# Novel Regulation of SRC Family Kinase Signalling by RASSF1 Isoforms

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# Abstract

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RASSF1A is a tumour suppressor, the silencing of which occurs through promoter methylation in a variety of human cancers. Loss of RASSF1A is associated with decreased sensitivity to DNA damaging agents and worse prognosis in breast, colon and lung cancers amongst others. RASSF1A functions in a number of cellular processes, promoting apoptosis in response to DNA damage or death receptor signalling, or cell cycle arrest at both G1/S and pro-metaphase checkpoints. As a scaffold protein, RASSF1A imparts these functions through direct interaction with target proteins. We have identified a novel interaction between RASSF1A and the SRC activator, OSSA. Further studies identify a role for RASSF1 in SRC signalling. We find that a second isoform of RASSF1, RASSF1C, the expression of which is maintained in cancers, is able to activate SRC. We also identify a novel tumour suppressor role for RASSF1A inhibiting SRC activation through binding of RASSF1C. SRC activation by RASSF1C expression promotes internalisation of adherens junctions leading to subsequent loss of tight junctions and cell polarity markers from sites of cell-cell contact. β-catenin is also found to be re-localised throughout the cells from where it is hypothesised to be able to upregulate pro-proliferative genes. In addition, we find that RASSF1C expression promotes cell motility in both scratch wound and transwell assays. Finally, we show that RASSF1C expression enhances tumour cell aggressiveness using a mammosphere growth assay. We conclude that RASSF1C is an oncogene that can promote EMT through the activation of SRC family kinases. This function is inhibited by the tumour suppressor RASSF1A. This work highlights why RASSF1A is lost through epigenetic mechanisms and not mutation and why loss of RASSF1A is associated with more aggressive, metastatic cancers.

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# Abbreviations

| | |
|---|---|
| **AJ** | Adherens junctions |
| **AJC** | Adhesion junction complex |
| **AKT** | RAC-alpha serine/threonine-protein kinase |
| **AMOT** | Angiomotin |
| **APC** | Adenomatous polyposis coli protein |
| **APC/C** | Anaphase promoting complex/cyclosome |
| **aPKC** | Atypical Protein kinase C |
| **Arf** | (e.g. ARF6) ADP-ribosylation factor 6 |
| **ARF** | (e.g. $p14^{ARF}$) Alternative reading frame |
| **ARP** | Actin-related protein |
| **ASPP** | Apoptosis-stimulating of p53 protein |
| **ATCC** | American Type Culture Collection |
| **ATF2** | Activating transcription factor 2 |
| **ATM** | Ataxia telangiectasia mutated |
| **ATP** | Adenosine Triphosphate |
| **ATR** | Ataxia telangiectasia and Rad3-related protein |
| **Bcl-2** | B-cell lymphoma 2 |
| **β-PIX** | PAK-interacting exchange factor beta |
| **BH3** | Bcl-2 homology domain 3 |
| **BME** | Beta mercaptoethanol |
| **BPB** | Bromophenol blue |
| **BRCA** | Breast cancer susceptibility protein |
| **BSA** | Bovine serum albumin |
| **Cas** | Crk-associated substrate |
| **cbl** | Casitas B-lineage lymphoma proto-oncogene |
| **CBP** | Calmodulin binding protein |
| **CDC** | Cell division control protein (e.g. cdc42, cdc20) |
| **CDK** | Cyclin-dependent kinase |
| **cDNA** | Complementary DNA |
| **CHK** | Checkpoint kinase |
| **CIM** | Cell invasion and migration (ExCELLigence plate) |
| **CK1** | Casein kinase 1 |

| | |
|---|---|
| **CLIP170** | Cytoplasmic linker protein CLIP-170. |
| **cm** | Centimetre |
| **CpG** | Dinucleotide consisting of cytosine followed by guanine |
| **CRUK** | Cancer Research UK |
| **Cs-137** | Caesium isotope 137. |
| **CSE1L** | Cellular apoptosis susceptibility protein |
| **CSK** | C-terminal SRC kinase |
| **CXCL12** | C-X-C motif chemokine 12 (Stromal cell-derived factor 1 (SDF1)) |
| **CXCR4** | C-X-C chemokine receptor type 4 |
| **DAG** | Diacylglycerol |
| **DAXX** | Death Domain-associated protein |
| **DDB1** | DNA Damage Binding protein 1 |
| **DDR** | DNA damage response |
| **Dlg** | Discs large |
| **DMEM** | Dulbecco's-Modified Eagles Medium |
| **DMP** | Dimethyl pimelimidate |
| **DMSO** | Dimethylsulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **DNA-PK$_{cs}$** | DNA protein kinase catalytic subunit. |
| **DOCK** | Dedicator of cytokinesis protein 1 |
| **DSB** | Double-stranded break |
| **DTT** | Dithiothreitol |
| **DVL** | Segment polarity protein dishevelled homolog |
| **ECL** | Enhanced chemiluminescence |
| **ECM** | Extracellular matrix |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EEA1** | Early Endosome Antigen 1 |
| **EFA6** | Exchange factor for Arf6 |
| **EGF** | Epidermal Growth Factor |
| **EGR1** | Early growth response protein 1 |
| **Emi1** | Early mitotic inhibitor 1 |
| **EMT** | Epithelial Mesenchymal transition |
| **ERK** | Extracellular signalling-regulated protein kinase |
| **EWS** | Ewing sarcoma breakpoint region 1 protein |

| | |
|---|---|
| **FAK** | Focal adhesion kinase |
| **FBS** | Fetal Bovine Serum |
| **FGF2** | Fibroblast growth factor 2 |
| **FilGAP** | Filamin-A-associated RhoGAP |
| **FLAG** | An epitope tag of the sequence DYKDDDDK |
| **FSG** | Fish Skin Gelatin |
| **GAP** | GTPase activating protein |
| **GDP** | Guanosine Diphosphate |
| **GEF** | Guanine nucleotide exchange factor |
| **Glu** | Glutamine |
| **GSK3** | Glycogen synthase kinase-3 |
| **GTP** | Guanosine Triphosphate |
| **Gy** | Gray |
| **$H_2O$** | Water |
| **HA** | An epitope tag of the sequence YPYDVPDYA |
| **HAUSP** | Herpes virus-associated ubiquitin-specific protease |
| **HCl** | Hydrogen Chloride |
| **HDAC** | Histone deacetylase |
| **Hippo** | *Drosophila* homolog of MST |
| **HPLC** | High-performance liquid chromatography |
| **HPV** | Human papillomavirus |
| **hr** | Hour |
| **$IC_{50}$** | Half maximal inhibitory concentration |
| **IGFBP-2** | Insulin-like growth factor-binding protein 2 |
| **IP** | Immunoprecipitation |
| **iPSC** | Induced pluripotent stem cells |
| **IR** | Irradiation |
| **JNK** | c-jun N-terminal kinase |
| **kb** | Kilobase (DNA). |
| **kDa** | Kilodalton |
| **KLF4** | Krueppel-like factor 4 |
| **l** | Litre |
| **LATS** | Large Tumour Suppressor |
| **LB** | Luria Broth |

| | |
|---|---|
| **LDL** | Low density lipoprotein |
| **LFA-1** | Lymphocyte function-associated antigen 1 |
| **Lgl** | Lethal giant larvae |
| **LIF** | Leukaemia inhibitory factor |
| **LOH** | Loss of Heterozygosity |
| **M** | Molar |
| **MAP1S** | Microtubule-associated protein 1S |
| **MAPK** | Mitogen-activated protein kinase |
| **MARK** | MAP/Microtubule affinity-regulating kinase |
| **MATS** | Mob as a tumour suppressor |
| **mDia** | Diaphanous homolog |
| **MDM2** | Mouse double minute 2 protein |
| **$MgCl_2$** | Magnesium Chloride |
| **min** | Minute |
| **miRNA** | MicroRNA |
| **MLC** | Myosin light chain |
| **MLCK** | Myosin light chain kinase |
| **MMP** | Matrix metalloprotease |
| **MOAP1** | Modulator of Apoptosis 1 |
| **MRE11** | Meiotic recombination 11 protein |
| **mRNA** | Messenger RNA |
| **MS** | Mass spectrometry |
| **MST** | Mammalian Sterile 20-like Protein (Also called KRS) |
| **MTOC** | Microtubule organising centre |
| **MYPT** | Myosin phosphatase-targeting subunit |
| **NaCl** | Sodium chloride |
| **NDR** | Nuclear Dbf2-related kinase 1 |
| **NER** | Nucleotide excision repair |
| **NF-2** | Neurofibromin-2 (Merlin) |
| **NHERF** | $Na^+/H^+$ exchange regulatory cofactor NHE-RF1 |
| **Nore1** | Novel Ras Effector (RASSF5 (RASSF isoform 5)) |
| **NP40** | Nonidet P-40 |
| **OSSA** | Oxidative Stress-associated SRC activator |
| **P/S** | Penicillin streptomycin |

| | |
|---|---|
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PAK** | p21 Activating kinase |
| **Par** | Partitioning defective (e.g. Par3, Par6) |
| **PBS** | Phosphate buffered saline |
| **PBST** | PBS with the addition of 0.1% Tween 20 |
| **PCR** | Polymerase chain reaction |
| **PEI** | Polyethylenimine |
| **Pen/Strep** | Penicillin/Streptomycin |
| **PI3K** | Phosphatidylinositide 3-Kinase |
| **PIWIL** | Piwi-like protein |
| **PML** | Promyelocytic leukaemia protein |
| **PMSF** | Phenylmethylsulfonyl fluoride |
| **pS** | Phosphoserine residue |
| **pT** | Phosphothreonine residue |
| **PTEN** | Phosphatidylinositol 3,4,5-triphosphate 3-phosphatase and dual specificity protein phosphatase. |
| **PTP** | Protein tyrosine phosphatase |
| **PUMA** | p53 upregulated modulator of apoptosis |
| **PVDF** | Polyvinylidene fluoride |
| **pY** | Phosphotyrosine residue |
| **RA** | Ras Association (domain) |
| **RACK1** | Receptors for Activated C-Kinases |
| **RASSF** | Ras Association Domain Containing Family of Proteins |
| **Rb (pRb)** | Retinoblastoma protein |
| **RNA** | Ribonucleic acid |
| **RNAi** | RNA interference |
| **ROCK** | Rho kinase |
| **RPA** | Replication protein A |
| **RT** | Room Temperature |
| **RT-PCR** | Reverse-transcriptase PCR. |
| **SARAH** | **Sa**v, **RA**SSF, **H**ippo |
| **Sav** | Salvador |
| **SBP** | Streptavidin binding protein |
| **SCF** | SKP1-CUL1-F-box |

| | |
|---|---|
| **SCID** | Severe combined immunodeficiency |
| **SDS** | Sodium dodecyl sulphate |
| **SEM** | Standard Error of the Mean |
| **SFK** | SRC family kinases |
| **siRNA** | Small interfering RNA molecule. |
| **Skp2** | S-phase kinase-associated protein 2 |
| **SNARE** | SNAP (Soluble NSF Attachment Protein) Receptor |
| **SNP** | Single Nucleotide Polymorphism |
| **SOCS** | Suppressor of cytokine signalling |
| **SRC** | Proto-oncogene tyrosine-protein kinase SRC (v-SRC – viral SRC) |
| **TAE** | Tris-acetate-EDTA |
| **TAZ** | Transcriptional co-activator with PDZ-binding motif |
| **TBS** | Tris buffered saline |
| **TBST** | TBS with the addition of 0.1% Tween 20 |
| **TCF/LEF** | T-cell factor/Lymphoid Enhancer Factor |
| **TEAD** | TEA domain family member |
| **Tet ON** | Tetracycline ON inducible vector. |
| **TIAM** | T-lymphoma invasion and metastasis-inducing protein |
| **TJ** | Tight junctions |
| **TLR2** | Toll-like Receptor 2 |
| **TNF** | Tumour Necrosis Factor |
| **TRAIL** | TNF-related apoptosis-inducing ligand |
| **UV** | Ultraviolet |
| **v/v** | Volume per volume |
| **w/v** | Weight per volume |
| **WW45** | 45 kDa WW domain protein (Salvador/Sav1) |
| **XP** | Xeroderma pigmentosum (e.g. XPA - complementation group A) |
| **YAP** | Yes-associated protein |
| **ZO-1** | Zonula Occludens protein 1 |

# Chapter 1 Cancer

In the year 2000, the collection of diseases collectively known as cancer were defined by a set of alterations to normal cells that allow tumourigenesis to occur. These were: enabling replicative immortality, sustaining proliferative signalling, evading growth suppressors, resisting cell death, inducing angiogenesis and activating invasion and metastasis (Hanahan and Weinberg 2000). Since this time two further hallmarks have emerged, deregulating cellular energetics and avoiding immune destruction. Two new enabling characteristics have also been characterised; genomic instability and mutation, and tumour-promoting inflammation (Hanahan and Weinberg 2011). Together these hallmarks lead towards tumour progression from benign to malignant and finally metastatic. It has been argued that tumour progression occurs via a succession of clonal expansions each developing from a chance acquisition of a mutation that confers a selective advantage (Hanahan and Weinberg 2011). These changes can occur via heritable means, i.e. mutation, or via epigenetic mechanisms, such as DNA methylation.

## 1.1 Oncogenes

Oncogenes are proteins that promote transformation of normal cells into cancer cells when they acquire an activating mutation or are overexpressed. Tumour suppressors are proteins that counter these processes and are often lost or inactivated in tumours. Originally, many oncogenes were identified as components of viruses that could transform cells, for example SRC from the Rous sarcoma virus and H- and K-Ras from Harvey sarcoma virus and Kirstin sarcoma virus respectively (Karnoub and Weinberg 2008). These oncogenes often have counterparts in normal cells, known as proto-oncogenes, which are mutated in cancers. In the context of RASSF1A, the most important oncogene identified is Ras. Ras belongs to a family of small G-proteins that are ubiquitously expressed and oscillate between an inactive, GDP-bound state, and an active, GTP-bound state, in response to diverse cellular signals

(Barbacid 1987; Malumbres and Pellicer 1998). Upstream regulators of Ras, for example, growth factor receptors, activate Ras by recruiting Guanine-nucleotide exchange factors (GEFs), which convert Ras-GDP to Ras-GTP. GTPase activating proteins (GAPs) inactivate Ras by promoting the hydrolysis of GTP to GDP (Karnoub and Weinberg 2008). Ras proteins regulate many biological functions, including: proliferation, differentiation, senescence, motility and apoptosis (Karnoub and Weinberg 2008). These functions are regulated by specific binding of effectors to the Ras-GTP form. The best characterised effectors are Raf kinases, phosphoinositide-3 kinase (PI3K) and RalGDS GEFs (Karnoub and Weinberg 2008). These effectors contribute to oncogenic transformation by Ras. Mutations of K-Ras that render it unable to hydrolyse GTP, such as G12V, are very frequent events in cancer, particularly in colorectal cancers, where it is present in greater than 30 % of tumours (Downward 2003; Karnoub and Weinberg 2008). The mutations result in a constitutively active Ras which lacks the requirement for upstream stimulation, driving continuous activation of downstream effector pathways and constitutive growth.

Mutant K-Ras is also able to promote apoptosis (Cox and Der 2003). Two potential mechanisms have been put forward to explain this phenomenon. The first is through the engagement of the pro-apoptotic protein, Bcl-$X_L$, which occurs in response to PKC phosphorylation of K-Ras (Bivona, Quatela et al. 2006). The second mechanism is through an interaction between K-Ras and RASSF1A leading to MST2-dependent activation of p53 (Matallanas, Romano et al. 2011).

## 1.2 Tumour Suppressor Genes

Mutation of oncogenes such as Ras or Raf are important to tumourigenesis as they drive tumour growth. However, loss of tumour suppressors, particularly those associated with genome maintenance can be argued to be more important. Loss of these genes results in increased sensitivity to mutagenic agents and compromised surveillance systems leading to increased mutation rates and genomic instability. Therefore, tumour suppressor loss drives tumour progression and limits the cells ability to

halt this progression. Surveillance genes monitor the genomic integrity and force damaged cells into senescence or apoptosis. The tumour suppressor p53 is central to this process and is often referred to as the 'guardian of the genome' (Lane 1992).

p53 is thought to be mutated in more than half of human tumours (Vogelstein, Lane et al. 2000; Petitjean, Mathe et al. 2007), and in many of the tumours that retain wild-type p53, its function is thought to be attenuated, for example by DNA tumour viruses (Levine 2009), or by upregulation of negative regulators such as the E3 ubiquitin ligase MDM2. MDM2, and it homolog MDMX are the major regulators of p53 levels in unstressed cells, targeting p53 for degradation (Poyurovsky and Prives 2006; Marine, Dyer et al. 2007; Manfredi 2010; Marine and Lozano 2010). Cellular stresses including: DNA damage, hypoxia and oncogene activation can activate p53 (Vousden and Lu 2002). Activation occurs by removal of MDM2/MDMX from p53, either by modification of p53, for example by phosphorylation by ATM, ATR, Chk1 and Chk2 (Siliciano, Canman et al. 1997; Chao, Saito et al. 2000; Saito, Goodarzi et al. 2002; Kurz and Lees-Miller 2004) or by the sequestration of MDM2, for example, by PML (Bernardi, Scaglioni et al. 2004). RASSF1A activation of p53 occurs through the inhibition of MDM2, discussed later. Activated p53 forms a tetramer and is a sequence specific transcription factor that: inhibits cell cycle progression, promotes senescence, induces apoptosis and regulates metabolic processes (Freed-Pastor and Prives 2012).

In contrast to mutations of other tumour suppressors such as Retinoblastoma (pRb) and Adenomatous polyposis coli (APC), mutation of p53 does not lead to reduced protein activity (Levine, Wu et al. 1995). Instead p53 mutants gain a dominant negative function due to the tetrameric nature of the active p53 protein. Mutant p53 can: block transcription of wild-type p53 target genes and also those of the p53 family member's p63 and p73; bind to proteins such as MRE11, promoting genomic instability; and promote the expression of a number of oncogenes (Freed-Pastor and Prives 2012).

Gene mutation is just one way tumour suppressors are inactivated. Other mechanisms include gene deletion, loss of heterozygosity (LOH), and epigenetic mechanisms. Epigenetics is defined as heritable

changes in the gene expression that do not alter the DNA sequence (Jones and Baylin 2007). Epigenetic alterations are controlled by three main mechanisms: DNA methylation, histone modification and post transcription regulation by micro-RNA (miRNA) molecules. In the context of this project it is DNA methylation that is important.

The majority of DNA methylation occurs on CpG dinucleotides and regions of large repetitive sequences, such as retrotransposon elements and centromeric repeats (Bird 2002; Takai and Jones 2002). It plays an important role in genomic imprinting, where one of the two parental alleles is hypermethylated and silenced to ensure monoallelic expression. It also maintains chromosome integrity by preventing chromosomal rearrangements, translocations, and gene disruptions by transposable elements within the genome (Bird 2002; Jones and Baylin 2002; Eden, Gaudet et al. 2003; Esteller 2007). CpG islands are associated with promoters of 'housekeeping' genes and are normally unmethylated allowing the binding of transcription factors to these sites (Lopez, Percharde et al. 2009). Methylation of cytosine residues within the CpG islands not only blocks transcription factor binding but also leads to the recruitment of methyl-binding domain proteins and histone deacetylases (HDACs) (Jones, Veenstra et al. 1998; Nan, Ng et al. 1998), leading to chromosome condensation and transcriptional inactivation of the targeted genes.

In human tumours a global loss of DNA methylation is seen, however tumours also acquire hypermethylation of specific promoters (Chuang and Jones 2007). The global loss of DNA methylation can be potentially harmful as it will result in the re-expression of areas of the genome normally kept silent such as the retrotransposons which promote genomic instability via chromosomal rearrangements (Lopez, Percharde et al. 2009). The promoters which become methylated in tumours are associated with tumour suppressor genes for example E-cadherin, RASSF1A and RASSF2A and many other members of the hippo pathway (Graff, Herman et al. 1995; Takahashi, Miyoshi et al. 2005; Donninger, Vos et al. 2007; Seidel, Schagdarsurengin et al. 2007; Richter, Pfeifer et al. 2009).

## 1.3 The RASSF family

The Ras-association domain containing family (RASSF) of proteins were originally designated on the basis of sequence homology to domains that associate with Ras-like small GTP binding proteins. These domains are known as Ras association (RA) domains [RalGDS (Ral guanine nucleotide dissociation stimulator)/AF6 (ALL-1 fusion partner from chromosome 6)] and are distinct from Ras binding domains (RBD) which bind an alternative set of Ras effectors (Ponting and Benjamin 1996; Yamamoto, Taya et al. 1999). There are 150 Ras-like proteins encoded in the human genome which can be grouped by homology, or functionality, as being similar to Ras, Rho, Rab, Arf (ADP-ribosylation factor) or Ran. Each mediate distinct biological responses by binding of effector proteins specifically to the GTP-bound state. While originally suggested to associate with Ras (Vavvas, Li et al. 1998), the RASSF family has a differential affinity for Ras-like GTPases, with NORE1(RAPL/RASSF5) displaying a much greater affinity for the closely related Ras homolog, Rap1B, than H-Ras itself (Wohlgemuth, Kiel et al. 2005). The RA domain of RASSF1 associates with K-Ras, rather than H-Ras or N-Ras (Matallanas, Romano et al. 2011). RASSF1A has also been described to associate with Ran (Dallol, Hesson et al. 2009). There are now 10 members in the RASSF family (RASSF1-10) sub-divided into two distinct sub-groups, the classical RASSF proteins (RASSF1-6) and the N-terminal RASSF proteins (RASSF7-10) based on the location of the RA domain (Sherwood, Manbodh et al. 2008). Little is known about the GTP binding proteins that may interact with the majority of the RASSF family or how they function but the potential exists for a greater number of signalling connections. In addition to an RA domain, the classical RASSF proteins also have a protein-protein interaction motif known as the SARAH domain that is responsible for scaffolding and regulatory interactions (Hwang, Ryu et al. 2007). This domain is a short coiled-coil region and so named due to its location in the extreme C-terminus of genetically linked *Drosophila* proteins; Salvador (hSav1/WW45), dRASSF and Hippo (hMST1/2) (SARAH: **Sa**lvador, **RA**ssf, **H**ippo) which can form both homo- and hetero-dimers (Scheel and Hofmann 2003). The N-terminal RASSFs lack an identifiable SARAH domain, although the SMART database predicts that RASSF7, 8 and 10 contain extensive coiled-coil regions, which can dimerise (Grigoryan and Keating 2008).

RASSF1A and RASSF5A [also known as NORE1A (**No**vel **R**as **E**ffector 1 isoform A)] also contain an N-terminal atypical diacylglycerol/phorbol ester-binding (DAG) domain also known as the protein kinase C conserved region (C1) domain that contains a central Zinc Finger (Zinc binding domain) (Newton 1995). The Zinc finger in the RASSF family members is denoted 'atypical' because it lacks critical residues required for binding of phorbol esters or DNA and therefore probably mediates protein-protein interactions. Indeed, structural analysis indicates that the C1 domain of NORE1A associates with the RA domain to occlude RAS association (Harjes, Harjes et al. 2006). As none of the family members have any known enzymatic activity they are thought to be scaffold/adaptor proteins using these binding domains to bring target proteins together to impart their functions.

## 1.4 RASSF1

It had long been suspected that the 3p21.3 region of the human genome harboured one or more important tumour suppressors because LOH was found at this locus in lung, breast and kidney tumours and genetic instability in this region is the earliest most frequently detected deficiency in lung cancers (Sundaresan, Ganly et al. 1992; Hung, Kishimoto et al. 1995; Kok, Naylor et al. 1997; Sekido, Ahmadian et al. 1998; Sekido, Fong et al. 1998; Wistuba, Behrens et al. 1999; Wistuba, Behrens et al. 2000). This 120 kb region contains 8 genes namely *CACNA2D2, PL6, 101F6, NPRL2/G21, ZMYND10/BLU, RASSF1/123F2, FUS1* and *MYAL2*. However, none of these candidate genes are frequently mutated in cancers (Sekido, Ahmadian et al. 1998; Lerman and Minna 2000). At the same time as these LOH studies, Dammann et al., (2000) identified RASSF1 as an interacting partner of the DNA damage repair protein Xeroderma Pigmentosum Complementation Group A (XPA) (Dammann, Li et al. 2000). While the role of RASSF1 in nucleotide excision repair could not be verified, it may yet prove to be significant given the emerging role of RASSF1 in the DNA damage response. The *RASSF1* gene consists of 8 exons spanning a region of about 11 kb. The C-terminal showed high sequence homology with NORE1, containing an RA domain and thus the gene was named *RASSF1* for **R**as **Ass**ociation Domain **F**amily Member 1 (Dammann,

Li et al. 2000). Alternative splicing generates 8 isoforms A-H from promoters held within 2 CpG islands (Figure 1.1). The upstream CpG island encompasses the promoter regions for RASSF1A, D, E, F and G. Epigenetic inactivation by DNA methylation at this CpG island is one of the most common events in human cancers (reviewed in (Donninger, Vos et al. 2007; Hesson, Cooper et al. 2007; Jones and Baylin 2007)). This methylation has recently been attributed to HOXB3 driven overexpression of the DNA methyltransferase, *DNMT3B* (Palakurthy, Wajapeyee et al. 2009). RASSF1B, C and H are generated from a promoter located within the larger downstream CpG island (van der Weyden and Adams 2007). This commonly remains unmethylated in cancers and consequently cells retain expression of these isoforms (Donninger, Vos et al. 2007). RASSF1A and RASSF1C are the major transcripts of the *RASSF1* gene and are expressed ubiquitously in normal tissues (Richter, Pfeifer et al. 2009).

Predicted orthologs have been reported by Ensembl (http://www.ensembl.org/index.html) in a wide range of organisms including: mammals, birds, fish, worms, flies and sea squirts (Hubbard, Aken et al. 2007). Of these research has investigated RASSF1 function in *M. musculus* (*Rassf1*), *C. elegans* (*T24F1.3a*), *D. rerio* (*zgc:92505*) and *D. melanogaster* (*CG4656*). The *C. elegans* gene product T24F1.3 is a 615 amino acid protein containing the C1 domain, RA domain and SARAH domain, but also has an extended C-terminal compared to RASSF1A. T24F1.3 is able to bind the proapoptotic kinase mammalian sterile 20-like kinase (MST) 1 and 2, but RNAi of T24F1.3 resulted in “no obvious phenotype” (Khokhlatchev, Rabizadeh et al. 2002; Kamath, Fraser et al. 2003; Sonnichsen, Koski et al. 2005). The expression of zgs:92505 was shown to have an expression pattern linked to cell proliferation. dRASSF shares the RA and SARAH domain with its mammalian counterparts, but does not contain a C1 domain. Instead the N-terminal contains a LIM domain, although this domain shares some similarities with the C1 domain. dRASSF has been observed to inhibit, not activate Hippo (discussed in Section 1.5.3) (Polesello, Huelsmann et al. 2006; Ribeiro, Josue et al. 2010).

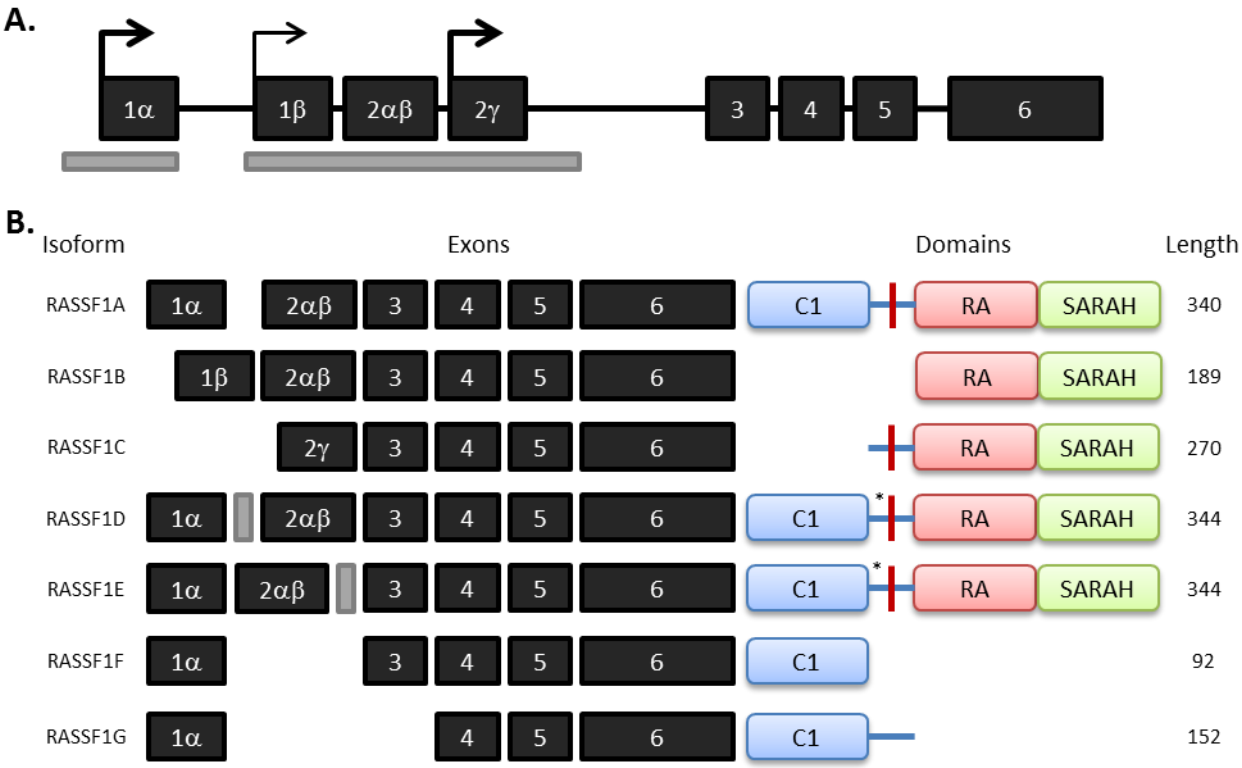


**Figure 1.1. RASSF1 Gene Structure and the Splice Variants Produced**. **A.** Diagram depicting the exons of the RASSF1 gene (black boxes), the two promoters (grey boxes) and the start sites used to produce the eight isoforms of RASSF1 (arrows). **B.** Arrangement of the exons present in each of the isoforms of RASSF1 (A-G), the domains present in each of the isoforms and the amino acid length of each polypeptide chain. Grey boxes seen in exons of RASSF1D and RASSF1E represent 4 amino acids added to these transcripts that are not present in RASSF1A. Their position in the protein structure is represented by an asterisk. Figure is adapted from (van der Weyden and Adams 2007).

## 1.5 RASSF1A

Exogenous expression of RASSF1A reduced colony formation in soft agar and reduced tumourigenicity in nude mice (Dammann, Li et al. 2000; Burbee, Forgacs et al. 2001; Dreijerink, Braga et al. 2001; Kuzmin, Gillespie et al. 2002). Similarly, re-expression of RASSF1A using demethyltransferase inhibitors such as Zebularine and 5-aza-2'-deoxycytidine caused significant growth arrest in ovarian cancer cell lines (Balch, Yan et al. 2005). Reciprocally, Rassf1A knock-out mice develop spontaneous tumours, particularly when combined with a knock-out of p53, highlighting the significance of Rassf1A in tumour development (Tommasi, Dammann et al. 2005; van der Weyden, Tachibana et al. 2005; Tommasi, Besaratinia et al. 2011). In addition these Rassf1A$^{-/-}$, p53$^{-/-}$ mice showed high levels of

aneuploidy/tetraploidy suggesting an important role for RASSF1A in maintaining genomic integrity. RASSF1A has been shown to have many roles in cell cycle control, microtubule organisation, promotion of apoptosis and the DNA damage response (Donninger, Vos et al. 2007; van der Weyden and Adams 2007; Hamilton, Yee et al. 2009). Understanding the interactions that RASSF1A makes is key to understanding its function and so we shall look at these in more detail.

## 1.5.1 RASSF1A and the DNA Damage Response

### 1.5.1.1 RASSF1A Phosphorylation

During the DNA damage response RASSF1A is phosphorylated on Serine 131 (S131) (Hamilton, Yee et al. 2009). The initial kinases that respond to breaks in DNA are the Phosphatidylinositol 3-kinase like kinases ATM (Ataxia Telangiectasia Mutated), ATR (ATM- and Rad3-Related) and DNA-$PK_{cs}$ (DNA-Dependent Protein Kinase catalytic subunit) (Durocher and Jackson 2001). RASSF1A has a consensus site for ATM phosphorylation on Serine 131 that is conserved in vertebrates and unique amongst family members and has recently been confirmed as a bone-fide target for ATM (Kim, Lim et al. 1999; Hamilton, Yee et al. 2009). Serine 131 phosphorylation appears important for RASSF1A activation and inactivating mutations of this site have been identified in human cancers (Shivakumar, Minna et al. 2002). Indeed, Shivakumar et al., (2002) showed that mutation of the predicted phosphorylation site, S131F, removed the ability to induce cell cycle arrest and block cell proliferation (Shivakumar, Minna et al. 2002). ATM-dependent phosphorylation at the 131 site is also restricted by S131F and disables the ability of RASSF1A to respond to various DNA damaging agents (Hamilton, Yee et al. 2009). Mutations near the ATM site are hypothesised to function by inactivating ATM phosphorylation. One of these is a non-synonymous single nucleotide polymorphism (SNP) at p.RASSF1A-A133S (rs2073498), which has significant allele frequencies in human populations (http://hapmap.ncbi.nlm.nih.gov/). The minor allele of the SNP encodes a Serine (A133S) and decreases the ability of RASSF1A to become phosphorylated

which, like S131F, results in a defective G1 arrest (Shivakumar, Minna et al. 2002). This suggests that sequence changes to the ATM consensus sequence (amino acids 125 – 138) may severely inhibit the function of RASSF1A by disrupting the phosphorylation of S131 and preventing the activation of RASSF1A.

RASSF1A promotes the formation of a Ran-GTP gradient between spindle poles and the metaphase plate which is important for the formation of mitotic spindle and for successful completion of mitosis. RASSF1A targets MST1/2 kinase activity towards the Ran-GEF (GTP Exchange Factor) RCC1, which inhibits its function and results in elevated Ran-GTP near the metaphase plate. Taken together, these studies indicate that RASSF1A is important for the maintenance of genomic stability by acting as an integrity checkpoint factor. Loss of RASSF1A is likely to weaken the pro-metaphase checkpoint and increase the potential to create genomic instability and DNA damage leading to cancer development. Indeed, the restriction of RASSF1A activity by modulation of the ATM site may be linked to numerous observations regarding the early onset of tumours in individuals carrying one minor allele of the p.RASSF1A-A133S polymorphism (Endoh, Yatabe et al. 2003; Gao, Xie et al. 2008). This has been controversially linked to the exacerbation of a BRCA1/2 genomic instability phenotype; however, the inconsistency may be due to confounding factors other than BRCA2 and may be due to genomic instability via defects in RASSF1A itself (Bergqvist, Latif et al. 2010). All this may indicate that DNA damage activation of RASSF1A may provide an extra level of regulatory response whereby the prometaphase checkpoint senses cells entering into mitosis with DNA damage.

