materials and Methods, then serum-starved for 6 hours, fixed and visualised. Results are representative of 2 individual experiments.

7.2.1.2 The effect of IKKβ siRNA gene silencing on MMP-1 and MMP-13 expression during IL-1+OSM stimulation in SW1353 cells

In order to determine whether NFκB regulated MMP-1 and -13 in chondrocytes, NFκB was inhibited and the mRNA expression of *MMP-1* and *-13* was observed. NFκB was inhibited through the silencing of IKKβ. As discussed in chapter 1, NFκB is inhibited through the binding of IκB. Upon stimulation, IKKβ phosphorylates IκB leading to its degradation, after which NFκB is active (Roman-Blas and Jimenez, 2006, Chakraborty *et al*, 2010). Therefore silencing of IKKβ will lead to binding of IκB to NFκB and its inhibition.

SW1353 cells were used as it was not possible to silence IKKβ in HAC; this was possibly due to the specific siRNA sequence not being effective in HAC. It was initially demonstrated that IKKβ could be silenced (Fig 7.2). IKKβ silencing resulted in a reduction in both *MMP-1* and *-13* mRNA expression during IL-1+OSM stimulation (Fig 7.3), suggesting that NFκB is involved in the regulation of MMP-1 and -13 in SW1353 cells.

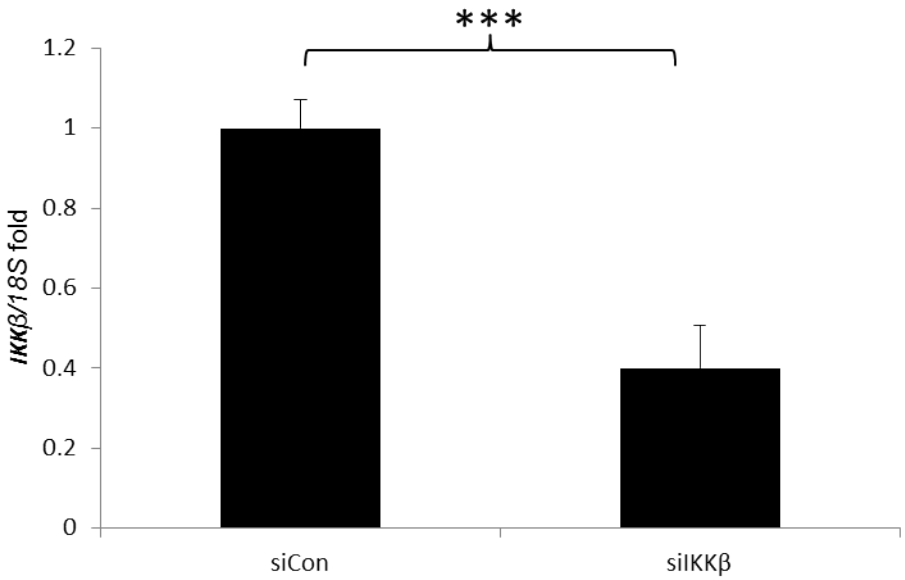

Figure 7.2. IKKβ siRNA gene silencing. mRNA expression analysis of *IKKβ* after transfection with IKKβ siRNA of SW1353 cells. Cells were transfected with IKKβ siRNA in 96 well plates (100 nmol/well) for 48 hours and serum-starved overnight before lysis. Total RNA was isolated, reverse transcribed to cDNA and *IKKβ* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean), ***p<0.001. Ct values for siCon were 15.26 +/- 0.17 for 18S and 26.11 +/- 0.13 for *IKKβ.* (+/- = standard error of the mean).

A B

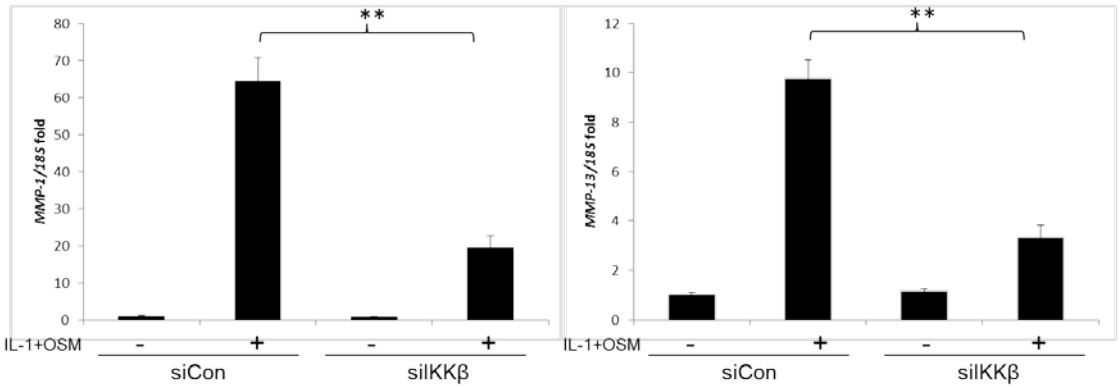

Figure 7.3. IKKβ siRNA gene silencing leads to a reduction in *MMP-1* and *MMP-13* mRNA expression during IL-1+OSM stimulation in SW1353 cells. mRNA expression analysis of *MMP-1* (A) and *MMP-13* (B) after transfection with IKKβ siRNA +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with IKKβ siRNA in 96 well plates (100 nmol/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 6 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *MMP-1* and *MMP-13* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 2 separate experiments, **p<0.01. Ct values for siCon were 15.72 +/- 0.09 for 18S, 25.87 +/- 0.51 for *MMP-1* and 28.12 +/- 0.64 for *MMP-13.* (+/- = standard error of the mean).

7.2.1.3 The effect of IKKβ siRNA gene silencing on IL-8 mRNA expression during IL-1+OSM stimulation in SW1353 cells

As it was shown that NFκB could regulate MMP-1 and -13, and that Trb1 could bind the p65 subunit of NFκB, it was then investigated whether Trb1 could regulate NFκB. In order to assess the effect of Trb1 on NFκB activation, IL-8 was used as a surrogate for NFκB activation, as it had previously been demonstrated that NFκB is an important mediator of IL-8 synthesis (Aupperle *et al*, 2001).

Initially it was confirmed that IL-8 could be used as a surrogate for NFκB activation, since IKKβ silencing in SW1353 cells showed a significant reduction in *IL-8* mRNA expression during IL-1+OSM stimulation (Fig 7.4).

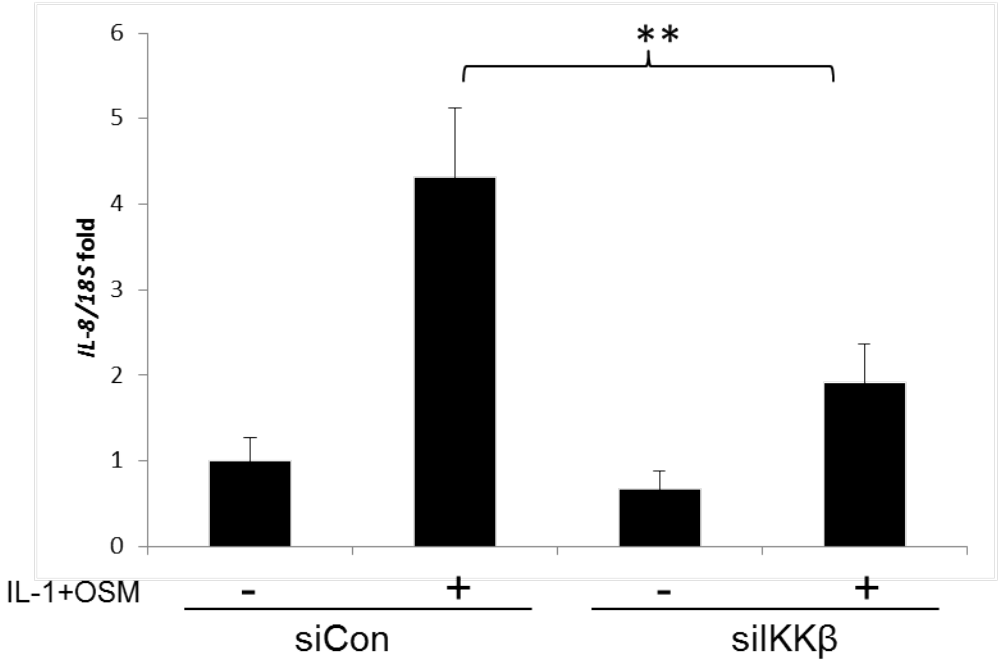

Figure 7.4. IKKβ siRNA gene silencing leads to a reduction in *IL-8* mRNA expression during IL-1+OSM stimulation in SW1353 cells. mRNA expression analysis of *IL-8* after transfection with IKKβ siRNA +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with IKKβ siRNA in 96 well plates (100 nmol/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 6 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *IL-8* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 2 separate experiments, **p<0.01. Ct values for siCon were 16.82 +/- 0.34 for 18S and 26.84 +/- 0.35 for *IL-8* (+/- = standard error of the mean).

7.2.1.4 The effect of Trb1 overexpression on IL-8 mRNA expression during IL-1+OSM stimulation in SW1353 cells and HAC

The effect of Trb1 overexpression on *IL-8* mRNA expression was next investigated in SW1353 cells. The overexpression of Trb1 showed a trend towards a reduction in *IL-8* mRNA expression during IL-1+OSM stimulation, however this did not prove to be significant (p = 0.107) (Fig 7.5).

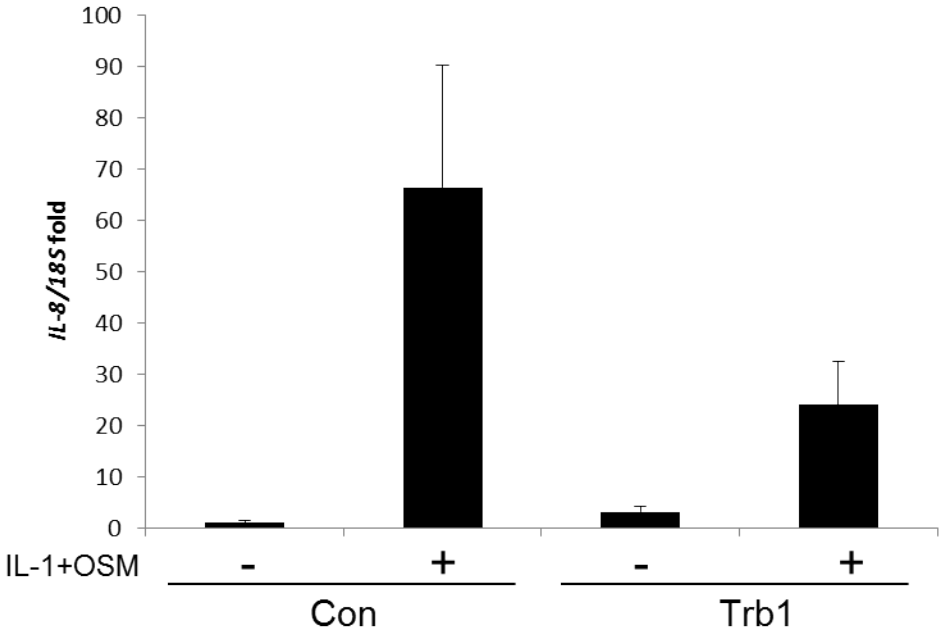

Figure 7.5. Trb1 overexpression shows a trend towards a reduction in *IL-8* mRNA expression during IL-1+OSM stimulation in SW1353 cells. mRNA expression analysis of *IL-8* after transfection with Trb1 overexpression plasmid +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb1 overexpression plasmid in 96 well plates (0.04 µg DNA/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 6 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *IL-8* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 2 separate experiments. Ct values were 19.53 +/- 0.21 for 18S and 28.11 +/- 0.56 for *IL-8.* (+/- = standard error of the mean).

The effect of Trb1 overexpression on *IL-8* mRNA expression was also investigated in HAC. As in SW1353 cells, the overexpression of Trb1 showed a trend towards a reduction in *IL-8* mRNA expression during IL-1+OSM stimulation, however this was not significant (p= 0.076) (Fig 7.6).

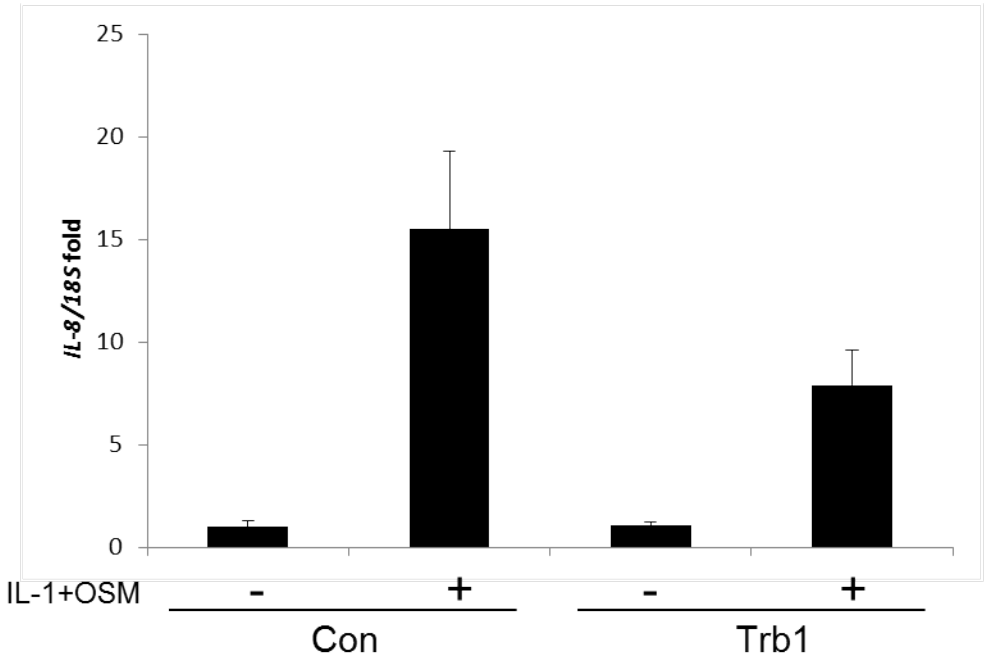

Figure 7.6. Trb1 overexpression shows a trend towards a reduction in *IL-8* mRNA expression during IL-1+OSM stimulation in HAC. mRNA expression analysis of *IL-8* after transfection with Trb1 overexpression plasmid +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb1 overexpression plasmid in 96 well plates (0.2 µg DNA/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 24 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *IL-8* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 3 separate donors. Ct values for siCon were 23.62 +/- 0.38 for 18S and 36.48 +/- 0.57 for *IL-8* (+/- = standard error of the mean).