### 1.5.1.2 RASSF1A Regulation by Domain Interaction

As a scaffold, RASSF1A must exert its tumour suppressor function through its interaction domains. The two most important domains in the context of DNA damage are the C1 domain and the SARAH domain. The most significant binding partners identified to interact with the C1 domain are the TNF-R1/TRAIL-R1 - Modulator of Apoptosis-1 (MOAP-1) complexes and the MDM2/DAXX/HAUSP/p53 complex (Foley,

Freedman et al. 2008; Song, Song et al. 2008) (Figure 1.2). MOAP-1 and RASSF1A are recruited to either TNF-R1 or TRAIL-R1 in response to TNF$\alpha$ stimulation. RASSF1A binds MOAP-1 causing an activating conformational change to the structure of MOAP-1. The active structure can bind to the pro-apoptotic Bcl-2 family member BAX which creates a pore in the outer mitochondrial membrane leading to the release of cytochrome C and induction of caspase-dependent apoptotic signalling pathways (Baksh, Tommasi et al. 2005; Foley, Freedman et al. 2008). BAX and the associated negative regulator BAK tightly regulate the cell's response to apoptotic signals and are often co-ordinated with other apoptotic signals such as DNA damage. It is reasonable to assume that RASSF1A-MOAP-1 may be affected by DNA damage but whether this contributes to the regulation of BAX/BAK at the mitochondria remains uncertain.

The response of tumour suppressor p53 to DNA damage results in a variety of outcomes including cell cycle arrest, apoptosis, and senescence, combining to protect the integrity of the genome (Vogelstein, Lane et al. 2000; Michael and Oren 2003). In unstressed cells p53 levels are low, being controlled by the RING domain-containing E3 ubiquitin ligase MDM2 (Mouse Double Minute 2) (Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997). Induction of DNA damage results in phosphorylation of p53 by the DNA damage checkpoint proteins ATM (on Serine 15) and CHK2 (Checkpoint Kinase 2) (on Serine 20) (Maya, Balass et al. 2001; Bode and Dong 2004). These phosphorylation events combine with an ATM-mediated restriction of MDM2 activity to stabilize p53. Song et al., (2008) have recently shown that the C1 domain of RASSF1A can bind and sequester MDM2 in an ATM-dependent manner (Song, Song et al. 2008). They describe a complex consisting of MDM2, DAXX (death-domain associated protein) and HAUSP1 (a de-ubiquitinating enzyme). HAUSP1 removes ubiquitin molecules from MDM2 and increases its stability. Upon DNA damage, ATM activates RASSF1A driving its association with MDM2, potentially through phosphorylation at S131. RASSF1A disrupts the MDM2-DAXX-HAUSP1 complex, sequestering MDM2 away from p53, and preventing HAUSP1 regulated de-ubiquitination of MDM2 to promote its degradation. Release of DAXX from the complex is thought to allow DAXX re-localisation to the plasma membrane where it can bind the death receptor Fas and activate c-Jun $NH_2$-terminal kinase (JNK) (Yang,

Khosravi-Far et al. 1997). Activated p53 exerts its tumour suppressor function by acting as a transcription factor. It has recently been shown that the *RASSF1* promoter is a target for p53 (Tian, Hou et al. 2011). Interestingly, p53 appears to downregulate the transcription of RASSF1A hinting at a second mechanism through which p53 can negatively regulate itself in addition to the upregulation of MDM2.

RASSF1A makes two significant interactions through its SARAH domain; the first with Mammalian Sterile 20-like kinases 1 and 2 (MST1/2) and the second to the scaffold protein Salvador (Figure 1.2). The RASSF1A interaction with MST1/2 leads to an increase in the local concentration of MST molecules allowing them to undergo trans-phosphorylation and auto-activation (Glantschnig, Rodan et al. 2002). The interaction further stabilises the MST1/2 kinase activity by preventing dephosphorylation of MST1/2 (Guo, Zhang et al. 2011). MST1/2 were initially cloned from a lymphoid cDNA library when looking for human relatives of *Saccharomyces cerevisiae* protein Ste20 and subsequently shown to be activated by a wide variety of cellular stresses (Creasy and Chernoff 1995; Creasy, Ambrose et al. 1996; Taylor, Wang et al. 1996). Of note is that both *Drosophila* dMST (Hippo) and MST2 are activated in response to DNA damage. In mammals, DNA damage induction of MST2 requires binding of RASSF1A and ATM mediated phosphorylation of S131. (Colombani, Polesello et al. 2006; Hamilton, Yee et al. 2009; Wen, Zhu et al. 2010). Interestingly MST1 was shown to be able to activate p53 in response to cisplatin-induced DNA damage by phosphorylating and inactivating Sirt1, a deacetylase that inactivates p53 (Yuan, Xie et al. 2011). Additional substrates of MST kinases that may prove subject to DNA damage are the histones H2B and H2AX, JNK and FOXO transcription factors (Cheung, Ajiro et al. 2003; Lehtinen, Yuan et al. 2006; Ura, Nishina et al. 2007; Praskova, Xia et al. 2008). However, a clear example of signalling through RASSF1A-MST after DNA damage is the recruitment and activation of the Large Tumour Suppressor kinases 1 and 2 (LATS1/2) (O'Neill, Matallanas et al. 2005; Guo, Tommasi et al. 2007).

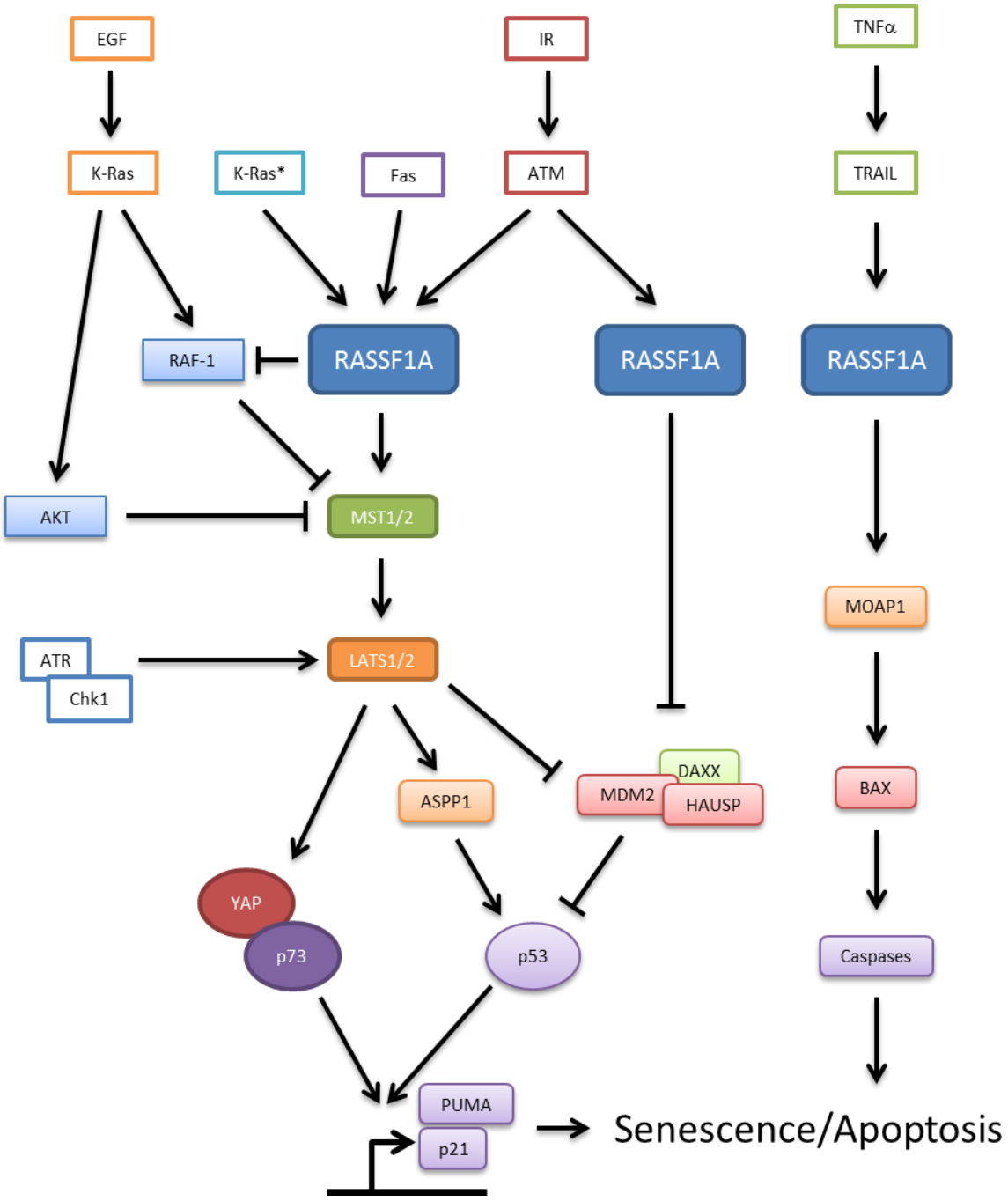


**Figure 1.2. Cartoon Depicting the Signalling Connections Made by RASSF1A and the Hippo Pathway to Regulate Apoptosis and Senescence.** RASSF1A is activated by a number of upstream signalling pathways in response to cellular stresses. Oncogenic K-Ras (K-Ras*), DNA damage and Fas signalling promote hippo pathway activation by RASSF1A leading to p53 and p73 stabilisation and activation. RASSF1A can also directly stabilise p53 in response to DNA damage through the sequestration of MDM2 away from the de-ubiquitinating protein HAUSP. TNFα stimulation of TRAIL also activates RASSF1A, stimulating apoptosis through MOAP1 and BAX. Wildtype EGF can inhibit RASSF1A activation of p53 through the inhibition of MST2 by AKT and Raf kinases.

Studies on the *Drosophila* homolog of MST1/2, Hippo, have discovered that the pathway through Warts (LATS1) is responsible for controlling proliferation and apoptosis and is conserved in both vertebrates and invertebrates. Mutations in pathway member's Hippo (MST1/2), Warts (LATS1/2), Salvador (WW45) or Mats (Mob1 as a tumour suppressor) result in vast tissue overgrowth. The pathway generates a signal to inhibit Yorkie (YAP). Yorkie mutants therefore inevitably show a reduced tissue growth phenotype (reviewed in (Reddy and Irvine 2008)). Yorkie is a non-DNA binding transcriptional co-activator that binds Scalloped (TEAD1-4) leading to the upregulation of proteins such as Cyclin E and Diap-1 to promote cell division and inhibit apoptosis. In this case Warts phosphorylates Yorkie creating a site for 14-3-3 binding. This sequesters Yorkie in the cytoplasm inhibiting its oncogenic activity (Zhao, Wei et al. 2007). In mammals, in the presence of RASSF1A and a DNA damage signal, LATS1 phosphorylation of YAP maintains a pool of YAP in the nucleus which switches binding partner from the anti-apoptotic, YAP-TEAD complex to a pro-apoptotic YAP-p73 complex (Yee and O'Neill 2010). The interaction between YAP1 and p73 stabilises p73 by preventing its nuclear export and subsequent degradation (Basu, Totty et al. 2003; Melino, Bernassola et al. 2004; Dobbelstein, Strano et al. 2005). YAP1 functions as a co-activator of p73 and this complex upregulates p73 responsive genes such as the pro-apoptotic BH3 only Bcl-2 family member, PUMA (Strano, Monti et al. 2005; Matallanas, Romano et al. 2007). This idea is in agreement with the finding that both LATS1 and LATS2 mediate apoptosis through p53. In certain cases LATS2 mediated apoptosis is p53-independent, potentially indicating a switch to YAP1 and p73 (Ke, Pei et al. 2004; Kawahara, Hori et al. 2008; Hamilton, Yee et al. 2009).

The pathway from RASSF1A to YAP was originally shown to be activated by Fas signalling (Matallanas, Romano et al. 2007). More recently, upstream activation of MST2 and LATS1 by RASSF1A was shown to be through oncogenic K-Ras (Matallanas, Romano et al. 2011). The activation of the pathway under these conditions was shown to lead, not to the activation of p73, but to the activation of p53, via the sequestration of MDM2 (Matallanas, Romano et al. 2011). The activation of MST2 by RASSF1A was shown to be blocked by wildtype K-Ras activation of AKT, which can phosphorylate MST2 (Matallanas, Romano et al. 2011). This phosphorylation limits MST2 activity by inhibiting its formation of an active

complex with RASSF1A and promoting the formation of an inhibitory complex with Raf-1 (Romano, Matallanas et al. 2010). Interestingly, caspase cleaved, constitutively active MST1 was shown to inhibit AKT, possibly promoting a positive feedback loop further activating MST1 (Cinar, Fang et al. 2007)

LATS2 has been shown to activate p53 both directly, by binding to and inhibiting MDM2 and indirectly by driving the nuclear accumulation of ASPP1 (Apoptosis-Stimulating Protein of p53) (Aylon, Yabuta et al. 2009; Aylon, Ofir-Rosenfeld et al. 2010). Interestingly, cytoplasmic ASPP1 appears to behave in an opposite manner and inhibits the ability of LATS1 to interact with YAP1 (Vigneron, Ludwig et al. 2010). As RASSF1A activates LATS1/2 in response to DNA damage this could potentially drive ASPP1 activation of p53 and contribute to the overall p53 response. Interestingly the *Drosophila* ASPP protein (dASPP) has also been shown to interact with dRASSF8 to regulate C-terminal SRC kinase (dCsk) and adherens junctions (Langton, Colombani et al. 2009); a site key to the regulation of the core hippo pathway (Pan 2010).

LATS2 has been implicated in the G1 tetraploidy checkpoint, a process that is thought to be driven by LATS2 activation by ATR and leads to direct stabilisation of p53 (Aylon, Michael et al. 2006; Aylon, Yabuta et al. 2009). Active p53 then creates a positive feedback loop with LATS2 by upregulating its activity further (Aylon, Michael et al. 2006). In response to UV radiation CHK1 activation by ATR has been shown to activate LATS2 (Okada, Yabuta et al. 2011).

Although not addressed in a RASSF1A-dependent manner, YAP forms an additional DNA damage promoted complex with the transcription factor Early Growth Response 1 (EGR1) (Zagurovskaya, Shareef et al. 2009). The interaction promotes enhanced transcriptional activity of EGR1 upregulating the Bcl-2-associated X (BAX) promoter. Thus, YAP can act as an oncogene and a tumour suppressor in a RASSF1A context dependent manner.

In Drosophila dRASSF and Salvador are known to compete for MST binding. Here Salvador acts as an adaptor to bring Hippo and Warts together to activate the hippo pathway, which is antagonised by

dRASSF (Polesello, Huelsmann et al. 2006). In mammals however, RASSF1A can bind both MST1/2 and Salvador at the same time using different regions with the SARAH domain. Using an L308P mutant of RASSF1A that cannot bind MST but remains bound to Salvador, Donninger et al., (2011) have shown that the RASSF1A Salvador interaction can activate p73 in an MST-independent manner (Donninger, Allen et al. 2011).

## 1.5.2 RASSF1A and the Cell Cycle

### 1.5.2.1 G1-S Checkpoint Regulation

Cells only require mitogenic stimuli during the first two thirds of G1 phase to decide to advance through the whole cell cycle (Pardee 1974). The point at which cells decide to progress through the cell cycle is termed the 'restriction point' (R point). The R point occurs at the G1/S checkpoint and is controlled by the Retinoblastoma protein (pRb). pRb enforces the R point primarily by sequestration of the E2F family of transcription factors which play a central role in governing cell cycle progression and DNA replication (Adams and Kaelin 1995; Macaluso, Montanari et al. 2005). pRb can also inhibit cell cycle progression through E2F-independent mechanisms including, binding and inhibiting cyclin-dependent kinase (CDK)-cyclin complexes and sequestration of Skp2, stabilising the CDK inhibitor p27 (Giacinti and Giordano 2006). This checkpoint is overcome by gradual CDK-mediated phosphorylation and inactivation of pRb. In early G1 phase, D-type cyclins with their binding partners, CDK4 and CDK6 phosphorylate pRb (Dowdy, Hinds et al. 1993). In middle and late G1 phase cyclins E and A complexed with CDK2 target pRb (De Luca, MacLachlan et al. 1997). Hyperphosphorylation of pRb is maintained until late mitosis when dephosphorylation of pRb occurs in a process dependent on PP1 phosphatase (Ludlow, Glendening et al. 1993). Phosphorylation of pRb by CDK-cyclin complexes occurs in the binding pocket used to bind E2F. Therefore phosphorylation of pRb releases E2F allowing it to upregulate target genes which promote cell cycle progression. As well as the E2F transcription factors the binding pocket of pRb also interacts

with viral oncoproteins which compete for the E2F binding pocket, limiting E2F sequestration and promoting uncontrolled cell cycle progression.

RASSF1A expression causes a cell cycle arrest at the G1-S checkpoint in a manner that is dependent on the pRb checkpoint activity. This was shown by the fact that the cell cycle arrest can be overridden by expression of the HPV17 E7 protein and also by cyclin A overexpression (Shivakumar, Minna et al. 2002). RASSF1A has been shown regulate this checkpoint by inhibiting the accumulation of cyclin D1 (Shivakumar, Minna et al. 2002). RASSF1A can inhibit cyclin D1 accumulation by regulating JNK activity and through interaction with Ewing sarcoma breakpoint protein (EWS) (Figure 1.3) (Whang, Kim et al. 2005; Whitehurst, Ram et al. 2008).

JNK is a stress-activated mitogen-activated protein kinase that is activated by exposure of cells to extracellular stresses. Its downstream signalling can lead to cell survival and tumour development as well as apoptosis (Liu, Hsu et al. 1996; Davis 2000; Vivo, Liu et al. 2003). RASSF1A expression leads to a downregulation of JNK signalling, observed by the ablation of phospho-JNK and subsequently loss of JNK phosphorylation of target proteins such as the transcription factors c-jun and Activating transcription factor-2 (ATF-2) (Derijard, Hibi et al. 1994; Gupta, Campbell et al. 1995). Cyclin D1 is induced by ectopic expression of c-jun. Therefore inhibition of JNK by RASSF1A will reduce cyclin D1 transcription.

EWS is a transcription factor best characterised for the fusion protein it forms with transcription factors for example FLi1 to cause Ewing's sarcoma (Arvand and Denny 2001). EWS was found to interact with RASSF1A through its C1 domain by yeast 2-hybrid. The interaction between RASSF1A and EWS was shown to negatively regulate EWS; however it was unclear how this interaction prevented the accumulation of cyclin D1. Two hypotheses were suggested, the first suggests that inhibition of EWS prevents it from positively regulating c-fos, which like c-jun can upregulate cyclin D1 (Kim, Denny et al. 2006). The second method of regulation fits more closely with the conclusions of Shivakumar et al. (2002) suggesting that EWS plays an active role in translation of cyclin D1, which is inhibited by RASSF1A expression.

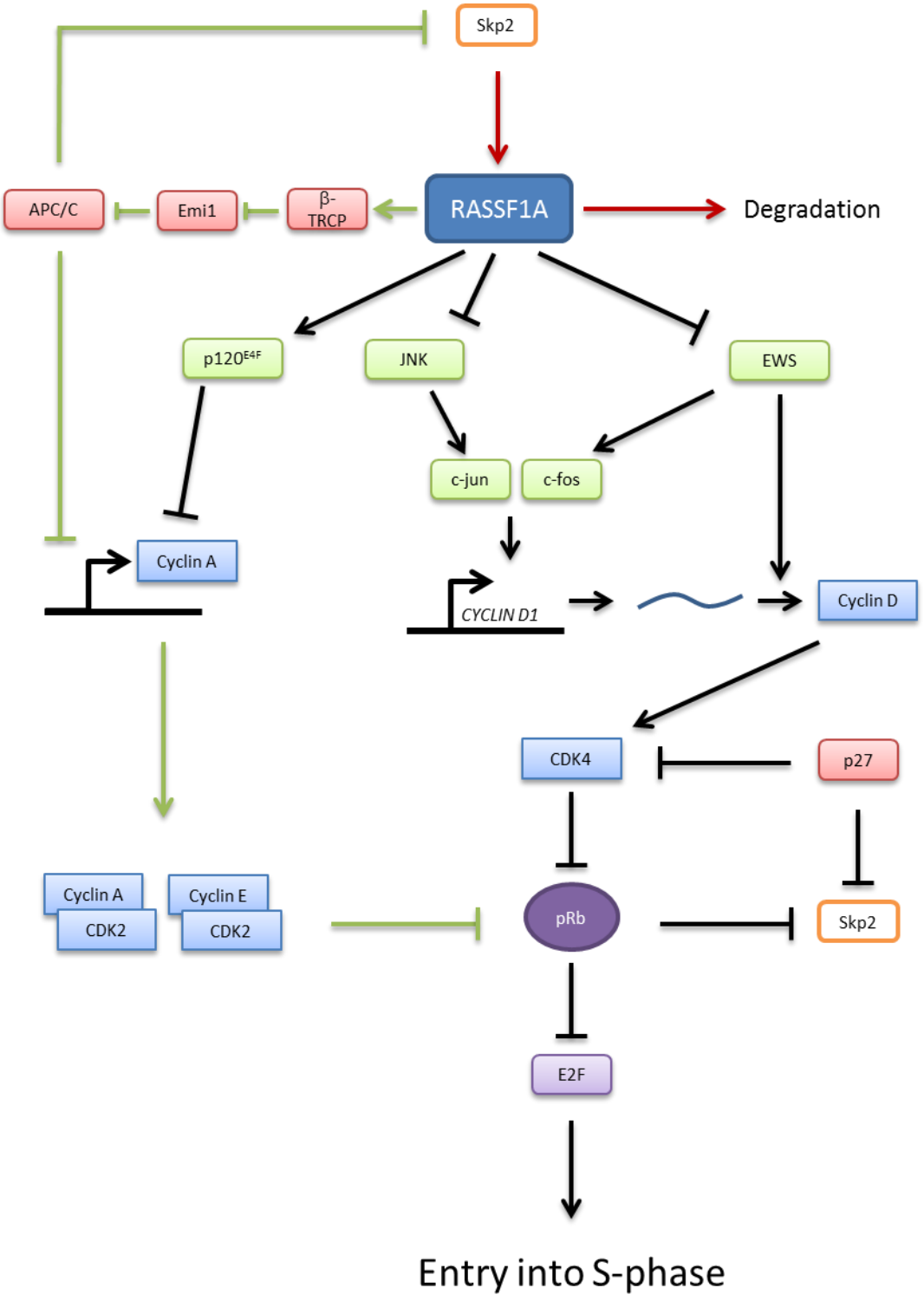


**Figure 1.3. Cartoon Depicting the Role of RASSF1A at the G1/S Checkpoint.** RASSF1A inhibits the transcription and translation of cyclin D1 to maintain CDK4 activity at a low level. This promotes sequestration of E2F by pRb preventing cells from entering S-phase (black arrows). Counter-intuitively, RASSF1A activation of the E3 ubiquitin ligase β-TRCP promotes progression through the G1/S transition by promoting the build-up of cyclin A, cyclin B and SKP2 through the inhibition of $APC^{CDH1}$ (green arrows). SKP2 forms part of a feedback loop which targets RASSF1A for ubiquitin-mediated degradation.

A second N-terminal interacting protein E1A-regulated transcription factor $p120^{E4F}$ when coexpressed with RASSF1A was shown to cause a G1-S cell cycle arrest (Fenton, Dallol et al. 2004). This was attributed to repression of the cyclin A2 promoter by $p120^{E4F}$, decreasing cyclin A expression which is essential for G1/S transition (Ahmed-Choudhury, Agathanggelou et al. 2005). $p120^{E4F}$ has also been associated with a number of other cell cycle regulators including: p53 (Sandy, Gostissa et al. 2000), pRb (Fajas, Paul et al. 2000) and $p14^{ARF}$ (Rizos, Diefenbach et al. 2003). Given that RASSF1A has also been shown to activate p53, this may provide an alternative mechanism through which the interaction between RASSF1A-$p120^{E4F}$ may promote a G1/S cell cycle arrest. RASSF1A expression has also been shown to cause a G1 arrest by stabilising the p53 target gene, p21, in a p53-independent manner through the activation of the MAPK kinase pathway (Thaler, Hahnel et al. 2009).

Given that RASSF1A has such a potent effect on cell cycle progression it must be regulated to ensure that cells can continue to pass through the cell cycle and proliferate. RASSF1A levels accumulate through the cell cycle until the G1-S checkpoint where there is a sudden drop in protein level. This drop has been attributed to ubiquitination mediated degradation of RASSF1A by the SKP2-CUL1-F-box (SCF) ubiquitin ligase complex. The CDK4-Cyclin D1 complex phosphorylates RASSF1A on Serine 203 creating a phospho-degron to which SKP2 binds leading to subsequent ubiquitination and degradation of RASSF1A (Song, Song et al. 2008) (Figure 1.3). The Serine 202/203 sites are also phosphorylated by the Aurora kinases at the G2/M checkpoint, which is covered below in section 1.5.2.2.

Surprisingly, RASSF1A appears to be required in order to pass through the checkpoint and into S-phase in HeLa cells. This observation comes from the fact that although overexpression of RASSF1A can lead to G1-S cell cycle arrest, loss of RASSF1A appears to also impair entry into S-phase as indicated by BrDU labelling (Whitehurst, Ram et al. 2008). Further investigation showed that RASSF1A could bind and inhibit the ubiquitin ligase β-TRCP, which normally inhibits the accumulation of Emi1. During the G1 phase of the cell cycle, Emi1 inhibits the activity of the CDH1 bound anaphase promoting complex/cyclosome ($APC/C^{CDH1}$) allowing the accumulation of G1-S promoting proteins such as SKP2, as

well as Cyclins A and B (Whitehurst, Ram et al. 2008). Once SKP2 has reached a threshold it can degrade RASSF1A, cyclin D1 can accumulate and the Rb checkpoint overcome, allowing entry into S phase (Figure 1.3).

### 1.5.2.2 The Pro-Metaphase Checkpoint

Unidirectional progression through Mitosis requires the timed destruction of proteins by the APC/C. Such proteins include: cyclin A, which is targeted for degradation in prometaphase after it has promoted nuclear envelope breakdown; cyclin B, which is targeted for degradation in anaphase; and securin, which is targeted for degradation once the spindle checkpoint has been overcome to allow chromosome segregation (den Elzen and Pines 2001; Geley, Kramer et al. 2001; Hagting, Den Elzen et al. 2002). The APC/C controls these events by forming a complex with one of two WD40 domain containing proteins, CDC20 and CDH1. CDC20 activates APC/C in early mitosis and CDH activates APC/C in late mitosis and throughout G1-phase of the cell cycle (Jaspersen, Charles et al. 1999; Kramer, Scheuringer et al. 2000; Pines 2006). Inactivation of APC/C, by inhibiting the interaction between APC/C and either CDC20 or CDH1 can cause cell cycle arrest during mitosis. This is employed by a number of proteins including RASSF1A and the spindle checkpoint proteins BUBR1 and MAD2 (Fang, Yu et al. 1998; Sudakin, Chan et al. 2001; Millband and Hardwick 2002; Tang, Shu et al. 2004).

One of the first identified functions of RASSF1A was to bind to and stabilise microtubules (Rong, Jin et al. 2004). This interaction was seen to be particularly strong at the centrosomes and on the spindles during mitosis. The interaction between RASSF1A and the microtubules requires a minimal binding region of amino acids 120-288 which covers the RA domain and appears to be very important for its tumour suppressor function during pro-metaphase. Here RASSF1A bound to microtubules, in association with C19ORF5 induces a pro-metaphase arrest by sequestering CDC20 (Dallol, Agathanggelou et al. 2004; Song, Song et al. 2004; Song, Chang et al. 2005). This prevents CDC20 from associating with the APC/C and thus it remains inactive. The RASSF1A-CDC20 interaction is inhibited by the binding of the helix-

loop-helix transcriptional repressor Id-1. Released CDC20 binds to the APC/C to form an active APC/$C^{CDC20}$ complex leading to cyclin B1 and securin degradation, resulting in premature mitosis (Wang, Di et al. 2008). In early mitosis Aurora A is able to bind to and be upregulated by RASSF1A, enabling Aurora A to phosphorylate RASSF1A on Threonine 202 and/or Serine 203 (Rong, Jiang et al. 2007; Liu, Guo et al. 2008; Song, Song et al. 2009). This phosphorylation, as with the binding of Id-1, has the effect of lowering the affinity of RASSF1A for CDC20 causing RASSF1A to dissociate from CDC20 (Song, Song et al. 2009) (Figure 1.4). These phosphorylation events have also been reported to promote RASSF1A dissociation from the microtubules, however this is still to be confirmed by another group (Rong, Jiang et al. 2007; Song, Song et al. 2009). Interestingly, Serine 203 has also been identified as a target for phosphorylation by PKC, along with Serine 197 (Verma, Ganesan et al. 2008). Verma et al. (2008) found that mutation of S203 did not affect the ability of RASSF1A to bind microtubules. However, the phosphorylation did prevent RASSF1A from being able to reorganise microtubules and as a consequence, cells failed to maintain golgi integrity. Aurora A phosphorylation of RASSF1A converts it from a repressor of APC/$C^{CDC20}$ to a target of it and leads to the ubiquitination and degradation RASSF1A (Chow, Wong et al. 2012). RASSF1A has also shown to be degraded through a second mechanism during M-phase by the Cullin 4A-DDB1 E3 ubiquitin ligase complex (Jiang, Rong et al. 2011). Degradation of RASSF1A by Cullin 4A-DDB1 severely inhibited the ability of RASSF1A to induce an M-phase arrest and was dependent on amino acids 165-200, which represents a basic region that is adjacent to the RA domain.

Aurora B has also been shown to phosphorylate RASSF1A in late mitosis (Song, Kim et al. 2009; Song, Song et al. 2009). This phosphorylation occurs in telophase when RASSF1A is located at the midbody (Song, Kim et al. 2009) and promotes RASSF1A binding to the t-SNARE Syntaxin 16. The interaction occurs though the C-terminal of RASSF1A and is required for successful cytokinesis (Figure 1.4).

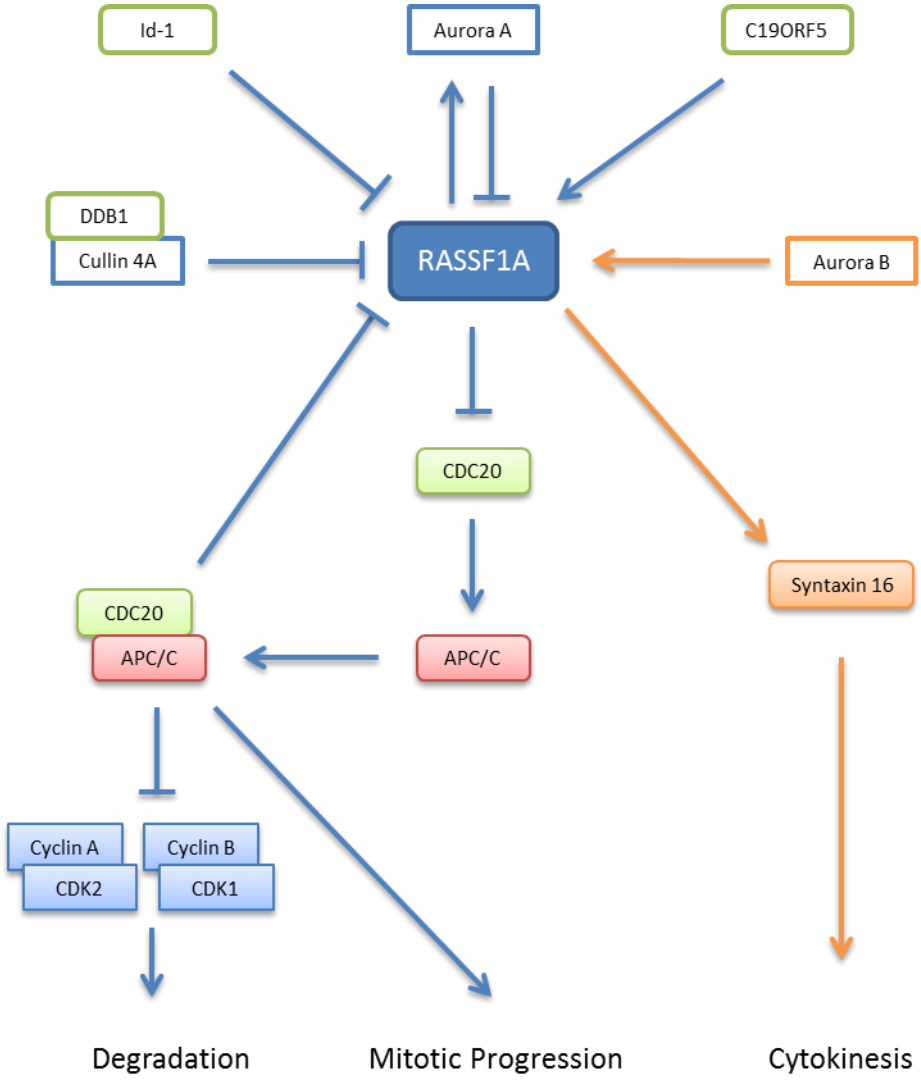


**Figure 1.4. The Role of RASSF1A in M-Phase.** RASSF1A forms a complex with C19ORF5 to stabilise microtubules and promote a cell cycle arrest at pro-metaphase by sequestration of CDC20 away from APC/C. This is countered by Id-1 and also by Aurora A phosphorylation of RASSF1A. Aurora A phosphorylation of RASSF1A prevents RASSF1A from inhibiting CDC20 and promotes RASSF1A degradation by APC/$C^{CDC20}$. This releases APC/$C^{CDC20}$ allowing it to promote progression into metaphase by degradation target proteins, such as cyclin A and cyclin B. RASSF1A is also targeted for degradation during mitosis by the Cullin 4A-DDB1 E3 ubiquitin ligase complex. In telophase Aurora B phosphorylates RASSF1A. This phosphorylation is required for RASSF1A to recruit Syntaxin 16 to the midbody and for successful completion of cytokinesis.

### 1.5.3 RASSF1, the Hippo Pathway, Cell Polarity and EMT

The Epithelial-Mesenchymal transition (EMT) is associated with epithelial cells acquiring the ability to invade, resist apoptosis and to disseminate throughout the body (Barrallo-Gimeno and Nieto 2005; Klymkowsky and Savagner 2009; Polyak and Weinberg 2009; Thiery, Acloque et al. 2009; Yilmaz and Christofori 2009). This process results in the loss of cell polarity, and subsequent tissue disorganisation. Originally, loss of cell polarity was considered to be a by-product of abnormal cell accumulation, however, recent evidence supports the idea that disruption of cell-polarity mechanisms plays a causal role in tumour initiation (Lee and Vasioukhin 2008). Apical-basal polarity is established and maintained by three protein complexes, two of which are apically located and one of which is basolaterally located. The three complexes are in a fine balance mutually antagonising each other. They consist of the apically located Par (Par3, Par6, aPKC and Cdc42) and Crumbs complexes (Crumbs, Pals1, PatJ and Lin-7) and the basolateral complex consisting of Scribble, Discs large (Dgl) and Lethal giant larvae (Lgl) (Bilder 2004; Margolis and Borg 2005; Suzuki and Ohno 2006). Their activities define the boundary position and size of the apical and basolateral membrane domains. Of the three complexes, the regulation of the Par complex is the best studied. It is known that Par6 and aPKC form a stable complex and can interact with Par3. aPKC can phosphorylate a number of the polarity proteins including: Par3, Lgl and Crumbs. Phosphorylation of Lgl inactivates it in the apical domain such that its activity is restricted to the basolateral domain (Betschinger, Mechtler et al. 2003; Plant, Fawcett et al. 2003). In return Dlg and Scribble inhibit the Par6-aPKC complex limiting its activity to apical regions. The polarity protein Par1 (mammals: MARK1-4) further regulates these complexes by phosphorylating Par3, which promotes its dissociation from Par6-aPKC (Benton and St Johnston 2003).