7.2.1.5 The effect of Trb1 overexpression on p65 protein abundance and activation during IL-1+OSM stimulation in SW1353 cells

Due to the trend towards Trb1 affecting the NFκB surrogate IL-8, and the ability of Trb1 to interact with p65, the effect of Trb1 overexpression on p65 protein abundance and also its activation was investigated in SW1353 cells. It was first demonstrated that Trb1 was successfully overexpressed. Trb1 overexpression led to a reduction in p65 protein abundance and a reduction in p65 activation during IL-1+OSM stimulation (Fig 7.7). This correlated with the data showing the effect of Trb1 overexpression on *IL-8* mRNA expression, which showed a reduction in *IL-8*. Trb1 overexpression had no effect on p65 protein abundance under basal conditions.

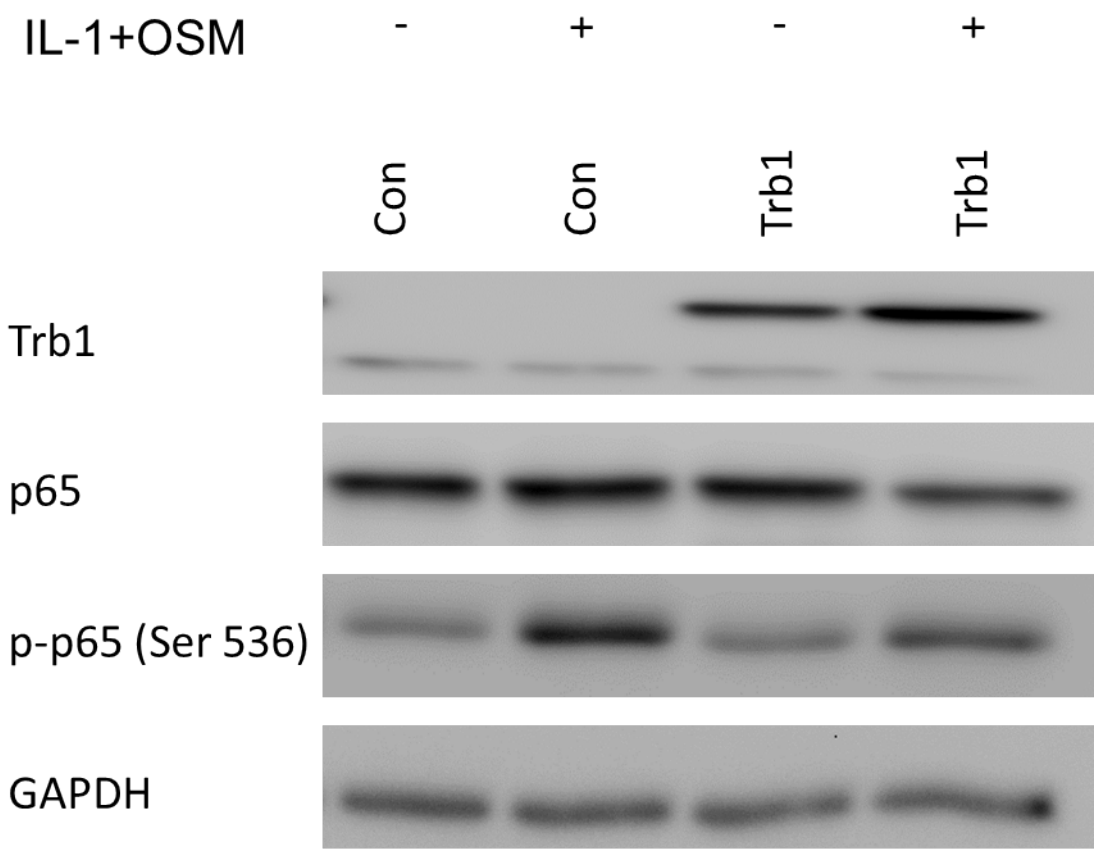

Figure 7.7. Trb1 overexpression leads to a reduction in p65 protein abundance and p65 activation during IL-1+OSM stimulation in SW1353 cells. Trb1, p65, p-p65 and GAPDH abundance after transfection with Trb1 overexpression plasmid +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb1 overexpression plasmid in 6 well plates (0.9 µg DNA/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 1 hour. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 4 independent experiments.

7.2.1.6 The interaction of Trb3 and the NFκB subunit p65 in SW1353 cells

It was initially assessed whether Trb3 could interact with the NFκB subunit p65 in SW1353 cells. As mentioned, it has previously been demonstrated that Trb3 has the ability to interact with p65 and regulate NFκB signalling (Duggan *et al*, 2010), and this may be somewhat cell type specific (Kiss-Toth *et al*, 2004).

Fluorescence microscopy demonstrated that Trb3 interacted with p65, with a diffuse staining pattern solely within the nucleus (Fig 7.8), as seen with Trb1 and p65.

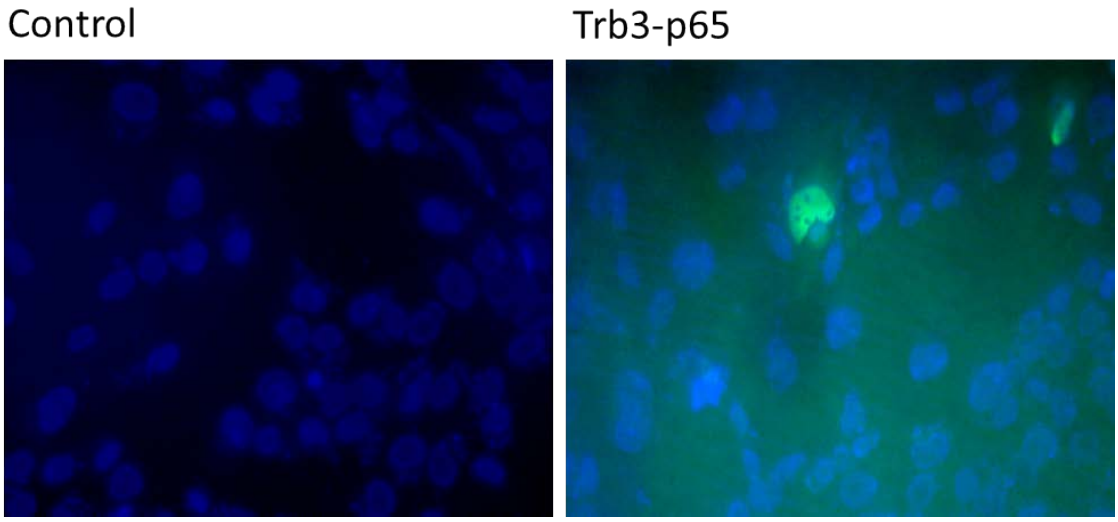

Figure 7.8. Trb3 interacts with the NFκB subunit p65 in SW1353 cells. Fluorescent images of p65 (V1) and Trb3 (V2) overexpression constructs in SW1353 cells. Cells were transfected with each construct in 8 well chamber slides (0.2 µg per construct) for 48 hours, as detailed in the materials and methods, then serum-starved for 6 hours, fixed and visualised. Results are representative of 2 independent experiments.

7.2.1.7 The effect of Trb3 siRNA gene silencing on IL-8 mRNA expression during IL-1+OSM stimulation in SW1353 cells and HAC

As it was demonstrated that Trb3 could bind to p65, it was then investigated whether Trb3 could regulate the NFκB surrogate IL-8.

Trb3 silencing showed a significant reduction in *IL-8* mRNA expression during IL-1+OSM stimulation in SW1353 cells (Fig 7.9).

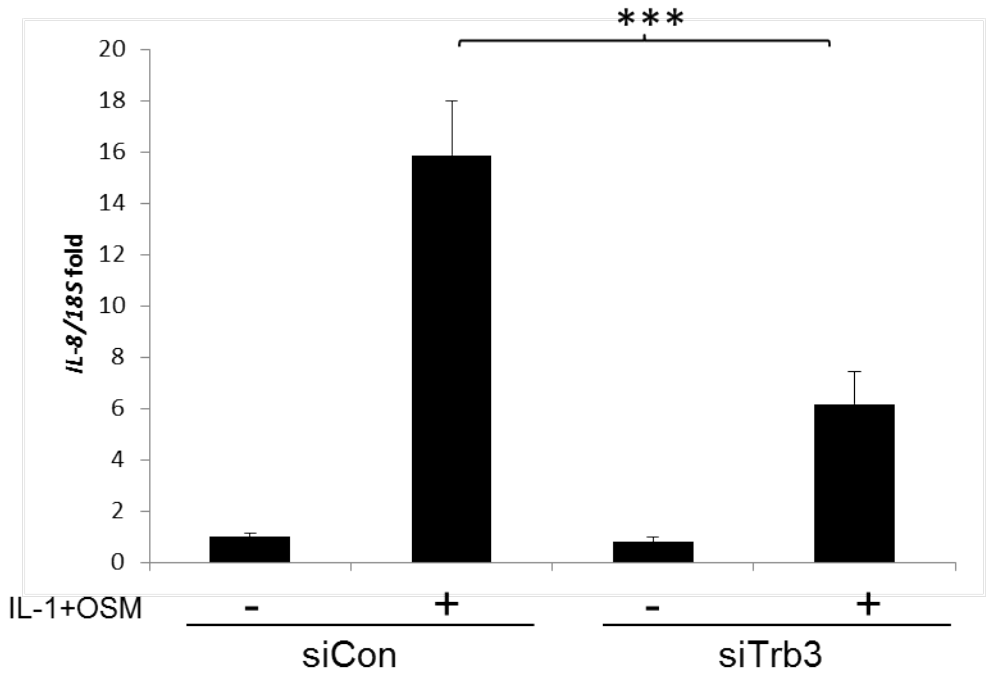

Figure 7.9. Trb3 siRNA gene silencing leads to a reduction in *IL-8* mRNA expression during IL-1+OSM stimulation in SW1353 cells. mRNA expression analysis of *IL-8* after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb3 siRNA in 96 well plates (100 nmol/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 6 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *IL-8* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 2 separate experiments, ***p<0.001. Ct values for siCon were 13.51 +/- 0.17 for 18S and 28.36 +/- 0.34 for *IL-8.* (+/- = standard error of the mean).

The effect of Trb3 silencing on *IL-8* mRNA expression was also investigated in HAC. Trb3 silencing led to a significant reduction in *IL-8* mRNA expression during IL-1+OSM stimulation (Fig 7.10), as seen in SW1353 cells.

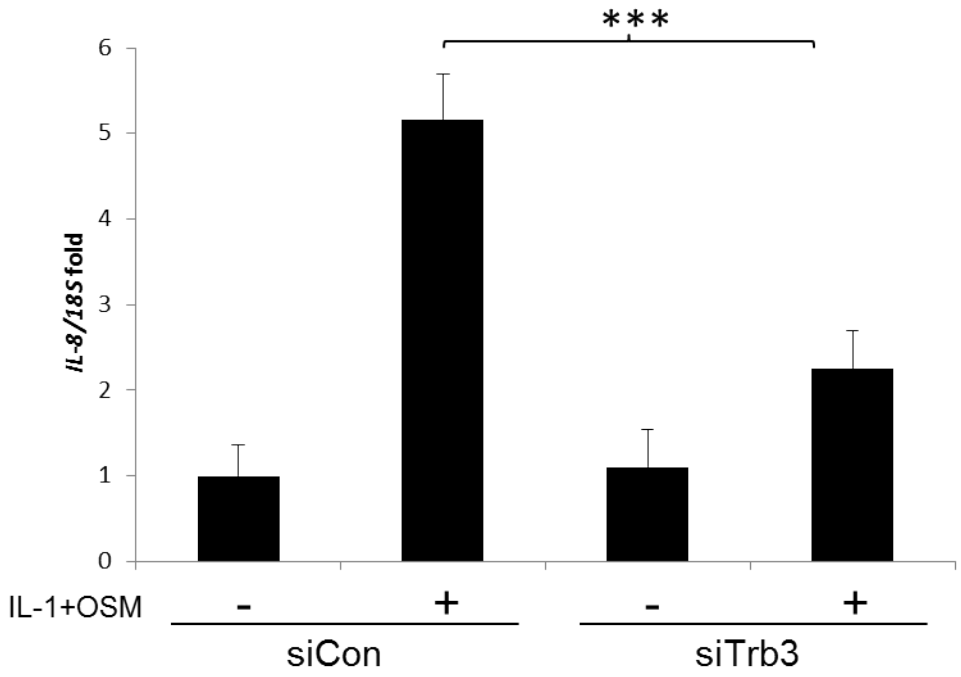

Figure 7.10. Trb3 siRNA gene silencing leads to a reduction in *IL-8* mRNA expression during IL-1+OSM stimulation in HAC. mRNA expression analysis of *IL-8* after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 siRNA in 96 well plates (100 nmol/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 24 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *IL-8* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 4 separate donors, ***p<0.001. Ct values for siCon were 20.68 +/- 0.23 for 18S and 28.63 +/- 0.45 for *IL-8* (+/- = standard error of the mean).

7.2.1.8 The effect of Trb3 overexpression on IL-8 mRNA expression during IL-1+OSM stimulation in SW1353 cells and HAC

As Trb3 silencing appeared to have an impact on *IL-8* mRNA expression, Trb3 was also overexpressed in order to see its effect on *IL-8*; this was initially performed in SW1353 cells. Trb3 overexpression led to a significant reduction in *IL-8* mRNA expression during IL-1+OSM stimulation (Fig 7.11). Both overexpression and silencing therefore resulted in a reduction in *IL-8* expression in SW1353 cells.