In epithelial cells the Adhesion Junction Complex (AJC) provides a physical link between these two domains. AJCs are major signalling centres that serve as biosensors of the external environment and mediate communication between the neighbouring cells in the normal organism. Their importance in suppressing carcinogenesis is supported by the fact that AJC components are frequently mutated in

human cancers (Royer and Lu 2011). This is particularly so for the major AJC component E-cadherin. Mutations in the gene encoding E-cadherin (*CDH1*) are found in more than 50% of stomach cancers (Graziano, Humar et al. 2003). The AJCs consist of: anchoring junctions composed of adherens junctions and desmosomes, which associate with the cortical cytoskeleton to mediate cell and tissue behaviour; tight junctions, which have an epithelial barrier function; and gap junctions, which serve as intercellular channels that permit direct cell-cell transfer of ions and small molecules (Green, Getsios et al. 2010). Of these adherens junctions are the first to form and their establishment is necessary for the subsequent formation of the tight junctions and the desmosomes (Adams, Nelson et al. 1996; Pokutta and Weis 2002; Gumbiner 2005). In mammalian epithelial cells tight junctions form a physical apical-basolateral barrier and are located apically to the adherens junctions. Components of the polarity complexes localise to the various junctions. The apically located Par-3 colocalises with the tight junction component ZO-1 and aids the formation and stabilisation of the tight junction (Izumi, Hirose et al. 1998; Joberty, Petersen et al. 2000; Chen and Macara 2005), whereas the basolateral component, Dlg colocalises with E-cadherin at adherens junctions, where it is stabilised (Reuver and Garner 1998; Ide, Hata et al. 1999; Mantovani and Banks 2003). This highlights that cell-cell contacts and the cell polarity are mutually dependent with each stabilising the other.

Epithelial adherens junctions are composed of E-cadherin in association with a number catenins ($\alpha$, $\beta$, $\gamma$ and p120) (Ozawa and Kemler 1992; Ozawa and Kemler 1998). E-cadherin is one of six classical cadherins, which also include: N (neural), VE (vascular-endothelial), P (placental), R (retinal) and K (Kidney) (Hulpiau and van Roy 2009). Classical cadherins are single-pass transmembrane proteins that form calcium-dependent homophilic interactions with cadherin molecules from adjacent cells (Pignatelli and Bodmer 1988; Takeichi 1993). The catenins bind the short cytoplasmic domain in a very specific manner. $\beta$-catenin and $\gamma$-catenin bind the very C-terminal 100 amino acids, $\alpha$-catenin links $\beta$-catenin to actin and p120 catenin binds the juxtamembrane portions, which stabilises cadherins at the cell membrane and promotes its correct functioning (Yap, Niessen et al. 1998). Binding of actin promotes adherens junction clustering and stabilisation of cell adhesion (Hirano, Kimoto et al. 1992; Reynolds and

Carnahan 2004). Loss of E-cadherin often occurs in the later stages of tumourigenesis and is thought to contribute to EMT, which represents a crucial step in metastasis (Royer and Lu 2011). E-cadherin is regulated both transcriptionally and post-translationally (reviewed in (Paredes, Figueiredo et al. 2012)). One example of this is that tyrosine kinases, such as SRC family kinases (SFKs) phosphorylate the E-cadherin-catenin complex resulting in: internalisation, ubiquitination by the E3 ubiquitin ligase HAKAI, and trafficking to the lysosome where it is degraded (Daniel and Reynolds 1997; Fujita, Krause et al. 2002; Palacios, Tushir et al. 2005). As well as loss of cell-cell contact and apical-basolateral polarity, downregulation of E-cadherin releases β-catenin from the cell junctions creating a cytoplasmic pool that is able to be activated by Wnt signalling (Hulsken, Birchmeier et al. 1994; Clevers and Nusse 2012). In the absence of Wnt signalling, a multiprotein complex composed of Axin, Adenomatous Polyposis Coli (APC), GSK3 and CK1, targets β-catenin for ubiquitination by β-TRCP leading to its degradation (Clevers and Nusse 2012). When Wnt binds to Frizzled receptors this complex is inactivated allowing β-catenin to enter the nucleus where it binds with T-cell factor (TCF)/Lymphoid Enhancer Factor (LEF) to upregulate target genes, which promote proliferation and invasion and inhibit apoptosis. Although β-TRCP mediated degradation of β-catenin is inhibited by Wnt signalling, it has also been shown that Wnt signalling upregulates β-TRCP (Spiegelman, Slaga et al. 2000). This is expected to accelerate the turnover of the less stable cytoplasmic Dlg (Mantovani and Banks 2003), which may lead to Dlg being titrated from the cell junctions where it is normally stabilised by E-cadherin. Thus a positive feedback cycle exists between loss of junctions and cell polarity, and promotion of proliferation and EMT. p120-catenin, like β-catenin, can also enter the nucleus upon release from the E-cadherin-catenin complex. Here it inhibits Kaiso (a transcriptional repressor) mediated repression of β-catenin/TCF target genes upregulating the β-catenin signal (Spring, Kelly et al. 2005).

Recently, a number of publications have elucidated the regulation of the hippo pathway by cell-cell junction components and cell polarity. Much of this work has been done in *Drosophila*; however, the mammalian pathways have also begun to be uncovered. In *Drosophila* the classical hippo pathway is

defined as comprising the core kinases; Hippo and Warts; the adaptors, Salvador and Mats; the transcriptional co-factor Yorkie, and a number of upstream modulators such as Expanded, Merlin, Fat and Dachsous (Genevet and Tapon 2011). Activation of the upstream components of the signalling cascade leads to the phosphorylation of Yorkie by Warts. Phosphorylated Yorkie is sequestered in the cytoplasm by 14-3-3 proteins (Pan 2010). Inactivation of the hippo pathway, therefore, leads to maintenance of Yorkie in the nucleus where it binds to scalloped to upregulate pro-proliferation and antiapoptotic genes such as *diap1, cyclin E* and the miRNA *bantam* (Tapon, Harvey et al. 2002; Wu, Huang et al. 2003; Nolo, Morrison et al. 2006; Thompson and Cohen 2006). Therefore, Yorkie promotes accelerated proliferation and resistance to apoptosis which is antagonised by the hippo pathway.

The first upstream regulators of the hippo pathway identified were the FERM domain adaptor proteins Merlin (human NF-2) and Expanded (Hamaratoglu, Willecke et al. 2006; Pellock, Buff et al. 2007). FERM domains are thought to link the membrane to the cytoskeleton. In the case of Expanded one of the connections made through its FERM domain is with the polarity protein Crumbs (Ling, Zheng et al. 2010). Overexpression of Expanded, or Merlin, was shown to promote Hippo/Warts activity (Hamaratoglu, Willecke et al. 2006). Two atypical cadherins, Fat and Dachsous, were also shown to function as non-catalytic activators of the hippo pathway (Bennett and Harvey 2006; Cho, Feng et al. 2006; Silva, Tsatskis et al. 2006; Willecke, Hamaratoglu et al. 2006). Finally, the protein Kibra was shown to be able to directly interact with and the upstream regulators, Merlin and Expanded and the core kinases Hippo and Warts linking the environmental receptors to the hippo pathway (Genevet, Wehr et al. 2010; Yu, Zheng et al. 2010) (Figure 1.5).

More recently Expanded has been shown to bind directly to Yorkie, sequestering Yorkie outside the nucleus (Badouel, Gardano et al. 2009; Oh, Reddy et al. 2009). This mechanism of regulation is thought to be conserved in mammals as the Angiomotin (AMOT) family proteins were also shown to bind to YAP and its paralog TAZ and sequester them to cell-cell junctions (Chan, Lim et al. 2011; Paramasivam, Sarkeshik et al. 2011; Zhao, Li et al. 2011) (Figure 1.5). AMOT has been reported to interact with a

number of tight junction proteins, such as ZO-1 (Zhao, Li et al. 2011) and polarity proteins including: Patj, Pals1 and Par3 (Wells, Fawcett et al. 2006). AMOT could provide a direct link between activation of the hippo pathway and formation of the cell-cell junctions and cell polarity. α-catenin is also known to sequester YAP at the adherens junctions. This interaction is indirect with α-catenin recruiting and sequestering 14-3-3 bound YAP (Schlegelmilch, Mohseni et al. 2011). This interaction therefore requires LATS phosphorylation of YAP, which is not required for the Expanded or AMOT interaction.

Other hippo pathway components that are apically localised include Hippo itself as well as Warts, Salvador and the negative regulator Ajuba, which colocalise to the adherens junctions (Badouel, Gardano et al. 2009; Grzeschik, Parsons et al. 2010). The apical localisation of Hippo is thought to be regulated by the polarity complexes. Loss of Lgl or overexpression of aPKC resulted in the re-localisation of hippo from an apical to a basolateral localisation. This allowed Hippo to interact with dRASSF, which is a negative regulator of the Hippo pathway in *Drosophila*. dRASSF inhibits Hippo in two ways, by competing with the positive regulator of Hippo, Salvador, and by bringing Hippo into close proximity with the dSTRIPAK-PP2A complex leading to dephosphorylation and inactivation of hippo (Polesello, Huelsmann et al. 2006; Ribeiro, Josue et al. 2010).

As cells become denser they make more cell-cell contacts. It is the formation of these contacts that is thought to activate the hippo pathway to control organ size. Indeed, Kim et al. (2011) recently showed that E-cadherin-dependent contact inhibition was dependent on the activation of the hippo pathway member LATS1/2 (Figure 1.5). This was thought to be MST1/2 independent. The connections between E-cadherin and LATS1/2 were not fully resolved, but the mechanism was dependent on α- and β-catenin. Signalling was thought to occur either directly through Kibra and Merlin or via a novel protein NHERF which was shown to be able to bind Merlin, β-catenin, TAZ and YAP (Kim, Koh et al. 2011) (Figure 1.5). NF-2 has previously been shown to regulate contact inhibition via activation of the hippo pathway (Zhao, Wei et al. 2007; Striedinger, VandenBerg et al. 2008; Zhang, Smolen et al. 2008). NF-2 is also known to play a role in tight junction maturation and links α-catenin to Par3 (Gladden, Hebert et al.

2010). Therefore, both adherens junctions and tight junctions could both signal via α-catenin to activate the hippo pathway to sequester YAP. Wada et al. (2011) also showed that the hippo pathway was activated by cell density, however, they reasoned that it was changes ain physical and mechanical stresses that were responsible. They have put this down to mechanical stress because low density cells, where YAP is nuclear and active, have higher numbers of F-actin stress fibres than high density cells, where YAP is cytoplasmic and inactive. Disruption of Actin also led to YAP inactivation by re-localisation to the cytoplasm. This is in agreement with Densham et al. (2009) who showed that MST1/2 could associate with actin and activate JNK in response to cytoskeleton disruption (Densham, O'Neill et al. 2009). Also, in agreement with Wada et al (2011), YAP and TAZ activation has been shown to be dependent on Actin stress fibre formation in response to Rho driven actomyosin cytoskeletal tension induced by ECM stiffness (Dupont, Morsut et al. 2011) (Figure 1.5). However, this was found to be hippo pathway independent.

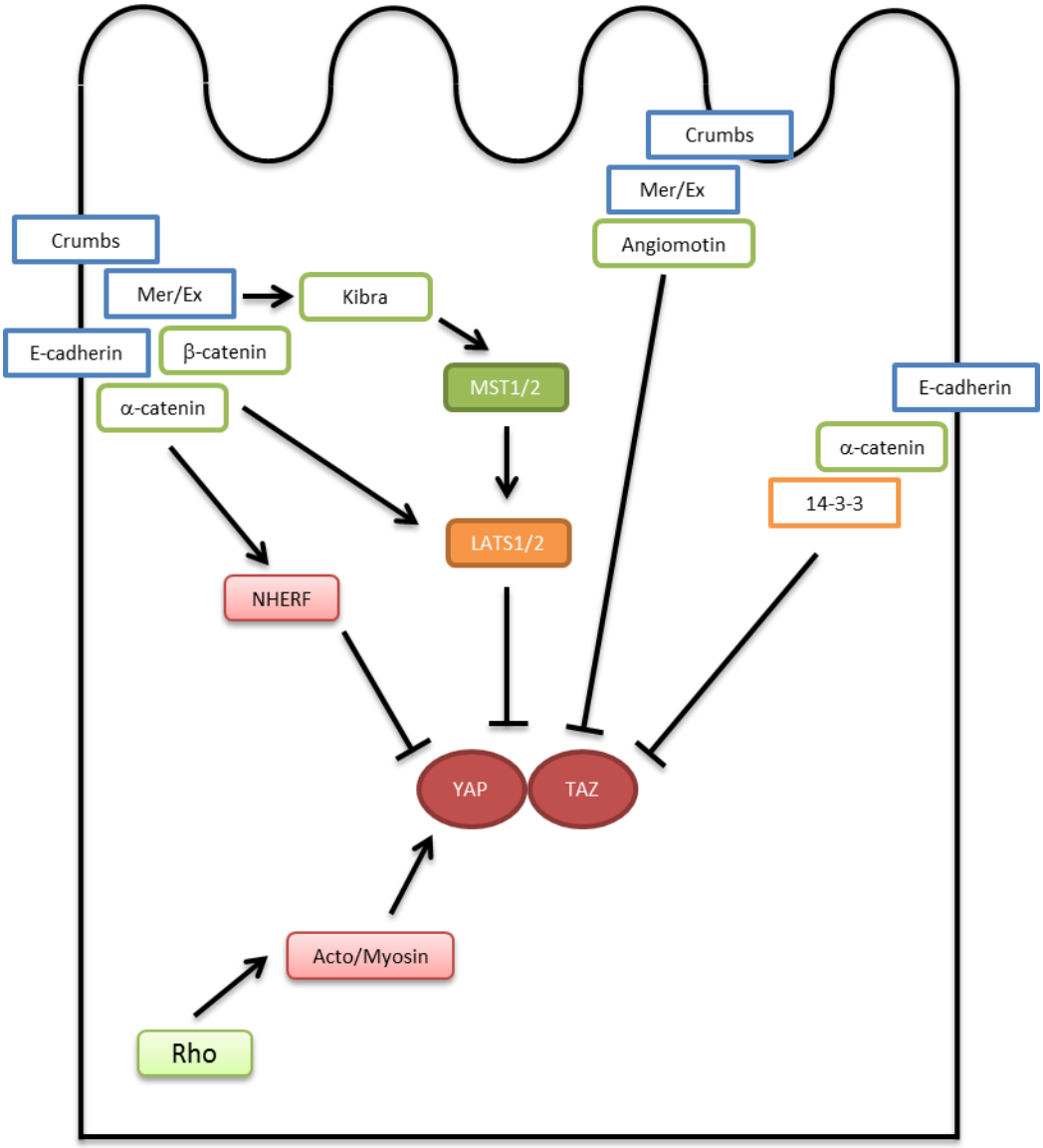


**Figure 1.5. Diagram Showing the Regulation of the Hippo Pathway by Cell-Cell Junctions and Cell Polarity in Mammals.** Cell density, sensed by cell-cell junctions, inhibits YAP/TAZ activation either through activation of the hippo pathway components MST1/2 and LATS1/2 or directly. In the context of cell density, hippo pathway activation leads to phosphorylation of YAP/TAZ and subsequent sequestration by 14-3-3 proteins in the cytoplasm of the cell. α-catenin sequesters phosphorylated YAP/TAZ at cell-cell junctions. E-cadherin also inhibits YAP/TAZ in an α- and β-catenin-dependent manner that is thought to occur either through LATS1/2 or directly through NHERF. The polarity protein Crumbs interacts with Merlin and Expanded. Activation of Merlin and Expanded can either activate the hippo pathway through Kibra or can directly sequester YAP/TAZ through Angiomotin. Sequestration prevents YAP/TAZ from entering the nucleus and transcribing pro-proliferative genes. Increased Rho-GTP-dependent actomyosin contractility activates YAP/TAZ in a hippo-independent manner.

## 1.6 RASSF1C

RASSF1C is the second ubiquitously expressed isoform of the *RASSF1* gene. Like RASSF1A, RASSF1C contains the ATM consensus sequence and has been shown to associate with K-Ras (Vos, Ellis et al. 2000). In RASSF1C the ATM phosphorylation site is at Serine 61. It has not yet been confirmed, but the sequence is identical between RASSF1A and RASSF1C at this site so it is plausible to suggest that RASSF1C is also phosphorylated and activated by ATM. Indeed, the Serine 61 to phenylalanine (S61F) mutant of RASSF1C was unable to block the genomic destabilising effects of Ras which can be ablated by overexpression of wildtype RASSF1C in the embryonic kidney cell line 293T and human lung tumour cell line NCI-H1299 (Vos, Martinez et al. 2004). This suggests that DNA damage activation of RASSF1C may require phosphorylation of Serine 61 (RASSF1A-131) site. Further to this, RASSF1C has recently been implicated in a DNA damage response pathway involving DAXX and JNK (Kitagawa, Kajiho et al. 2006). In unstressed conditions RASSF1C is shown to be in a complex with DAXX in the nucleus, recently resolved by NMR (Escobar-Cabrera, Lau et al. 2010). Upon ultraviolet radiation or MMS-induced DNA damage this interaction is lost allowing RASSF1C to move to the cytoplasm where it aids the activation of SAPK/JNK signalling (Kitagawa, Kajiho et al. 2006). DAXX however remains in the nucleus concentrating at PML bodies. The signal that leads to release of RASSF1C from DAXX is unknown, however, it would be interesting to see if the signal relies upon the ATM phosphorylation site. RASSF1A and RASSF1C levels are also thought to be regulated by the oncogenic protein CSE1L by a mechanism that is independent of promoter methylation. Loss of CSE1L in RASSF1A negative cells leads to the upregulation of RASSF1C, which sensitises cells to cisplatin treatment promoting apoptosis (Lorenzato, Martino et al. 2012). Conversely, another study has identified that RASSF1C, far from being activated by DNA damage, is targeted for degradation under stress conditions. Exposure to UV radiation or treatment of cells with doxorubicin leads to RASSF1C phosphorylation by GSK3β creating a phosphodegron at S19/23 which is bound to by $SCF^{\beta\text{-}TRCP}$ targeting RASSF1C for degradation (Zhou, Li et al. 2011). This GSK3β-dependent degradation was shown to be inhibited by the PI3K/AKT pathway. Since AKT activity can lead to RASSF1C

upregulation it suggests that RASSF1C could function as an oncogene. This is in keeping with several recent reports showing RASSF1C increased cell proliferation in lung cancer and osteoblast cells and migration in breast cancer cell lines (Amaar, Minera et al. 2006; Reeves, Baldwin et al. 2010; Reeves, Baldwin et al. 2012). In these studies the pro-proliferative effect of RASSF1C was identified as being through upregulation of Extracellular-signal-regulated kinase 1/2 (ERK1/2), potentially through an interaction with Insulin growth factor binding partner -2 (IGFBP-2). Downstream target genes upregulated by RASSF1C also suggest an oncogenic role, for example; In breast cancer cell lines CXCR4 is upregulated, which enhances cell proliferation and metastasis (Reeves, Baldwin et al. 2010); and in lung cancer cell lines the stem cell renewal factor PIWIL1 was shown to be upregulated (Reeves, Baldwin et al. 2012).

As well as being a target for β-TRCP mediated degradation, RASSF1C has been shown to bind and inhibit β-TRCP re-localising it from the nucleus to the cytoplasm, where it is unstable (see chapter 1.5.3). RASSF1C inhibition of β-TRCP prevents it from interacting with β-catenin leading to β-catenin nuclear accumulation and transcriptional transactivation (Estrabaud, Lassot et al. 2007). RASSF1C has also been shown to interact with the tumour suppressor Tissue factor pathway inhibitor-2 in the nucleus although the functional outcome of this interaction is unknown (Chen, Li et al. 2012).

Clinical studies appear to support the role of RASSF1C as an oncogene. Multiple groups have shown that not only is RASSF1C not methylated in human cancers it is in fact upregulated. These studies have analysed tumours form a number of different sources including Pancreatic endocrine tumours, large cell neuroendocrine carcinomas and small cell lung carcinomas (Pelosi, Fumagalli et al. 2010; da Costa Prando, Cavalli et al. 2011; Malpeli, Amato et al. 2011). In fact in the lung cancers RASSF1C expression was shown to be an adverse prognostic factor (Pelosi, Fumagalli et al. 2010).

## 1.7 Therapeutic Implications of RASSF1A Loss

One of the most common and widespread events to occur during cancer development is the loss of RASSF1A expression. This loss is due to methylation of the upstream CpG island in the *RASSF1* gene (Dammann, Li et al. 2000; Burbee, Forgacs et al. 2001). The frequency of epigenetically driven loss of RASSF1A correlates well with the increasing grade of the tumour. Methylation has been reported in over 37 tumour types (comprehensively reviewed in (Dammann, Schagdarsurengin et al. 2005; Hesson, Cooper et al. 2007)) and is thought to be an early event in Breast and Thyroid tumourigenesis, childhood neoplasia and endometrial carcinogenesis (Donninger, Vos et al. 2007; van der Weyden and Adams 2007).

Univariate analysis has correlated RASSF1A methylation with poor prognosis in all cancers studied. Significance is maintained when more stringent multivariate analysis is carried out in breast, lung, colorectal, bladder (muscle-invasive bladder cancer, clear cell renal cell carcinoma and renal cell carcinoma), pancreatic endocrine neoplasms, hepatocellular carcinoma and hepatoblastoma (House, Herman et al. 2003; Fischer, Ohnmacht et al. 2007; Sugawara, Haruta et al. 2007; Yanagawa, Tamura et al. 2007; Honda, Haruta et al. 2008; Kawai, Sakano et al. 2010; Huang, Hu et al. 2011; Tommasi, Pinto et al. 2011; Wang, Wang et al. 2011; de Martino, Klatte et al. 2012; Jiang, Cui et al. 2012; Kim, Chae et al. 2012). As well as tumour grade RASSF1A methylation has also been associated with metastasis (Fendri, Masmoudi et al. 2009; Hu, Chen et al. 2010; Tommasi, Pinto et al. 2011; Kim, Chae et al. 2012).

Meta-analysis of a number of different cancers including, breast and non-small cell lung cancer and colorectal carcinoma have now been done, each enhancing the significance of RASSF1A methylation as a prognostic marker (Tommasi, Pinto et al. 2011; Wang, Wang et al. 2011; Jiang, Cui et al. 2012). The clinical significance of RASSF1A methylation has been best studied in breast cancer, where it has been shown to be a better prognostic marker than standard prognostic parameters (Muller, Widschwendter et al. 2003). The recent meta-analysis analysed data from 1795 patients from 8 studies and used multivariate analysis to investigate disease free survival and overall survival in breast cancer patients

with methylated RASSF1A, showing a significant correlation between these parameters (Jiang, Cui et al. 2012). A similar study involving 2802 patients from 19 studies with non-small cell lung carcinoma made similar conclusions (Wang, Wang et al. 2011).

Colorectal carcinogenesis is often associated with constitutive activation of Ras or downstream pathway members B-Raf and PI3K (Velho, Corso et al. 2010; De Roock, De Vriendt et al. 2011). The observed methylation of RASSF1A in colorectal cancers varies enormously, from 12 % (Yoon, Dammann et al. 2001), to 81 % (Sakamoto, Terai et al. 2004). One interesting observation seen in a number of these studies is that RASSF1A methylation rarely occurs together with K-Ras mutation (reviewed in (Fernandes, Carneiro et al. 2012)). Since oncogenic, but not wildtype K-Ras can induce apoptosis through RASSF1A cells with oncogenic K-Ras must inactivate RASSF1A signalling. It has recently been shown that to counter this cancer cells inhibit the apoptotic signal downstream of RASSF1A, either by maintaining one copy of wildtype K-Ras or downregulating MST2 (Matallanas, Romano et al. 2011). However, another study focusing on colorectal cancers with microsatellite instability showed that methylation of RASSF1A was associated with increased genomic instability and an accumulation of mutation in *KRAS* and *BRAF* genes (Oliveira, Velho et al. 2005).

RASSF1A methylation also correlates with a decreased responsiveness to DNA damaging therapies (Catto, Azzouzi et al. 2005; Dote, Cerna et al. 2005; Honda, Haruta et al. 2008). The DNA methyltransferase (DNMT) inhibitor Zebularine has been used to effectively re-express RASSF1A and show an increase in cancer cell sensitivity to radiation-induced damage *in vitro* and *in vivo* (Dote, Cerna et al. 2005) as well as to cisplatin (Balch, Yan et al. 2005). Dote et al., (2005) showed that 48 hr treatment with Zebularine, which corresponded to the maximum re-expression of RASSF1A increased the radiosensitivity of PaCa, DU145 and U251 cancer cell lines by 1.5 times and caused increased tumour delay in U251 xenograft models in mice (Dote, Cerna et al. 2005). A 48 hr treatment with Zebularine also increased cancer cell sensitivity to DNA damage and a 16-fold reduction in $IC_{50}$ of cisplatin in resistant ovarian cancer cell lines (Balch, Yan et al. 2005). Sensitivity of testicular germ cell tumours to cisplatin

could also be enhanced by another DNMT inhibitor that is in clinical trials, 5-aza-2'-deoxycytidine (Beyrouthy, Garner et al. 2009). Interestingly, they noted that effectiveness of the 5-aza-2'-deoxycytidine treatment was dependent on the level of DNMT3B levels. The higher the DNMT3B level the greater the effect. One of the most significant target genes for DNMT3B was shown to be RASSF1A (as mentioned above). Thus it can be extrapolated that the increase in sensitivity to cisplatin is due to the re-expression of RASSF1A. Re-expression of RASSF1A using 5-aza-2'-deoxycytidine or re-introduction of RASSF1A into the hepatocellular carcinoma cell line, SMMC-7721, was also shown to increase sensitivity to chemotherapeutics such as fluorouracil, mitomycin as well as cisplatin (Zhao and Dou 2007). Together these results support a clinically relevant role for RASSF1A in the DNA damage response that is backed up by phase I and II clinical trials in myelodysplasia and leukaemia patients where 5-aza-2'-deoxycytidine has shown efficacy both alone and in combination with the histone deacetylase (HDAC) inhibitor valproic acid (Garcia-Manero, Kantarjian et al. 2006; Ruter, Wijermans et al. 2006). Therapeutic failure upon RASSF1A loss can also be counteracted by targeting the downstream DNA damage responsive signalling pathway. Direct activation of BAX via the BH3 mimetic ABT-737 has recently put forward as a potential treatment for RASSF1A methylated medulloblastoma (Levesley, Lusher et al. 2011). The role of RASSF1A in checkpoint activation and maintenance of genomic integrity is highlighted in a study by Zhang et al (2002) which showed a significant increase in DNA damage caused by aflatoxin $B_1$ in tumour tissues where RASSF1A has been lost due to DNA methylation (Zhang, Ahsan et al. 2002).

## 1.8 Project Aims

The aims of this project are to investigate roles of RASSF1A as a tumour suppressor in human cancer cell lines and the mechanisms through which these roles are achieved in order to better understand how loss of RASSF1A contributes to tumourigenesis. I shall fulfil these aims by identifying novel interacting partners of RASSF1A and investigating how these interactions contribute to the tumour suppressor activity of RASSF1A. A novel role for RASSF1C will be assessed and the consequences of this will be

analysed in the context of RASSF1A loss. The data generated in this project will provide new evidence to suggest why patients that have lost RASSF1A by promoter methylation have a worse prognosis and why loss of RASSF1A is associated with a more malignant, metastatic phenotype.

# Chapter 2 Materials and Methods

## 2.1 Materials

All chemicals were purchased from Sigma-Aldrich or Fisher Scientific unless stated and used according manufacturer's guidelines. Tissue culture plastic was purchased from Sarstedt and Corning. Glassware was purchased from Fisher and VWR.

## 2.2 Cell Culture

All cell lines (Table 2.1) were maintained in complete media (Dulbecco's Modified Eagle Medium (DMEM) containing 10 % (v/v) Fetal Bovine Serum (FBS), 2 mM Glutamine and 100 U/ml Penicillin/streptomycin (Pen/strep)). All media and supplements were purchased from Gibco® (Life Technologies). Cells were maintained in a humidified incubator at 37 $^{o}$C, with 5 % $CO_2$.

Cells were thawed rapidly in a water bath at 37 $^{o}$C after removal from liquid Nitrogen. Once thawed cells were diluted in complete media and centrifuged at 500 x g, 5 min. Cell pellets were resuspended in complete media and seeded into a T75 flask (Sarstedt).

Cells were passaged upon reaching confluency. Cells were rinsed in sterile Phosphate Buffered Saline (PBS) before 0.25 % Trypsin-EDTA solution (Gibco®, Life Technologies) was added to the cells. Media was added to the trypsinised cells and a proportion of the cells reseeded into a new flask containing fresh complete media.

In order to freeze cells, trypsinised cells resuspended in complete media were centrifuged 500 x g, 5 min. The resulting pellet was resuspended in freezing media (90 % (v/v) FBS, 10 % (v/v) Dimethyl sulfoxide (DMSO)), before being aliquoted into 1.5 ml cryovials (Thermo Scientific). Cryovials were

placed into a Nalgene® Mr Frosty containing 200 ml isopropanol at room temperature (RT), and placed at -80 $^{o}$C for 24 hr prior to long term storage in liquid Nitrogen.

Cells were regularly tested for mycoplasma using the PlasmoTest kit (Invivogen). This kit uses Toll-like Receptor 2 (TLR2) expressing cells to detect the presence of mycoplasma within cultured cells. The Mycoplasma binds TLR2 activating NF-κB signalling and leading to the upregulation of secreted alkaline phosphatase. This is detected by a blue colour in the detection media.

In order to guarantee that the cancer cell lines that were being used were authentic we bought our cell lines from official sources, namely ATCC and CRUK. Although, we have not currently assessed the validity of cell lines acquired from non-official sources, this can be done by sending a sample of cell lysate to LGC Standards for genetic analysis.

## 2.3 Transfection

Commercially available siRNA was purchased from Dharmacon (Thermo Scientific) or Ambion. Other siRNA was purchased from Eurofins MWG Operon (Table 2.2). siRNA was resuspended in nuclease-free water (Qiagen) to form a 20 μM stock, aliquoted and stored at -80 $^{o}$C.

Appropriate volumes of siRNA or plasmid DNA and Lipofectamine 2000 transfection reagent (Life Technologies) (Ratio: 1 μl siRNA to 1 μl Lipofectamine 2000 or 1 μg DNA to 2 μl Lipofectamine 2000) were diluted in separate 1.5 ml microcentrifuge tubes in OptiMEM (Gibco® Life Technologies), and incubated at room temperature for 5 min before mixing. Once mixed the siRNA or DNA/Lipofectamine 2000 mixture was incubated for 25 min at RT with occasional gentle mixing, and then added in a dropwise fashion to the cells. After 6 hr the media containing the transfection mix was aspirated and cells were cultured in fresh complete media until harvesting. Unless stated elsewhere, cells were treated with 50 nM siRNA.

Polyethylenimine (PEI) (Polysciences, Inc.) transfection reagent was used for the transfection of cells for large scale experiments for mass spectrometry. Plasmid DNA was mixed with OptiMEM before PEI was added to the solution. The DNA/PEI solution was gently mixed and incubated for 25 min at RT before being added in a dropwise fashion to the cells. The ratio of PEI to DNA was 10 μl PEI to 1 μg DNA. The volume of the transfection mix for a 15 cm plate was 1 ml. Stock PEI was 1 mg/ml.

The efficiency of transfection for experiments that were analysed by western blot or immunoprecipitation was determined by expression of the tagged construct or loss of the endogenous protein. However, true transfection efficiency is defined as the percentage of cells that have been successfully transfected with the vector. Only, the transfection efficiency of immunofluorescence experiments was determined in this manner. In these experiments the percentage of cells expressing either EGFP or Zs-Green empty vector plasmid was greater than or equal to 80 %. The transfection efficiency of cells transfected with FLAG-RASSF1C, EGFP-RASSF1C or Zs-Green-RASSF1C appears much lower than this. Although we cannot rule out that the addition of the RASSF1C sequence to the vector may have affected the transfection efficiency we have observed the same expression pattern in both stable and inducible cell lines. This observation is actively being investigated.

## 2.4 Stable Cell Line Production and Maintenance

The correct concentration of selection agent required to produce each stable cell line was determined using a kill curve. Cells were cultured in a range of concentrations of selection agent around concentrations identified from the literature for a particular cell line. The optimal concentration was determined as the lowest concentration that killed all of the cells in the dish.

H1299 cells stably expressing a Tet ON regulatory plasmid were transfected with the required expression vector (Table 2.3) and pBabe-PURO vector in a 10:1 ratio. Cells that were resistant to Puromycin were considered to have been transfected with both plasmids. MDA-MB-231 cells were

transfected only with the expression vector (Table 2.3) which contained a Puromycin resistance gene that could be used to select transfected cells. Resistant cells were seeded at approximately 1000 cell/ml for clone selection. 50 μl trypsin was added to each colony and the resuspended cells collected with a p200 pipette. Clones were expanded and a sample of total cell lysate examined for expression. Cell lines were maintained in complete media supplemented with appropriate selection agent. Table 2.3 shows the stable cell lines used in this project and the selection agent used to maintain them. Figure 8.5 and Figure 8.6 show the expression detected in selected clones. H1299 Tet ON clones were selected on the basis that they had clear induction of expression upon addition of doxycycline, but no detectable expression in the absence of doxycycline. MDA-MB-231 cells were selected by the level of expression. Expression was determined by western blot.

## 2.5 Clonogenic Survival Assay

Cells were plated at 400 cells per 6 cm dish and allowed to attach before exposure to 0 or 2 Gy ionising radiation from a Cs-137 source (GSRD1 Gamma-service Medical GmbH). Cells were incubated at 37 $^{o}$C, 5 % $CO_2$ in a humidified incubator overnight before fresh media was applied to the cells. Cells were incubated 37 $^{o}$C, 5 % $CO_2$ in a humidified environment for 14 days to allow colonies to form. Colonies were fixed and stained using crystal violet solution (0.5 % (w/v) crystal violet, 20 % (v/v) Ethanol, 10 % (v/v) methanol), before the number of colonies was determined. The surviving fraction was derived using the formula,

$$(\#\ \text{Colonies} / \#\ \text{of cell plated})_{\text{irradiated}} / (\#\ \text{colonies} / \#\ \text{of cell plated})_{\text{unirradiated)}}.$$

## 2.6 Immunoprecipitation

Cells were washed two times with PBS before lysis into immunoprecipitation lysis buffer (Table 2.9) supplemented with protease inhibitor cocktail (Roche) and 50 mM sodium fluoride, 10 mM β-glycerophosphate and 0.5 mM sodium orthovanidate. Cells were scraped from the dish using a rubber cell scraper, collected into a 1.5 ml microfuge tube and incubated end over end at 4 $^{o}$C for 10 min. Lysates were then cleared by centrifugation (20817 x g, 10 min, 4 $^{o}$C). The protein concentration of the lysates was then determined by Bradford assay, such that an equal amount of protein was loaded into each immunoprecipitation.