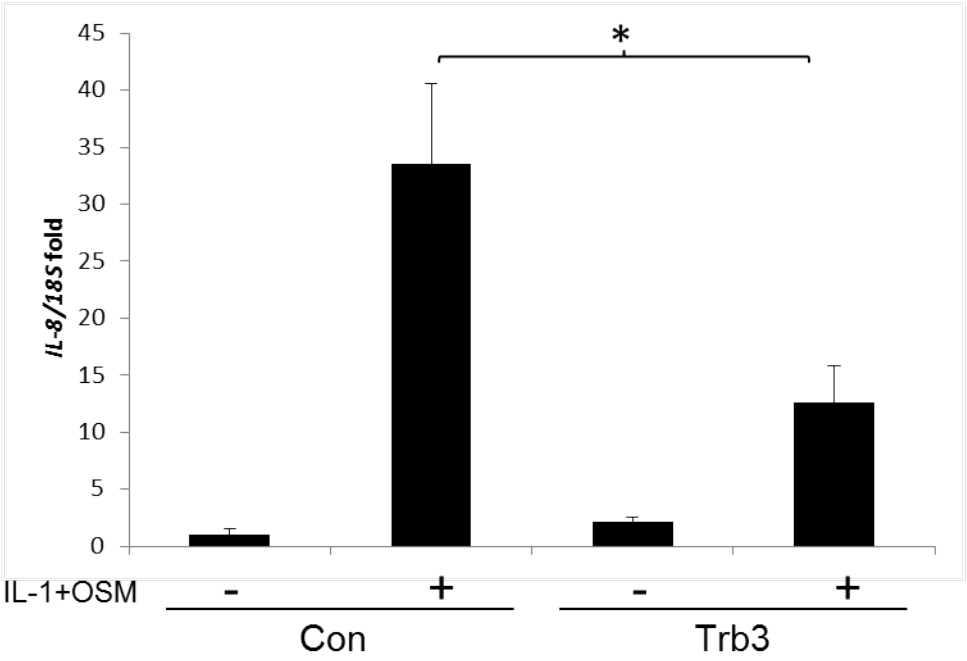

Figure 7.11. Trb3 overexpression leads to a reduction in *IL-8* mRNA expression during IL-1+OSM stimulation in SW1353 cells. mRNA expression analysis of *IL-8* after transfection with Trb3 overexpression plasmid +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb3 overexpression plasmid in 96 well plates (0.04 µg DNA/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 6 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *IL-8* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 3 separate experiments, *p<0.05. Ct values for siCon were 13.59 +/- 0.18 for 18S and 25.90 +/- 0.88 for *IL-8* (+/- = standard error of the mean).

Trb3 was also overexpressed in HAC and *IL-8* mRNA expression investigated. Trb3 overexpression showed a trend towards a reduction in *IL-8* mRNA expression during IL-1+OSM stimulation (Fig 7.12), however, this result was not significant (p=0.098). Both overexpression and silencing therefore suggest a trend towards a reduction in *IL-8* in HAC.

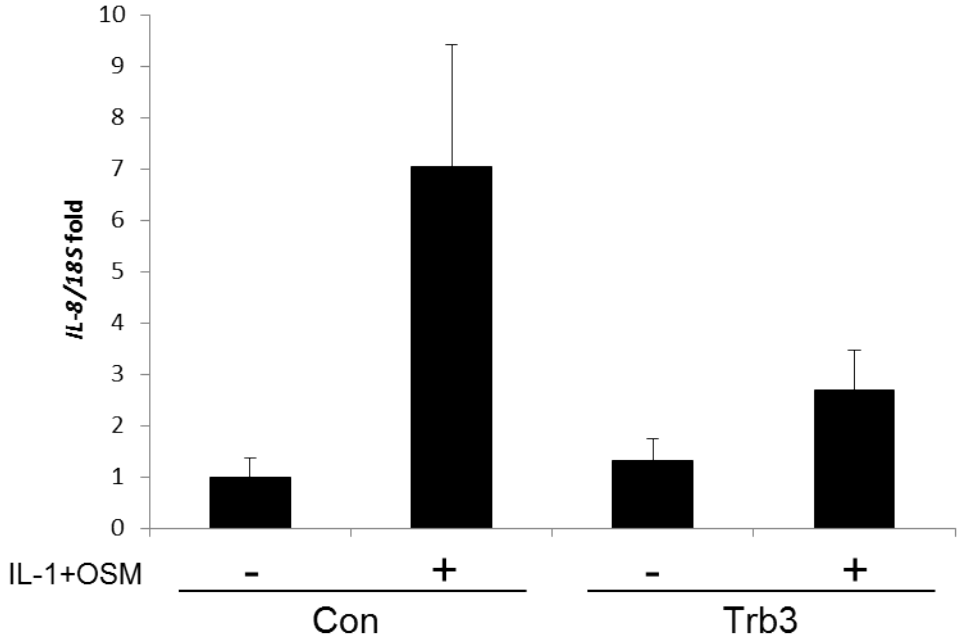

Figure 7.12. Trb3 overexpression shows a trend towards a reduction in *IL-8* mRNA expression during IL-1+OSM stimulation in HAC. mRNA expression analysis of *IL-8* after transfection with Trb3 overexpression plasmid +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 overexpression plasmid in 96 well plates (0.2 µg DNA/well) for 48 hours and serum-starved overnight before addition of IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 24 hours before lysis. Total RNA was isolated, reverse transcribed to cDNA and *IL-8* expression assessed by real-time RT-PCR. Values were normalised to the housekeeping gene 18S rRNA and the mean value plotted. Results are measured in hextuplicate, (+/- standard error of the mean). Results are combined data from 3 separate donors. Ct values for siCon were 24.44 +/- 0.35 for 18S and 36.73 +/- 1.18 for *IL-8* (+/- = standard error of the mean).

7.2.1.9 The effect of Trb3 siRNA gene silencing on p65 protein abundance and activation during IL-1+OSM stimulation in SW1353 cells and HAC

Due to the effect of Trb3 silencing and overexpression on *IL-8* mRNA expression, and the ability of Trb3 to interact with p65, Trb3 was silenced and the protein abundance of p65 as well as its activation was analysed in SW1353 cells. The silencing of Trb3 protein expression was initially demonstrated. Trb3 silencing showed a reduction in p65 protein abundance, as well as activation during IL-1+OSM stimulation (Fig 7.13). This would concur with that seen for IL-8, where Trb3 silencing led to a reduction in *IL-8* mRNA expression. Trb3 silencing had no effect on p65 protein abundance under basal conditions.

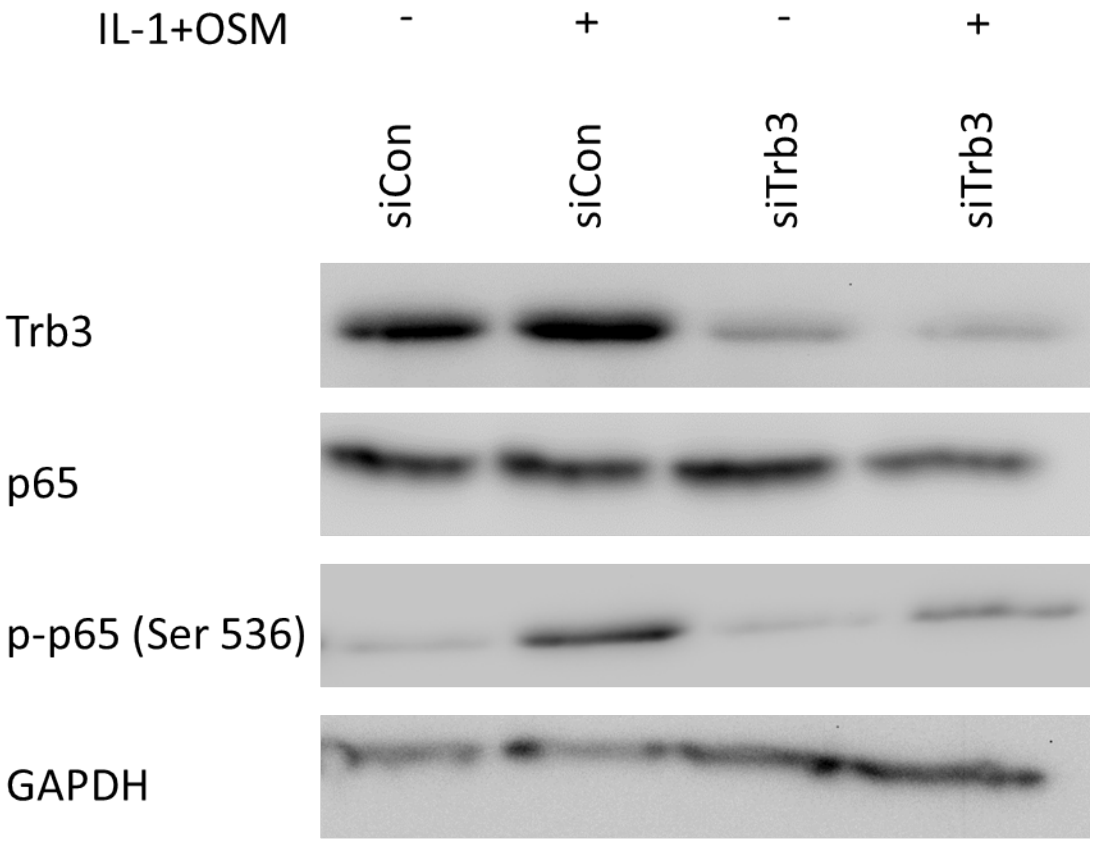

Figure 7.13. Trb3 siRNA gene silencing leads to a reduction in p65 protein abundance and p65 activation during IL-1+OSM stimulation in SW1353 cells. Trb3, p65, p-p65 and GAPDH abundance after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb3 siRNA in 96 well plates (100 nmol/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 1 hour. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments.

The effect of Trb3 silencing on p65 protein abundance and activation was also investigated in HAC. The silencing of Trb3 protein expression was initially established. As in SW1353 cells, Trb3 silencing showed a reduction in p65 protein abundance and activation during IL-1+OSM stimulation (Fig 7.14). This also correlated with the IL-8 data in HAC, where Trb3 silencing showed a reduction in *IL-8* mRNA expression. As in SW1353 cells, Trb3 silencing had no effect on p65 protein abundance under basal conditions.

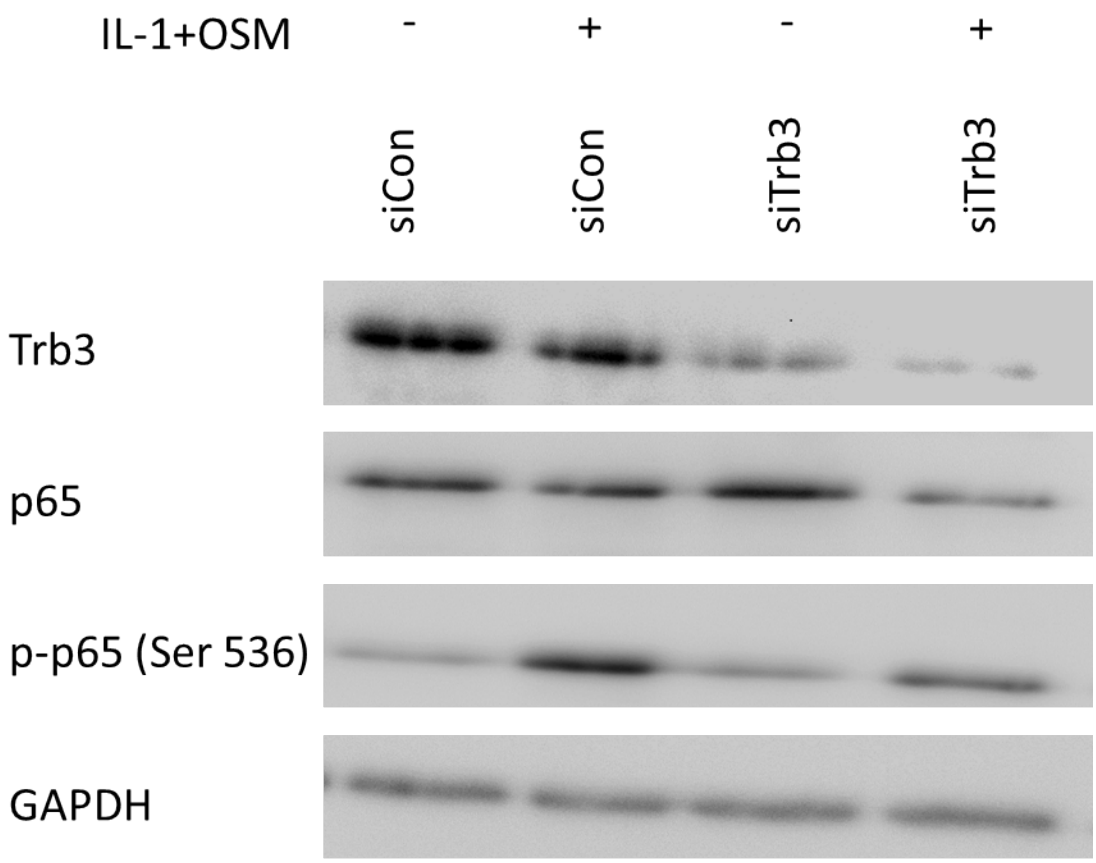

Figure 7.14. Trb3 siRNA gene silencing leads to a reduction in p65 protein abundance and p65 activation during IL-1+OSM stimulation in HAC. Trb3, p65, p-p65 and GAPDH abundance after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 siRNA in 6 well plates (100 nmol/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 1 hour. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments, from 3 separate donors.

7.2.1.10 The effect of Trb3 overexpression on p65 protein abundance and activation during IL-1+OSM stimulation in SW1353 cells

Due to the effect of Trb3 silencing on p65, Trb3 was overexpressed and the effect on p65 protein abundance and p65 activation investigated in SW1353 cells. It was initially demonstrated that Trb3 was overexpressed through its c-Myc tag. As was observed with Trb3 silencing, overexpression of Trb3 led to a reduction in p65 protein abundance as well as activation during IL-1+OSM stimulation (Fig 7.15). This is consistent with the IL-8 data, where both silencing and overexpression of Trb3 showed a reduction in *IL-8* mRNA expression. As

shown with Trb3 silencing, Trb3 overexpression had no effect on p65 protein abundance under basal conditions.

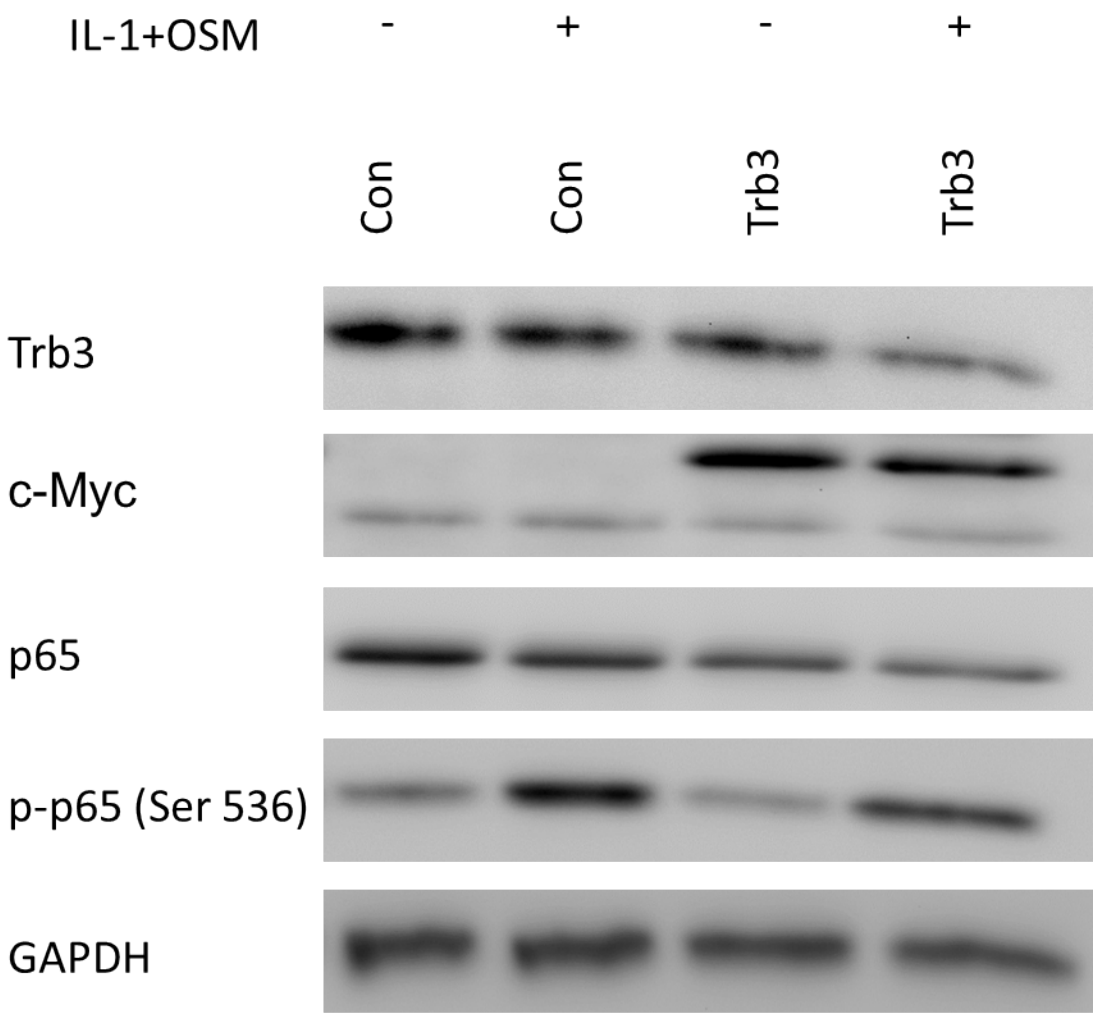

Figure 7.15. Trb3 overexpression leads to a reduction in p65 protein abundance and p65 activation during IL-1+OSM stimulation in SW1353 cells. Trb3, c-Myc, p65, p-p65 and GAPDH abundance after transfection with Trb3 overexpression plasmid +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb3 overexpression plasmid in 6 well plates (1.2 µg DNA/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 1 hour. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 2 independent experiments.

7.2.2 Akt

7.2.2.1 The effect of Trb1 overexpression on Akt activation during IL-1+OSM stimulation in SW1353 cells

It was initially investigated whether Trb1 regulated Akt activation through phosphorylation in SW1353 cells. It was firstly established that Trb1 protein was overexpressed. Trb1 overexpression had no effect on Akt activation both under basal conditions and during IL-1+OSM stimulation (Fig 7.16).

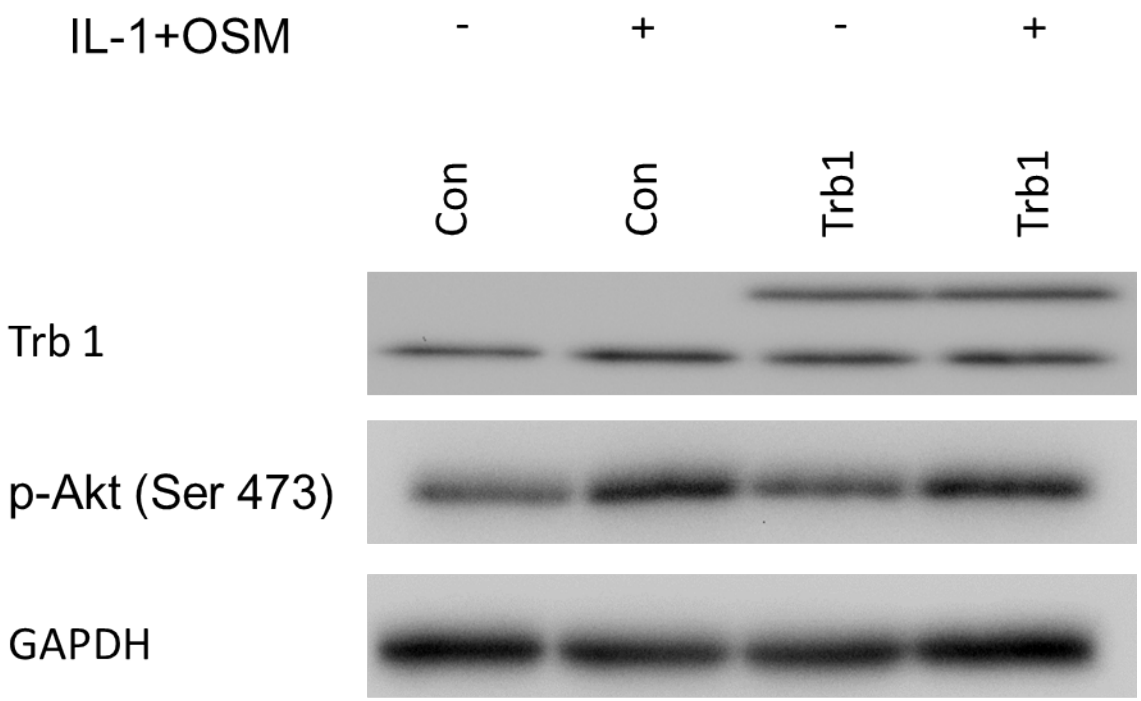

Figure 7.16. Trb1 overexpression does not affect Akt activation during IL-1+OSM stimulation in SW1353 cells. Trb1, p-Akt, and GAPDH abundance after transfection with Trb1 overexpression plasmid +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb1 overexpression plasmid in 6 well plates (0.9 µg DNA/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 20 minutes. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments.

7.2.2.2 The effect of Trb3 siRNA gene silencing on Akt activation during IL-1+OSM stimulation in SW1353 cells and HAC

The effect of Trb3 silencing on Akt activation was also assessed; this was initially performed in SW1353 cells. It was firstly shown that Trb3 was silenced. Trb3 silencing led to an increase in Akt activation basally, however, there was no effect with IL-1+OSM stimulation (Fig 7.17).

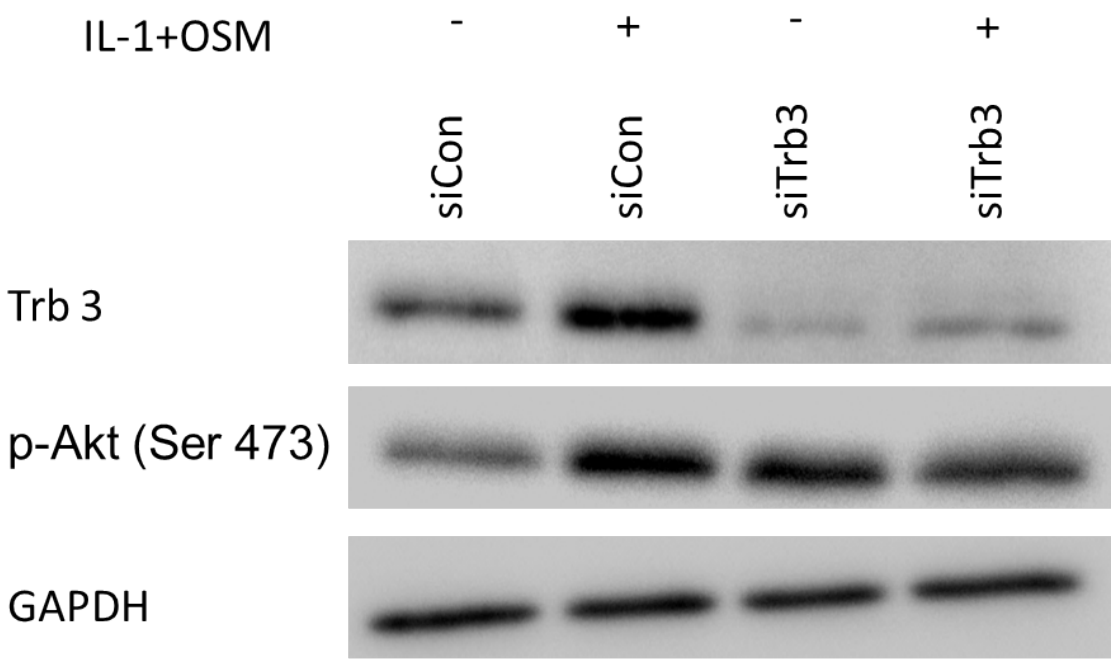

Figure 7.17. Trb3 siRNA gene silencing leads to an increase in Akt activation in SW1353 cells. Trb3, p-Akt, and GAPDH abundance after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb3 siRNA in 6 well plates (100 nmol/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 20 minutes. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments.

The effect of Trb3 silencing on Akt activation was also assessed in HAC. It was initially established that Trb3 was silenced. The silencing of Trb3 had no effect on Akt activation either under basal conditions or during IL-1+OSM stimulation (Fig 7.18).

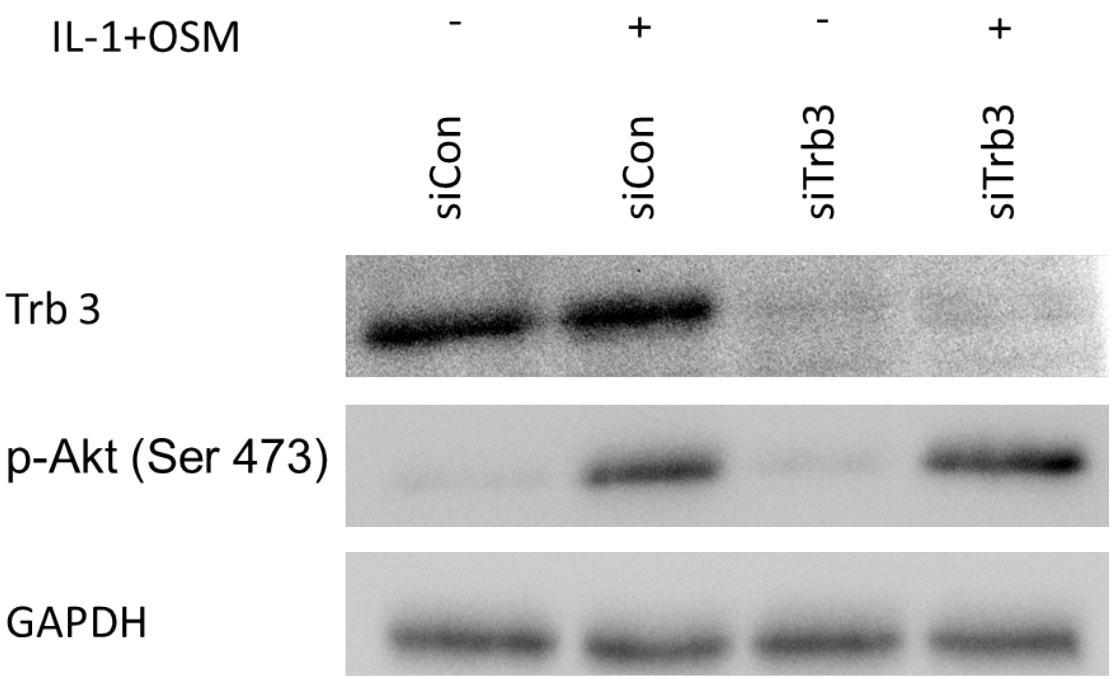

Figure 7.18. Trb3 siRNA gene silencing has no effect on Akt activation in HAC. Trb3, p-Akt, and GAPDH abundance after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 siRNA in 6 well plates (100 nmol/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 20 minutes. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 4 independent experiments, from 4 separate donors.

7.2.2.3 The effect of Trb3 overexpression on Akt activation during IL-1+OSM stimulation in SW1353 cells

Due to Trb3 silencing in SW1353 cells having an effect on Akt activation, Trb3 was also overexpressed and the effect on Akt activation investigated in SW1353 cells. It was initially shown that Trb3 was overexpressed, demonstrated through the c-Myc tag. Trb3 overexpression led to a decrease in Akt activation during IL-1+OSM stimulation (Fig 7.19).

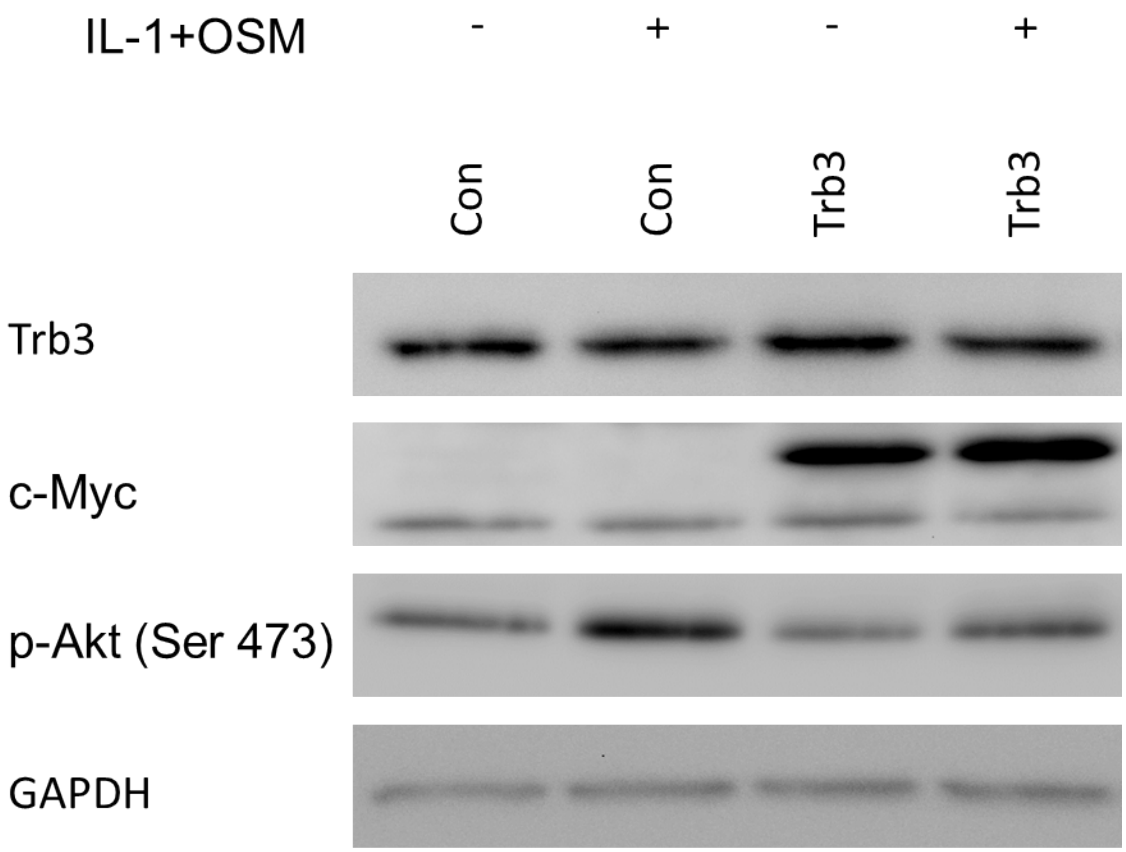

Figure 7.19. Trb3 overexpression leads to a decrease in Akt activation during IL-1+OSM stimulation in SW1353 cells. Trb3, c-Myc, p-Akt and GAPDH abundance after transfection with Trb3 overexpression plasmid +/- IL-1+OSM stimulation of SW1353 cells. Cells were transfected with Trb3 overexpression plasmid in 6 well plates (1.2 µg DNA/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.5 ng/ml) + OSM (10 ng/ml) for 20 minutes. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments.