Anti-FLAG M2-conjugated agarose (Sigma), protein G sepharose (Millipore) and protein G metallic (Millipore) beads were used during this project. All beads were washed 3 times in immunoprecipitation lysis buffer before being aliquoted into 1.5 ml microfuge tubes. 1-2 μg antibody was added per 10 μl of the protein G sepharose or protein G metallic beads before lysate was added to the tubes and incubated end over end at 4 $^{o}$C for 3 hr. Samples containing anti-FLAG M2-conjugated agarose or protein G sepharose beads were washed three times by collecting the beads by centrifugation (2150 x g, 4 $^{o}$C, 2 min) before aspirating the supernatant and then resuspending the beads. Protein G metallic beads were washed three times by immobilising the beads using a magnet, aspirating the supernatant and resuspending the beads. Protein was removed from the antibody by boiling at 100 $^{o}$C for 5 min in 1 x SDS-PAGE sample buffer (Table 2.9). An equal volume of each sample was then loaded onto a gel for electrophoresis and western blot (Section 2.10).

One limitation to the immunoprecipitation protocol presented here is that the experiments lack IgG controls. An IgG control is used to eliminate the possibility that the interaction between two proteins of interest is non-specific. Proteins that interact will only be immunoprecipitated by an antibody with an epitope against one of the proteins of interest. Proteins that bind non-specifically to the antibody or the beads will still be present in an immunoprecipitation where the epitope of the antibody does not

recognise either of the proteins of interest. Therefore, without IgG controls the interactions cannot be fully confirmed as specific.

### 2.6.1 Optimised Immunoprecipitation Protocol for Mass Spectrometry

MCF7 cells were seeded at a density of 5 x $10^6$ cells per dish. A total of 10 x 15 cm dishes were used for each condition. Cells were transfected with 5 μg pcDNA3 or FLAG-RASSF1A per dish using PEI transfection reagent. Cell lysates were prepared 48 hr post transfection by scraping cells with a rubber cell scraper into 0.5 ml lysis buffer (300 mM NaCl, 1 % (v/v) Nonidet P40 (NP40), 20 mM Hepes pH 7.5 supplemented with protease inhibitor cocktail (Roche), 50 mM sodium fluoride, 10 mM β-glycerophosphate and 0.5 mM sodium orthovanidate) per plate. Lysates were rotated end over end for 10 min at 4 $^{o}$C. before being cleared by centrifugation (20817 x g, 10 min, 4 $^{o}$C). 10 μl anti-FLAG M2-conjugated protein G agarose beads (Sigma) per plate were cross linked for 1 hr at RT with DMP solution (50 mM DMP, 200 mM Hepes pH7.5). The cross linking reaction was terminated with 50 mM Tris pH 7.5. Cross-linked beads were washed with lysis buffer prior to mixing with the cleared lysates. The antibody and lysate mixture was incubated for 2 hr at 4 $^{o}$C on a rotating wheel. The beads were washed four times with wash buffer (300 mM NaCl, 1 % Nonidet P40 (NP40), 20 mM Hepes pH 7.5) before transfer into a clean 1.5 ml microfuge tube. Protein was eluted from the antibody with two 25 min washes of 1 mg/ml, 3 x FLAG peptide (Sigma) at 4 $^{o}$C. Eluates were then concentrated to approximately 30 μl using Amicon Ultra Centrifugal filter units (Millipore) before being loaded onto a NuPAGE 10 % SDS-PAGE gel and separated by electrophoresis (Section 2.10). The gel was then stained with colloidal coomassie blue stain and destained with distilled $H_2O$ to identify protein bands for excision. The gel was imaged on the Licor Odyssey and bands of interest excised using a clean scalpel in a lamina flow hood.

## 2.7 Mass Spectrometry

Gel pieces excised from the SDS-PAGE gel were subjected to tryptic digest and the resulting peptides extracted from the gel following the *in-gel* digest protocol from the Kessler lab (www.ccmp.ox.ac.uk/protocols-and-tools). Briefly, samples were incubated overnight at RT in wash buffer (50 % (v/v) methanol, 5 % (v/v) acetic acid) before dehydration with acetonitrile. Samples were reduced (10 mM DTT, 10 min, RT) and alkylated (50 mM iodoacetamide, 10 min, RT) prior to digestion overnight at 37 $^{o}$C with 20 μg/μl trypsin (Promega) (in 50 mM ammonium bicarbonate). Excess trypsin was removed and gel pieces incubated at 37 $^{o}$C overnight. Trypsinised protein was extracted from the gel with extraction buffer 1 (50 % (v/v) acetonitrile, 5 % (v/v) formic acid, 10 min, RT) followed by extraction buffer 2 (85 % (v/v) acetonitrile, 5 % (v/v) formic acid, 10 min RT). Extracted peptides were analysed by online nanoflow liquid chromatography tandem mass spectrometry using a Dionex U300 (fitted with a Pepmap C18 column and eluted with a linear gradient of acetonitrile) connected to a Bruker HCTultra ETD II ion trap trough a nanoelectrospray ion source.

Identified peptides were analysed using Mascot MS/MS ion search (www.matrixscience.com).

## 2.8 HPLC

A mix of standard proteins (Ovalbumin (43 kDa), Conalbumin (75 kDa), Aldose (158 kDa) and Thyroglobulin (669 kDa)) (GE healthcare) were used to generate a standard curve. Both the standard proteins and the eluted protein from 2.6.1 were filtered through a Millipore Millex® GV 0.22 μm filter before injection onto Agilent Zorbax GF-450 column. The column was fitted to an Agilent 1200 series machine and equilibrated with running buffer (10 mM Tris, 150mM NaCl, pH 7.0). HPLC was carried out at 4 $^{o}$C. Samples were separated at a flow rate of 1 ml/min and the absorbance was measured continuously at 210 nm. Protein samples were collected at a rate of 10 fractions per minute into 96 well plates. Fractions were stored at -20 $^{o}$C.

## 2.9 Preparation and Quantitation of Cell Lysates

Cells were washed two times with PBS before being lysed directly in the plate by mixing cells with laemmli lysis buffer (Table 2.9) supplemented with 1 x protease inhibitor (Roche) using a rubber cell scraper (Sarstedt). Lysates were transferred to a 1.5 ml microfuge tube and boiled at 100 $^{o}$C for 10 min. Lysates were either used straight away or stored at -20 $^{o}$C for later use.

The protein concentration of cell lysates was determined using Bradford assay. A standard curve of concentrations was generated using Bovine Serum Albumin (BSA) (Thermo Scientific), ranging from 0 – 2000 μg/ml. Lysates were diluted 1/10 with water and 5 μl of each sample was added to the wells of a 96 well plate. 250 μl of Bradford reagent (BioRad) was added to each of the samples and the absorbance of each well determined at wavelength 595 nm using a plate reader (BMG POLARstar Omega). The standard curve was used to calculate the actual concentration of the sample in μg/μl allowing normalisation of the samples.

An equal amount of protein from each sample was combined with an appropriate volume of 4 x SDS-PAGE sample buffer (Table 2.9) and the volume of each sample equalised with Laemmli lysis buffer. Samples were boiled for 5 min, 100 $^{o}$C before loading onto an SDS-PAGE gel for electrophoresis. Samples were stored at -20$^{o}$C.

## 2.10 SDS-PAGE and Western Blot

Protein samples were loaded onto a gel and separated by SDS-PAGE. NuPAGE® pre-cast gels (10 % or 4-12 %) (Life Technologies) were loaded into a Novex® mini cell tank filled with Novex® MOPS running buffer. Self-cast gels made with a 10 or 15 % resolving gel (10 or 15 % final concentration of 30 % (v/v) acrylamide: 0.8 % (v/v) Bis solution, 375 mM Tris pH 8.8, 0.1 % (w/v) SDS, 0.1 % (w/v) APS, 0.01 % (v/v)

TEMED) and a 4 % stacking gel (4 % final concentration of 30 % (v/v) acrylamide: 0.8 % (v/v) Bis solution, 125 mM Tris pH 6.8, 0.1 % (w/v) SDS, 0.1 % (w/v) APS, 0.01 % (v/v) TEMED) (Laemmli 1970) and were loaded into BioRad Mini Protean® TetraCell gel tanks filled with SDS-PAGE running buffer (Table 2.9). Samples were loaded using gel loading tips (alpha labs), along with See Blue® plus 2 prestained markers (Life Technologies) for the NuPAGE® pre-cast gels and Fermentas PageRuler™ prestained protein ladder (Thermo) for the self-cast gels. Gels were run at a constant voltage of 120 V.

After being separated using gel electrophoresis, the protein samples were transferred from the gel onto a polyvinylidene fluoride (PVDF) membrane (Immobilon™ from Millipore). Two different PVDF membranes were used. PVDF-P was used for membranes developed by chemiluminescence and PVDF-F was used for membranes developed by fluorescence. Both membranes were activated using 100 % methanol before the transfer was performed using the BioRad mini gel blotting system, in which the gel and membrane were sandwiched between two sheets of Whatmann paper and compacted using sponges. The transfer was performed in transfer buffer (Table 2.9) at a voltage of 100 V for between 65 min and 80 min depending on the mass of protein being blotted for.

Following transfer, PVDF-P membranes were blocked for 1 hr on an orbital shaker at room temperature in 5 % skimmed milk (w/v) PBS-T (Table 2.9). PVDF-F membranes were blocked in Licor blocking buffer diluted 1:1 with PBS. After blocking the primary antibody was added to the membrane. The primary antibodies were used as outlined in Table 2.4. All primary antibodies were incubated with the membrane overnight at 4 $^{o}$C.

Following primary antibody incubation, membranes were washed (4 x 5 min) in PBS-T on an orbital shaker before being incubated with either HRP-conjugated secondary antibody for PVDF-P or fluorescently-tagged secondary antibody for PVDF-F (Table 2.4). Membranes were incubated at room temperature for 1.5 hr prior to being washed (4 x 5 min in PBS-T). PVDF-F membranes were kept in the dark after addition of the secondary antibody to prevent bleaching of the fluorescence signal.

PVDF-F membranes were washed once in PBS before being developed using the Licor Odyssey. Images were quantified using Licor Odyssey software where indicated.

PVDF-P membranes were exposed to either Enhanced chemiluminescence (ECL) solution (Thermo) for normal exposure or ECL advance (GE healthcare) for low abundance proteins according to manufacturer's protocol. Membranes were exposed to film (KODAK) and developed using a XoGraph processor (XoGraph Imaging Systems) before being scanned onto a computer. Blots were quantified using Image J software where indicated.

In order to allow membranes to be probed with multiple primary antibodies, membranes were stripped for 30 min at 50 $^{o}$C, using stripping buffer (Table 2.9). Membranes were washed twice with PBS-T before re-blocking and incubation with primary antibody.

## 2.11 Immunofluorescence

Cells plated onto glass coverslips (Fisher), were washed twice with PBS and fixed in 4 % (w/v) Paraformaldehyde solution (16 % (w/v) Paraformaldehyde diluted in PBS) for 15 min at RT. Coverslips were washed twice with PBS before the cells were permeabilised with 0.2 % (v/v) Triton-X solution (in PBS) for 5 min at RT. Cells were washed with PBS and blocked using 0.2 % (v/v) Fish Skin Gelatin (FSG) (in PBS) for 30 min at RT. Cells were incubated for 2 hr at 37 $^{o}$C with primary antibody (Table 2.4). Cells were washed with PBS (3 x 5 min) before the secondary antibody was added (Table 2.4) and the cells incubated for a further 1 hr at 37 $^{o}$C. Cells were washed with PBS (3 x 5 min) and the coverslips mounted onto microscope slides using Prolong® Gold antifade reagent with DAPI (Life Technologies). Slides were sealed with clear nail varnish and analysed by microscopy. Light microscopy was done using a Nikon 90i microscope using the NIS elements software or a Leica DM IRBE microscope using Simple PCI6 software. Confocal microscopy was done using a Zeiss LSM710 using Zeiss ZEN software.

## 2.12 Migration Assays

### 2.12.1 Scratch Wound Assay

Confluent cell monolayers were wounded with a p200 pipette tip and washed three times with PBS to remove any floating cells. Cells were imaged at 0 hr and then at 24 hr for HeLa cells or 14 hr for MDA-MB-231 cells using a Nikon TE-2000 Eclipse inverted microscope. The size of the wound was calculated by measuring at 4 points per image. The size of the wound was measured at 0 hr and the end time point by hand or using Nikon NIS elements software. These measurements were used to calculate the distance that the cells had migrated. Statistical analysis of wound closure was carried out using a Student's T-test.

### 2.12.2 Transwell Assay

Cells were trypsinsed, collected by centrifugation (500 x g, 5 min) and resuspended in DMEM without supplements. Complete DMEM was added to the bottom chamber of the transwell plate before 2500 cells were plated into the top chamber. Transwell plates (BD biosciences) with an 8 μm pore size were used in each experiment. Cells were allowed to migrate at 37 $^{o}$C, 5 % $CO_2$ for 18 hr in a humidified incubator before cells were fixed in 100 % methanol for 10 min at RT. Transwell membranes were removed from the bottom of the top chamber and mounted onto microscope slides using Prolong® Gold antifade reagent with DAPI (Life Technologies). Cells that had migrated through the membrane were then counted using a Nikon 90i microscope at 20 X magnification. Five fields of view were counted per membrane and three membranes were used in each experiment.

### 2.12.3 ExCELLigence Transwell Assay

160 μl complete DMEM with or without doxycycline (2 μg/ml) (SIGMA) was added to the bottom chamber of an exCELLigence CIM plate. The top chamber was then attached to the bottom chamber and 50 μl of DMEM without supplements was added to each well. The plate was loaded into the exCELLigence analyser and a blank run was done to zero the machine.

Cells were trypsinised, collected by centrifugation (500 x g, 5 min) and resuspended in DMEM without supplements in the presence or absence of doxycycline (2 μg/ml). 40,000 cells were plated into the top chamber and allowed to settle for 30 min at room temperature before the plate was loaded into the exCELLigence analyser. Cells were incubated in the machine at 37 $^{o}$C, 5 % $CO_2$ in a humidified incubator and measurements were taken every 15 min for 25 hr. The exCELLigence analyser measures electrical impedance across a circuit that is positioned between the two chambers. As cells migrate towards the bottom chamber they come into contact with the circuit. This creates a signal that is measured by the analyser. Measurement of the change in the signal over time is used to determine the rate of migration.

## 2.13 Mammosphere Growth Assay

Matrigel (BD Biosciences) was thawed overnight on ice at 4 $^{o}$C. Thawed matrigel was mixed in a ratio of 1:1 with ice cold DMEM without supplements on ice using pipettes stored at -20 $^{o}$C overnight. 300 μl of the Matrigel mix was then pipetted into each well of a pre-cooled 24 well plate. Matrigel was then allowed to solidify by incubating at 37 $^{o}$C for 30 min.

MDA-MB-231 cells were trypsinised, collected by centrifugation (500 x g, 5 min) and resuspended in complete DMEM before 2500 cells were plated onto the solidified Matrigel in complete media. Plates were incubated at 37 $^{o}$C, 5 % $CO_2$ in a humidified incubator to allow mammosphere growth. The media was changed every 3 days for 9 days at which point images were taken using a Nikon TE2000 Eclipse microscope. Image J was used to quantify the number of mammospheres per field, average size of the

mammospheres and the aggressiveness of the mammospheres. An aggressive mammosphere was defined as one with greater than five projections into the surrounding matrigel (Figure 6.10B).

## 2.14 Cloning

The constructs in Table 2.5 were produced using standard cloning techniques. Sequences of interest were amplified by PCR using Platinum *Pfx* (Life Technologies) (PCR mix Table 2.6, PCR conditions Table 2.7) using cloning primers in Table 2.8 and subcloned into the required vector. Amplified DNA was purified using agarose gel purification (Qiagen Gel purification kit) following manufacturer's protocol. Restriction endonucleases, purchased from Roche, were used to digest purified PCR product and the vector into which the PCR product was to be ligated following manufacturer's instructions (Table 2.5). Digested DNA was purified by gel purification before the vector was dephosphorylated and the insert and vector ligated using the Roche rAPid DNA dephosphorylation and ligation kit. Ligated DNA was transformed by heat shock (42 $^{o}$C, 30 sec) into DH5$\alpha$ *E.coli* (Life Technologies) and plated onto agar plates containing an appropriate antibiotic selection agent. Single clones were picked and the DNA purified using a mini-prep kit (Qiagen). Correct insertion of the PCR product into the vector was tested by double restriction digest, to show reconstitution of the cut sites, and also by Sanger sequencing (Source Biosciences). Large scale DNA purification was done by Maxi-prep (Qiagen).

Site-directed mutagenesis was carried out using Agilent QuickChange II Site-directed Mutagenesis Kit following the manufacturer's protocol using Site directed mutagenesis primers in Table 2.8. Positive clones were confirmed by Sanger sequencing for the presence of the introduced mutation.

## 2.15Reverse-Transcriptase PCR (RT-PCR)

RNA was extracted from cells using Qiagen RNeasy® mini kit. Briefly, cells were lysed by mixing with lysis buffer using a rubber cell scraper. Lysates were homogenised using Qiagen QIAshredder spin column. RNA was then purified using an RNeasy column and eluted into nuclease-free water.

RT-PCR was done using the Superscript III one-step RT-PCR kit (Life Technologies), which used a two-step process to convert mRNA to cDNA before amplifying a specific cDNA sequence. 1 μg RNA was amplified using RT-PCR primers (Table 2.8). Amplified cDNA was resolved by agarose gel electrophoresis.

## 2.16 Statistics

Data for statistical analysis was entered into a spread sheet (Microsoft Excel). Data was analysed using standard Excel formulae for the mean, standard deviation and ρ-values were generated using a student's T-test. Standard Error Mean (SEM), which was used for error bars, was calculated by dividing the standard deviation by the square root of the number of measurements made.

## 2.17 Tables

| Name | Type | Source |
|---|---|---|
| MCF7 | Breast adenocarcinoma | CRUK |
| HeLa | Cervix adenocarcinoma | CRUK |
| 293T | Human Embryonic Kidney | Madalena Tarsounas |
| H1299 | Non-small cell lung carcinoma | CRUK |
| U2-OS | Osteosarcoma | ATCC |
| SW480 | Colorectal adenocarcinoma | ATCC |
| MDA-MB-231 | Breast adenocarcinoma | Ester Hammond |
| RKO | Colon Carcinoma | Ester Hammond |

**Table 2.1. Details of the Cell Lines Used in this Study.** Table indicates the cell line, the tissue from which they are derived and where they were acquired from.

| siRNA | Sequence(s) (sense) | Supplier |
|---|---|---|
| Non-targeting sequence 2 | Not available | Thermo Scientific (D-001210-02) |
| siGENOME SMARTpool Human RASSF1 (11186) | ACGCACAAGGGCACGUGAA CAAGGACGGUUCUUACACA GCAAGAAGCCACCCUCCUU CUACAUAACUUCCUACGUA | Thermo Scientific (M-017219-01) |
| RASSF1_1 | ACGCACAAGGGCACGUGAA | Eurofins MWG OPERON |
| RASSF1_2 | CAAGGACGGUUCUUACACA | Eurofins MWG OPERON |
| siGENOME SMARTpool Human FAM120A (OSSA) (23196) | GCGUAUGACUCUGAUUAUG GUUAUUCGAUUUAAGAGAG AGGCAGCUGUCUUAAAUAA GCUAUCAGCUCUCUUCAUG | Thermo Scientific (M-014186-01) |
| RASSF1A | GACCUCUGUGGCGACUUCA | Eurofins MWG OPERON |
| RASSF1C1 | UGACCUGGAGCAGCACGACtt | Eurofins MWG OPERON |
| RASSF1C2 | GAGGACUCGGACUCGGAGCtt | Eurofins MWG OPERON |
| MST2 | GGAUAGUUUUUCAAAUAGGtt | Ambion |
| LATS1 | UAGCAUGGAUUUCAGUAAUUU | Thermo Scientific |
| TP53 | GACUCCAGUGGUAAUCUACtt | Eurofins MWG OPERON |

**Table 2.2. siRNA Sequences Used During this Project.** RASSF1_1 and RASSF1_2 are the two sequences from the RASSF1 SMARTpool siRNA used in Figure 6.7 and Figure 8.3.

| Cell Line | Expression | Vector | Selection agent |
|---|---|---|---|
| H1299 (containing Tet ON plasmid, produced by Dr Karen Yee) | Inducible Empty vector (KY) | pTET Tight empty | Puromycin (1 μg/ml) |
| | Inducible FLAG-RASSF1A (KY) | pTre Tight FLAG-RASSF1A | Puromycin (1 μg/ml) |
| | Inducible FLAG-RASSF1C (SS) | pTre Tight FLAG-RASSF1C | Puromycin (1 μg/ml) |
| | Inducible FLAG-RASSF1C A63S (SS) | pTre Tight FLAG-RASSF1C A63S | |
| MDA-MB-231 | Empty vector (SS) | pcDNA3 | Puromycin (0.6 μg/ml) |
| MDA-MB-231 | MYC-RASSF1C (SS) | pCMV-MYC-RASSF1C | Puromycin (0.6 μg/ml) |

**Table 2.3. Stable Cell Lines Produced That Were Used in the Project**. H1299 cells stably expressing a regulatory plasmid (pTET ON) were transfected with expression plasmid containing a Tetracycline response element. KY indicates cell lines made by Dr Karen Yee, SS indicates cell lines made by Simon Scrace. pTET ON and pTre Tight vectors were purchased from Clontech (BD Biosciences). The pCMV-MYC-tag vector was purchased from Stratagene (Agilent).

| Antibody | Company | Catalogue No. | Use (Dilution) |
|---|---|---|---|
| **Primary Antibodies** | | | |
| RASSF1 | Santa Cruz Biotechnology | Sc-58470 | WB (1/500) IP (1/250) |
| KRS1/2 | Santa Cruz Biotechnology | Sc-6211 | IP (1/250) |
| MST2 (N-term) | Epitomics | 1943-1 | WB (1/2000) |
| Phospho-MST1 Thr183 MST2 Thr180 | Cell Signalling Technology | #3681 | WB (1/1000) |
| Anti-phosphotyrosine (4G10) | Millipore | 05-1050 | IP (1/250) |
| ZO-1 | Invitrogen | 339100 | IF (1/100) |
| CBLL1 (M-25) (HAKAI) | Santa Cruz Biotechnology | Sc-101912 | WB (1/1000) |
| RASSF1C | Abcam | Ab24419 | WB (1/1000) |
| GM130 | Abcam | Ab52649 | IF (1/100) |
| Csk (C74C1) | Cell Signalling Technology | #4980 | WB (1/1000) IP (1/250) |
| Partitioning-defective 3 (PAR3) | Millipore | 07-330 | IF (1/100) |
| DYKDDDK Tag (FLAG) | Cell Signalling Technology | #2368 | WB (1/2000) |
| Phospho-catenin δ-1 Tyr 228 | Cell Signalling Technology | #2911 | WB (1/1000), IF (1/100) |
| phospho-Myosin Light Chain 2 Ser19 | Cell Signalling Technology | #3671 | IF (1/100) |
| FAK | BD | 610087 | WB (1/1000) |
| Phospho-FAK Y397 | Abcam | Ab4803 | WB (1/1000) |
| E-Cadherin | Cell Signalling Technology | #4065 | WB (1/1000), IP (1/250), IF (1/100) |
| Anti-p120 catenin | BD | 610134 | WB (1/1000), IF (1/100) |
| β–catenin | Santa Cruz Biotechnology | Sc-7199 | WB (1/1000), IF (1/100) |
| FAM120A (OSSA) | Abcam | Ab83909 | WB (1/1000) |
| Occludin (H-279) | Santa Cruz Biotechnology | Sc-5562 | IF (1/100) |
| Phospho-Paxillin Tyr118 | Cell Signalling Technology | #2541 | WB (1/1000) |
| Phospho-SRC Family Tyr416 | Cell Signalling Technology | #2101 | WB (1/1000), IF (1/50) |
| SRC Family Kinase | Cell Signalling Technology | #2108 | WB (1/1000), IP (1/250), IF (1/100). |
| SRC (L4A1) | Cell Signalling Technology | #2110 | WB (1/1000) |
| Csk (C-20) | Santa Cruz Biotechnology | Sc-286 | WB (1/1000) |
| FLAG M2 | Agilent | 200472-21 | WB (1/2000), IF (1/100) |
| GAPDH | Epitomics | 2251-1 | WB (1/6000 Licor) (1/10000 (X-ray) |

| | | | |
|---|---|---|---|
| HA Tag | Millipore | 05-904 | WB (1/1000), IP (1/250) |
| MYC Tag | Millipore | 05-724 | WB (1/1000), IP (1/250) |
| DDB1 | Abcam | Ab9194 | WB (1/1000) |
| XPA | Santa Cruz Biotechnology | Sc-853 | WB (1/1000) |
| **Secondary Antibodies** | | | |
| HRP conjugated anti-Mouse | Jackson ImmunoResearch | 315-035-048 | WB (1/2500-5000) |
| HRP conjugated anti-Rabbit | Jackson ImmunoResearch | 711-035-152 | WB (1/2500-5000) |
| Goat $\alpha$-mouse 680 nm | Licor | 926-32210 | WB (1/5000) |
| Goat $\alpha$-rabbit 800 nm | Licor | 926-32221 | WB (1/5000) |
| Alexa fluor® goat $\alpha$-mouse 488 nm | Invitrogen, Life Technologies | A-11001 | IF (1/500) |
| Alexa fluor® goat $\alpha$-rabbit 488 nm | Invitrogen, Life Technologies | A-11008 | IF (1/500) |
| Alexa fluor® goat $\alpha$-mouse 594 nm | Invitrogen, Life Technologies | A-11005 | IF (1/500) |
| Alexa fluor® goat $\alpha$-rabbit 594 nm | Invitrogen, Life Technologies | A-11012 | IF (1/500) |
| Alexa fluor® goat $\alpha$-rabbit 633 nm | Invitrogen, Life Technologies | A-31576 | IF (1/500) |
| Alexa fluor® goat $\alpha$-mouse 568 nm | Invitrogen, Life Technologies | A-21422 | IF (1/500) |

**Table 2.4. Antibodies Used During This Project.** Table indicates the technique the antibody was used for and dilution of the antibody used for that technique. WB: western blot, IP: immunoprecipitation, IF: immunofluorescence.

| Construct | Plasmid | 5' cloning site | 3' cloning site |
|---|---|---|---|
| pCMV4-OSSA-FLAG | pCMV-Tag 4 (stratagene) | BamH1 | HindIII |
| pCMV3-Myc-OSSA | pCMV-Tag 3 (Stratagene) | BamH1 | HindIII |
| pCMV3-Myc-RASSF1A (1-340 or Full Length (FL)) | pCMV-Tag 3 (Stratagene) | BamH1 | HindIII |
| pCMV3-Myc-RASSF1A 1-119 | pCMV-Tag 3 (Stratagene) | BamH1 | HindIII |
| pCMV3-Myc-RASSF1A 1-287 | pCMV-Tag 3 (Stratagene) | BamH1 | HindIII |
| pCMV-Myc-RASSF1A 120-287 | pCMV-Tag 3 (Stratagene) | BamH1 | HindIII |
| pCMV-Myc-RASSF1A 120-340 | pCMV-Tag 3 (Stratagene) | BamH1 | HindIII |
| pCMV-Myc-RASSF1A 287-340 | pCMV-Tag 3 (Stratagene) | BamH1 | HindIII |
| pCMV-Myc-RASSF1A A133S | pCMV-Tag 3 (Stratagene) | BamH1 | HindIII |
| pCMV-Myc-RASSF1C | pCMV-Tag 3 (Stratagene) | BamH1 | HindIII |
| pCMV-Myc-RASSF1C 1-50 | pCMV-Tag 3 (Stratagene) | BamH1 | HindIII |
| pCMV-Myc-RASSF1C 1-216 | pCMV-Tag 3 (Stratagene) | BamH1 | HindIII |
| pTre-Tight-FLAG-RASSF1C | pTre-Tight | BamH1 | HindIII |
| pTre-Tight-FLAG-RASSF1C A63S | pTre-Tight | BamH1 | HindIII |
| (pCMV)FLAG-RASSF1C A63S | pCMV-Tag 5 from Ortiz-vega et al. 2002 | n/a | n/a |
| peGFP-RASSF1C | peGFP-C1 | HindIII | BamH1 |
| pCMV5-FLAG-RASSF1A resist 1 | pCMV-Tag 5 from Ortiz-vega et al. 2002 | n/a | n/a |
| pCMV5-FLAG-RASSF1A resist 2 | pCMV-Tag 5 from Ortiz-vega et al. 2002 | n/a | n/a |
| pCMV5-FLAG-RASSF1C resist 1 | pCMV-Tag 5 from Ortiz-vega et al. 2002 | n/a | n/a |
| pCMV5-FLAG-RASSF1C resist 2 | pCMV-Tag 5 from Ortiz-vega et al. 2002 | n/a | n/a |

**Table 2.5. Constructs Produced During D.Phil Project.** Table shows the name of the construct produced, the backbone of the vector used to produce the construct and the cloning sites between which the insert was ligated. n/a replaces cut site information where construct was made by site-direct mutagenesis.

| Component | Amount (μl) | Final Concentration |
|---|---|---|
| dsDNA template (10 ng/ml) | 2 | 0.4 ng/ml |
| Forward primer (10 ng/ml) | 1.5 | 0.3 ng/ml |
| Reverse primer (10 ng/ml) | 1.5 | 0.3 ng/ml |
| Reaction buffer (10 x) | 5 | 1 x |
| dNTP mix (25 mM) | 0.6 | 0.3 mM |
| 50 mM $MgSO_4$ | 1 | 1 mM |
| Platinum *Pfx* enzyme (2500 U/ml) | 0.4 | 1 unit |
| Nuclease-free water | 33 | |

**Table 2.6. Standard PCR Mix for Cloning With Platinum *Pfx* PCR Kit** (Invitrogen).

| Step | Temperature ($^oC$) | Time (sec) | Cycles |
|---|---|---|---|
| Denaturation | 94 | 60 | 1 |
| Amplification | 94 | 30 | 30 |
| | 55 | 30 | |
| | 68 | 60 per 1 kb | |
| Final Extension | 68 | 300 | 1 |

**Table 2.7. Standard PCR Protocol Used for Platinum *Pfx* PCR Kit.**

| Primer Name | Sequence | Construct |
|---|---|---|
| **Cloning Primers** | | |
| OSSA_FWD | AATAATGGATCCATGGGCGTGCAGGGCTTCCAGGAC | pCMV4-OSSA-FLAG |
| OSSA_REV | AATAATAAGCTTCTCTTCTTTATTTAAGACAGCTGCCTCC | pCMV4-OSSA-FLAG |
| MYCOSSA rev | AATAATAAGCTTTTACTCTTCTTTATTTAAGACAGCTGCCTC | |
| MYCR1Astart_1 | AATAATGGATCCATGTCGGGGGAGCCTGAGCTC | RASSF1A truncation |
| MYCR1Astart_120 | AATAATGGATCCGACGAGCCTGTGGAGTGGGAG | RASSF1A truncation |
| MYCR1Astart_288 | AATAATGGATCCTCTGGGGAGGTGAACTGGGACGCC | RASSF1A truncation |
| MYCR1Aend_119 | AATAATAAGCTTCTTCACGTTCGTGTCCCGCTCCACCG | RASSF1A truncation |
| MYCR1Aend_287 | AATAATAAGCTTGTCATTTTCCTTCAGGACAAAGCTCAG | RASSF1A/C truncation |
| MYCR1Aend_340 | AATAATAAGCTTCCCAAGGGGGCAGGCGTGCAGGGC | RASSF1A/C truncation |
| BAMH1R1Cfor | AATAATGGATCCATGGGCGAGGCGGAGGCGCCT | RASSF1C truncation |
| R1CFLAGBamH1 | GACGGATCCCGCACCATGGACTACAAGGACGACGATGACAAAGGCGAGGCGGAGGCG | pTre-Tight FLAG-R1C |
| GFP-R1CFOR | AATAATAAGCTTCGATGGGCGAGGCGGAGGCGCCT | peGFP-RASSF1C |
| GFPR1CREV | AATAATGGATCCTCACCCAAGGGGGCAGGCGTG | peGFP-RASSF1C |
| **Site-Directed Mutagenesis** | | |
| RASSF1CA63S FOR | ACACCTGACCTTTCTCAA**T**CTGAGATTGAGCAGAAGATC | FLAG-RASSF1C A63S |
| RASSF1CA63SREV | GATCTTCTGTGCTCAATCTC**A**GATTGAGAAAGGTCAGGTGT | FLAG-RASSF1C A63S |
| siRESISTR1For1 | GTGCTGTCACGCACAAG**A**GCACGTGAAGTCATTGAGG | FLAG-RASSF1A Resist 1 |
| siRESTSTR1Rev1 | CCTCAATGACTTCACGTGC**T**CTTGTGCGTGACAGCAC | FLAG-RASSF1A Resist 2 |
| siRESISTR1For2 | AGCTTGAACAAGGACGG**A**TCTTACACAGGCTTCATC | FLAG-RASSF1C Resist 1 |
| siRESISTR1Rev2 | GATGAAGCCTGTGTAAGA**T**CCGTCCTTGTTCAAGCT | FLAG-RASSF1C Resist 2 |
| **RT-PCR primers** | | |
| RASSF1A For | AGCGTGCCAACGCGCTGCGCAT | |
| RASSF1A Rev | CAGGCTCGTCCACGTTCGTGTC | |
| RASSF1C For | GACCTGGAGCAGCACGACGAG | |
| RASSF1C Rev | AACAGCTTCCGCAAGTACAC | |
| GAPDH For | CAGACCTACTCAGGGATTC | |
| GAPDH Rev | GAGCCAGACGCTGCTTTGT | |

**Table 2.8. Primers Used For Cloning, Site-Directed Mutagenesis and RT-PCR.** Table shows the name of primer, the primer sequence and the construct generated using the primer. Site-directed mutagenesis target site is highlighted in bold and underlined.

| Buffer Name | Contents | For 1 l |
|---|---|---|
| 4 X DNA Loading Buffer | 50 % (v/v) Glycerol<br>1 X TAE<br>Orange G | 500 ml Glycerol<br>500 ml 1 x TAE<br>Orange G to colour. |
| 4 x SDS-PAGE Sample Buffer | 40 % (v/v) Glycerol<br>250 mM Tris-HCl pH 6.8<br>8 % SDS<br>11.56 mM Bromophenol Blue<br>8 % (v/v) 2-mercaptoethanol | 400 ml Glycerol<br>250 ml Tris-HCl pH 6.8<br>80 g SDS<br>2.5 g Bromophenol Blue<br>80 ml 2-mercaptoethanol |
| 5 % skimmed Milk blocking Solution | 5 % (w/v) skimmed milk powder in 1 x PBS-T | 50 g skimmed milk powder<br>1 l PBS-T |
| Immunoprecipitation lysis buffer | 150 mM NaCl<br>0.5 % (v/v) NP40<br>20 mM Hepes pH7.5 | 30 ml of 5 M NaCl<br>50 ml of 10 % (v/v) NP40<br>50 ml of 1 M Hepes pH7.5 |
| Laemmli Lysis Buffer | 50 mM Tris-HCl pH 6.8<br>2 % (w/v) SDS<br>1 x Protease inhibitor cocktail | 20 ml Tris-HCl pH 6.8<br>20 g SDS |
| LB Broth | 2 % (w/v) LB broth | 20 g LB broth |
| Licor Blocking Buffer Solution | 50 % (v/v) Licor Blocking Buffer Solution<br>50 % (v/v) PBS | 500 ml Licor blocking buffer<br>500 ml PBS |
| PBS (Phosphate-buffered Saline) | 137 mM NaCl<br>2.7 mM KCl<br>10 mM $Na_2HPO_4$<br>1.76 mM $KH_2PO_4$<br>pH7.4 | 8.01 g NaCl<br>0.2 g KCl<br>1.78 g $Na_2HPO_4$<br>0.27 g $KH_2PO_4$ |
| PBS-T (PBS Tween) | PBS as above<br>0.1 % (v/v) Tween 20 | 1 ml Tween per l of PBS |
| SDS-PAGE Running Buffer | 25 mM Tris<br>190 mM Glycine<br>0.1 % (w/v) SDS | 3.03 g Tris<br>14.4 g Glycine<br>1 g SDS |
| SDS-PAGE Sample Buffer | 10 % (v/v) Glycerol<br>62.5 mM Tris-HCl pH 6.8<br>2 % (w/v) SDS<br>2.89 mM Bromophenol Blue<br>2 % (v/v) 2-mercaptoethanol | 100 ml Glycerol<br>62.5 ml Tris-HCl pH 6.8<br>20 g SDS<br>0.625 g Bromophenol blue<br>20 ml 2-mercaptoethanol |
| Stripping Buffer | 62.5 mM Tris-HCl pH 6.8<br>2 % (w/v) SDS<br>0.7 % 2-Mercaptoethanol | 62.5 ml Tris-HCl pH 6.8<br>20 g SDS<br>7 ml 2-mercaptoethanol |
| TAE Buffer | 40 mM Tris<br>18 mM Glacial acetic acid<br>1 mM EDTA pH 8.0 | 4.48 g Tris<br>1.14 ml Glacial acetic acid<br>2 ml 0.5 M EDTA pH 8 |
| Western Blot Transfer Buffer | 25 mM Tris<br>190 mM Glycine<br>20 % (v/v) Methanol | 3.03 g Tris<br>14.4 g Glycine<br>200 ml Methanol |

**Table 2.9. Common Buffers Used During This Thesis**. All solutions are diluted in water unless stated.

# Chapter 3 Identification of Novel RASSF1A Binding Partners

## 3.1 Introduction

RASSF1A is a tumour suppressor involved in the regulation of a range of cellular functions from maintenance of cell cycle checkpoints to control of migration (Donninger, Vos et al. 2007). RASSF1A also promotes apoptosis through the activation of BAX by MOAP-1 and through MST1/2 (Baksh, Tommasi et al. 2005; Matallanas, Romano et al. 2007; Foley, Freedman et al. 2008). At the beginning of this project, characterisation of the roles of the ATM phosphorylation site at S131 of RASSF1A was in progress. This work, now published, showed that ATM was able to phosphorylate RASSF1A in response to ionising radiation (IR) induced DNA double-strand breaks (DSB). Phosphorylated RASSF1A directly interacted with MST2 leading to its activation and subsequently, stabilisation of a transcription factor complex involving p73 and the upregulation of the proapoptotic gene *PUMA* (Hamilton, Yee et al. 2009). At the same time as this another group identified a second, MST1/2-independent, DNA damage activated pathway through RASSF1A leading to the activation of p53 (Song, Song et al. 2008). A common feature of these signal pathways is the requirement for direct binding of RASSF1A to its target protein. We therefore wanted to identify novel proteins that interact with RASSF1A that may contribute to its role as a tumour suppressor.