7.2.3 JAK/STAT

7.2.3.1 The effect of Trb3 siRNA gene silencing on STAT activation during IL-1+OSM stimulation in HAC

The effect of Trb3 silencing on STAT activation was investigated in HAC. IL-1+OSM stimulation was performed at both 20 minutes and 60 minutes in order to investigate the effect of Trb3 on both STAT serine and STAT tyrosine phosphorylation. Both phosphorylation sites were investigated, as both sites

have been shown to be involved in MMP regulation (Zugowski *et al*, 2001; Litherland *et al*, 2010). It was initially determined that Trb3 was successfully silenced. Trb3 silencing led to a reduction in STAT1 serine phosphorylation at both 20 and 60 minutes of IL-1+OSM stimulation (Fig 7.20). No effect was seen on either STAT3 serine, STAT1 tyrosine or STAT3 tyrosine phosphorylation during Trb3 silencing.

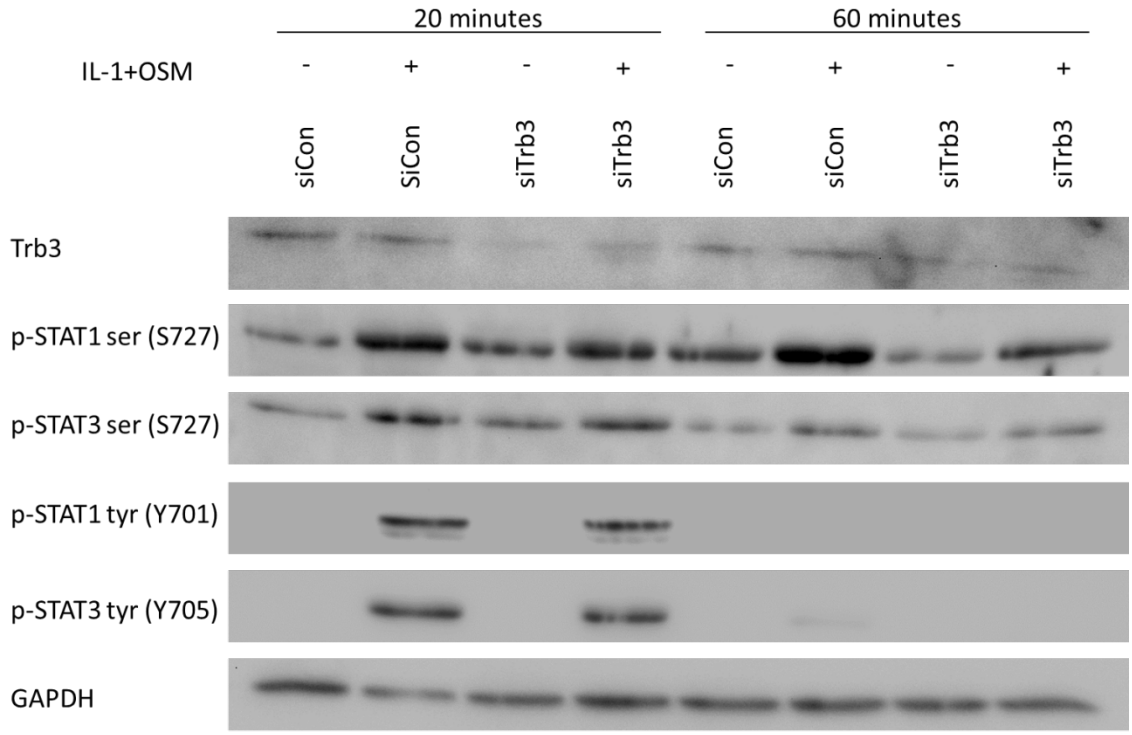

Figure 7.20. Trb3 siRNA gene silencing leads to a reduction in STAT1 serine activation during IL-1+OSM stimulation in HAC. Trb3, p-STAT1 ser, p-STAT3 ser, p-STAT1 tyr, p-STAT3 tyr and GAPDH abundance after transfection with Trb3 siRNA +/- IL-1+OSM stimulation of HAC. Cells were transfected with Trb3 siRNA in 6 well plates (100 nmol/well) for 48 hours and serum-starved overnight before stimulation with IL-1 (0.05 ng/ml) + OSM (10 ng/ml) for 20 minutes and 60 minutes. Cells were then lysed and proteins separated by SDS-PAGE and analysed by immunoblotting for the indicated proteins. Results are representative of 3 independent experiments, from 3 separate donors.

7.3 Discussion

7.3.1 NFκB

NFκB has been shown to have the ability to regulate MMP-1 and -13. This has been demonstrated in the literature (Vincenti and Brinckerhoff, 2002, Mengshol *et al*, 2000), and also within this study. NFκB has also been shown to cooperate with AP-1 in order to regulate MMPs (Barchowsky *et al*, 2000). In addition, NFκB has been shown to cooperate with ATF3 (however this is as a co-repressor rather than activator) (Liu *et al*, 2011), as well as regulating ATF3 expression (Jack *et al*, 2006). Therefore, along with the MAPK pathway, NFκB may be important in the ability of Trb1 and Trb3 to regulate MMPs.

Trb1 was shown to bind to the NFκB subunit p65 in SW1353 cells. This has previously been demonstrated in other cell types (Ostertag *et al*, 2010). The fluorescent staining pattern of this interaction was entirely nuclear. This possibly suggests that Trb1 may be involved in regulating the transcriptional activity of NFκB, as NFκB remains in the cytoplasm when inactive whilst post-activation it subsequently translocates to the nucleus where it is transcriptionally active (Roman-Blas and Jimenez, 2006, Chakraborty *et al*, 2010). This however may be an anomaly, as the PCA method involves overexpression of the proteins of interest, and it has previously been demonstrated that overexpression of NFκB can lead to its translocation to the nucleus without activation (Carlotti *et al*, 1999).

As mentioned, in order to study the effect of Trb1 and Trb3 on NFκB, IL-8 was used as a surrogate for NFκB activation. IL-8 was used as NFκB has been shown to be important in the mediation of IL-8 synthesis (Aupperle *et al*, 2001). This was further demonstrated through the silencing of IKKβ, which led to a reduction in NFκB activation, and demonstrated a significant reduction in *IL-8* mRNA expression. However, although NFκB appears to be the key regulator of IL-8, it has also been shown that JNK can regulate IL-8 expression independently of NFκB (Krause *et al*, 1998), and also to a lesser extent, p38 has been shown to regulate IL-8 induction (Suzuki *et al*, 2000). Therefore, these results cannot be solely accepted to be an effect on NFκB without further

verification, especially as it was shown in chapter 6 that Trb1 and Trb3 have the ability to regulate the MAPK pathway.

As had been suggested previously, it appeared that Trb1 may act in an anti-inflammatory fashion within this pathway. Overexpression of Trb1 both in SW1353 cells and HAC showed a trend towards a reduction in *IL-8* mRNA expression during pro-inflammatory IL-1+OSM stimulation. However, this was not significant. To support this anti-inflammatory effect of Trb1 on NFκB, suggested by the IL-8 data, Trb1 overexpression led to a reduction in p65 protein abundance and p65 activation in SW1353 cells. This is in contrast to that shown by Ostertag *et al*. (2010), who demonstrated that Trb1 was pro-inflammatory and a co-activator of NFκB transcriptional activity. This may be due to cell type specificity of Trb1. Trb1 only had an effect on p65 protein abundance during IL-1+OSM stimulation, suggesting that its anti-inflammatory action only occurs during this pro-inflammatory stimulation, and in a resting state Trb1 does not affect p65 expression. Due to the ability of Trb1 to bind p65 in the absence of stimulation, one may have expected it to also affect p65 protein abundance under basal conditions. Without the ability to silence Trb1, its anti-inflammatory affects cannot be confirmed, especially in light of the Trb3 results on NFκB (discussed below), and other pathways. It is unknown whether Trb1 is exerting its effect on p65 at the transcriptional level or at the protein level. However, the fact that Trb1 binds p65 could suggest that Trb1 is exerting its effect at the protein level by affecting factors such as protein stability or degradation.

Trb3 was also able to interact with p65 in SW1353 cells. This had previously been demonstrated in a different cell type (Duggan *et al*, 2010). The staining pattern was similar to that for Trb1, suggesting that Trb3 may also influence NFκB transcriptional activity.

Using IL-8 as a surrogate for NFκB activation (discussed previously), the results suggested that Trb3 regulates NFκB in a similar way to MMP-13, ATF3 and MAPKs, in that both silencing and overexpression lead to a reduction in *IL-8* mRNA expression; this was consistent for both HAC and SW1353 cells. In addition, both silencing and overexpression of Trb3 in SW1353 cells, as well as silencing of Trb3 in HAC led to a reduction in p65 protein abundance and p65

activation, which would also concur with previous data, as well as confirming the IL-8 data. This may suggest that the level of Trb3 within the cell is critical for NFκB regulation, and deviation from a specific level leads to a dysregulation of signalling. As with Trb1, Trb3 only had an effect on p65 protein abundance during IL-1+OSM stimulation, suggesting that its effect on NFκB only occurs during this pro-inflammatory stimulation, and in a resting state Trb3 does not affect p65 abundance. In order to further investigate the effect of Trb1 and 3 on NFκB activation their effect on IκB-α abundance should be investigated, this would allow activation to be more thoroughly studied. As discussed previously, in order for NFκB activation to occur IκB is phosphorylated and degraded through the proteasome.

These results suggest that Trb3 in both SW1353 cells and HAC regulates NFκB in a similar manner. In addition, these results lend credence to the fact that NFκB may be one of the transcription factors, along with AP-1 and ATF3, in which Trb3 regulates in order to regulate MMP-13 expression.

7.3.1.1 Synopsis

Both Trb1 and Trb3 have the ability to regulate NFκB, and this may be through interaction with the p65 subunit. Trb1 and Trb3 may regulate NFκB in a similar manner, whereby an alteration in Trb1 and Trb3 levels leads to a dysregulation in signalling. However, due to the inability to silence Trb1 this cannot be established. It may therefore be the case that Trb1 is anti-inflammatory and simply negatively regulates NFκB.

7.3.2 Akt

In terms of Trb1 and Trb3 regulation of signalling pathways, the PI3K/Akt pathway has been studied the most prolifically. This is in part due to the role in which this pathway plays in diabetes, and the negative way in which Trb3, in particular, regulates this pathway through prevention of Akt phosphorylation (Du *et al*, 2003). The dysregulation of this pathway by Trb3 has been suggested by

Cravero *et al*. (2009), to be important in chondrocyte survival, proteoglycan production, and therefore OA. With this in mind, and the ability of this pathway to regulate MMP-1 and -13 (Litherland *et al*, 2008), and cooperate with the MAPK pathway (Yart *et al*, 2009), the PI3K/Akt pathway may be an important mediator of Trb1 and Trb3 regulation of MMPs.

Trb1 overexpression did not appear to have any effect on Akt activation in SW1353 cells. It has not been previously shown that Trb1 has any effect on Akt activation, only Trb2 and Trb3 have been shown to play a role in Akt activation (Du *et al*, 2003, Cravero *et al* 2009); this therefore concurred with previous literature. The PI3K/Akt pathway is regulated by OSM (Godoy-Tundidor *et al*, 2005) perhaps more than the other pathways investigated so far. In addition, it has been shown previously that PI3K and Akt inhibition led to a reduction in OSM induced MMP-13 expression (Mabrouk *et al*, 2007). This result may therefore suggest that Trb1 is more important in regulating IL-1 mediated pathways, rather than those stimulated by OSM. In order to investigate this more thoroughly, the effect of Trb1 overexpression on signalling pathways in the presence of separate stimulations of IL-1 and OSM would be required.

Trb3 appeared to negatively regulate Akt activation in SW1353 cells. Trb3 silencing showed an increase in Akt activation under basal condition, whilst overexpression of Trb3 showed a reduction in Akt activation during IL-1+OSM stimulation. This concurred with what has previously been shown in the literature, where Trb3 is known to prevent Akt phosphorylation (Du *et al*, 2003, Cravero *et al* 2009). IL-1+OSM stimulation had no effect on Akt activation during Trb3 silencing; the level of phosphorylated Akt was the same both under basal conditions or with IL-1+OSM stimulation. This possibly suggested that optimal activation had been reached during stimulation, and therefore silencing of Trb3 could not enhance phosphorylation anymore, however under basal conditions this was not the case. The effect of Trb3 on Akt appeared to be cell type specific, as in HAC Trb3 silencing had no effect on Akt phosphorylation. This result was contradictory to that seen by Cravero *et al*. (2009), which stated that Trb3 overexpression led to a reduction in Akt phosphorylation in chondrocytes. However, Akt phosphorylation was investigated on Threonine 308 in the Cravero paper, whilst in this study Akt phosphorylation on Serine 473 was investigated, which may account for the difference. Nevertheless, it is

generally thought that the Serine 473 phosphorylation site is more important for full Akt activation (Martelli *et al*, 2010).

7.3.2.1 Synopsis

The regulation of Akt activation by Trb3 appeared to be cell type specific. Trb3 appeared to negatively regulate Akt activation in SW1353 cells; however, it had no effect in HAC. Trb1 did not appear to regulate Akt activation when investigated in SW1353 cells.

7.3.3 JAK/STAT

The JAK/STAT pathway has been shown to be an important regulator of MMPs, and this has been reported to occur through the AP-1 subunit c-Fos (Litherland *et al*, 2010). As well as this, it has been suggested that the JAK/STAT pathway may work in conjunction with AP-1 to enhance MMP expression (Korzus *et al*, 1997). Previous work in chapter 5 demonstrated that Trb3 appeared to regulate c-Fos protein abundance. The JAK/STAT pathway was therefore investigated.