In order to fully investigate how signalling pathways are integrated to elicit a cellular response we needed to accumulate information about the constituents of individual signalling complexes, their protein modifications and how they respond to stimuli. As scaffold protein that integrates a number of upstream and downstream signalling pathways, RASSF1A represents an ideal candidate for identifying links between distinct signalling pathways activated by DNA damage. We therefore sought to develop a mechanism through which we would be able to separate and identify RASSF1A complexes from cells.

## 3.2 The role of RASSF1A in the DNA Damage Response

Our group recently showed that in response to IR and cisplatin induced DNA damage, RASSF1A is phosphorylated and activated by ATM (Hamilton, Yee et al. 2009). Phosphorylated RASSF1A binds to and promotes the activation of MST2 initiating a signalling cascade through LATS1, leading to the stabilisation of a complex between YAP and p73. The YAP-p73 complex promotes apoptosis through the upregulation of p73 responsive genes such as *PUMA*. As part of this work a clonogenic cell survival assay in response to IR was done to confirm that RASSF1A did indeed regulate cell survival and cell growth in response to DNA damage. In a clonogenic cell survival assay loss of pro-apoptotic proteins will lead to a decrease in radiosensitivity. Loss of pro-survival proteins will have the opposite effect. We therefore predicted that loss of RASSF1A or components of the downstream hippo pathway, MST2 and LATS1, would lead to a decrease in radiosensitivity due to the loss of pro-apoptotic signals in response to ATM activation. HeLa cells were treated with non-targeting siRNA or siRNAs targeting RASSF1, MST2 or LATS1 before cells were exposed to 2 Gy IR and plated to allow colony formation (Figure 3.1). As expected, depletion of RASSF1, MST2 or LATS1 had no effect on colony formation in untreated cells, but led to a significant decrease in sensitivity of HeLa cells to IR. We also noted that loss of RASSF1 led to a greater decrease in radiosensitivity than the other hippo pathway members suggesting that other pathways from RASSF1, independent of MST2, may be activated in response to DNA damage. This is in keeping with the MST1/2 independent role for RASSF1A in activating p53 in response to DNA damage (Song, Song et al. 2008). We therefore sought to identify novel RASSF1A binding partners using an immunoprecipitation (IP) and mass spectrometry (MS) based screen.

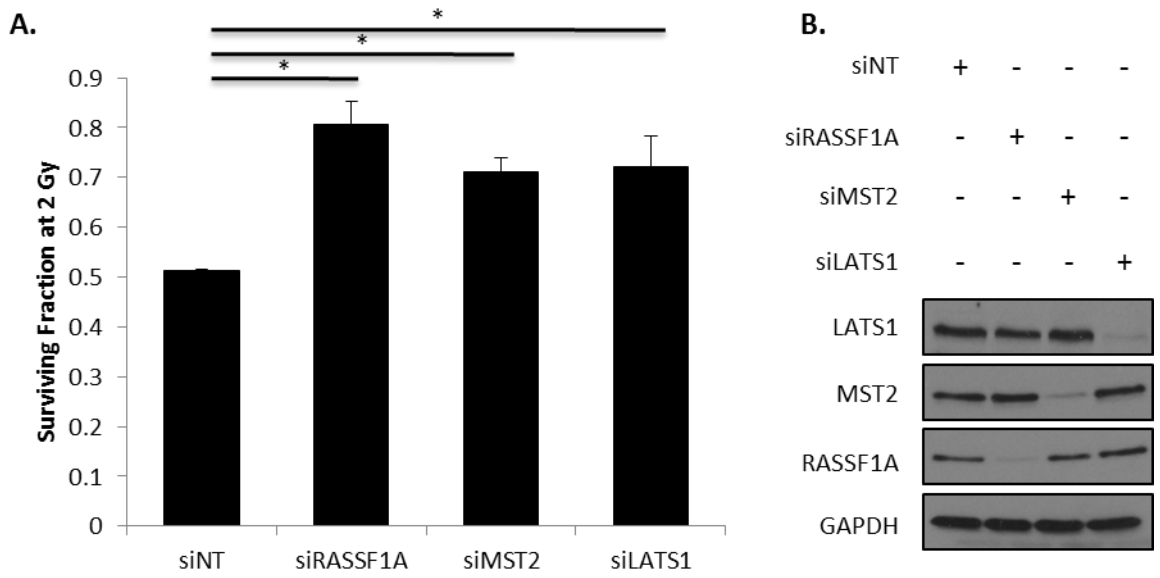


**Figure 3.1. The Hippo Pathway Promotes Apoptosis in Response to Ionising Radiation.** HeLa cells were transfected with non-targeting siRNA or siRNA targeting RASSF1, MST2 or LATS1. 24 hrs post transfection cells were re-plated at low density and allowed to settle before being exposed to 2 Gy IR. Cells were cultured in complete media to allow colonies to form. Colonies were fixed and stained with crystal violet solution before the clonogenic survival was calculated. **A.** Bar chart depicting the clonogenic survival of cells exposed to 2 Gy ionising radiation. Loss of RASSF1A, MST2 or LATS1 resulted in decreased radiosensitivity to IR. Data is from 3 independent experiments, each with 3 replicates. **B.** Cells not plated for colony formation were lysed with laemmli lysis buffer and analysed by SDS-PAGE and western blot for knockdown of target gene expression using antibodies against endogenous RASSF1A, MST2 and LATS1. GAPDH is a loading control. All three siRNA molecules efficiently knockdown expression of the target genes, but have no effect on colony formation in untreated cells. Blots are representative of three independent experiments.

## 3.3 Optimisation of Immunoprecipitation Protocol

Given that RASSF1A is a tumour suppressor that can regulate a number of MST-dependent and independent pathways in response to DNA damage we decided to identify novel DNA damage regulated binding partners of RASSF1A via large scale IP and MS. MCF7 cells were chosen as they lack endogenous RASSF1A, due to promoter methylation. This was considered preferential because it allowed the exogenous expression of FLAG-tagged RASSF1A. This would be beneficial because the anti-FLAG-tag antibody is much more efficient than the existing antibodies to endogenous RASSF1A and therefore the likelihood of identifying false positives due to non-specific binding to the antibody would be reduced.

Before carrying out the screen we confirmed that we could transfect the MCF7 cells with FLAG-RASSF1A. MCF7 cells were transfected with pcDNA3 or FLAG-RASSF1A before whole cell lysates were generated and analysed by western blot for FLAG-RASSF1A expression (Figure 3.2). A high level of expression of FLAG-RASSF1A was seen indicative of efficient transfection.

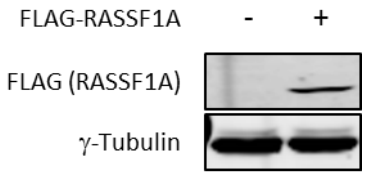


**Figure 3.2. FLAG-RASSF1A Expression in MCF7 Cells.** MCF7 cells ($2x10^5$ cells/plate) were transfected with empty vector or FLAG-RASSF1A using Lipofectamine 2000 transfection reagent and lysed 48 hrs post transfection with Laemmli lysis buffer. Lysates were resolved by SDS-PAGE and expression was confirmed by western blot with anti-FLAG antibodies. γ-tubulin is a loading control. Data is representative of one independent experiment.

Previous work in the lab had shown that phosphorylation of RASSF1A by ATM in response to DNA damage occurred by 30 min and that it lasted for at least 2 hrs when treated with 10 Gy IR. RASSF1A-dependent MST1/2 activity was also maintained for 2 hrs (Hamilton, Yee et al. 2009). Therefore, we chose 2 hrs post IR as the time point at which cells would be lysed for IP. ATM is activated by DSB and IR is known to create DSB in a dose dependent manner (Sachs, Hahnfeld et al. 1997). In order to generate maximum pathway activation through ATM and in keeping with the previous work, 10 Gy IR was chosen.

We aimed to optimise an IP protocol, maximising sensitivity and minimising background contamination to enable the identification of native protein complexes. The starting point for the IP was a scaled up version of a generic IP protocol used in our laboratory (See Chapter 2.6) Protocol optimisation covered four key areas: **1. Minimising Keratin Contamination**; **2. Increasing Sensitivity**, optimising the amount of protein precipitated; **3. Background**, removing contaminating proteins; and **4. Elution**, optimising FLAG-peptide elution efficacy. The steps taken to optimise each of these areas is detailed below and summarised in Figure 3.3.

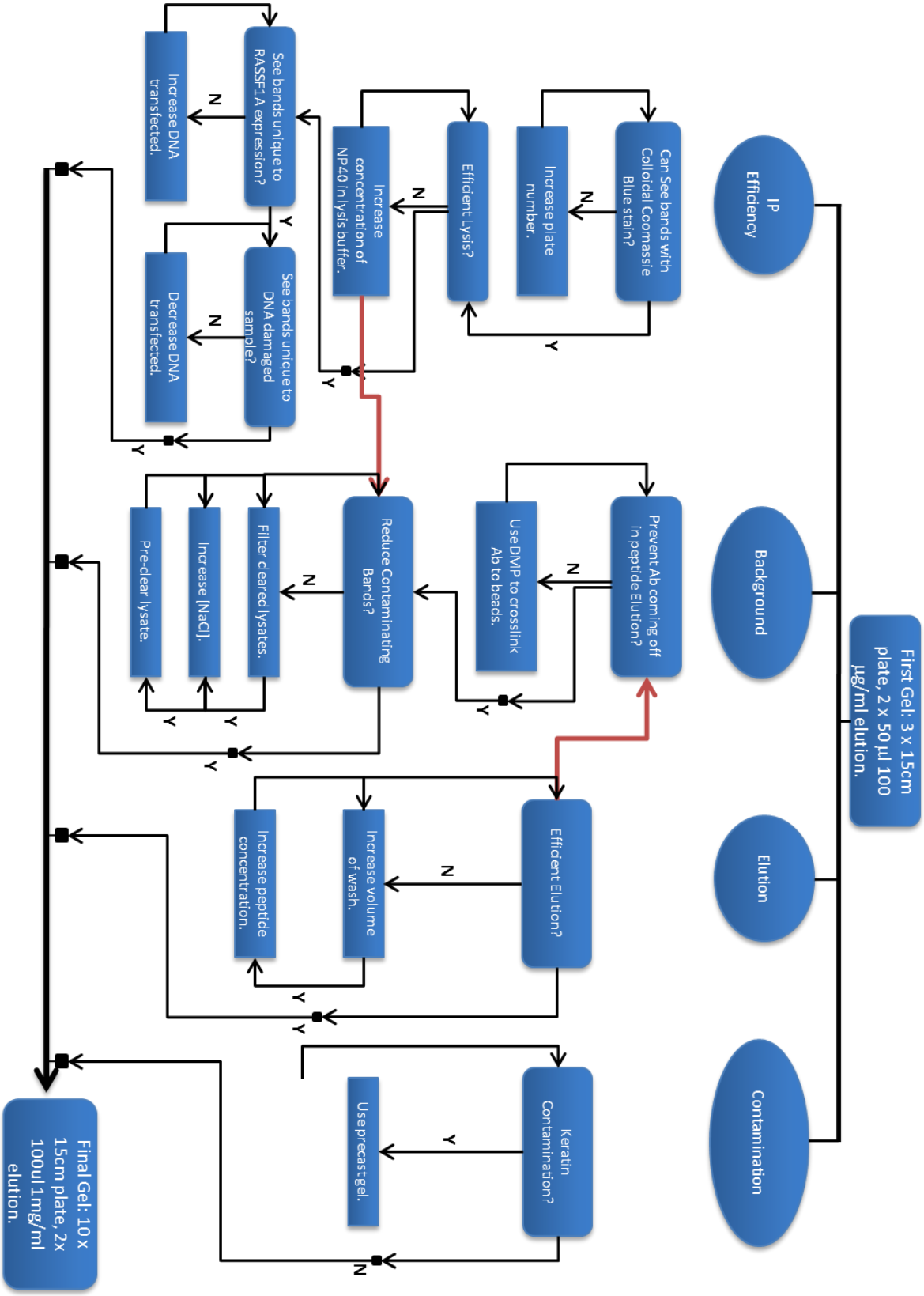


**Figure 3.3. Summary of the Immunoprecipitation-Mass Spectrometry Protocol Optimisations.** Flow diagram depicting the steps taken to optimise the immunoprecipitation used to pull down RASSF1A-associated proteins and identify them by mass spectrometry. The optimisation focused on increasing the efficiency of the immunoprecipitation and FLAG peptide elution, decreasing background contamination, and minimising the environmental keratin contamination of the mass spectrometry samples.

Keratin peptides are very abundant therefore can suppress the signal from lower abundance proteins in the mass spectrometer. Most Keratin contamination comes from dust particles in the air and from the chemicals used to make SDS-PAGE gels. The use of Pre-cast gels and excision and trypsinisation of identified bands within a lamina flow hood efficiently cut this down which was reflected in the MS data collected (data not shown).

Initially, 1 x $10^7$ MCF7 cells were seeded onto 3 x 15 cm dishes per condition and transfected with 10 µg of pcDNA3 or FLAG-RASSF1A. Cells were immunoprecipitated for 2 hrs with anti-FLAG M2-conjugated agarose beads. Two 25 min washes with 100 µg/ml, 1 x FLAG peptide were used to elute the protein. Under these conditions the protein level was too low because identification of many of the protein bands on the SDS-PAGE gel was only possible using silver stain. For confident identification of proteins by MS, protein bands should ideally be visualised by colloidal coomassie blue stain. This is because colloidal coomassie blue stain is less sensitive and so more protein is required for visualisation. Also, silver stain covalently crosslinks proteins preventing the extraction of trypsinised peptides from the gel and therefore preventing them from being able to be identified by MS. To counter this, the number of plates was increased to 10 per condition. Originally, the antibody was removed from the beads during the FLAG peptide elution. This masked a number of legitimate RASSF1A binding partners on the SDS-PAGE gel. To resolve this issue the homobifunctional imido ester, DMA, was used to covalently crosslink the antibody to the beads preventing the antibody from coming off the beads during the elution. This significantly reduced the background without affecting the binding of RASSF1A to the antibody.

To facilitate increased confidence in the MS identification by achieving greater sequence coverage and to aid the identification of low affinity interactions we aimed to maximise protein concentration in the IP. To increase the yield of protein in the IP the NP40 concentration of the lysis buffer was increased from 0.5 % to 1 %. The increase in NP40 was associated with an increase in the amount of protein seen in the gel (Before: Figure 3.4, After: Figure 3.5). However, it also increased the level of contaminating bands. This was addressed by increasing NaCl concentration from 150 mM to 300 mM which reduced

the background beyond that left by the previous steps (Figure 3.6). This is probably due to a greater disruption of weaker non-specific ionic interactions between contaminating proteins and the antibody or agarose beads. Further removal of background contamination was achieved by filtering the lysate to remove particles and aggregates (data not shown).

Elution efficiency was evaluated by the percentage of protein that was eluted compared to that left on the beads. Elution efficiency was improved by switching from a short competitive peptide to one with three tandem repeats of the FLAG sequence (3 x FLAG peptide). Increasing the volume of the wash and increasing the concentration of the peptide improved efficiency of the elution (Before: Figure 3.4, After: Figures 3.5 and 3.6).

We aimed to identify alterations that occur upon DNA damage. Initially no variation was seen in the banding pattern. It is known that when RASSF1A is expressed at a high level, binding partners that are normally induced by a stimulus are able to be immunoprecipitated in the absence of stimulus. Decreasing the amount of RASSF1A transfected from 10 μg to 5 μg per 15 cm dish enabled us to visualise differences between constitutive and DNA damage induced RASSF1A binding partners.

The final optimised protocol gave a clean gel with efficient elution of target proteins in which DNA damage associated RASSF1A binding proteins could be identified (Figure 3.6). The final protocol can be seen in Chapter 2.6.1. Figure 3.6A also shows a potential phospho-shift of RASSF1A (Figure 3.6A Bands 13), increasing our confidence that the binding partners identified are interacting with the DNA damage activated, ATM phosphorylated, RASSF1A protein.

Throughout the IP optimisation three conditions were analysed, control, RASSF1A and RASSF1A plus DNA damage. We confirmed that the proteins precipitating in the RASSF1A plus DNA damage condition were specific to RASSF1A by repeating the IP with a fourth condition control plus DNA damage (Figure 3.7). As expected no further proteins were precipitated in the control plus DNA damage sample.

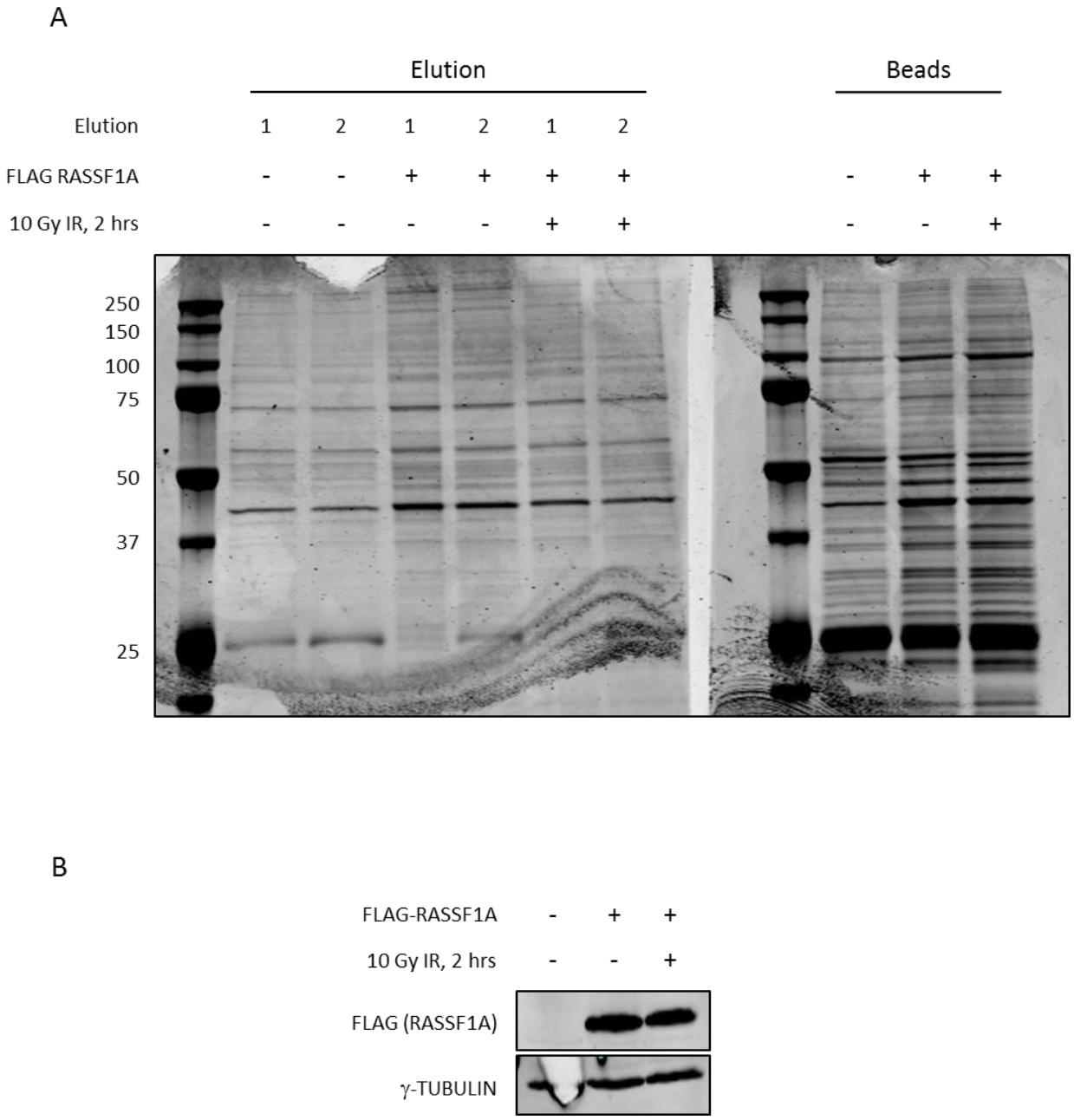


**Figure 3.4. Optimisation of Immunoprecipitation Protocol Gel 1.** MCF7 cells (5 x $10^6$ cells/plate) were transfected with 10 μg of either pcDNA3 or FLAG-RASSF1A, 10 x 15 cm plates per condition. Cells were cultured in media containing 0.1 % FBS for 16 hrs prior to being lysed by scraping into 0.5 % NP40, 150 mM NaCl lysis buffer. Cells were immunoprecipitated for 2 hrs with anti-FLAG M2-conjugated agarose beads. Samples were eluted from the antibody, 2 x 25 min with 100 μl 1 mg/ml 1 x FLAG peptide. **A.** Eluates and beads were resolved by SDS-PAGE and proteins were stained using colloidal coomassie blue stain. **B.** Inputs were analysed by SDS-PAGE and western blot for RASSF1A expression using anti-FLAG antibodies. GAPDH is a loading control.

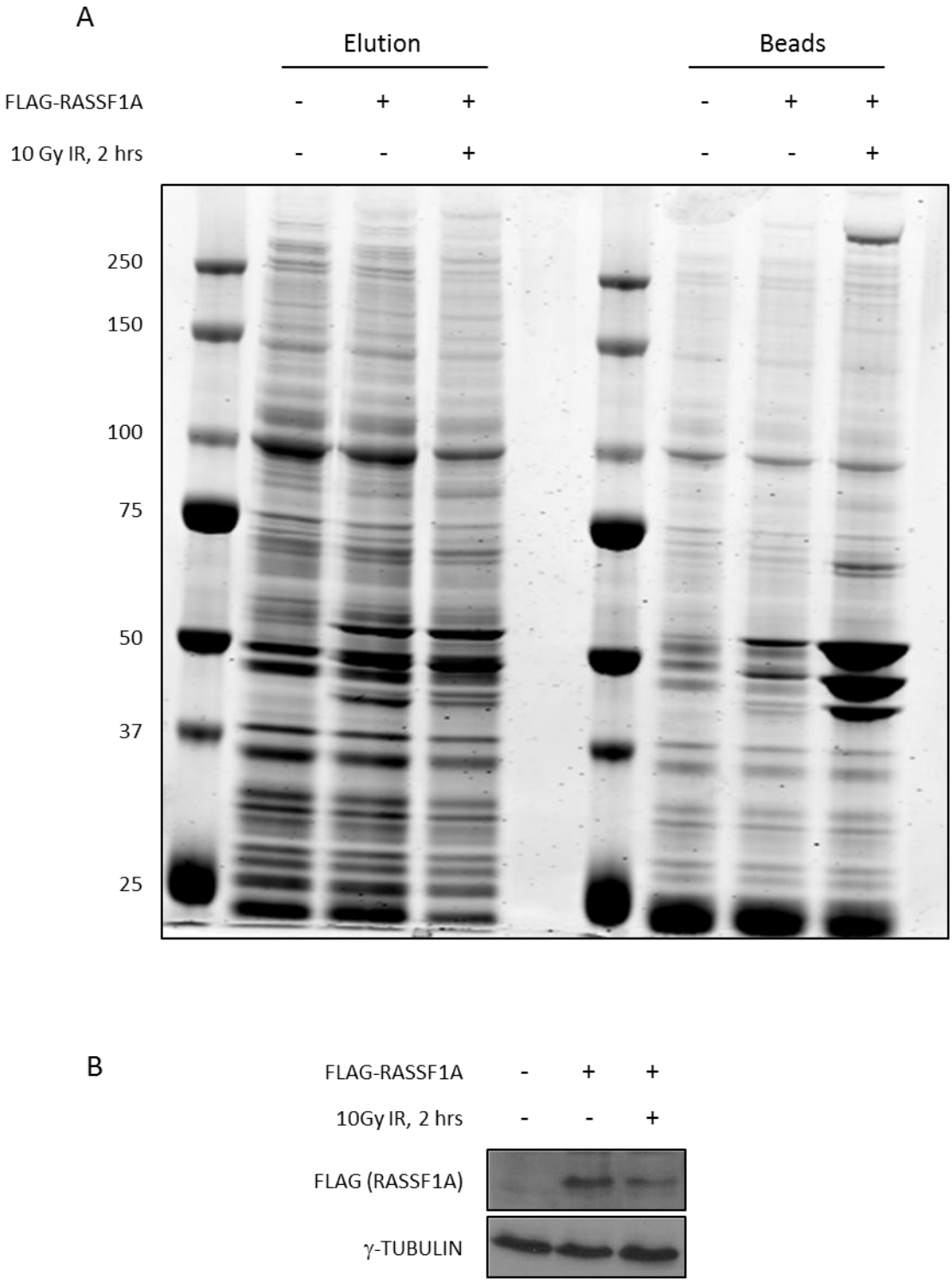


**Figure 3.5. Optimisation of Immunoprecipitation Protocol Gel 2**. MCF7 cells (5 x $10^6$ cells/plate) were transfected with 5 µg of either pcDNA3 or FLAG-RASSF1A, 10 x 15 cm plates per condition. Cells were cultured in media containing 0.1 % FBS for 16 hrs prior to being lysed by scraping into 1 % NP40, 150 mM NaCl lysis buffer. Cells were immunoprecipitated for 2 hrs with anti-FLAG M2-conjugated agarose beads. Samples were eluted from the antibody, 2 x 25 min with 100 µl 1 mg/ml 3 x FLAG peptide. Eluates were combined into one sample. **A.** Eluates and beads were resolved by SDS-PAGE and proteins were stained using colloidal coomassie blue stain. **B.** Inputs were analysed by SDS-PAGE and western blot for RASSF1A expression using anti-FLAG antibodies. GAPDH is a loading control.

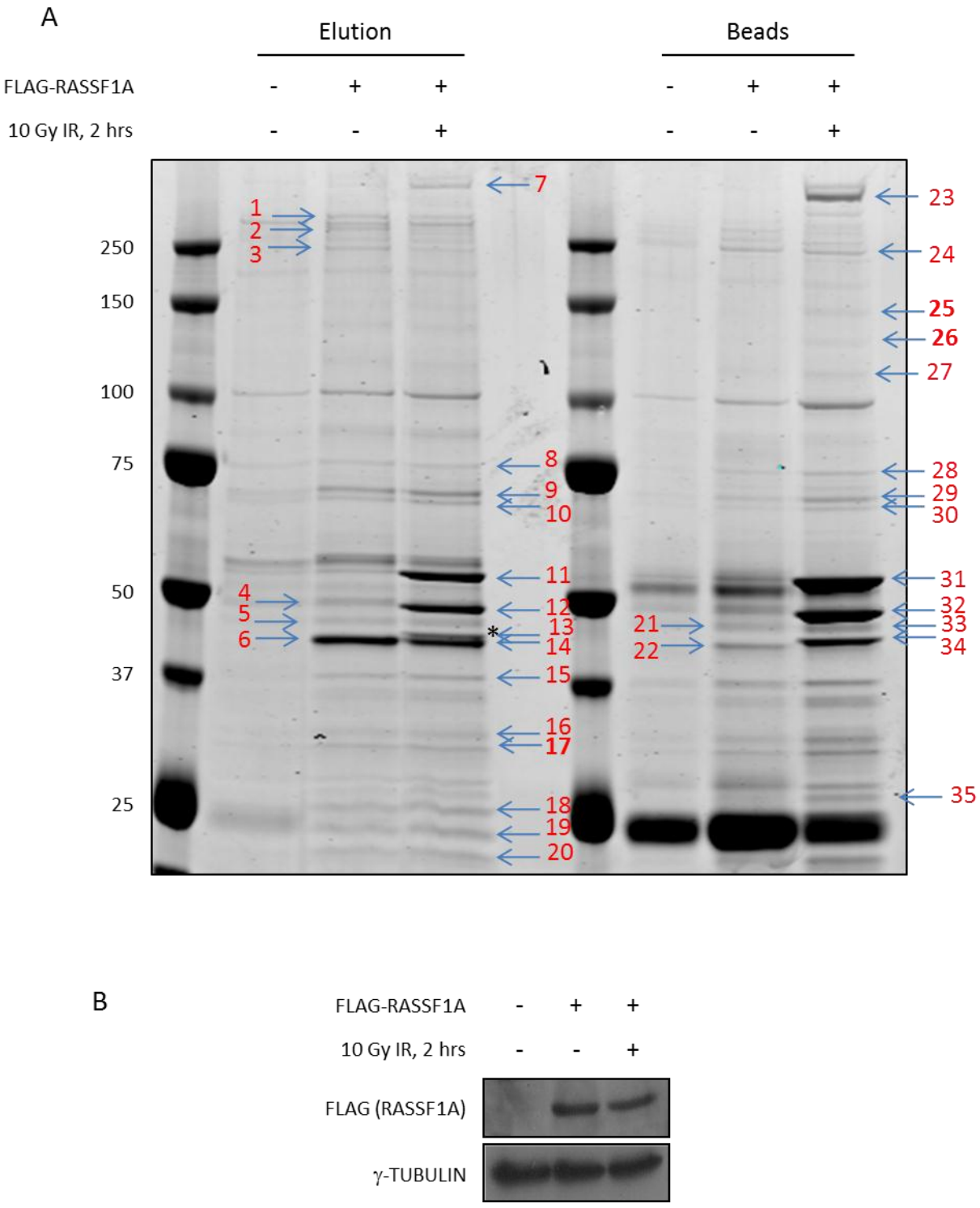


**Figure 3.6. Optimisation of Immunoprecipitation Protocol Gel 3: Final Gel.** MCF7 cells (5 x $10^6$ cells/plate) were transfected with 5 µg of either pcDNA3 or FLAG-RASSF1A, 10 x 15 cm plates per condition. Cells were cultured in media containing 0.1 % FBS for 16 hrs prior to being lysed by scraping into 1 % NP40, 300 mM NaCl lysis buffer. Cells were immunoprecipitated for 2 hrs with anti-FLAG M2-conjugated agarose beads. Samples were eluted form the antibody, 2 x 25 min with 100 µl 1 mg/ml 3 x FLAG peptide. Eluates were combined into one sample. **A.** Eluates and beads were boiled, separated by SDS-PAGE and proteins stained using colloidal coomassie blue stain. Arrows indicate excised protein bands. Numbers correspond to Table 8.1, which lists the top hits identified in the mass spectrometry. Selected hits for further investigation are in bold. 17 – XPA, 25 – OSSA and 26 – DDB1. Asterisk represents RASSF1A identified by mass spectrometry with decreased mobility in the gel. **B.** Inputs were analysed by SDS-PAGE and western blot for RASSF1A expression using anti-FLAG antibodies. GAPDH is a loading control.

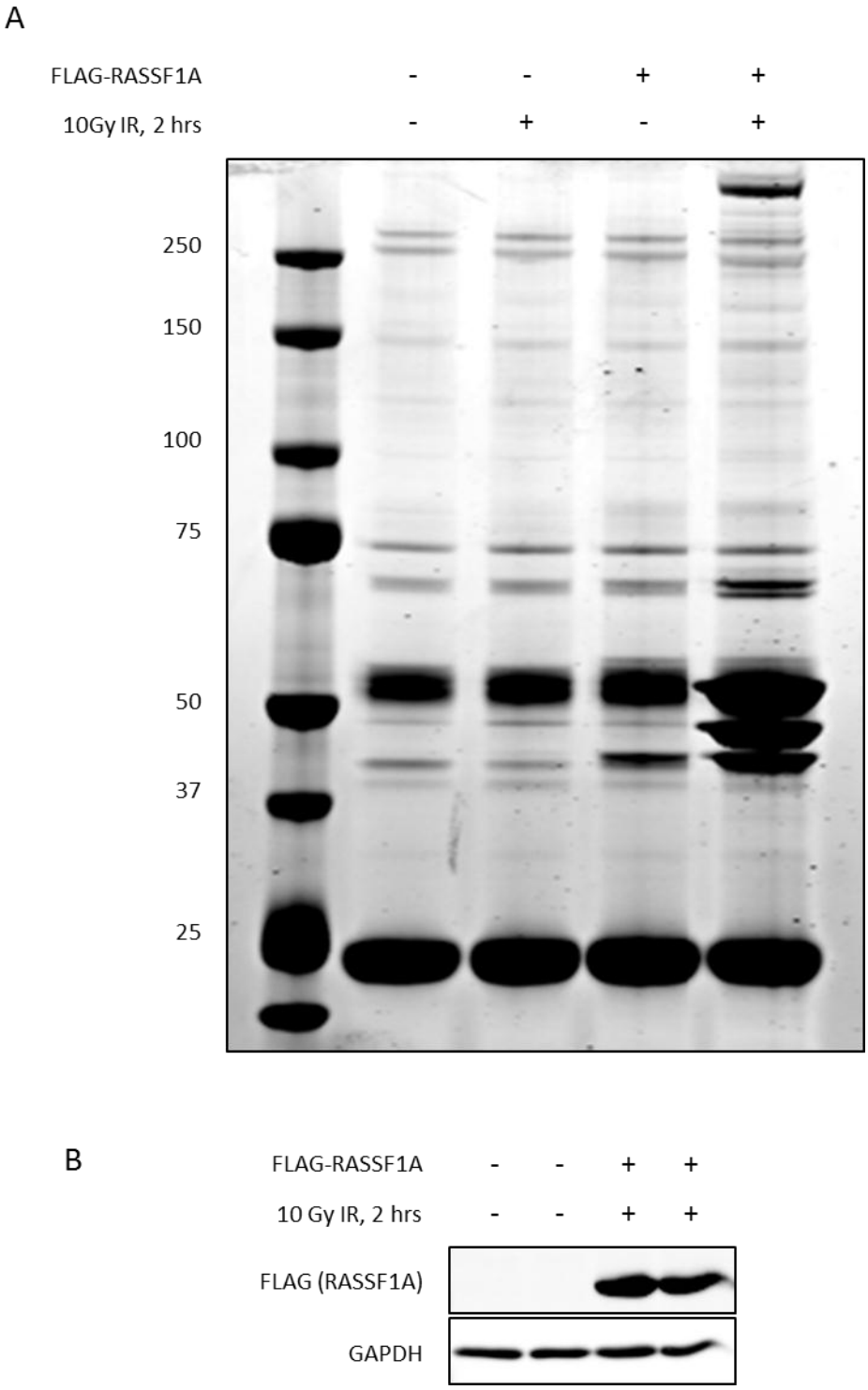


**Figure 3.7. Optimisation of Immunoprecipitation Protocol Gel 4**: DNA damage specifically induces RASSF1A protein interactions. MCF7 cells (5 x $10^6$ cells/plate) were transfected with 5 µg of either pcDNA3 or FLAG-RASSF1A, 5 x 15 cm plates per condition. Cells were cultured in media containing 0.1 % FBS for 16 hrs prior to being lysed using 1 % NP40, 300 mM NaCl lysis buffer and immunoprecipitated for 2 hrs using anti-FLAG M2-conjugated agarose beads. **A.** Protein was boiled from the antibody and resolved by SDS-PAGE before being stained with colloidal coomassie blue stain for the presence of protein bands. DNA damage specific bands were only seen in cells expressing RASSF1A. **B.** Inputs were analysed by SDS-PAGE and western blot for RASSF1A expression using anti-FLAG-tag antibodies. GAPDH is a loading control.

## 3.4 Separation of RASSF1A Containing Complexes by HPLC

Identification of native complexes is advantageous when compared to identifying proteins from a denaturing SDS-PAGE gel because it provides an opportunity to identify connections between protein complexes, potentially highlighting novel regulatory pathways. In a traditional IP the protein is recovered from the antibody by boiling in a denaturing buffer, which disrupts the interactions between the proteins. To prevent denaturation of the RASSF1A containing complexes whilst recovering the protein from the beads we eluted FLAG-RASSF1A from the beads using a competitive FLAG peptide. This would allow the eluted protein to be run over a size exclusion column and distinct RASSF1A containing fractions collected, digested and analysed by MS. An Agilent Zorbax GF-450 column was chosen for the complex separation as it efficiently separates proteins in a mass range of 10 – 900 kDa. Although, the column is capable of separating proteins at a flow rate of up to 3 ml/min better separation of standard proteins was achieved at a slower flow rate of 1 ml/min. We also maximised the number of fractions collected (10 per min). Together these steps increase our chances of resolving complexes with similar mass. Prior to loading the eluted protein onto the HPLC a set of four standard proteins were run (Table 3.1). From the absorbance trace we can predict that a monomer of RASSF1A would elute at approximately 13 min and a heterodimer of RASSF1A and MST2 to elute at approximately 11 min (Figure 3.8A).

| Protein | Molecular Mass (Mr, kDa) | Elution Time (min) |
|---|---|---|
| **Ovalbumin** | 43 | 12.772 |
| **Conalbumin** | 75 | 11.134 |
| **Aldose** | 158 | 10.601 |
| **Thyroglobulin** | 669 | 8.627 |

**Table 3.1. Standard Proteins Used in the HPLC.** Table indicates the mass and elution time of the proteins used.

We first loaded the eluates produced by following the IP protocol used for IP MS onto the HPLC, but we were unable to identify significant peaks on the HPLC. We concluded that level of protein in the sample was likely to below the level of detection. To combat this, the protocol was scaled up to 100 x 15 cm dishes per condition. From Figure 3.6 it can be seen that exogenous expression of RASSF1A can precipitate a number of proteins without the addition of a stimulus. Therefore, we were confident that we would be able to identify RASSF1A containing complexes from the collected fractions. In order to facilitate growing this number of cells whilst minimising any differences in the conditions in which they were grown we opted for H1299 cells containing a Tetracycline inducible RASSF1A construct as opposed to transient transfection (see Appendix Figure 8.1 for expression). Figure 3.8B shows the expression of RASSF1A in the lysates used for the IP was efficient and that RASSF1A was precipitated by the anti-FLAG M2-conjugated agarose beads. The eluates obtained from this IP were loaded onto the column (Figure 3.8C: control, Figure 3.8D: RASSF1A). Both samples gave a small peak with an elution time of around 7-8 minutes and another larger peak at 11 min. This suggested that the concentration of the protein in the sample was still too low for detection.

In order to identify whether RASSF1A was present in the eluted samples we took 5 μl from each fraction collected and combined 6 fractions together to create a sample of 30 μl. These samples were analysed by SDS-PAGE gel and western blot with anti-FLAG antibody. We were unable to detect a distinct FLAG-RASSF1A band in any of these samples. However, there was a large smear on the blot in a lane composed of samples from approximately 2 min (Figure 3.8E). This potentially represents a large complex of mega Dalton size that could be caused by RASSF1A binding to the microtubule network. We also saw a set of bands that appeared to be the heavy and light chain of the antibody at approximately 11 min (data not shown). This data suggests that a large proportion of the RASSF1A is contained within one mega-complex and that other RASSF1A containing complexes are too dilute in the eluted fraction to be detected on the absorbance trace or by western blot. To test this, samples composed of six fractions evenly collected from every 12 representing minutes 7 to 16 of the HPLC run were pooled and concentrated to 30 μL samples and analysed by western blot for FLAG-RASSF1A. Using this technique we

were successfully able to identify FLAG-RASSF1A in two of the 10 samples run (Figure 3.8F). The bands represented an elution time of between 10 and 12 minutes. These elution times could represent monomers, dimers as well as larger complexes of RASSF1A. We therefore re-blotted these membranes with an antibody against a MST2, a known RASSF1A binding partner. However, we were unable to detect MST2 in either of the two lanes where RASSF1A was seen (data not shown).

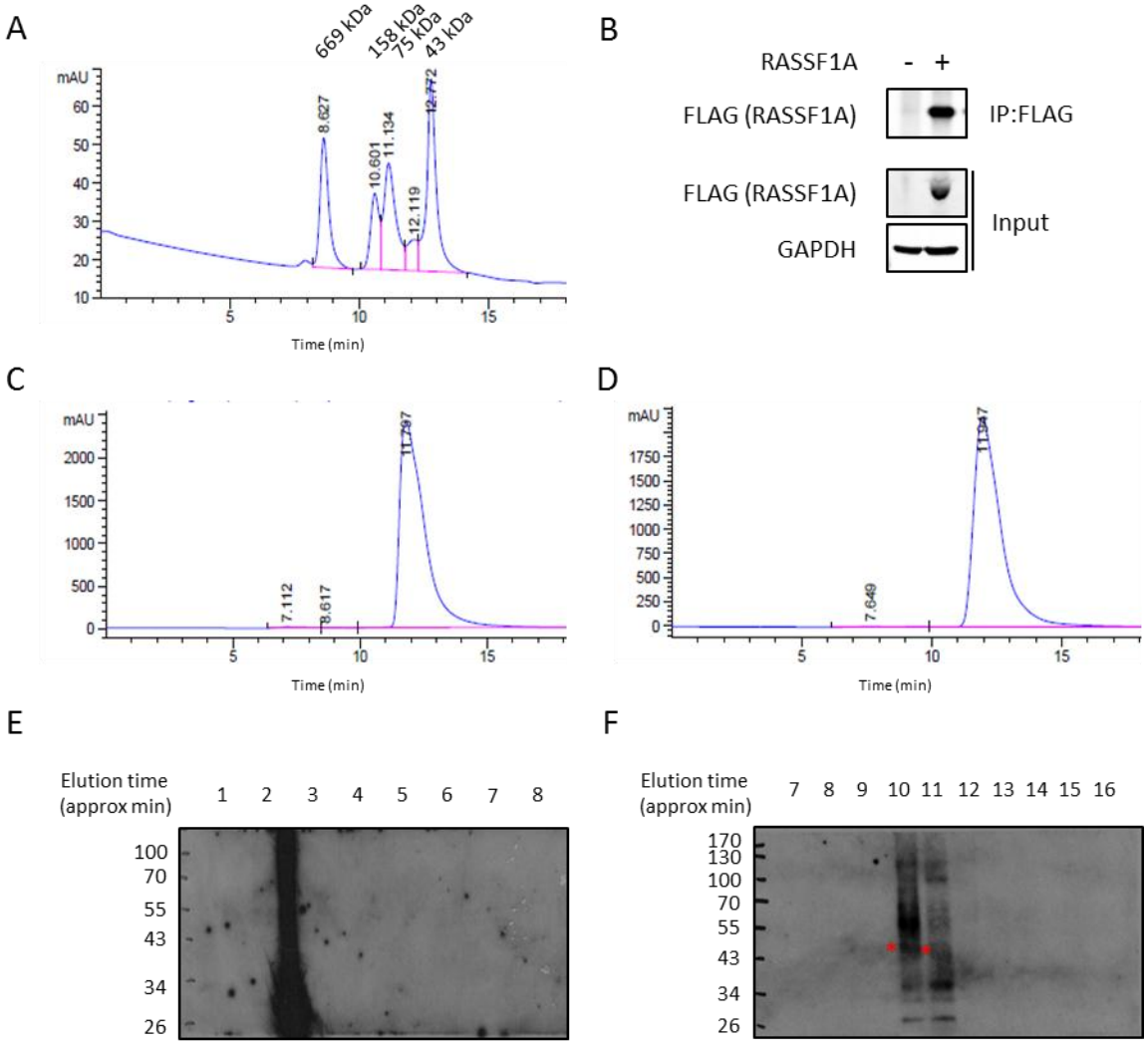


**Figure 3.8. Separation of Complexes by Size Exclusion HPLC. A.** Standard proteins, Ovalbumin (43 kDa), Conalbumin (75 kDa), Aldose (158 kDa) and Thyroglobulin (669 kDa), were loaded onto a Zorbax GF-450 column with a flow rate of 1 ml/min. Protein was measured by absorption at 210 nm. Clear separation of each protein can be detected. **B-F.** H1299 TET ON control and RASSF1A inducible cells were plated (5 x $10^6$ cells/plate, 100 plates/condition) and expression was induced with 2 μg/ml doxycycline. Cells were cultured for 24 hrs to allow expression to occur before cells were put into media containing 0.1 % FBS for 16 hrs in the presence of doxycycline. Cells were lysed using 1 % NP40, 300 mM NaCl lysis buffer and immunoprecipitated for 2 hrs with anti-FLAG M2-conjugated agarose beads. Protein was eluted from the antibody using 2 x 25 min washes with 1 mg/ml 3 x FLAG peptide. **B.** Inputs and protein boiled from the beads post elution were analysed by SDS-PAGE and western blot for FLAG-RASSF1A expression using anti-FLAG antibodies. GAPDH is a loading control. **C.** Eluted protein from anti-FLAG immunoprecipitation from control H1299 Tet ON cells was run over the column using conditions outlined in (**A**). **D.** Eluted protein from anti-FLAG immunoprecipitation from RASSF1A H1299 Tet ON cells using conditions outlined in (**A**). **E.** 5 μl of each set of 6 fractions collected in (**D**) were combined and analysed by SDS-PAGE and western blot for FLAG-RASSF1A expression. One band was detected in a lane representing an elution time of approx. 2 min. **F.** Six fractions in every 12, from fractions representing minutes 7 to 16 from (**D**) were pooled, concentrated and analysed by SDS-PAGE and western blot for FLAG-RASSF1A. RASSF1A was detected in 2 lanes representing an elution time of approx. 10 – 11 minutes. These samples also appear to contain the antibody. The membrane was also probed for MST2, which was not detected (data not shown). Data represents one independent experiment.

## 3.5 Identification of Hits

It was clear that the HPLC sensitivity was an issue and while we were able to successfully elute and detect RASSF1A further optimisation would be required. Protein bands excised from the gel in Figure 3.6 were identified and had revealed a number of interesting hits and so the decision was taken to focus on the characterisation of these. Protein bands that appeared in the RASSF1A or RASSF1A plus DNA damage samples, but not in the control samples in colloidal coomassie blue stained SDS-PAGE gels were excised from the gel and subjected to tryptic digest. The digested samples were loaded into the mass spectrometer to identify the peptides in the sample by MS/MS tandem mass spectrometry. The resulting data was analysed using the Mascot MS/MS ion search programme (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS).

The mascot programme works by searching a data base of protein sequences to match them against the peptides identified. A probability score is then given to each protein identified. This score takes into account: the number of peptides identified matching a hit, the quality of these peptides (i.e. how many of the ions in a predicted peptide sequence could be identified), the percentage coverage of the protein sequence the peptides identified represent and whether the peptides identified are unique to the protein or not. Within the mass spectrometer the peptides are further fragmented to produce ions with either a positive or negative charge. Only the mass of the positively charged ion is measured. In MS/MS mass spectrometry, the peptides are fragmented in such a way that they break at the peptide bond. Here, the positive charge can be on the carboxyl group, forming what are known as b-ions, or the amino group, which are called y-ions. The more b- and y-ions that can be identified for a particular peptide the greater the confidence that the amino acid sequence identified is correct. Taken together, hits with a large number of identified peptides, covering a significant proportion of the total protein sequence are considered to be confident hits with a high probability of being a genuine RASSF1A interacting protein. In addition to this analysis we also assessed whether the mass of the matched protein fitted the

predicted mass of the protein band excised from the gel. Table 8.1 shows a list of the top hits identified from Figure 3.6.

Following identification of the proteins within the bands we selected hits for further analysis based on the strength of the hit and its relevance to the DNA damage response (DDR). Also, links to interesting or novel pathways or prior relationships with hippo pathway members was taken into consideration. The proteins selected for confirmation and further investigation were XPA, DDB1 and OSSA. XPA and DDB1 were chosen because there function in the Nucleotide Excision Repair (NER) pathway, potentially linking RASSF1A to a novel role in DNA damage repair. XPA had also been previously identified in a yeast 2-hybrid screen as a binding partner of RASSF1A, but the function consequence of this interaction has never been pursued (Dammann, Li et al. 2000). OSSA was chosen because it links RASSF1A to a novel DNA damage regulated pathway regulating the oncogene SRC (Tanaka, Sasaki et al. 2009).

## 3.6 RASSF1A Interacting Proteins

Despite optimising the screen to minimise the amount of non-specific binding in the IP, false positive results are inevitable. Therefore, there is a need to confirm the mass spectrometry data with independent experiments. The screen aimed to identify novel binding partners of RASSF1A that were induced to bind RASSF1A in response to DNA damage. Two such proteins identified in the screen were the nucleotide excision repair proteins XPA (Xeroderma pigmentosum group A-complementing protein) and the DDB1 (DNA Damage-Binding protein 1). XPA and DDB1 are two members of the nucleotide excision repair pathway. XPA, with its binding partners XPG and RPA form part of the preincision complex. They are thought to bind to the undamaged strand and promote the cleavage of the damaged DNA (de Laat, Appeldoorn et al. 1998; Hermanson-Miller and Turchi 2002; Andressoo, Hoeijmakers et al. 2006; Coin, Oksenych et al. 2008). DDB1 acts upstream of XPA, at the DNA damage recognition stage. DDB1 and XPC are thought to scan the DNA for damage recognising changes in secondary DNA structure

(Sugasawa, Okamoto et al. 2001; Sugasawa, Shimizu et al. 2002; Min and Pavletich 2007). When found, DDB1 helps XPC to bind to the damaged DNA in order to initiate the recruitment of the nucleotide excision repair proteins (Sugasawa, Okuda et al. 2005; Hoogstraten, Bergink et al. 2008). To confirm that XPA bound to RASSF1A, MCF7 cells were transiently transfected with pcDNA3 or FLAG-RASSF1A. Cells were treated with 25 μM cisplatin for 2 hrs and immunoprecipitated for 2 hrs with anti-FLAG M2-conjugated agarose beads. We were unable to confirm XPA as a binding partner of RASSF1A, suggesting that it is a false positive (Figure 3.9A). We next looked to confirm if DDB1 is a binding partner of RASSF1A. The same protocol was used for this as for XPA except cells were exposed to 10 Gy IR, and lysed after 2 hrs incubation as was done in the screen (Figure 3.9B). We could confirm the interaction between DDB1 and FLAG-RASSF1A. However, this interaction appeared constitutive and failed to be induced further by IR.

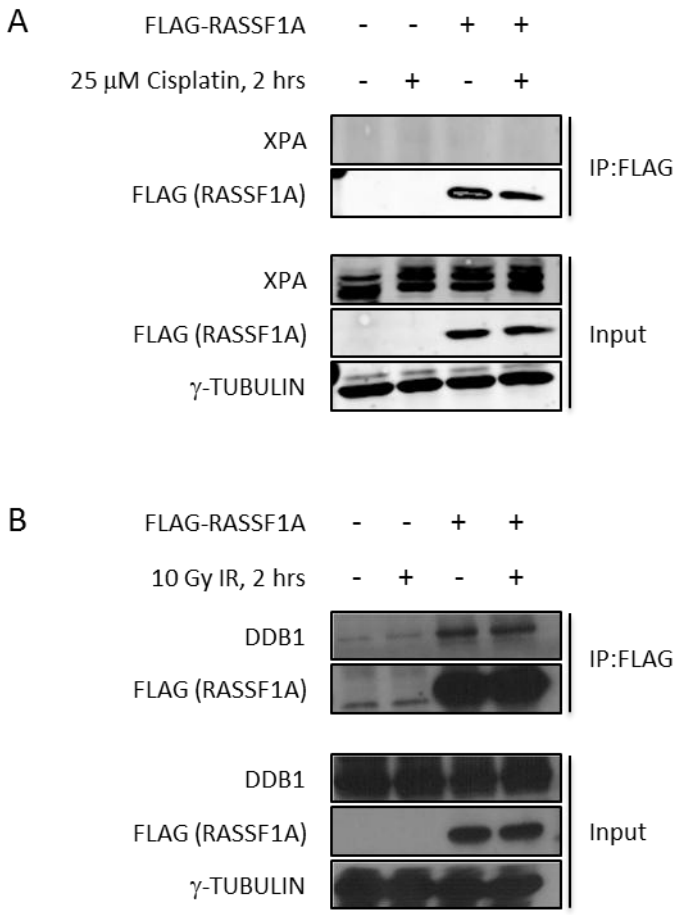


**Figure 3.9. Analysis of Potential RASSF1A Interacting Partners.** MCF7 cells (1 x $10^6$ cells/plate) were transfected with 5 μg empty vector or FLAG-RASSF1A and lysed 48 hrs post transfection using a 1 % NP40, 300 mM NaCl lysis buffer before being immunoprecipitated with anti-FLAG M2-conjugated agarose beads. Inputs and precipitates were analysed by SDS-PAGE and western blot for interaction using anti-FLAG antibodies and anti-XPA (**A.**) or anti-DDB1 (**B.**) antibodies. Tubulin was used as a loading control. **A.** XPA does not bind FLAG-RASSF1A. **B.** DDB1 was confirmed as a binding partner, but the interaction is unaffected by exposure to 10 Gy IR. Data represents one independent experiment.

The third protein we looked at, OSSA (Oxidative Stress-associated SRC Activator), had also been shown to be activated by DNA damage (Tanaka, Sasaki et al. 2009). In response to UV radiation, OSSA translocated from the nucleus to the plasma membrane, where it interacted with and activated SRC family kinases. This interaction was shown to occur within 3 mins of the induction of damage stimulating survival signalling. Presumably, this forms part of a mechanism to prevent the cell from immediately undergoing apoptosis whilst the level of DNA damage is assessed. OSSA and SRC were shown to dissociate approximately 30 mins post exposure to oxidative stress. In our screen the cells were lysed 2 hrs post IR and therefore we were interested to see whether the tumour suppressor RASSF1A was responsible for inhibiting this survival signal by disrupting the interaction between OSSA and SRC.

We first confirmed the interaction between OSSA and RASSF1A using exogenously expressed constructs. 293T cells were transiently transfected with pcDNA3, FLAG-RASSF1A, MYC-OSSA or both FLAG-RASSF1A and MYC-OSSA (Figure 3.10A). MYC-OSSA co-immunoprecipitated with FLAG-RASSF1A, but FLAG-RASSF1A did not precipitate with MYC-OSSA (Figure 3.10A). As a control to rule out epitope association 293T cells were then transfected with pcDNA3, HA-RASSF1A, FLAG-OSSA or HA-RASSF1A and FLAG-OSSA (Figure 3.10B). HA-RASSF1A co-immunoprecipitated with FLAG-OSSA, but again this was not reciprocal. As with DDB1, the interaction with OSSA appeared to be constitutive. The interaction was confirmed to be constitutive by precipitating endogenous RASSF1A from HeLa cells transfected with non-targeting siRNA or siRNA targeted to OSSA (Figure 3.11). Although the interaction was not induced by DNA damage, we were intrigued by the association as it potentially highlighted a novel tumour suppressor function for RASSF1A in regulating the oncogene SRC.

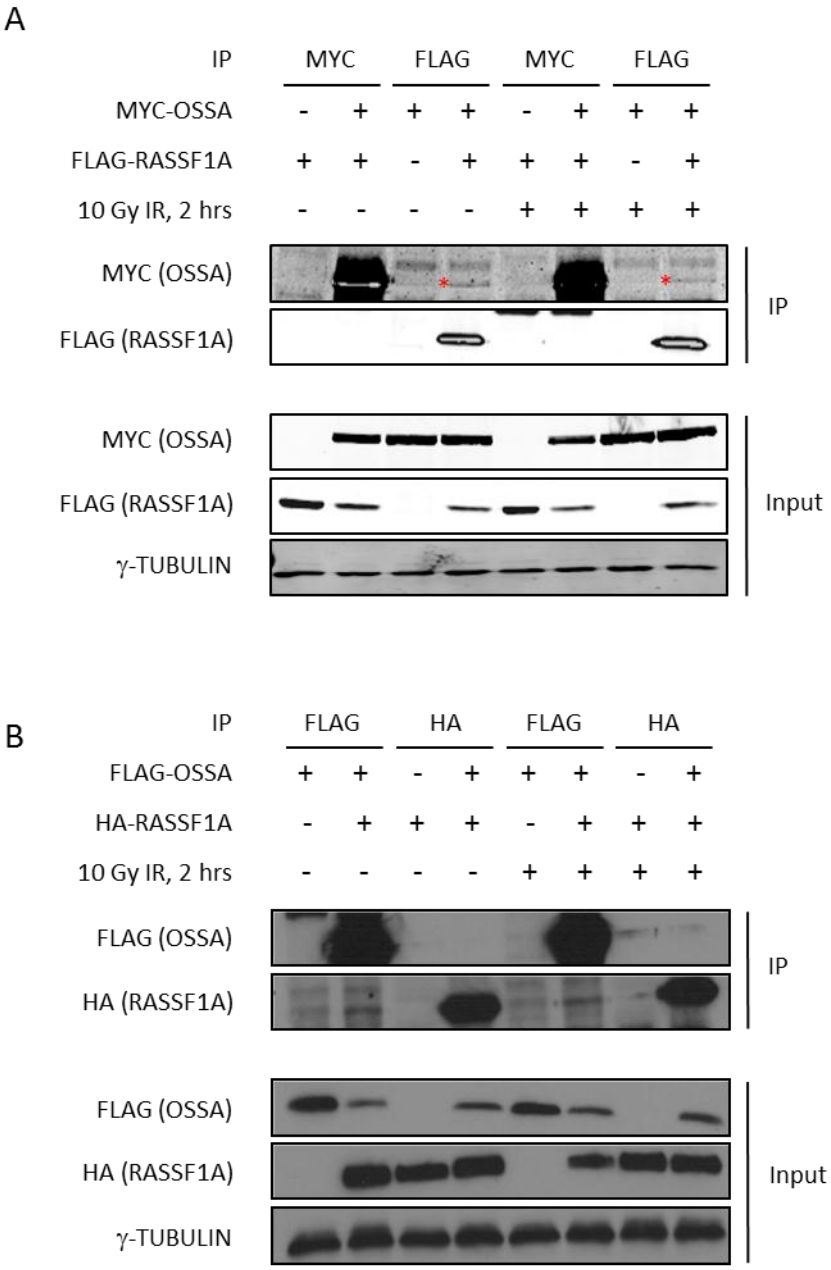


**Figure 3.10. Analysis of Potential RASSF1A Interacting Partners: OSSA Binds RASSF1A.** 293T cells (5 x $10^5$ cells/plate) were transfected with either 4 μg MYC-OSSA and FLAG-RASSF1A (**A.**) or FLAG-OSSA and HA-RASSF1A (**B.**). DNA transfected was equalised using empty vector DNA. Cells were lysed 48 hrs post transfection using a 1 % NP40, 300 mM NaCl lysis buffer and immunoprecipitated with anti-MYC-tag (**A**) or anti-HA-tag (**B**) antibodies bound to protein G sepharose beads, or anti-FLAG M2-conjugated agarose beads. Inputs and precipitates were analysed by SDS-PAGE and western blot with antibodies against the protein tags. Tubulin was used as a loading control. **A.** MYC-OSSA precipitated with FLAG-RASSF1A. **B.** HA-RASSF1A precipitated with FLAG-OSSA. Enhancement of the RASSF1A-OSSA interaction with exposure to 10 Gy IR was not detected in either experiment. Data represents one independent experiment.

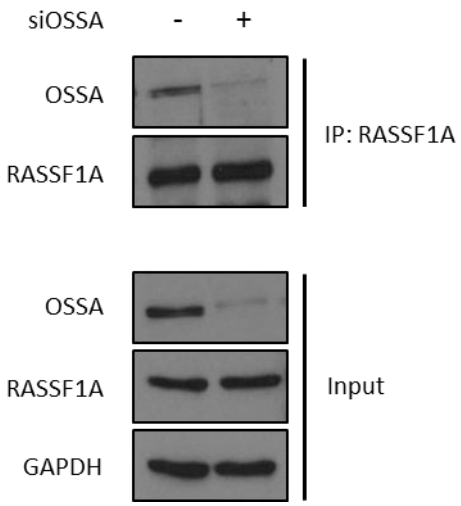


**Figure 3.11. Endogenous RASSF1A and OSSA Interact.** HeLa cells (1 x $10^6$ cells/plate) were transfected with either 50 nM non-targeting siRNA or siRNA targeting OSSA and cultured for 48 hrs before being lysed with a 0.5 % NP40, 150 mM NaCl lysis buffer and immunoprecipitated with anti-RASSF1A antibodies bound to protein-G sepharose beads. Inputs and precipitates were analysed by SDS-PAGE and western blot for RASSF1A and OSSA with antibodies against endogenous protein. GAPDH was used as a loading control. RASSF1A and OSSA specifically interact. The Interaction is lost with the ablation of OSSA expression. Data represents two independent experiments.

Having confirmed the interaction between RASSF1A and OSSA we decided to map the interaction further using RASSF1A deletion constructs, which could be used to determine binding to each of the three known interaction domains of RASSF1A, the C1 domain, the RA domain and the SARAH domain (Figure 3.12A). Despite attempts to equilibrate levels, the expression of the constructs varied. The two shortest constructs, C1 domain only (a.a. 1-119) and SARAH domain only (a.a. 288-340) constructs showed limited expression. In contrast, constructs consisting of the RA domain alone (a.a. 120-287) or the C1 and RA domain together (a.a. 1-287) showed much higher expression than either the full length (a.a. 1-340) or the construct containing the RA domain and SARAH domain (a.a. 120-340). To investigate the binding of each of these constructs to endogenous OSSA, MCF7 cells were transiently transfected with each construct and immunoprecipitated with anti-OSSA antibodies. Full length MYC-RASSF1A and a construct consisting of the C1 and RA domains (a.a. 1-287) immunoprecipitated with endogenous OSSA (Figure 3.12B). This data shows that the interaction requires the RA domain or the C1 domain. This is because both constructs precipitated by OSSA contain both of these interaction domains. The poor

expression of the C1 domain-only construct in this experiment means that the interaction cannot be further refined.

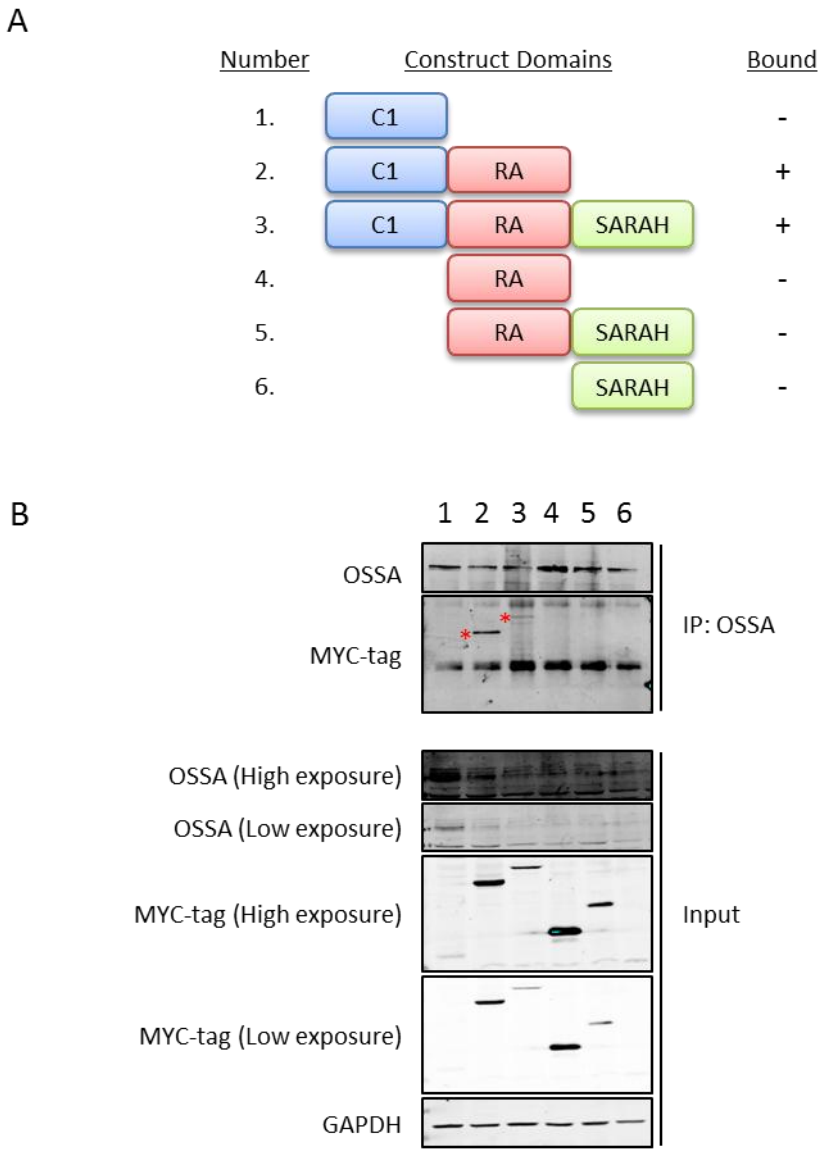


**Figure 3.12. Mapping the interaction site between RASSF1A and OSSA. A.** Graphical representation of the binding domains present in each truncation construct and an indication of whether that construct precipitated with endogenous OSSA. **B.** MCF7 cells (1 x $10^6$ cells/plate) were transfected with MYC-tagged constructs of RASSF1A numbered 1-6 (1. amino acids 1-119 (C1 domain), 2. amino acids 1-287 (C1 domain and RA domain), 3. amino acids 1-340 (full length), 4. amino acids 120-287 (RA domain), 5. amino acids 120-340 (RA domain and SARAH domain) and 6. amino acids 288-340 (SARAH domain). Cells lysed with a 0.5 % NP40, 150 mM NaCl lysis buffer and immunoprecipitated with an anti-OSSA antibody bound to protein-G sepharose beads. Inputs and precipitates were analysed by SDS-PAGE and western blot for OSSA and MYC-tag construct interaction. GAPDH was used as a loading control. The interaction can be mapped to a region encompassing the C1 and RA domains. Expression of construct 1 and 6 was weak and undetectable, respectively. Asterisks indicate the presence of a truncation construct in the immunoprecipitation. Data represents one independent experiment.

## 3.7 The Role of RASSF1 in SRC Activation

Having shown that OSSA bound to RASSF1A and knowing that OSSA activates SRC, we decided to investigate the role of RASSF1A in the activation of SRC. It was suggested that OSSA activated SRC to create a survival signal in response to oxidative stress to prevent the cell undergoing apoptosis whilst the severity of DNA damage was assessed. As a proapoptotic protein activated by DNA damage we hypothesised that RASSF1A would inhibit the interaction between SRC and OSSA switching off the survival signal to promote apoptosis when severe DNA damage was detected. HeLa cells were transfected with non-targeting siRNA or siRNA targeting RASSF1 or OSSA before being exposed to cisplatin or hydrogen peroxide and lysed. Cisplatin was used to generate a DDR that has previously been shown to activate RASSF1A (Hamilton, Yee et al. 2009). Hydrogen peroxide was used to generate the oxidative stress response known to activate OSSA (Tanaka, Sasaki et al. 2009). SRC activity was assessed in these cells by western blotting for phospho-SRC Y419, which represents the autophosphorylation site of SRC and is indicative of active SRC (Hunter 1987). We hypothesised that loss of OSSA would lead to a decrease in the levels of SRC phosphorylated on Y419. As expected we saw a slight decrease in the level of activated SRC in cell treated with siRNA targeting OSSA (Figure 3.13A). Our hypothesis predicted that loss of the tumour suppressor RASSF1A would lead to an increase in the levels of active SRC and therefore, we were surprised to see that in fact the opposite was true. In untreated cells, targeting RASSF1 with siRNA completely ablated the levels of active SRC (Figure 3.13A), suggesting that RASSF1A, a tumour suppressor, was required for the activation of SRC, the first oncogene ever to be characterised (Guarino 2010).

SRC has previously been shown to be activated by oxidative stress (Abe, Takahashi et al. 1997). However, in Figure 3.13A we were unable to show the activation of SRC by the DNA damaging agents, cisplatin or hydrogen peroxide. We reasoned that this was because the cells were cultured in DMEM containing 10 % FBS, which has a high concentration of growth factors, which also activate SRC. Given

that loss of RASSF1 completely ablated SRC activity under these stimulatory conditions, it suggests a requirement for RASSF1 in the activation of SRC by growth factors.