Trb3 silencing demonstrated a reduction in STAT1 serine phosphorylation during IL-1+OSM stimulation. Trb3 silencing had no effect on either STAT3 serine, STAT1 tyrosine or STAT3 tyrosine phosphorylation, suggesting that Trb3 was specific for STAT1 serine. Whether this specificity was through JAK dependent phosphorylation or through another pathway remains to be elucidated. Trb3 may therefore regulate MMP-13 expression through regulation of STAT1 activation.

7.3.3.1 Synopsis

Trb3 appeared to regulate the JAK/STAT pathway in HAC. This occurred through the serine phosphorylation of STAT1, and it appeared to be specific to this phosphorylation site.

7.4 Summary

- Trb1 and Trb3 interact with the NFκB subunit p65.

- Trb1 overexpression showed a trend towards a reduction in *IL-8* mRNA expression in HAC and SW1353 cells, as well as a reduction in p65 protein abundance and activation in SW1353 cells.

- Both silencing and overexpression of Trb3 led to a reduction in *IL-8* mRNA expression in HAC and SW1353 cells, as well as leading to a reduction in p65 protein abundance and p65 activation in SW1353 cells.

- Trb3 silencing also led to a reduction in p65 protein abundance and activation in HAC.

- Trb1 overexpression had no effect on Akt activation in SW1353 cells.

- Trb3 silencing led to an increase in Akt activation in SW1353 cells but had no effect in HAC. Overexpression of Trb3 led to a decrease in Akt activation in SW1353 cells.

- Trb3 silencing led to a reduction in STAT1 serine phosphorylation, but had no effect on STAT1 tyrosine, STAT3 serine or STAT3 tyrosine phosphorylation in HAC.

Chapter 8: General Discussion

Trb1-3 are a group of 3 proteins which have been shown to play a role in a number of cellular pathways, and are involved in a number of diseases. Trb1-3 have previously been shown to play a role in a number of signalling pathways which are known to regulate MMPs including the MAPK pathway (Kiss-Toth *et al*, 2004), TGF-β/Smad pathway (Chan, 2010) and the PI3K/Akt pathway (Du *et al*, 2003). They have also been shown to regulate a number of transcription factors known to be involved in MMP regulation such as AP-1 (Kiss Toth *et al*, 2004; Eder *et al*, 2008), ATF (Bowers *et al*, 2003) and NFκB (Duggan *et al*, 2010; Ostertag *et al*, 2010). Trb3 has been implicated in OA and chondrocyte death (Cravero *et al*, 2009). Trb3 has also been shown to be up regulated in stress conditions such as ER stress and hypoxia (Ohoka *et al*, 2005; Bowers *et al*, 2003), which have been implicated in arthritis (Yudoh *et al*, 2005; Takada *et al*, 2011).

Due to the ability of Trb1 and Trb3 to regulate signalling pathways and transcription factors involved in the regulation of the two major collagenases implicated in arthritis, MMP-1 and -13, as well as evidence of Trb3 being involved in arthritis, it was hypothesised that Trb1 and Trb3 proteins may regulate MMP-1 and -13 in order to regulate cartilage degradation in arthritis.

The role of Trb1 and Trb3 in arthritis was studied in two cell types both primary Human Articular Chondrocytes (HAC), and the SW1353 human chondrosarcoma cell line. Ideally all experiments would have been performed in HAC, however, due to certain constraints such as cell availability and transfection efficiency, SW1353 cells were used as a model of HAC. Trb2 was not assessed within this thesis as previous work within the lab demonstrated that Trb2 did not regulate *MMP-1* and *-13* expression (Duncan, 2010).

Throughout this thesis the effect of Trb1 on the various signalling pathways could only be assessed through its overexpression, as Trb1 could not be effectively and specifically silenced. Trb1 silencing was attempted through siRNA and shRNA, both of which proved unsuccessful or non-specific. The reason for this unsuccessful silencing was unknown, however, it was hypothesised that it was possibly due to *Trb1* mRNA having a very short half-life

of less than 1 hour (Sharova *et al*, 2009). This would prevent silencing of Trb1, as any of the mRNA that was being silenced would quickly be degraded, and new functional mRNA synthesised before an effect could be seen. This would correlate with the fact that Trb3 has been shown to have a longer half-life and was successfully silenced.

Pro-inflammatory cytokines have been shown to play a major role in both OA and in particular RA. They mediate signalling pathways, including those mentioned above, which lead to cartilage degradation. There are many cytokines involved in this with a major one being IL-1 (Feldman *et al*, 1996; Scott *et al*, 2010; McInnes and Schett, 2007; Goldring *et al*, 1994). OSM a member of the IL-6 family has also been shown to have a role in arthritis (Plater-Zyberk *et al*, 2001). IL-1 and OSM have been shown to work synergistically in terms of cartilage degradation and MMP expression (Cawston *et al*, 1995; Cawston *et al*, 1998; Rowan *et al* 2003; Fearon *et al*, 2006), therefore this is a potent cytokine stimulus. This stimulus was therefore used throughout this thesis in order to mimic a pro-inflammatory environment *in vitro*.

As well as these cytokines being able to regulate various signalling pathways, it was demonstrated within this thesis that IL-1+OSM stimulation also regulates Trb1 and Trb3, leading to an increase in Trb1 and Trb3 at both the mRNA and protein level in HAC. In addition, IL-1+OSM stimulation led to an increase in both Trb1 and Trb3 protein abundance within the cytoplasm in SW1353 cells. This suggested that Trb1 and Trb3 were responsive to pro-inflammatory stimuli and may therefore play a role in modulating the response of these signalling cascades during arthritis. Both Trb1 and Trb3 were also responsive to stress conditions, demonstrating an up-regulation in protein abundance during serum starvation in HAC. Both Trb1 (Ostertag *et al*, 2010) and, as mentioned previously, Trb3 (Ohoka *et al*, 2005; Bowers *et al*, 2003) have been shown to be responsive to cellular stress, which is a feature of arthritis (Yudoh *et al*, 2005; Takada *et al*, 2011).

It was initially assessed whether Trb1 and Trb3 had the ability to regulate MMP-1 and -13 during pro-inflammatory stimulation with IL-1 and OSM. The ability of Trb1 and Trb3 to regulate these two MMPs appeared to be somewhat cell type specific, with Trb1 overexpression and Trb3 silencing leading to a reduction in

both *MMP-1* and *-13* in SW1353 cells, but only affecting *MMP-13* in HAC. This is not surprising as Trb1 and Trb3 have often been shown to be cell type specific (Hegedus *et al*, 2006; Kiss-Toth *et al*, 2004; Duggan *et al*, 2010), and it has previously been shown that there are differences in MMP signalling pathways between primary human chondrocytes and SW1353 cells (Borden *et al*, 1996, Mengshol *et al*, 2000). These results, however, suggested that Trb1 and Trb3 may be somewhat specific for MMP-13 in HAC. Trb3 overexpression also led to a reduction in *MMP-13*. This was somewhat unexpected as one would expect overexpression and silencing to have opposing effects. It is therefore hypothesised that within the cellular environment, for normal homeostasis and signalling to occur, a specific level of Trb3 is required within the cell. When this level is altered, either increased or decreased, there is dysregulation within the pathways, leading to a reduction in signalling.

In order to regulate MMP-13 expression in HAC both Trb1 and Trb3 regulate the MMP transcription factor AP-1. Trb1 overexpression showed a reduction in mRNA expression of both AP-1 subunits *c-fos* and *c-jun*, whilst Trb3 silencing had no effect on the mRNA expression but showed a decrease in c-Fos nuclear protein abundance and c-Jun activation. Although both Trb1 and Trb3 appeared to regulate AP-1, the above result suggests the way in which they regulate AP-1 may be different. Both Trb1 and Trb3 were also shown to regulate the MMP transcription factor ATF3. Trb1 overexpression showed a reduction in *ATF3* mRNA expression whilst both silencing and overexpression of Trb3 led to a reduction in *ATF3* mRNA expression, as well as silencing showing a reduction in ATF3 nuclear protein abundance. AP-1 is not specific for MMP-13 (Litherland *et al*, 2010), therefore this specificity of MMP-13 regulation by Trb1 and Trb3 may come from the ATF3 transcription factor, as work in our lab demonstrated that ATF3 silencing led to a reduction in *MMP-13* mRNA expression, but had no effect on *MMP-1* (Macdonald, 2013). Further evidence for this is the fact that both overexpression and silencing of Trb3 led to a reduction in *ATF3*, much in the same way Trb3 affects *MMP-13*.

It was hypothesised that Trb1 and Trb3 may regulate AP-1, which subsequently regulates ATF3; these two transcription factors may combine to regulate MMP-13, as it has been previously demonstrated that c-Jun can induce ATF3

expression (Mei *et al*, 2008). In addition, work within our lab demonstrated that IL-1+OSM-induced ATF3 is AP-1-dependent (Macdonald, 2013).

It was unknown how Trb1 and Trb3 may be regulating AP-1 and through which pathways. The MAPK pathway was investigated, as this has previously been shown to be important in MMP regulation (Han *et al*, 2001; Raymond *et al* 2006; Mengshol *et al* 2000), and one way in which they regulate MMPs is through the AP-1 transcription factor (Minden *et al*, 1997; Leppa *et al*, 1998; Han *et al*, 2001). In addition, the MAPK pathways have also been implicated in ATF3 regulation (Lu *et al*, 2007; Hamdi *et al*, 2008). Both Trb1 and Trb3 were shown to regulate the MAPK pathways, with Trb1 overexpression leading to a reduction in both p38 and JNK activation in SW1353 cells. Trb3 appeared to once again demonstrate cell type specificity, with silencing of Trb3 showing a reduction in the activation of all 3 MAPK pathways in HAC, but only affecting p38 in SW1353 cells. However, interestingly, overexpression of Trb3 in SW1353 cells did reduce activation of all 3 MAPK pathways. This perhaps suggests that Trb3 does have the ability to regulate all of the MAPKs in SW1353 cells, as it does in HAC. This is further supported by the fact that the 3 MAPK pathways have very similar activation profiles during IL-1+OSM stimulation in both HAC and SW1353 cells, suggesting they may be regulated in a similar manner. A possible reason why Trb3 silencing does not reduce the activation of all 3 MAPK pathways in SW1353 cells may be due to the fact that, as discussed, the level of Trb3 within the cell may be important. Trb3 silencing may produce a particular level of Trb3 that does not inhibit JNK or ERK activation, but does affect p38 in SW1353 cells. This has been previously demonstrated by Kiss-Toth *et al*. (2004), whereby alterations in Trb3 levels had differing effects on the MAPK pathway. This result may suggest that p38 is more responsive to changes in Trb3 levels than JNK or ERK in SW1353 cells.

Trb1 and Trb3 may regulate MAPK activation through the binding of MAP2Ks, as it was demonstrated that both Trb1 and Trb3 have the ability to bind MEK1, MKK4, MKK6 and MKK7 in SW1353 cells. Collectively these MAP2Ks are involved in all 3 MAPK pathways, further supporting the hypothesis that Trb1 and Trb3 have the ability to regulate all 3 MAPK pathways. The reason why Trb1 was not seen to regulate ERK was unknown, however, as suggested with Trb3, the level of Trb1 may be critical in its ability to regulate pathways. In

addition, the ERK pathway is also regulated by the MAP2K MEK2, therefore there may be redundancy whereby ERK is regulated through MEK2, although it is possible that Trb1 may also bind MEK2. These were the only MAP2Ks tested, therefore Trb1 and Trb3 may have the ability to bind all of the MAP2Ks. It is possible that the binding of Trb3 to the MAP2Ks stabilises Trb3, as combined silencing of MEK1, MKK4, MKK6 and MKK7 showed a reduction in Trb3 protein abundance. This has been demonstrated previously by Kiss-Toth *et al*, (2004), who showed binding of Trb1 and Trb3 to the MAP2Ks increased their protein levels. Further to this, inhibition of MAPK activation through MAP2K inhibition also showed a reduction in Trb3 protein levels. Interestingly however, this was not the case for Trb1, which was not affected by MAP2K silencing or inhibition of MAP2Ks (data not shown), suggesting Trb1 and Trb3 may be regulated in different ways. This was further demonstrated by the fact that Trb3 appears to be degraded through the proteasome, as demonstrated by addition of the proteasome inhibitor MG132, whereas Trb1 does not.

Trb1 and Trb3 may also regulate AP-1, in particular the subunit c-Fos, through the JAK/STAT pathway. Trb3 silencing was shown to reduce STAT1 activation, and the JAK/STAT pathway has been shown to regulate c-Fos (Litherland *et al*, 2010). STATs have also been shown to regulate MMPs directly through binding to their promoters (Zugowski *et al*, 2011), which may be another way in which Trb1 and Trb3 regulates MMP expression. The regulation of this particular pathway by Trb1 and Trb3 has not been previously discussed within the literature.

In addition to their ability to regulate the AP-1 and ATF3 transcription factors in order to regulate MMP expression it was investigated whether Trb1 and Trb3 could also regulate NFκB. Trb1 and Trb3 have previously been shown to regulate NFκB (Duggan *et al*, 2010; Ostertag *et al*, 2010). The NFκB transcription factor has been shown to be important in arthritis, acting as a key mediator of pro-inflammatory pathways (Kapoor *et al*, 2011), as well as being associated with MMP expression (Vincenti and Brinckerhoff, 2002). The MAPK pathway has also been shown to be an important regulator of NFκB (Mukkherjee and Sikka, 2006; Larsen *et al* 2005), it was therefore hypothesised that through their role in MAPK activation Trb1 and Trb3 may regulate NFκB. The role of Trb1 and Trb3 in regulating NFκB appeared to be similar to the way

in which they regulate other pathways discussed. Trb1 overexpression showed a trend towards a reduction in the NFκB surrogate *IL-8* in HAC and SW1353 cells, as well as showing a reduction in p65 protein abundance and activation in SW1353 cells. Whilst Trb3 silencing and overexpression in SW1353 cells and HAC showed a reduction in *IL-8* (overexpression was not significant in HAC but showed a strong trend), as well as a reduction in p65 protein abundance and activation (overexpression data only shown in SW1353 cells). This result for Trb3 further supports the hypothesis that it is the level of Trb3 within the cell that is critical for correct signalling to occur, and divergence above or below a certain level leads to a dysregulation in signalling. Trb1 and Trb3 may also regulate NFκB through direct interaction with the transcription factor, as both Trb1 and Trb3 were shown to interact with the NFκB subunit p65. This interaction was solely isolated to the nucleus, possibly suggesting that Trb1 and Trb3 may play a role in regulating the transcriptional ability of NFκB.