We were interested to check whether RASSF1A plays a role in the activation of SRC in response to DNA damaging agents, therefore, we transfected HeLa cells with non-targeting siRNA or siRNA targeting RASSF1. Cells were incubated overnight in the absence of serum before being lysed 2 hrs post cisplatin treatment or 30 mins post exposure to IR. We were able to see the activation of SRC by IR and that this was ablated in the cells treated with siRASSF1 (Figure 3.13B). Therefore RASSF1 is important for the activation of SRC in response to growth factors and IR.

Although the loss of OSSA had only a minor effect on the activity of SRC compared to that seen with loss of RASSF1, we knew that RASSF1A could bind OSSA (Figure 3.10 and 3.11) and that OSSA could bind and activate SRC. Therefore, we wished to see whether the activation of SRC by OSSA was dependent on RASSF1A. HeLa cells were transfected with non-targeting siRNA or siRNA targeting RASSF1 and subsequently transfected a second time with pcDNA3 or FLAG-OSSA. We observed that ectopic expression of FLAG-OSSA was able to activate SRC and that this was enhanced by exposing the cells to IR (Figure 3.14). However, neither FLAG-OSSA expression or exposure to IR were able to activate SRC in cells transfected with siRNA targeting RASSF1, suggesting that the mechanism by which OSSA activates SRC maybe be RASSF1 dependent (Figure 3.14). Another interesting observation from this experiment was that the stability of OSSA is dependent on the levels of RASSF1. In the lanes where RASSF1 has been depleted the levels of OSSA are also lower.

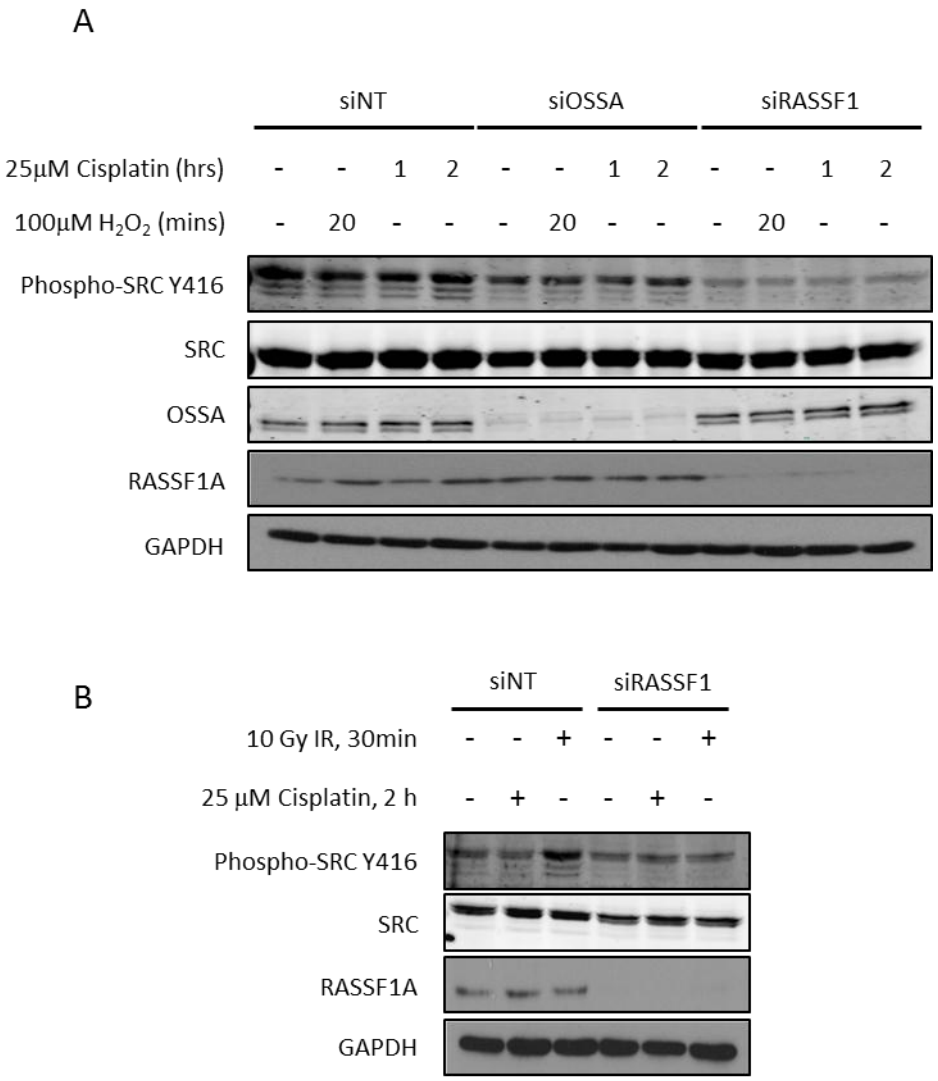


**Figure 3.13. RASSF1 Expression is Required for SRC Activation by Growth Factors or DNA Damaging Agents. A.** HeLa cell (2.5 x $10^5$ cells/plate) were transfected with 50 nM non-targeting siRNA or siRNA targeting RASSF1 or OSSA. Cells were lysed using Laemmli lysis buffer and analysed by SDS-PAGE and western blot for SRC activity using anti-phospho-SRC Y419 and total SRC antibodies. RASSF1A and OSSA expression were confirmed using antibodies to the endogenous protein. GAPDH is a loading control. SRC activity is decreased in cells transfected with siRNA to OSSA; however, it is completely ablated in cells transfected with siRNA to RASSF1. **B.** HeLa cells (2.5 x $10^5$ cells/plate) were transfected with non-targeting siRNA or siRNA targeting RASSF1. Cells were cultured in media containing 0.1 % FBS 16 hrs prior to exposure to 25 mM Cisplatin for 2 hrs, or 10 Gy IR and lysed after 30 mins, using Laemmli lysis buffer. Lysates were analysed by SDS-PAGE and western blot for SRC activation using anti-phospho-SRC Y419 and total SRC antibodies. Knockdown of RASSF1A expression was confirmed with antibodies to endogenous protein and GAPDH is a loading control. Exposure to 10 Gy IR is seen to activate SRC. This activity is ablated by knockdown of RASSF1. Data represents one independent experiment.

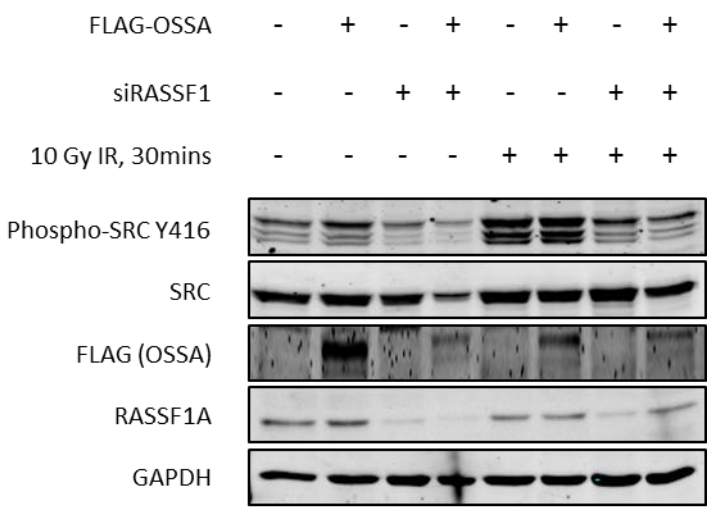


**Figure 3.14. OSSA overexpression is unable to activate SRC in the absence of RASSF1 expression**. HeLa cells (2 x $10^5$ cells/plate) were transfected with 50 nM non-targeting siRNA or siRNA targeting RASSF1 before being transfected with 1 μg empty vector or FLAG-OSSA. Cells were cultured in DMEM containing 0.1 % FBS for 16 hrs prior to exposure to 10 Gy IR. Cell were lysed with laemmli lysis buffer 30 mins post exposure to IR and analysed by SDS-PAGE and western blot for SRC activity with anti-phospho-SRC Y419 and total SRC antibodies. Anti-RASSF1A and anti-FLAG-tag antibodies were used to confirm knockdown of RASSF1A and exogenous expression of OSSA respectively. GAPDH is a loading control. Exogenous OSSA expression induced SRC activation, as does exposure to ionising radiation. Loss of RASSF1 expression prevents SRC activation by these stimuli. Data represents one independent experiment.

## 3.8 Discussion

In this chapter, I have shown an involvement for RASSF1A and the hippo pathway members MST2 and LATS1 in the DNA damage response. Following this, an immunoprecipitation protocol was optimised and constitutive and DNA damage induced binding partners were identified by mass spectrometry. DNA damage-related proteins, DDB1 and OSSA were confirmed as RASSF1A binding partners. Finally, a novel role for RASSF1 in the activation of the oncogene SRC kinase was identified.

At the beginning of this project it was known that RASSF1A could induce a cell cycle arrest at both the G1/S and G2/M transitions and promote apoptosis through both hippo-dependent and hippo-independent mechanisms in response to TNF$\alpha$ and Fas signalling or activated K-Ras (Shivakumar, Minna et al. 2002; Rong, Jin et al. 2004; Vos, Martinez et al. 2004; Baksh, Tommasi et al. 2005; Matallanas, Romano et al. 2007). This project stemmed from work in the laboratory investigating whether RASSF1A played a role in the activation of MST1 and MST2 in response to DNA damage. ATM was shown to phosphorylate RASSF1A on Serine 131 promoting RASSF1A binding to and activation of MST1/2 leading to p73-dependent apoptosis (Hamilton, Yee et al. 2009). As part of this work, a clonogenic survival assay was performed to assess the how the radiosensitivity of HeLa cells would be affected when RASSF1, MST2 or LATS1 expression was ablated (Figure 3.1). As expected, loss of expression of each these three proteins led to a decrease in sensitivity to radiation indicating that they played a role in the DDR. This experiment also highlighted that loss of RASSF1 had a greater effect than loss of MST2 or LATS1, suggesting that RASSF1A was responsible for the activation of other hippo-independent pathways in response to DNA damage. This formed the premise for a screen designed to identify the novel DNA damage activated pathways emanating from RASSF1A. RASSF1A has since been shown to able to disrupt the MDM2-DAXX-HAUSP complex leading to the stabilisation of p53 in response to DNA damage (Song, Song et al. 2008), further suggesting that in response to DNA damage RASSF1A activates hippo-independent pathways.

The screen was carried out by immunoprecipitating RASSF1A binding partners and then identifying them by mass spectrometry. The immunoprecipitation was optimised for two reasons. The first reason was to be able to identify novel proteins that interact with RASSF1A in response to DNA damage, which we were able to achieve. DNA damage induced interactions were clearly identifiable using the optimised protocol (Figure 3.6A), which was not the case prior to optimisation (Figure 3.4A). Further to this, we observed a shift in the band identified to be RASSF1A in the cells exposed to IR (Figure 3.6A band 13). This decrease in mobility through the gel is probably caused by the phosphorylation of RASSF1A by ATM. The second reason for optimising the IP, with particular reference to the removal of the background and the elution efficiency, was because we wished to run the eluted complexes over a size exclusion column and then identify native complexes.

We were successful in fractionating RASSF1A containing native complexes using the HPLC. RASSF1A was detected by western blot in fractions corresponding to retention times of approximately 2 min and between 10-11 min. The first elution may represent RASSF1A complexed with microtubules as part of a megadalton complex. The second elution had a shorter retention time than we expect for monomeric RASSF1A, but can be explained by the presence of the antibody in the RASSF1A containing fractions, which would add approximately 125 kDa to the mass of RASSF1A or more likely that we were indeed separating distinct RASSF1A complexes. Future work would concentrate on optimising the recovery of the protein from the beads. Even at its most efficient the elution only removed approximately half of the protein from the beads (Figure 3.6). More efficient elution may be achieved by using either SBP (streptavidin binding protein) tag or a CBP (calmodulin binding protein) tag. SBP is efficiently eluted by addition of biotin to the IP and CBP is eluted by addition of calcium to the IP. Alternatively, the proteins in the IP could be cross-linked after the final wash step, chemically binding the complexes together. All the protein could be extracted from the beads by denaturing the proteins. Whether proteins treated in this manner can be identified by mass spectrometry remains unclear as the mass of the cross linker could confuse the mascot software, which has been designed to identify the specific masses of the

amino acids. Improving the resolution in the 10 – 13 minute range may also aid the identification of individual RASSF1A containing fractions.

The hits identified from the mass spectrometry cover a variety of functions and consist of both known and novel interacting proteins (Table 8.1). The proteins identified that were already known to bind, or have been shown to bind in publications since the screen was done are: MST2 (Figure 3.6 Band 10 and 30), MAP1S (Figure 3.6 band 24), NDR1 (Figure 3.6 band 11), Tubulin (Figure 3.6 band 12), 14-3-3 (Figure 3.6 band 17), Aurora A (Figure 3.6 band 4) DDB1 (Figure 3.6 band 26) and XPA (Figure 3.6 band 17). The presence of previously identified binding partners of RASSF1A in the screen increased our confidence that the novel proteins identified are likely to be bona fide interactions. Many of the previously identified proteins have been shown to have a role in the DNA damage response or DNA repair, providing further evidence that the conditions used in the screen were able to successfully identify DNA damage induced RASSF1A interacting proteins.

The proteins identified in the screen can be sorted into roughly three groups: Known binding partners of RASSF1A and/or other hippo pathway members; cytoskeletal proteins, particularly proteins associated with Actin and Tubulin; and novel proteins that are known to function in response to oxidative stress.

Proteins associated with members of the hippo pathway were expected in the screen as the MST kinases are some best characterised binding partners of RASSF1A, and RASSF1A is known to form a multicomponent complex with MST kinase, LATS kinase and WW45 (*Drosophila* Salvador) (Guo, Tommasi et al. 2007). These proteins include: Adenomatous polyposis coli (Figure 3.6 band 1), a tumour suppressor also known to bind microtubules and is involved in the Wnt pathway, which can regulate and is regulated by the hippo pathway members YAP and TAZ (Varelas, Miller et al. 2010; Heallen, Zhang et al. 2011; Imajo, Miyatake et al. 2012; Konsavage, Kyler et al. 2012); Peroxiredoxin 1 (Figure 3.6 band 35), has been shown to be required for p53-dependent MST1 kinase activation in response to oxidative stress (Morinaka, Funato et al. 2011); and LATS1 phosphorylation of YAP recruits Casein kinase 1 (CK1) (Figure 3.6 band 15), which is known to phosphorylate YAP in order to target it for degradation (Zhao, Li

et al. 2010). It was expected that hippo pathway members would be seen in the screen because of the importance of the hippo pathway to RASSF1A signalling. The presence of these other proteins linked to the hippo pathway may indicate that the effect of RASSF1A on the hippo pathway is not limited to YAP-p73 stabilisation, but also extends to controlling the other pathways, for example, growth control that the hippo pathway has also been shown to regulate.

The presence of proteins associated with Tubulin were expected due to the fact that RASSF1A is able to bind to and stabilise microtubules (Liu, Tommasi et al. 2003). The function of RASSF1A has been reported to depend on microtubule binding proteins (Song, Chang et al. 2005). The association between RASSF1A and Actin and proteins that regulate Actin polymerisation and stabilisation is less well understood. Loss of RASSF1A has been shown to lead to an increase in polymerised F-actin stress fibres and MST1/2 is known to act as a sensor for Actin stability, activating JNK and subsequently p21 when actin is depolymerised (Dallol, Agathanggelou et al. 2005; Densham, O'Neill et al. 2009). However, RASSF1A has not been shown to bind Actin and RASSF1A activity has not been shown to be regulated by Actin stability. Also in this group of proteins there are cytoskeleton associated proteins that are thought to coordinate signalling molecules at the cell membrane by scaffolding the cytoskeleton, membrane proteins and signalling proteins to facilitate interactions, for example Filamin A (Figure 3.6 band 24), scaffolds Actin to the membrane and regulates signalling pathways and cell motility (Zhou, Hartwig et al. 2010). It does this through interactions with: cell adhesion molecules, such as integrins; small GTPases including, the Rho family of GTPases and RalA; and transcription factors such as the SMAD proteins and p73. Some of these proteins are already linked to RASSF1A for example p73 which RASSF1A activates through the hippo pathway (Matallanas, Romano et al. 2007). Also, the integrins, and the Rho family of GTPases (RhoA, Rac1 and Cdc42) regulate cell motility, a cellular activity that RASSF1A has been previously implicated in regulating (Dallol, Agathanggelou et al. 2005).

In the final group, there were a number of interesting novel proteins. The hits included proteins such as the E3 Ubiquitin ligase HUWE1 (Figure 3.6 band 1), which promotes the ubiquitination and degradation

of p53 (Chen, Kon et al. 2005). RASSF1A is able to activate p53 in response to DNA damage by binding to MDM2 and DAXX (Song, Song et al. 2008). Recently, RASSF1C has also been shown to interact with HUWE1. HUWE1 is thought to be responsible for the steady state turnover of RASSF1C (Zhou, Li et al. 2011). We do not know whether RASSF1C was in the immunoprecipitation and whether the interaction with HUWE1 identified was through RASSF1C. However, the immunoprecipitation was with anti-FLAG antibodies and therefore, if the interaction with HUWE1 was through RASSF1C then the complex must also contain RASSF1A. Other proteins identified include proteins that regulate mRNA stability and also DNA damage associated proteins such as XPA, DDB1 and the scaffold protein OSSA (Figure 3.6 band 25).

Three proteins were chosen to from this list, all of which were novel at the time and associated with DNA repair or the DNA damage response. These proteins were XPA, DDB1 and OSSA. Of these three we were able to confirm DDB1 and OSSA, but not XPA as RASSF1A interacting proteins (Figures 3.9, 3.10 and 3.11). Interestingly, RASSF1A was originally identified to bind XPA in a yeast 2-hybrid screen (Dammann, Li et al. 2000). Two proteins that have been linked to DNA damage and showed a very obvious induction of binding to RASSF1A in response to DNA damage were the proteins Plectin-1 and Epiplakin (Figure 3.6 bands 7 and 23). These two large cytoskeletal proteins were identified as very strong hits in the mass spectrometry however they were not taken forward for further investigation because their mass was prohibitive of routine analysis. Little is known about Epiplakin, a 555 kDa protein, but Plectin-1, a 532 kDa protein is known to form part of the desmosome linking intermediate filaments to the cell junctions (Koster, Geerts et al. 2003). Plectin-1 then, along with Filamin A may have provided links between RASSF1A localisation and function at cell-cell junctions.

Like RASSF1A, DDB1 has a number of functions varying from regulating DNA replication and transcription, cell cycle regulation and initiation of nucleotide excision repair in response to UV-induced DNA damage (Hayes, Shiyanov et al. 1998; Fitch, Cross et al. 2003; Wang, Zhu et al. 2004; Bevilacqua, Iovine et al. 2005; Chu and Yang 2008; Iovine, Iannella et al. 2011). DDB1 performs all these functions by ubiquitinating target proteins, such as $p27^{Kip1}$ at the G1/S checkpoint (Bondar, Kalinina et al. 2006;

Iovine, Iannella et al. 2011). The targeting of these proteins appears to either be through direct binding of DDB1 and Cul4A, with which it forms an E3 ubiquitin ligase complex, or through binding of associated factors that target the DDB1-Cul4A complex to substrates. One of these targeting factors is DDB2, which targets the complex towards the histones acetyl transferase p300. This leads to histones H2A, H3 and H4 modification allowing DNA remodelling, loading of the NER protein XPC, and subsequent DNA repair (Datta, Bagchi et al. 2001; Sugasawa 2009). Interestingly, DDB2 is also a substrate for DDB1-Cul4A ubiquitination (Chen, Zhang et al. 2001; Fitch, Cross et al. 2003; Wang, Zhu et al. 2004). Given these roles for DDB1 in identifying and responding to DNA damage and also its roles in the G1/S checkpoint it made for an interesting binding partner for RASSF1A because RASSF1A also has similar functions. However, we were unable to see any change in RASSF1A binding to DDB1 in the IP with IR. Although traditionally DDB1 is known to respond to UV induced damage, it is unlikely that we would have seen an induction in RASSF1A binding to DDB1 under these conditions as the binding appeared constitutive. The constitutive binding may be explained by the overexpression of RASSF1A inducing interactions that normally only occur after stimulation. This is something that we found during the optimisation of the screen as decreasing the amount of RASSF1A transfected into cells led to a dramatic difference between the binding partners seen in cells exposed to IR and those that were not (Figure 3.4 before Figure 3.5 and 3.6 after reduction of transfected RASSF1A). Since performing this screen, RASSF1A has been shown to be targeted for ubiquitin-mediated degradation by the DDB1-Cul4A ubiquitin ligase during M-phase (Jiang, Rong et al. 2011). This reveals that despite the crossover of roles in the DNA damage response, the DDB1-RASSF1A interaction is actually cell cycle dependent. It is already known that RASSF1A is targeted for degradation by the APC/$C^{CDC20}$ ubiquitin ligase, triggered by Aurora A phosphorylation. A trigger for DDB1-Cul4A dependent degradation of RASSF1A was not proposed, but it could potentially be something similar. The paper provides another explanation as to why we saw constitutive binding of DDB1 to RASSF1A. In the IP we performed, the cells were unsynchronised and thus some will have been in M-phase.

The protein that was selected for further investigation was another oxidative stress activated protein, OSSA. OSSA is a scaffold protein and, like DDB1, was shown to be activated by UV radiation, however, unlike DDB1, this activation did not take OSSA into the nucleus, but to the plasma membrane where it was shown to bind to and activate the SRC family kinase (SFK) oncogenes (Tanaka, Sasaki et al. 2009). The reported activation of SFK occurred very early after damage, activating SRC after only 3 min. Once active, SRC was shown to phosphorylate OSSA creating a site to which the SH2 domain of the p85 subunit of PI3-Kinase can bind, become activated and create a survival signal through AKT that precedes the activation of the apoptosis signal from ATM and p53. A secondary role for OSSA as an mRNA binding protein was also identified. OSSA was shown to bind insulin-like growth factor II (IGF-II) mRNA through its C-terminal, stabilising the mRNA and enhancing the extracellular secretion of IGF-II (Tanaka, Sasaki et al. 2009).

A number of factors influenced our decision to investigate OSSA further. Firstly, OSSA has high levels of expression and is prominently phosphorylated by SFK in invasive gastric cancer cells (Tanaka, Sasaki et al. 2009). Loss of RASSF1A is associated with more aggressive cancers (Fendri, Masmoudi et al. 2009; Hu, Chen et al. 2010; Tommasi, Pinto et al. 2011; Wang, Wang et al. 2011; Jiang, Cui et al. 2012; Kim, Chae et al. 2012), suggesting that inhibition by RASSF1A may regulate SRC activation and therefore the invasive potential of a tumour by inhibiting OSSA. OSSA is localised in the cytosol, as is RASSF1A, and also at protruding cell edges. This may suggest that OSSA plays a role in the epithelial-mesenchymal transition (EMT), which YAP, the hippo pathway member is also known to promote (Overholtzer, Zhang et al. 2006). RASSF1A influences the activity of YAP (Matallanas, Romano et al. 2007). YAP stands for Yes-associated protein. Of the three ubiquitous isoforms of SRC (c-SRC, c-YES and c-FYN), OSSA was found to bind most strongly to c-YES (Tanaka, Sasaki et al. 2009).

We failed to detect any change in the binding of RASSF1A to OSSA with exposure to IR suggesting that either the binding was constitutive, or that the stimulus was not correct (Figure 3.10). An endogenous immunoprecipitation in HeLa cells confirmed that the interaction was constitutive (Figure 3.11). Given

that OSSA is a potential oncogene and RASSF1A a tumour suppressor we speculated that RASSF1A sequesters OSSA, potentially to microtubules preventing it from being able to activate SFK. This hypothesis is supported by Figure 3.14 which clearly highlights that the stability of OSSA is affected by RASSF1A levels. Here, expression of FLAG-OSSA was more efficient in cells expressing RASSF1A than in cells where the expression has been lost.

One point of note from this set of immunoprecipitations was that although endogenous RASSF1A could pull down endogenous OSSA efficiently (Figure 3.11), the precipitation of exogenously expressed RASSF1A and OSSA constructs was less efficient (Figure 3.10). This may be due to the interaction being occluded by the tags on the proteins. All of the protein tags except the FLAG-OSSA tag were N-terminal and the interaction was narrowed down an N-terminal fragment consisting of the C1 domain and the RA domain of RASSF1A. It is possible to infer that the interaction only requires the C1 domain because the construct composed of only the RA domain was not found to be precipitated by OSSA despite the expression of the construct being very efficient. This is not the case for the N-terminal (1-119) construct, which expressed poorly and therefore the results were inconclusive.

We were more intrigued by the prospect that RASSF1A could regulate the activity of one of the most important oncogenes in cancer, SRC, therefore we decided to investigate the role of RASSF1A in SFK activation further. Given the opposing roles of SRC and RASSF1A our hypothesis was that as a tumour suppressor RASSF1A would inhibit SFKs and consequently, ablating RASSF1A expression would result in upregulation of SFK activity. However, loss of RASSF1 expression far from resulting in an upregulation of SRC, led to a complete ablation of activity. This was the case for both serum stimulation and activation induced by exposure to IR (Figure 3.13 A and B). This was a very interesting result as it suggested RASSF1 expression was required for SFK activity and bought up questions concerning why RASSF1A, which is lost in almost every tumour type studied (Donninger, Vos et al. 2007), would be lost if it was so important to the activation of SFK.

Given that our link between RASSF1A and SRC was OSSA, we wanted to see if OSSA lay upstream or downstream of RASSF1 in a cascade that leads to the activation of SFK. If OSSA is downstream of RASSF1 then exogenous expression of OSSA, which is normally able to activate SRC, would fail to activate SRC when RASSF1 expression was ablated. We found that this was indeed the case. From this we concluded that RASSF1 is essential for SRC activation and probably lies upstream of a number of factors, one of which is OSSA (Figure 3.15). It is this model that provides the basis for the remainder of the research carried out in this thesis. The data highlights a very exciting, unexplored oncogenic role for RASSF1 and thus has a great deal of potential to provide insight into novel functions of RASSF1 in cancer and understanding of why RASSF1A is lost in cancer.

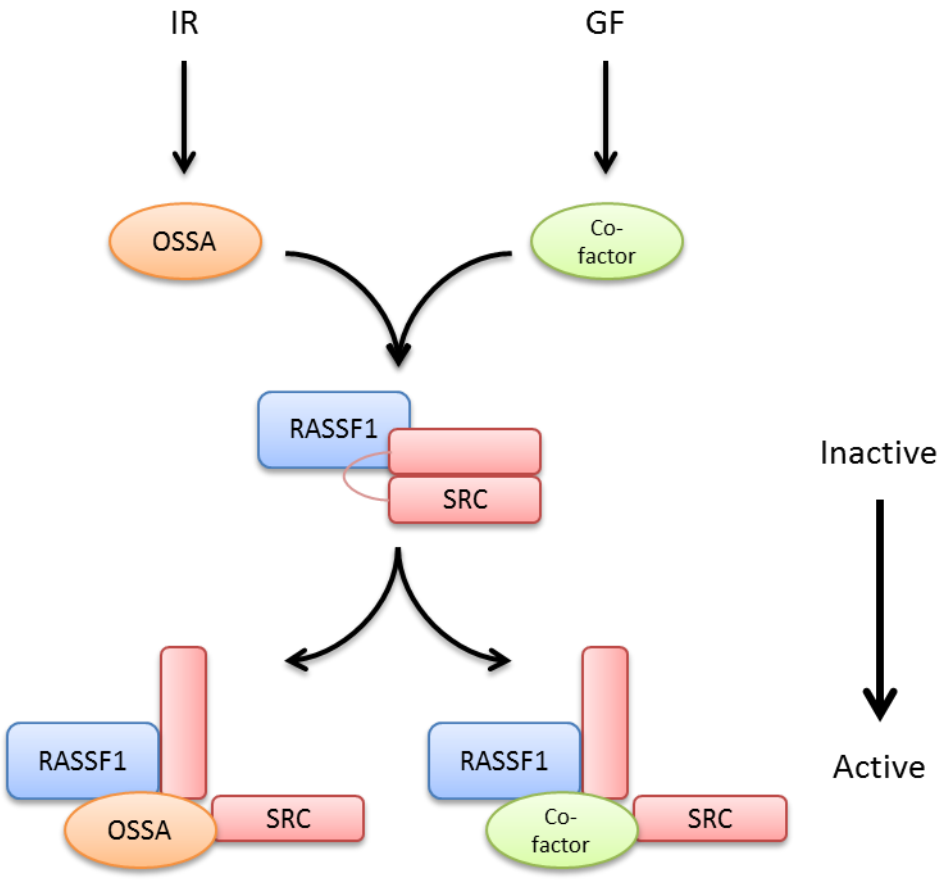


**Figure 3.15. Model Depicting the Hypothesis Made from the Work in Chapter 3.** RASSF1 is required for SRC activation. In response to oxidative stress (IR) or growth factor stimulation (GF), RASSF1 scaffolds OSSA and/or other cofactors enabling their binding to and activation of SRC family kinases.

# Chapter 4 RASSF1A Inhibits the Activation of SRC by RASSF1C

## 4.1 Introduction

In Chapter 3, we aimed to identify novel RASSF1A interacting proteins by mass spectrometry. The hits included DDB1 and OSSA which were shown to be bona fide interacting proteins of RASSF1A. Further analysis of the OSSA interaction with RASSF1A revealed that there was a requirement for the N-terminal domain of RASSF1A and that the interaction stabilised OSSA. Finally, whilst investigating the role of RASSF1A on the activation of SRC by OSSA it was shown that expression of RASSF1 is required for SRC activation in response to an array of stimuli including serum stimulation and DNA damage which additionally may require a number of cofactors of which OSSA maybe one.

### 4.1.1 SRC Family Kinases and Their Activation

The SRC Family Kinases are non-receptor tyrosine kinases. v-SRC the viral counterpart of the cellular proto-oncogene c-SRC was the first oncogene to be discovered. The gene sequence and the signalling properties of SRC were identified in the 1970s; however, it was not until the 1990s when the 3D structure of c-SRC was revealed that the regulation of SRC and its biological functions were truly understood (Xu, Harrison et al. 1997; Kim, Song et al. 2009). There are now nine members of the SRC family kinases, three of which are ubiquitously expressed (SRC, FYN and YES). The other six have a more-restricted expression (reviewed in (Thomas and Brugge 1997)).

SRC family kinases have multiple biological functions, including: regulation of cell adhesion complex assembly and turnover, motility, proliferation and survival, all of which are important in cancer progression. In fact elevated expression and/or activity of SRC is associated with the development of

tumours, particularly colon and breast tumours (Cartwright, Kamps et al. 1989; Verbeek, Vroom et al. 1996). It also correlates with advanced stage and metastatic potential of disease (Cartwright, Meisler et al. 1990; Irby, Mao et al. 1999), as is observed with the epigenetic loss of RASSF1A (Fendri, Masmoudi et al. 2009; Tommasi, Pinto et al. 2011). In a subset of advanced metastatic colon cancers activation of SRC has been shown to be caused by mutations to the C-terminus of c-SRC, analogous to constitutive v-SRC activation (Irby, Mao et al. 1999). All of these factors make SRC an attractive target for therapy and, not surprisingly, in 2010 a number of SRC inhibitors were in phase 1, 2 and 3 clinic trials (Kim, Song et al. 2009; Aleshin and Finn 2010).

The structure of SRC family kinases consists of six functional domains including: an SH3 and SH2 domains; a kinase domain, which contains the autophosphorylation site at Y419; and a regulatory C-terminal, which contains an inhibitory phosphorylation site at Y530 (Sandilands and Frame 2008). Phosphorylation of Y530 by C-terminal SRC kinase (CSK) causes a conformational change allowing the SH2 domain to associate and the SH3 domain to interact with a proline-rich sequence within a linker region between the SH2 domain and the kinase domain (summarised in Figure 4.1). This promotes the dephosphorylation of the Y419 site, rendering the kinase inactive in what is referred to as the 'closed confirmation' (Xu, Harrison et al. 1997). This process is reversed by dephosphorylation of Y530 by protein tyrosine phosphatase alpha (PTP$\alpha$). Overexpression of PTP$\alpha$ or loss of the C-terminal regulatory region can lead to cell transformation and tumourigenesis (Zheng, Wang et al. 1992). OSSA has been hypothesised to bind to the SH3 domain of SRC, opening up the structure and allowing autophosphorylation and activation to occur (Tanaka, Sasaki et al. 2009).

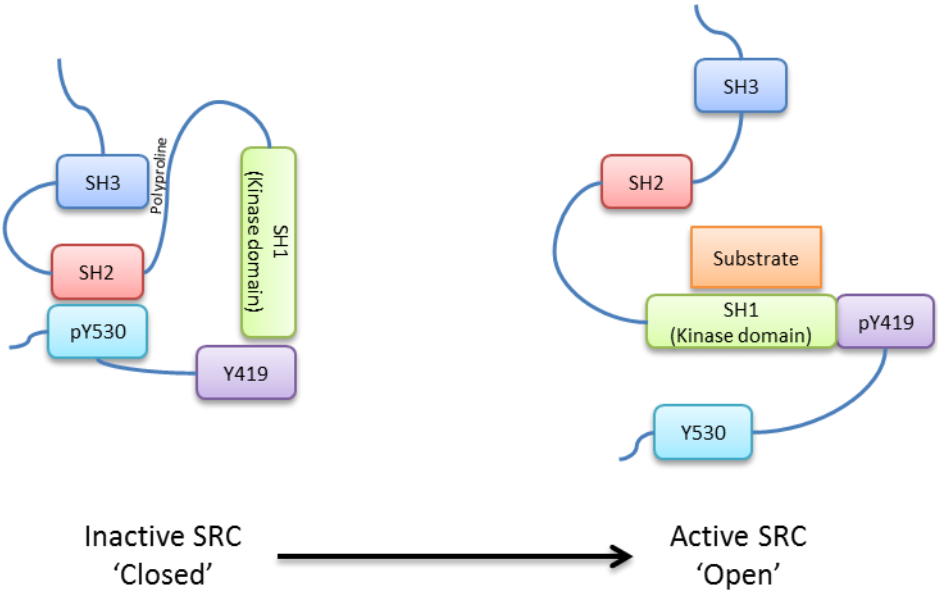


**Figure 4.1. SRC Tyrosine Kinase Structure and Activation.** SRC kinases consists of 6 domains: a myristylation domain, a domain unique to each family member, a SRC homology 3 (SH3) domain, an SH2 domain, the kinase domain containing the autophosphorylation site (Y419) and a C-terminal regulatory domain containing the inhibitory phosphorylation site (Y530). Phosphorylation on the Y530 site by CSK leads SRC to form the inactive, closed conformation. Here the phosphorylated Y530 site forms an internal interaction with the SH2 domain and the SH3 domain interacts with a polyproline sequence in a linker region between the SH2 domain and the kinase domain. Upon activation the Y530 site is dephosphorylated, releasing the constraints on the kinase domain allowing the kinase to form the open conformation. Autophosphorylation now occurs on the Y419 site activating the protein and the SH2 and SH3 domains are free to form interactions with proteins involved in intracellular signalling.