It is interesting however that the effect of Trb3 on Akt activation did not show both overexpression and silencing reducing Akt activation, as has been seen with other pathways. Trb3 acted solely as a negative regulator of Akt activation in SW1353 cells, with silencing of Trb3 increasing Akt activation and overexpression reducing it. This was not surprising in the sense that Trb3 is known to negatively regulate Akt activation (Du *et al*, 2003), and this has been proposed to be an important factor in insulin signalling and diabetes (Liu *et al*, 2010). In addition, this regulation of Akt by Trb3 has been suggested to play a role in OA development and chondrocyte cell death (Cravero *et al*, 2009). Again Trb3 showed cell type specificity, as it did not appear to affect Akt activation in HAC, but did in SW1353 cells. This result would therefore contradict the Cravero data, however Akt phosphorylation was only investigated on serine 473 within this thesis, whilst the phosphorylation of threonine 308 was investigated in the Cravero paper, which may account for the difference. Unlike many of the other pathways discussed, in which Trb1 and Trb3 have both been shown to regulate, the Akt pathway did not appear to be one of them, as Trb1 overexpression did not have an effect on Akt phosphorylation in SW1353 cells.

Throughout this thesis the effect of Trb1 and Trb3 on various signalling pathways has been investigated in both SW1353 cells and HAC. As mentioned previously, SW1353 cells are used as a model for HAC, though there are

limitations. It has previously been demonstrated that within the presence of IL-1β SW1353 cells have reduced expression levels of anabolic genes compared to chondrocytes. However, in terms of catabolic genes they more closely resemble one another, in particular MMP-1 and -13, as well as NFκB and IL-6, which would positively reflect the use of SW1353 cells within this study (Gebauer *et al,* 2005).

Within this thesis, there have been differences seen between SW1353 cells and HAC in terms of the ability of Trb1 and Trb3 to regulate signalling pathways. Perhaps the most striking difference being between the regulation of Akt by Trb3, which showed no effect in HAC, whereas Akt was negatively regulated in SW1353 cells. It is possible that the other differences seen between HAC and SW1353 cells in relation to the other pathways studied may be due to the levels of Trb1 and Trb3 required to influence the various pathways which differ between HAC and SW1353 cells, as overexpression and silencing appeared to have differing effects, as in the case for MAPK and MMP regulation. It would appear however that tribbles may be more specific for MMP-13 than MMP-1 in HAC, whilst in SW1353 cells they may regulate both MMP-1 and -13. However, overall in terms of the way that Trb1 and Trb3 regulate the signalling pathways investigated there appears to be many similarities between HAC and SW1353 cells.

This thesis has demonstrated the ability of Trb1 and Trb3 to regulate a number of different signalling pathways within a pro-inflammatory disease context. It was shown that Trb1 and Trb3 through regulation of these signalling pathways can regulate transcription factors important in MMP regulation, and as such regulate MMPs, suggesting their ability to regulate cartilage degradation. The ability of Trb1 and Trb3 to regulate MMPs, and how they regulate MMPs has not been previously investigated. There were however limitations to this thesis; it was not possible to silence Trb1, therefore the true nature of Trb1 regulation could not be investigated. This may be important in light of the way in which Trb3 can regulate many pathways with both silencing and overexpression having similar effects. In addition, SW1353 cells were used for some experiments due to certain constraints, and as discussed above, these cells do not always perfectly mimic HAC.

Throughout this thesis the Trb1 results would suggest that it is anti-inflammatory and has the ability to down regulate MMP expression. It is hypothesised that this is through interaction with MAP2Ks, leading to an inhibition of MAPK activation, which inhibits the MMP transcription factor AP-1 subunits *c-fos* and *c-jun* mRNA expression, as well as ATF3 and NFκB (Fig 8.1). It is possible that these transcription factors cooperate together in order to regulate MMP expression, but may also work independently. The regulation of ATF3 by Trb1 may be through its regulation of AP-1. In addition, Trb1 may regulate NFκB directly through interaction with p65. The anti-inflammatory ability of Trb1 would be in agreement with previous work within our lab that suggested that Trb1 may be involved in the IL-4 pathway (Duncan, 2010). This pathway is a known anti-inflammatory cytokine pathway that has been shown to reduce both MMP-1 and -13 levels (Borghaei *et al*, 1998). Trb1 may work through this pathway in order to reduce MMP expression. It is therefore postulated that overexpression of Trb1 within an *in vivo* model of arthritis may be protective. However, as mentioned previously, Trb1 could not be silenced, therefore without the ability to silence Trb1 expression its anti-inflammatory capability cannot be confirmed.

The role of Trb3 in regulating many of the pathways discussed appears to be more complex than simply up regulating or down regulating signalling. In many instances both overexpression and silencing of Trb3 led to a down regulation of signalling. As mentioned, the levels of Trb3 within the cell may be critical, and the level of Trb3 required may vary for different pathways, as some pathways may be more responsive than others. This may suggest that Trb3 is acting as a scaffold protein, as both overexpression and silencing of scaffolds has been shown to lead to inhibition of signalling (Morrison and Davis, 2003). As discussed previously, the reason behind this is that low levels of the scaffold protein do not allow the proteins within the pathway to combine, and high levels could segregate the components of this pathway, preventing them interacting with other proteins within a particular pathway. It is therefore hypothesised that Trb3 through the binding of MAP2Ks has the ability to regulate MAPK activation, which can regulate the MMP transcription factors AP-1, ATF3 and NFκB, which then leads to the regulation of MMP-13 and cartilage degradation (Fig 8.1). Trb3 may also regulate NFκB directly by binding to its p65 subunit. Trb3 could also regulate MMP expression through an alternative pathway, as it

has been shown to regulate the JAK/STAT pathway, which can subsequently regulate MMP-13 expression. Regulation of MMP expression by this pathway may be through regulation of AP-1, or by direct transcriptional regulation. It is also possible there is cross talk between these 2 pathways. It is therefore theorised that both silencing and overexpression of Trb3 in an *in vivo* model of arthritis would lead to protection.

A recent study performed within this lab investigated the effect of Trb3 on cartilage degradation *in vivo*. Trb3 was silenced in a DMM mouse model. Trb3 silencing demonstrated reduced MMP-13 expression, as well as protection to cartilage degradation. This study therefore compliments the *in vitro* Trb3 data seen within this thesis.

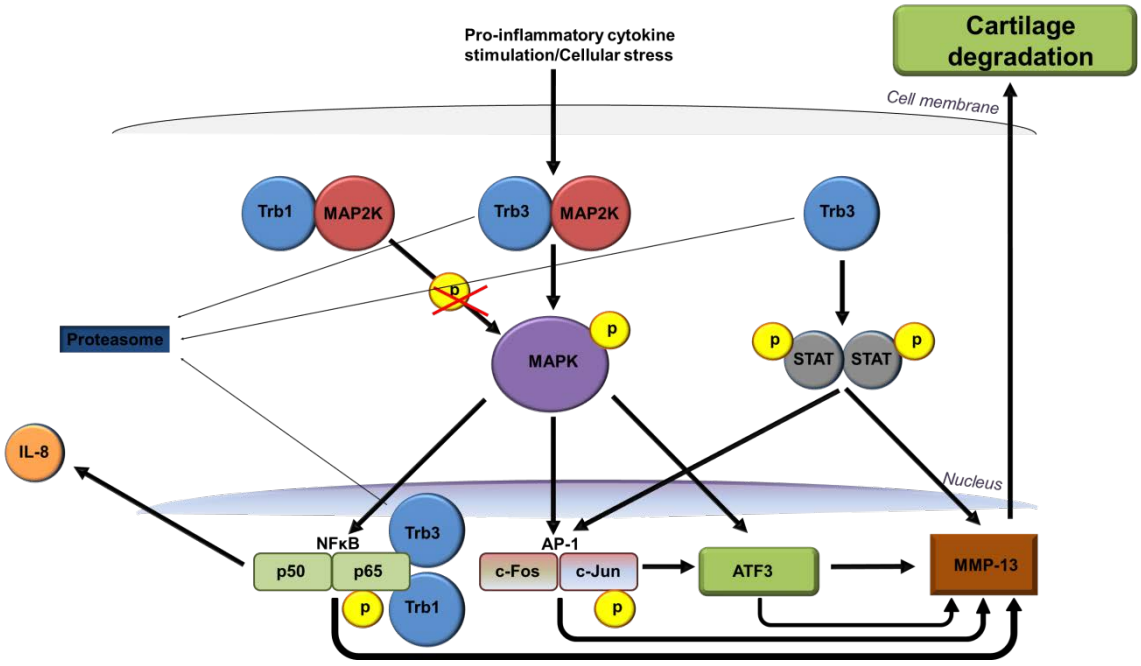

Figure 8.1. The hypothetical pathway of Trb1 and Trb3 regulation of MMP-13 in HAC. Trb; Tribbles, MAP2K; Mitogen activated protein kinase kinase, IL-8; Interleukin-8, MAPK; Mitogen activated protein kinase, p; phosphorylation, NFκB; Nuclear factor κ B, AP-1; Activating protein-1, ATF3; Activating transcription factor, MMP-13; Matrix metalloproteinase-13, STAT; Signal transducers and activators of transcription.

The results within this thesis demonstrate that both Trb1 and Trb3 have the ability to regulate a number of important pathways involved in MMP regulation in chondrocytes, within a pro-inflammatory context, and they appear to regulate many of the same pathways. It is possible that they have opposing roles within this context with Trb1 being anti-inflammatory and Trb3 being pro-inflammatory. This knowledge of how Trb1 and Trb3 may be involved in signalling pathways which regulate MMP expression and therefore cartilage degradation may help in future treatments for arthritis.

8.1 Summary of possible future studies

- Investigate the effect of Trb1 and Trb3 on other transcription factors known to regulate MMPs, in particular MMP-13, such as RUNX2 and C/EBP-α.

- Silence individual MAPK pathways using combinations of specific MAP2K siRNAs in order to decipher if specific MAPK pathways effect Trb1 and Trb3 expression.

- Investigate the effect of Trb1 and Trb3 on MAP2K mRNA expression and protein abundance.

- Investigate the effect of Trb1 and Trb3 to regulate the signalling pathways discussed in the presence of other cytokine stimuli such as TNF-α, in order to investigate whether Trb1 and Trb3 regulate signalling pathways through other cytokine pathways.

- Determine the role which Trb1 and Trb3 play within the individual IL-1 and OSM pathways, by stimulating separately and investigating the

effects on the pathways discussed, this may be relevant in pathways such as Akt and JAK/STAT, which have been shown to be regulated through the OSM pathway.

- Co-immunoprecipitation for Trb1 and Trb3 with the MAP2Ks and NFκB, in order to verify their interaction.

- Mutation of Trb1 and Trb3 binding sites and protein complementation assay with MAP2K and NFκB in order to verify their interaction.

- Investigate the effect of IL-1+OSM stimulation on Trb1 and Trb3 interaction, as well as the interaction of Trb1 and Trb3 with the MAP2Ks and NFκB. Including real-time observation of these interactions and cellular localisation with and without stimulation. This will allow investigation of Trb1 and Trb3 interactions within a pro-inflammatroy context, which may differ compared to basal conditions. This will also be more relevant in terms of an arthritic context.

- Investigate the effect of IL-1+OSM stimulation on Trb1 and Trb3 proteasomal degradation through treatment with MG132.

- Immunofluorescence in order to verify the cellular distribution of Trb1 and Trb3.

- Investigate the effect of Trb1 and Trb3 on the other major collagenase MMP-8. This will give a greater indication on the specificity of Trb1 and Trb3 for MMP-13, or whether they act in a broader context.

- Investigate the effect of Trb1 and Trb3 on TIMPs, in order to greater understand how Trb1 and Trb3 may regulate MMPs.

- Investigate the effect of Trb1 and Trb3 on ADAMTS-4 and -5, in order to investigate wether Trb1 and Trb3 can regulate proteoglycan degradation as well as collagen type II degradation.

- Investigate the effect of Trb1 and Trb3 overexpression on STAT activation.

- Investigate the effect of Trb1 and Trb3 on JAK regulation.

- Investigate the effect of Trb3 on PI3K.

- Investigate the effect of Trb1 and Trb3 on the various pathways on collagen type II coated plates, in order to give a greater physiological significance.

- Investigate the effect of Trb1 and Trb3 on other proteins within cartilage, such as fibronectin and anchorin.

- Investigate the effect of Trb3 overexpression, as well as Trb1 silencing and overexpression on cartilage degradation and MMP expression in a mouse model.

Each of these experiments will further provide a greater understanding of the roles in which Tribbles may play in regulating cellular pathways in order to regulate proteases involved in cartilage degradation. This greater understanding may help in finding future treatments for arthritis.

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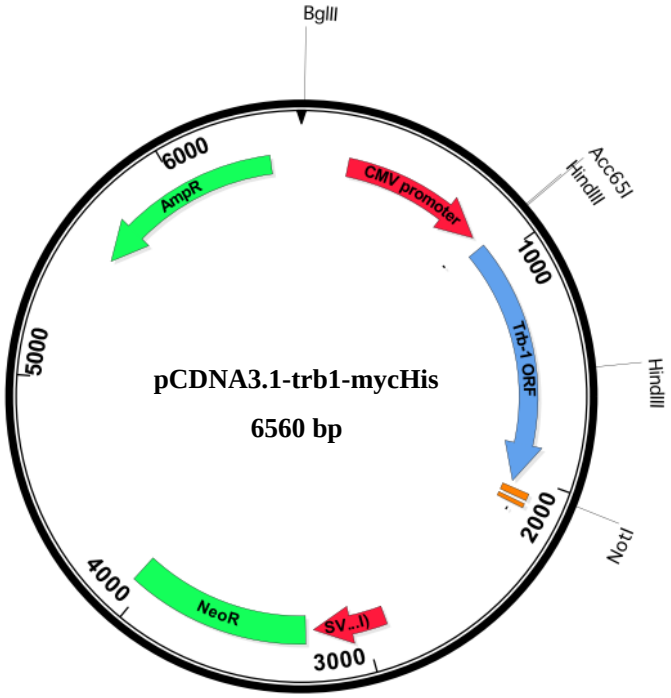
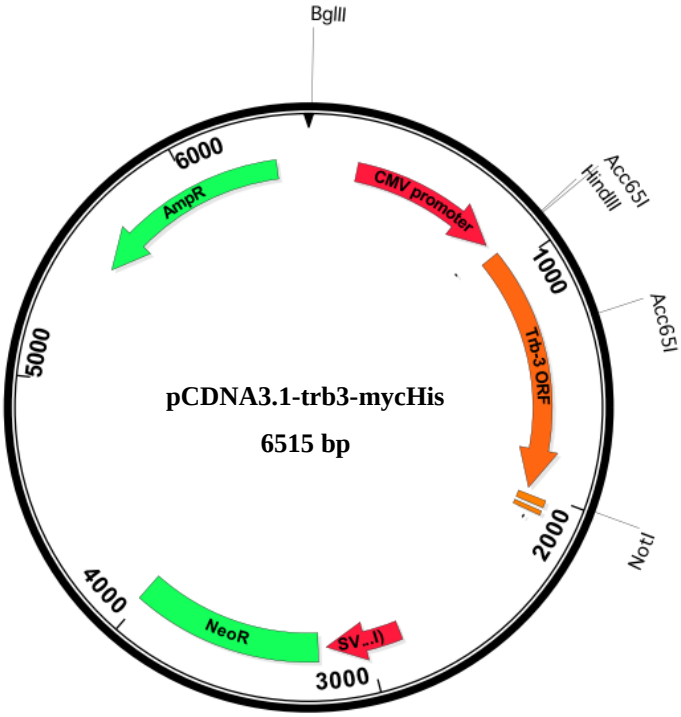
Appendix A - Overexpression plasmid maps

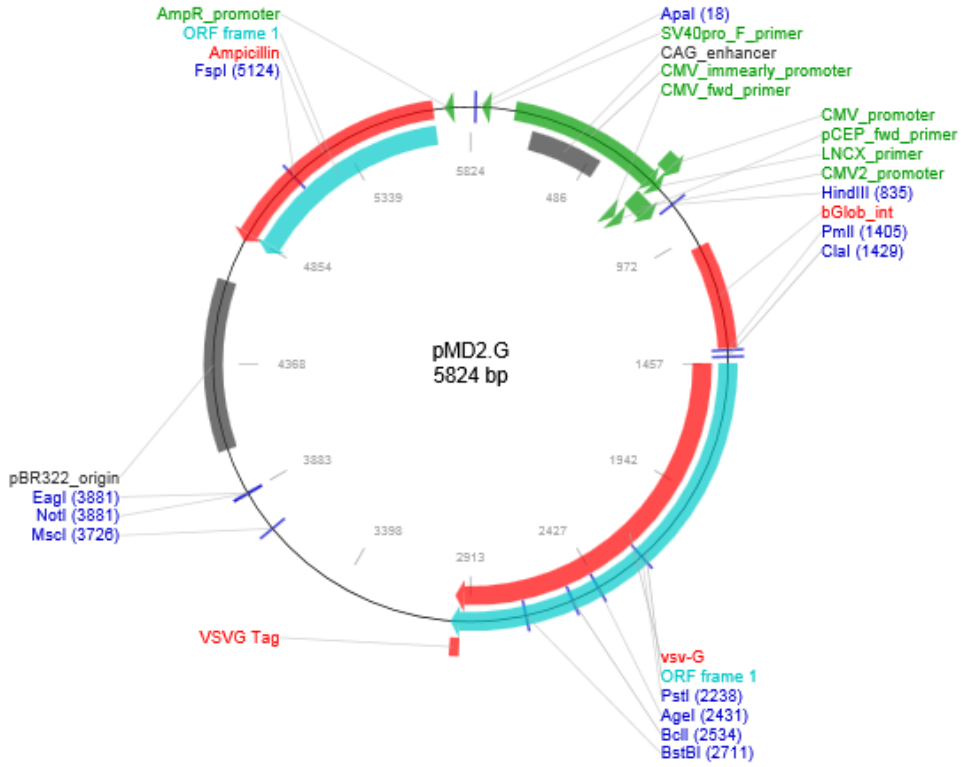
A



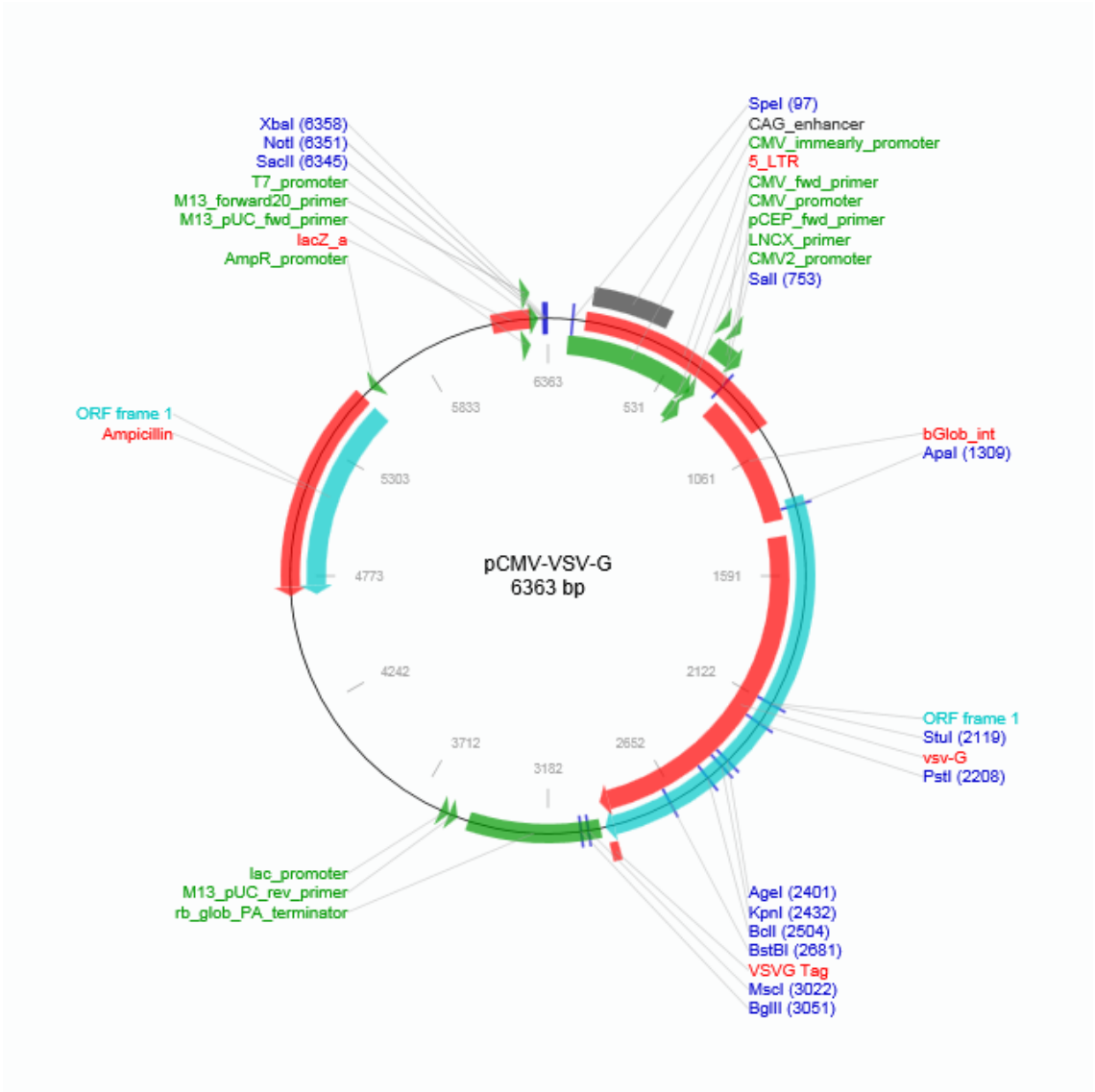
B



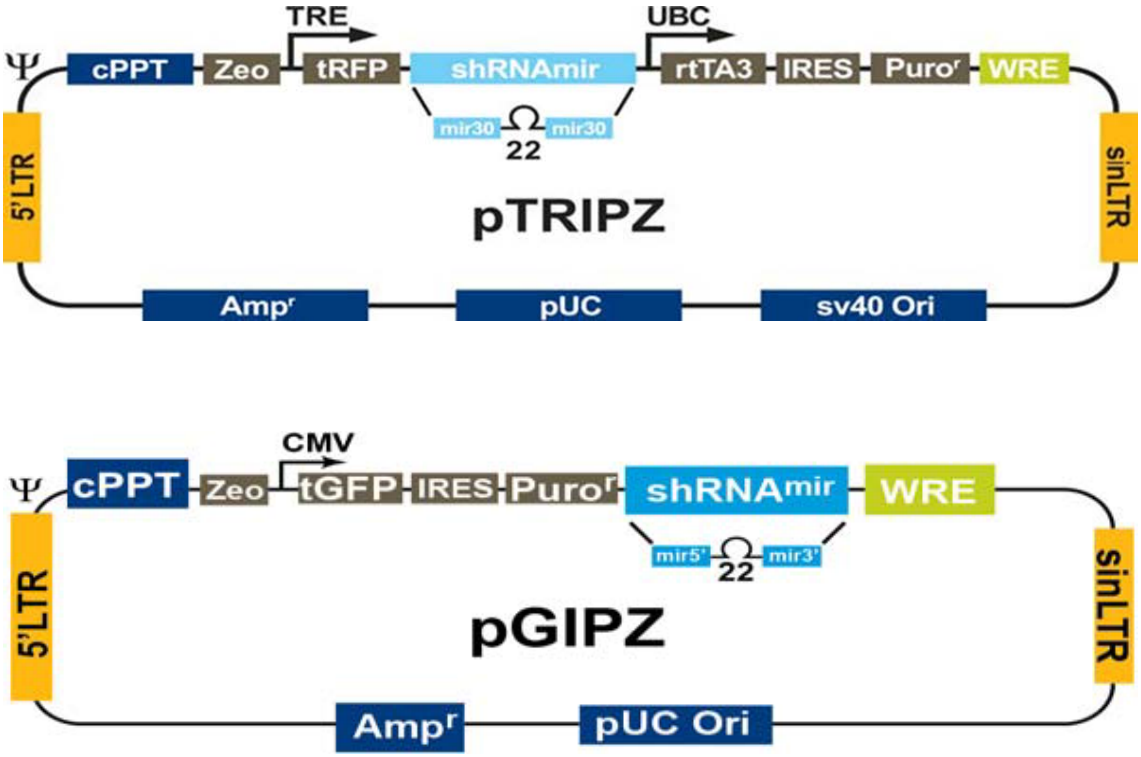
Trb1 (A) and Trb3 (B) overexpression plamid maps. Trb; Tribbles, AmpR; Ampicillin resistance, CMV; Cytomegalovirus, ORF; Open reading frame, SV; Simian virus, NeoR; Neomycin resistance, His; Histidine.



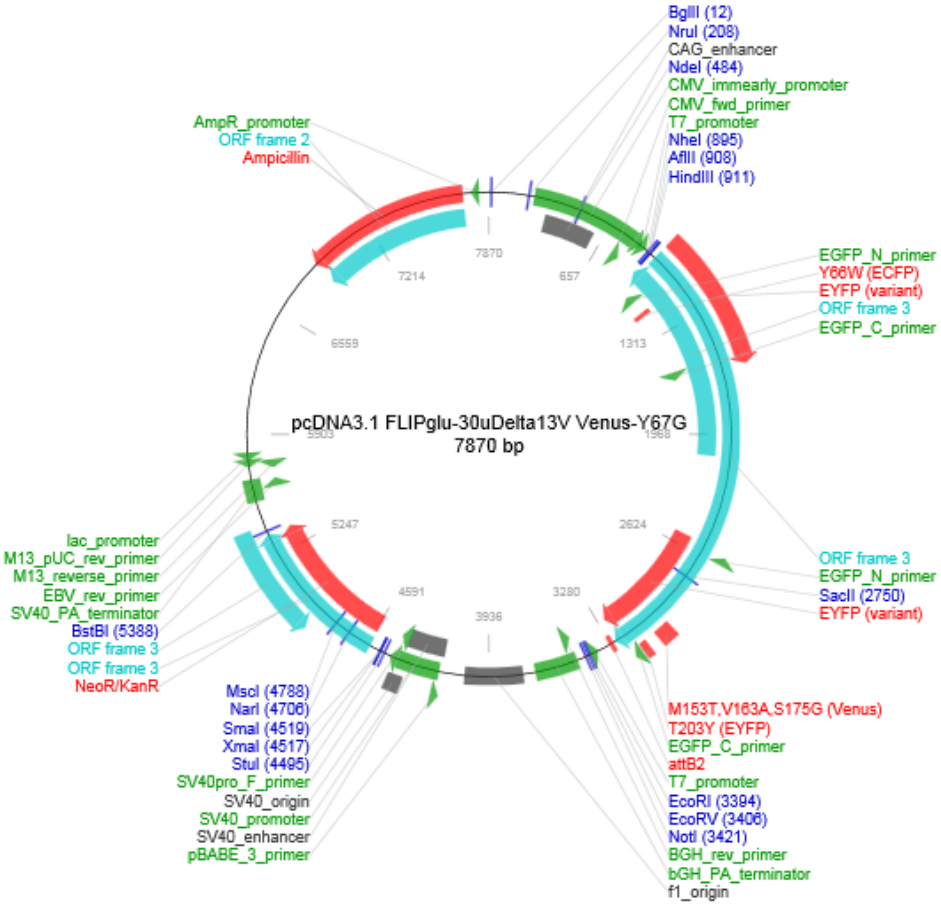
pMD2 lentiviral envelope plasmid. AmpR; Ampicillin resistance, ORF; Open reading frame, Fsp; Fusarium oxysporum, CMV; Cytomegalovirus, SV40; Simian virus, bGlob; β globin, MscI; Murine stem cell virus, Eag; E. agglomerans, NotI Nocardia otitidis-caviarum, VSVG; Vesicular stomatis virus glycoprotein, PstI; Providencia Stuartii.



pCMV lentiviral packaging plasmid. AmpR; Ampicillin resistance, ORF; Open reading frame, Fsp; Fusarium oxysporum, CMV; Cytomegalovirus, SV40; Simian virus 40, bGlob; β globin, MscI; Murine stem cell virus, Eag; E. agglomerans, NotI; Nocardia otitidis-caviarum, VSVG; Vesicular stomatis virus glycoprotein, PstI; Providenia Stuartii.



TRIPZ and GIPZ lentiviral plasmids. Ampr; Ampicillin resistance; rtTA3; reverse tetracycline transactivator 3, Puror; Puromycin resistance, LTR; Long terminal repeat, cPPT; Central polypurine tract, Zeo; Zeomycin, CMV; Cytomegalovirus, GFP; Green fluorescent protein, IRES: Internal ribosome entry site, RFP; Red fluorescent protein, SV40; Simian virus 40.



Venus fluorescent protein plasmid. AmpR; Ampicillin resistance, ORF; Open reading frame, GFP; Green fluorescent protein, YFP; Yellow fluorescent protein, EcoRI; E.coli restriction enzyme I, SV40; Simian virus 40, MSCV; Murine stem cell virus, NeoR; Neomycin resistancance, KanR; Kanamycin resistance, Lac; Lactose, EBV; Epstein Bar virus, BGH; Bovine growth hormone, NotI; Nocardia otitidis-caviarum, CMV; Cytomegalovirus.