As well as structural regulation, SRC family kinases are spatially regulated within cells. Initial studies showed that v-SRC existed in two pools in the cell, an inactive perinuclear pool and an active plasma membrane associated pool (Rohrschneider 1979; Courtneidge, Levinson et al. 1980; Welham and Wyke 1988). The activity of c-SRC was found to be regulated in the same way. The majority of wildtype c-SRC was located in the perinuclear region directly associated with the microtubule organising centre (MTOC) and co-localized with endosomal markers (Kaplan, Swedlow et al. 1992). Active c-SRC was found at the cell periphery associated with focal adhesions (Kaplan, Bibbins et al. 1994). Trafficking of SRC from the perinuclear region to the plasma membrane has been shown to be dependent on Rho-GTPases, endosomal trafficking and actin polymerisation (Kaplan, Swedlow et al. 1992; Timpson, Jones et al. 2001; Gasman, Kalaidzidis et al. 2003; Sandilands, Cans et al. 2004; Sandilands, Brunton et al. 2007; Sandilands and Frame 2008).

### 4.1.2 RASSF1 Isoforms

In chapter 1.4 the RASSF1 gene and its isoforms were introduced (Figure 1.1). Briefly, RASSF1 can be spliced into eight isoforms which are transcribed from two different promoters. The upstream promoter codes for RASSF1A, D, E, F and G and is often methylated and epigenetically inactivated in cancer. The downstream promoter codes for RASSF1B, C and H and is rarely silenced in cancer (Agathanggelou, Cooper et al. 2005). Of these isoforms, only RASSF1A and RASSF1C have been characterised extensively. RASSF1A is a tumour suppressor that has been shown to play a role in the DNA damage response, apoptosis, cell cycle (promoting G1/S and pro-metaphase arrest) and migration (reviewed in (Donninger, Vos et al. 2007)). Initially, RASSF1C was also thought to be a tumour suppressor because it was shown to induce apoptosis and could reduce tumourigenicity of KRC/Y RCC cells in SCID mice (Vos, Ellis et al. 2000; Li, Wang et al. 2004; Lorenzato, Martino et al. 2012). However, some recent reports suggest that it is an oncogene, promoting growth in Lung and Breast cancer cell lines (Vos, Ellis et al. 2000; Amaar, Minera et al. 2006; Reeves, Baldwin et al. 2010; Reeves, Baldwin et al. 2012). Neither RASSF1A nor RASSF1C have any enzymatic activity and so are thought to exert their function by acting as scaffold proteins.

### 4.1.3 Aims

The work in Chapter 3 highlighted a novel role for RASSF1 in the regulation of SRC family kinases. One problem with this work was the use of an siRNA targeting RASSF1 to investigate the role of RASSF1A. A dependence of SRC activation on RASSF1A does not correlate with its characterisation as a tumour suppressor. Recent work has begun to elucidate functions for the other isoforms of RASSF1, particularly RASSF1C. Therefore, in this chapter we shall investigate the role of the major RASSF1 isoforms, RASSF1A and RASSF1C, in the activation of SRC.

## 4.2 RASSF1A Expression is Unable to Activate SRC

Previously, we have shown that loss of RASSF1 led to a complete ablation of SRC activity (Figure 3.13). This did not fit with the role of RASSF1A as a tumour suppressor. We reasoned that if RASSF1A was indeed required for SRC activation then re-expression of RASSF1A into RASSF1A-negative cancer cells would lead to an increase in SRC activity. To address this we transiently transfected FLAG-RASSF1A into MCF7 cells, which are methylated for the RASSF1A promoter and considered to be RASSF1A null. We found that SRC activity was unaffected by expression of RASSF1A in these cells (Figure 4.2). Interestingly, we also found that SRC was unable to be activated by DNA damaging agents, something we had been able to detect in RASSF1A expressing HeLa cells (Figure 3.13B).

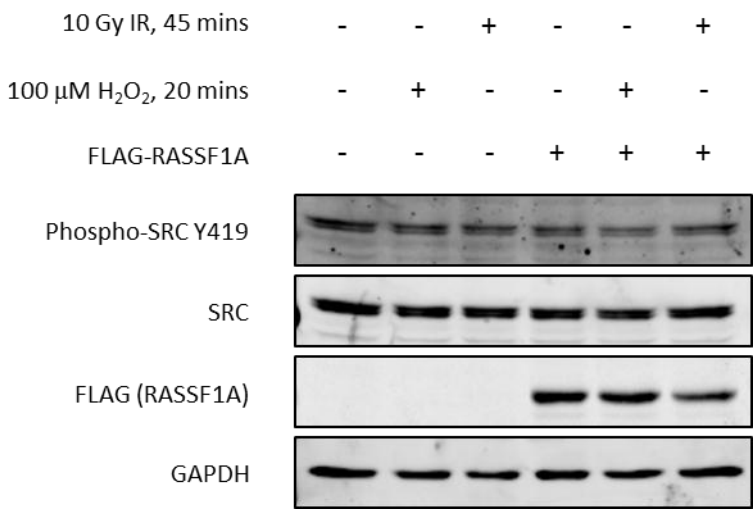


**Figure 4.2. . Expression of RASSF1A is Unable to Activate SRC Family Kinases.** MCF7 cells (2 x $10^5$ cells/plate) were transfected with 1 μg pcDNA3 or FLAG-RASSF1A. Cells were cultured in DMEM containing 0.1 % FBS 16 hrs prior to exposure to 100 μM $H_2O_2$ for 20 mins, or 10 Gy IR and lysed 45 mins post exposure with Laemmli lysis buffer. Lysates were analysed by SDS-PAGE and western blot for activated SRC with antibodies against phospho-SRC Y419 and total SRC. Anti-FLAG-tag antibodies were used to confirm RASSF1A expression and GAPDH represents a loading control. The level of phosphorylated SRC does not increase with RASSF1A expression. SRC stimulation by IR or $H_2O_2$ also appears to be limited in MCF7 cells. Data is representative two independent experiments.

In chapter 3 we utilised siRNA targeting RASSF1, which silences all RASSF1 isoforms, with the assumption being any affects we saw would be due to loss of RASSF1A as it is the major isoform expressed from the *RASSF1* gene. To test whether the activation of SRC was indeed due to loss of RASSF1A we transfected HeLa cells with non-targeting siRNA, RASSF1 siRNA or with a RASSF1A isoform specific siRNA. We found that, as we had seen previously, loss of RASSF1 led to a decrease in SRC activity, however, loss of RASSF1A did not, suggesting that RASSF1A was not the isoform of the RASSF1 gene that was responsible for the activation of SRC (Figure 4.3).

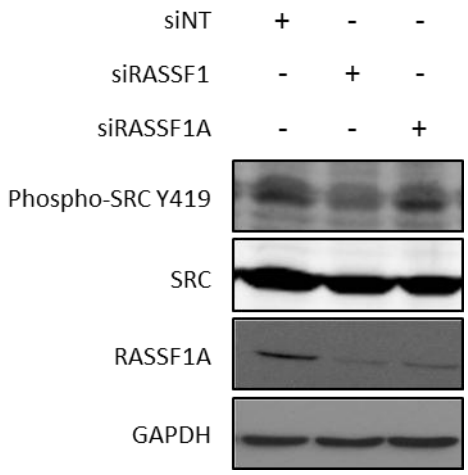


**Figure 4.3. SRC Family Kinases Are Activated in Cells Specifically Ablated of RASSF1A, But Not All RASSF1 Isoforms.** HeLa cells (2 x $10^5$ cells/plate) were transfected with non-targeting siRNA, an siRNA to RASSF1 or an isoform specific siRNA to RASSF1A. Cells were lysed with Laemmli lysis buffer and analysed by SDS-PAGE and western blot for SRC activity using antibodies against phospho-SRC Y419 and total SRC. Antibodies against endogenous RASSF1A were used to confirm knock down of expression and GAPDH was used as a loading control. Levels of phosphorylated SRC are decreased in cells treated with siRNA to RASSF1, but are unaffected in cells treated with the isoform specific RASSF1A siRNA. Data is representative of three independent experiments.

Taken together the data suggests that RASSF1A is not the isoform of RASSF1 that is required for SRC activation, because neither RASSF1A overexpression or specific ablation of RASSF1A expression affected SRC activity. This led us to investigate the roles other RASSF1 isoforms, namely RASSF1C in regulating SRC activation.

## 4.3 RASSF1C Expression Can Activate SRC Only When RASSF1A is Absent from the Cells

As loss of RASSF1A did not affect SRC activity we decided to address the role of RASSF1C. RASSF1C is the second major isoform expressed form the *RASSF1* gene. RASSF1C is transcribed from an internal downstream promoter within the RASSF1 gene (Figure 1.1). This promoter remains unmethylated in cancers and thus the expression of RASSF1C is maintained. RASSF1C has been shown to drive proliferation and migration in lung and breast cancer cells respectively, two biological functions that are also associated with SRC activity (Amaar, Minera et al. 2006; Reeves, Baldwin et al. 2010). This makes RASSF1C a plausible candidate for the isoform of RASSF1 responsible for the activation of SRC. We transiently transfected H1299 cells, which like MCF7 cells, are RASSF1A null, with FLAG-RASSF1C. Cells were cultured in 0.1 % FBS containing medium overnight to remove growth factor stimulation of SRC before determining SRC activity by western blot. As expected we saw that expression of RASSF1C led to an increase in the levels of phosphorylated, active, SRC (Figure 4.4A). RASSF1A is associated with the hippo pathway, which is known to be regulated by cellular density (Zhao, Wei et al. 2007; Ota and Sasaki 2008). We therefore sought to test whether cell density played a role in the RASSF1C activation of SRC. H1299 cells were plated at high and low cell densities such that at the time of lysis cells would be either very confluent or approximately 70 – 80 % confluent. Under both conditions RASSF1C was shown to activate SRC to a similar level, ruling out cell density as a regulator of this novel pathway (Figure 4.4B). Given that RASSF1A binds OSSA and OSSA was reported to activate SRC we wished to address the role of OSSA in RASSF1C-dependent activation of SRC. H1299 cells were transfected with non-targeting siRNA or OSSA siRNA before transfecting the cells with increasing amounts of RASSF1C. As in chapter 3, OSSA stability was shown to be positively affected by RASSF1 isoform expression (Figure 4.4B), and RASSF1C was able to activate SRC even in the absence of OSSA, again suggesting that OSSA may require RASSF1C to activate SRC.

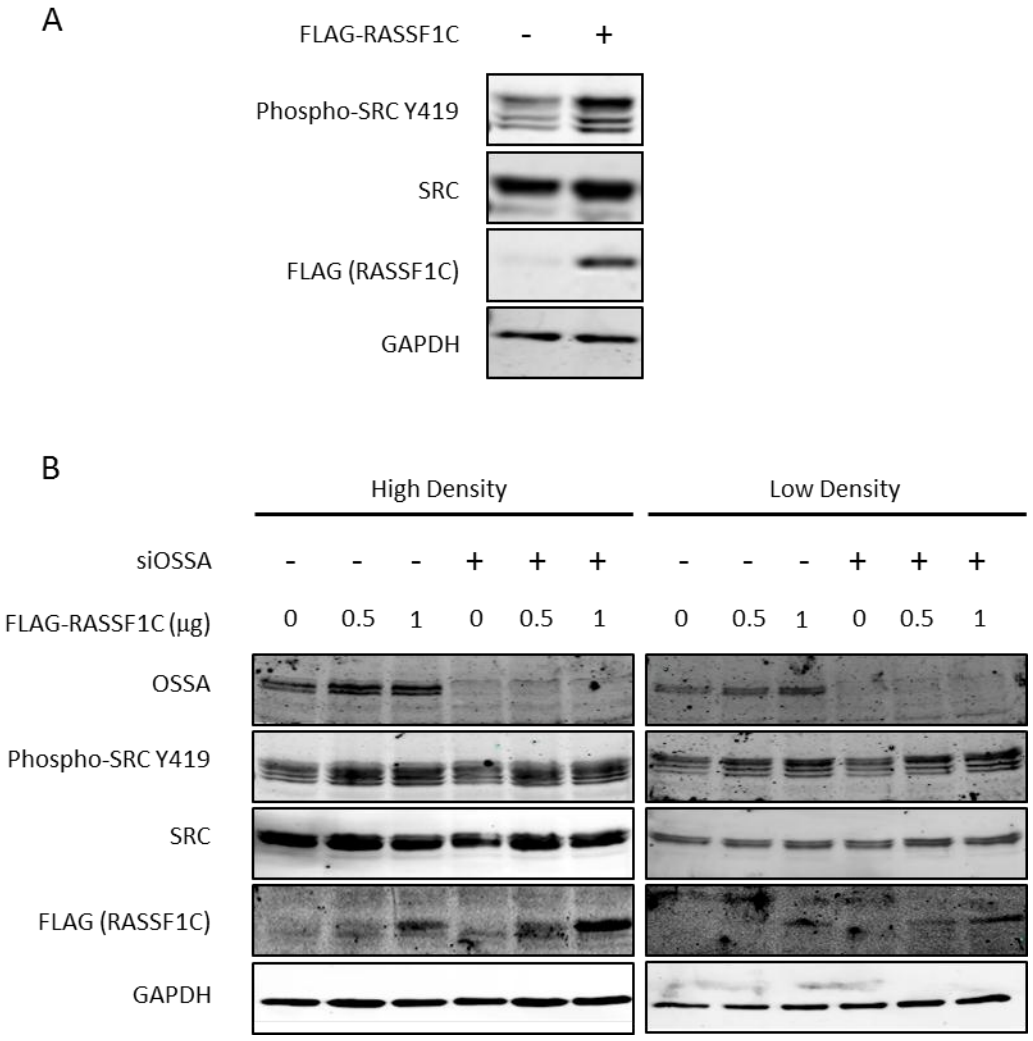


**Figure 4.4. RASSF1C Expression Can Activate SRC in H1299 cells in an OSSA-Independent Fashion. A.** H1299 cells (2 x $10^5$ cells/plate) were transfected with 0.5 µg pcDNA3 or FLAG-RASSF1C. Cells were cultured in DMEM containing 0.1 % FBS 16 hrs before lysis with laemmli lysis buffer. Lysates were analysed by SDS-PAGE and western blot for activated SRC using antibodies against phospho-SRC Y419 and total SRC. Anti-FLAG antibodies were used to confirm RASSF1C expression. GAPDH is a loading control. Phosphorylated, active, SRC clearly increases with RASSF1C expression. Data is representative of three independent experiments. **B.** H1299 cells were plated at high (3 x $10^5$ cells/plate) or low (1.5 x $10^5$ cells/plate) density and transfected with non-targeting siRNA or siRNA targeting OSSA. Subsequently, cells were transfected 0, 0.5 or 1 µg FLAG-RASSF1C. DNA transfected was equalised using empty vector. Cells were cultured in DMEM containing 0.1 % FCS for 16 hrs prior to lysis with Laemmli lysis buffer. Lysates were analysed with SDS-PAGE and western blot for activated SRC using antibodies against phospho-SRC Y419 and total SRC. Endogenous OSSA antibodies were used to confirm OSSA knockdown and anti-FLAG antibodies confirmed RASSF1C expression. GAPDH is a loading control. Phosphorylated SRC increased with RASSF1C expression in an OSSA- and cell density-independent manner. Data represents one independent experiment.

We next wanted to confirm that the activation of SRC by RASSF1C was cell line independent. We transiently transfected FLAG-RASSF1C into RASSF1A expressing (U2-OS, SW480, and HeLa), or RASSF1A null (MCF7 and MDA-MB-231) cells. Cells were incubated in 0.1 % FBS medium overnight, lysed and blotted for SRC activity. In U2-OS, HeLa and SW480, which all express RASSF1A, we were unable to detect an increase in SRC activity upon exogenous expression of RASSF1C. However, in the MCF7 and MDA-MB-231, RASSF1A null cells, we detected RASSF1C induced SRC activation (Figure 4.5). Given that the cell line used to test whether RASSF1C could activate SRC, H1299, are RASSF1A negative, we reasoned that cell lines with RASSF1A expression could not activate SRC upon RASSF1C expression.

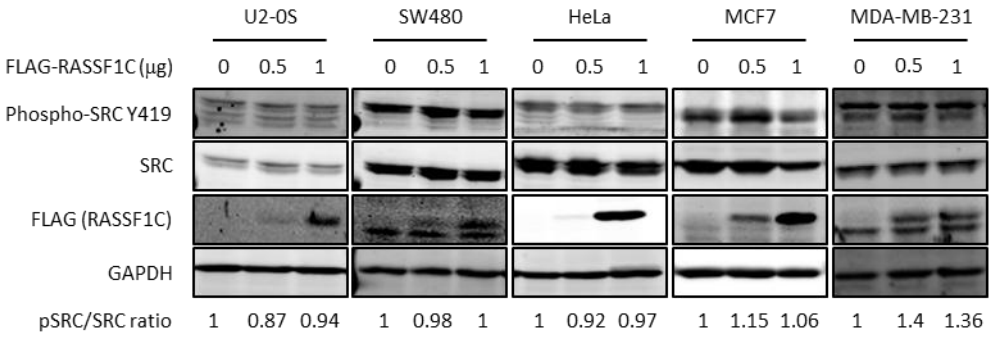


**Figure 4.5. The Expression of RASSF1C can Only Activate SRC in RASSF1A-Negative Cell Lines.** RASSF1A expressing cell lines (U2-0S, SW480 and HeLa) and RASSF1A negative (RASSF1A methylated) cell lines (MCF7 and MDA-MB-231) (2 x $10^5$ cells/plate) were transfected with 0, 0.5 or 1 μg FLAG-RASSF1C. Cells were cultured in DMEM containing 0.1 % FBS for 16 hrs prior to lysis with Laemmli lysis buffer. Lysates were analysed by SDS-PAGE and western blot for activated SRC with antibodies against phospho-SRC Y419 and total SRC. Anti-FLAG antibodies confirmed RASSF1C expression and GAPDH is a loading control. Phosphorylated SRC increases with RASSF1C expression in MCF7 and MDA-MB-231 cells, but not in U2-OS, SW480 or HeLa cells as indicated by the Phospho-SRC to SRC ratio (pSRC/SRC). Blots were quantitated using Licor Odyssey software. Data represents one independent experiment.

To test that RASSF1C mediated activation of SRC was inhibited by RASSF1A we transfected a cell line that was known to be 100 % methylated at the RASSF1A promoter with FLAG-RASSF1C and investigated the ability of RASSF1C to activate SRC. RKO cells were chosen for this purpose as they are known to be

100 % methylated for RASSF1A (Eric O'Neill, personal communication). As expected, expression of RASSF1C into RKO cells led to a dose-dependent increase in SRC activity (Figure 4.6).

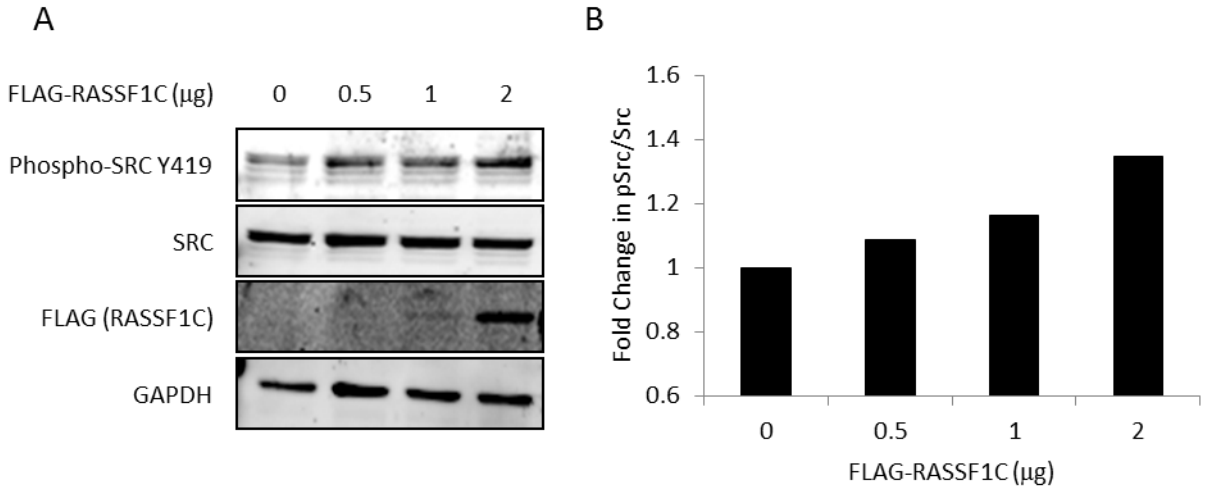


**Figure 4.6. Expression of RASSF1C can activate SRC in the Colon Carcinoma cell line RKO.** RKO cells (2 x $10^5$ cells/plate) were transfected with 0, 0.5, 1 or 2 μg FLAG-RASSF1C. DNA transfected was equalised with pcDNA3. Cells were cultured in DMEM containing 0.1 % FBS for 16 hrs prior to lysis with Laemmli lysis buffer. **A.** Lysates were analysed with SDS-PAGE and western blot for activated SRC with antibodies against phospho-SRC Y419 and total SRC. Anti-FLAG antibodies were used to confirm RASSF1C expression and GAPDH is a loading control. Phospho-SRC levels increase in a RASSF1C expression-dependent manner. Western blot is representative of 2 independent experiments. **B.** Quantification of phospho-SRC levels relative to total SRC of western blot shown using the Licor Odyssey software.

To further confirm that RASSF1A inhibited RASSF1C activation of SRC we generated a cell line that stably expressed a Tetracycline inducible form of RASSF1A. This 'Tet ON' system would allow us to induce the expression of RASSF1A with the addition of doxycycline to the cell culture medium (Figure 8.1). H1299 cells were chosen for this as we knew that expression of RASSF1C would lead to a robust activation of SRC (Figure 4.4). Transient transfection of RASSF1C into these Tet ON H1299 cells only led to an activation of SRC when doxycycline, and thus RASSF1A expression was absent (Figure 4.7).

Taken together the data indicates that RASSF1C is the isoform of RASSF1 that is responsible for the activation of SRC. Activation of SRC by RASSF1C can only occur in cells that have lost RASSF1A expression as re-introduction of RASSF1A into RASSF1A-negative cells can inhibit this activation.

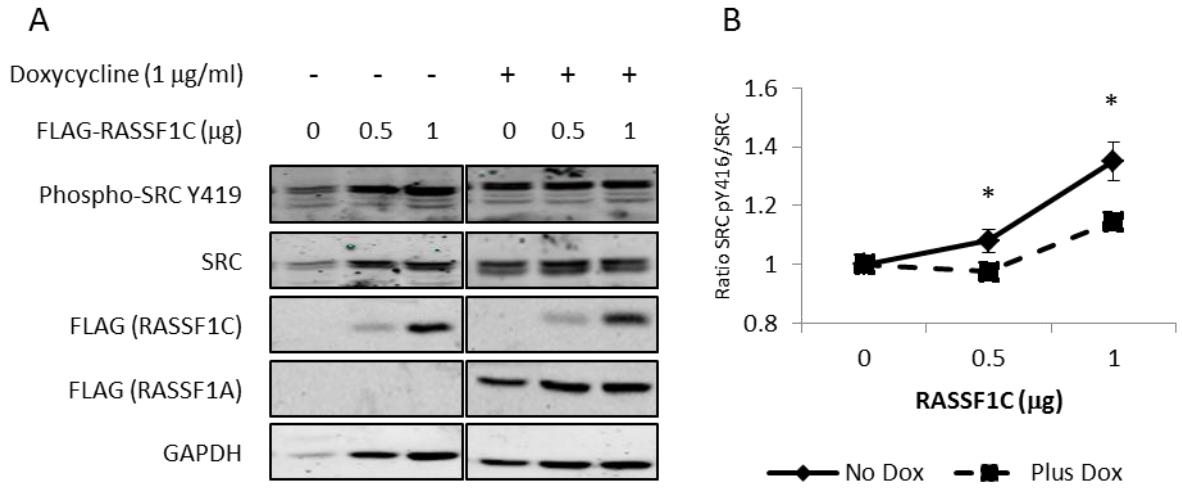


**Figure 4.7. Expression of RASSF1A in Doxycycline Inducible RASSF1A H1299 Cells Inhibits the Activation of SRC by RASSF1C. A.** Doxycycline inducible RASSF1A expressing H1299 cells (2 x $10^5$ cells/plate) were transfected with 0, 0.5 or 1 μg FLAG-RASSF1C and 24 hrs later 1 μg/ml Doxycycline was added to the tissue culture medium. After 8 hrs the medium was changed to DMEM containing 0.1 % FBS and the doxycycline re-added to the medium. Cells were incubated overnight cells were lysed with laemmli lysis buffer. Lysates were analysed by SDS-PAGE and western blot for activated SRC using anti-phospho-SRC Y419 and total SRC antibodies. Levels of phosphorylated SRC increase with the addition of RASSF1C only in the absence of RASSF1A expression. **B.** Quantification of levels of phosphorylated SRC against the levels of total SRC. Quantification of three independent experiements was done using the Licor Odyssey. Asterisks indicate a ρ value of <0.05 in a one-tailed student's T-test. (ρ-values: at 0.5 μg RASSF1C is 0.01, at 1 μg RASSF1C is 0.04).

## 4.4 RASSF1A Interacts with RASSF1C to Inhibit its Function.

Having identified that RASSF1A was inhibiting RASSF1C we next wished to investigate how this might be occurring. As scaffolds RASSF1A and RASSF1C activate or inhibit their target proteins through direct interactions. The hypothesis therefore is that RASSF1A may bind RASSF1C and sequester it away from SRC. To address this we performed a co-immunoprecipitation with exogenously expressed HA-RASSF1A

and FLAG-RASSF1C in H1299 cells. We were able to see that HA-RASSF1A did indeed immunoprecipitate with FLAG-RASSF1C and that FLAG-RASSF1C immunoprecipitated with HA-RASSF1A (Figure 4.8). This was not surprising given that both RASSF1A and RASSF1C have a SARAH domain, which has previously been shown to be responsible for interactions between RASSF1A, MST and WW45 (Guo, Tommasi et al. 2007; Hwang, Ryu et al. 2007; Donninger, Allen et al. 2011).

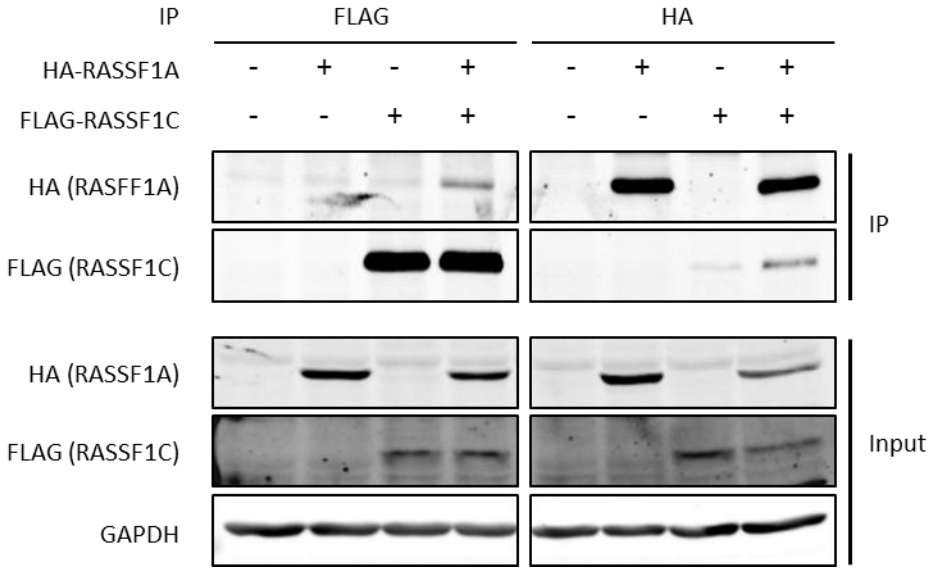


**Figure 4.8. RASSF1A and RASSF1C Can Interact.** H1299 cells (1 x $10^6$ cells/plate) were transfected with HA-RASSF1A and FLAG-RASSF1C either alone or together. Empty vector was used to balance the amount of DNA transfected. Cells were cultured for 48 hrs before cells were lysed using a 0.5 % NP40, 150 mM NaCl lysis buffer and immunoprecipitated with either anti-HA antibodies bound to protein G sepharose beads or with anti-FLAG M2-conjugated agarose beads. Inputs and precipitates were analysed by SDS-PAGE and western blot for RASSF1A and RASSF1C with anti-HA or anti-FLAG antibodies respectively. Data is representative of two independent experiments.

To further characterise the binding between RASSF1A and RASSF1C, H1299 cells were transiently transfected with HA-RASSF1C along with FLAG-RASSF1A or FLAG-RASSF1A constructs with point mutations at the ATM consensus sequence (S131A) (Hamilton, Yee et al. 2009), and a common polymorphic variant (A133S) (Yee, Grochola et al. 2012), or MYC-RASSF1A deletion constructs (Full length, 1-119, 1-287, 120-340). FLAG-RASSF1C, MYC-RASSF1C, MYC-RASSF1A A63S or a MYC-RASSF1C

deletion construct (1-216) were also co-transfected with HA-RASSF1C to gain more information as to whether RASSF1C can form homodimers, a property that RASSF1A has (Hamilton, Yee et al. 2009). Mutation of the ATM phosphorylation site (S131) had no effect on the interaction between RASSF1C and RASSF1A, but mutation of A133 site led to a decrease in the amount of RASSF1A precipitating with RASSF1C (Figure 4.9). Full length RASSF1A precipitated with RASSF1C as did an N-terminal construct, consisting of C1 and RA domains and a C-terminal construct, consisting of RA and SARAH domains. From this we can conclude that the RA domain is important for the interaction as it is common to all three constructs. The poor expression of the N-terminal construct (1-119) meant we were unable to rule out an interaction between the C1 domain of RASSF1A and RASSF1C. It was surprising to see that the interaction between RASSF1A and RASSF1C was SARAH domain independent. Expression of a construct containing only the RA domain could confirm this.

The experiment also allowed us to confirm that RASSF1C can form homodimers and that the interaction was also SARAH domain independent, as the N-terminal construct (1-216) which contains the unique N-terminal domain and the RA domain was able to interact with HA-RASSF1C (Figure 4.9).

Taken together, this data shows that RASSF1C can form both homodimers and heterodimers with RASSF1A. Both interactions required the RA domain. A secondary interaction site in the C1 or SARAH domain may also exist. The binding of RASSF1C by RASSF1A provides a mechanism through which RASSF1A can inhibit RASSF1C, preventing RASSF1C from activating SRC by sequestering it away from SRC. We therefore, decided to test this hypothesis.

We also noticed that the expression of HA-RASSF1C appeared to be differentially expressed when co-expressed with the FLAG-tag and MYC-tag constructs. Further investigation showed that this was due to competition between the expression of the proteins from different vector backgrounds (Figure 8.2).

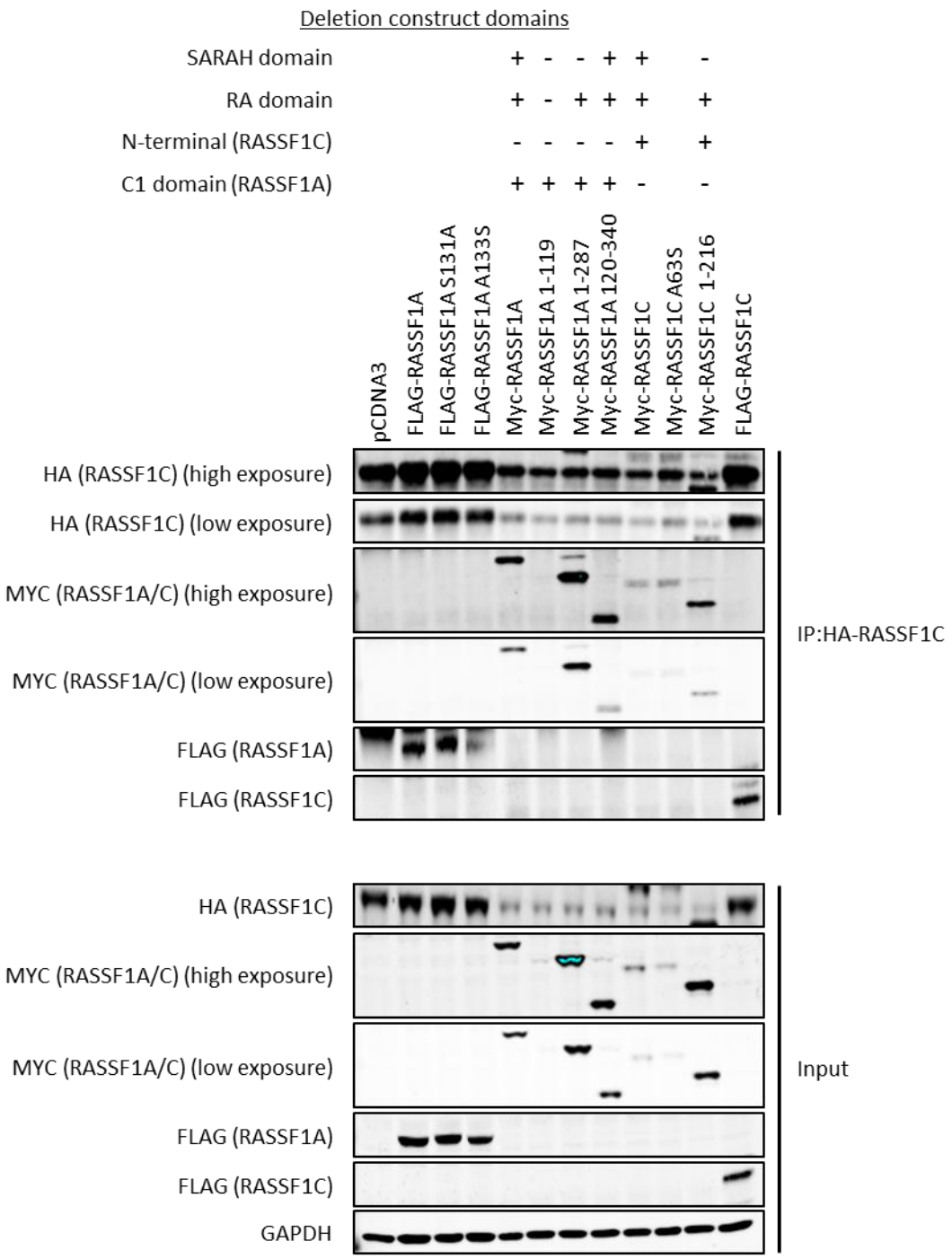


**Figure 4.9. The Interaction Between RASSF1A and RASSF1C or RASSF1C and RASSF1C requires the RA domain.** H1299 cells (1 X $10^6$ cells/plate) were transfected with HA-RASSF1C along with FLAG-RASSF1A and FLAG-RASSF1C constructs. The constructs include mutations surrounding the ATM phosphorylation site as well as truncation mutations (domain present in each truncation construct shown in table above blot). Cells were lysed using a 0.5 % NP40, 150 mM NaCl lysis buffer and immunoprecipitated using an anti-HA antibodies bound to protein G sepharose beads. Inputs and precipitates were analysed by SDS-PAGE and western blot for the presence of RASSF1 proteins using antibodies against HA-tag, FLAG-tag and MYC-tag. GAPDH was used as a loading control. FLAG-RASSF1A and FLAG-RASSF1C immunoprecipitated with HA-RASSF1C. FLAG-RASSF1A binding was unaffected by the S131A mutation, but is reduced by the A133S mutation. The interaction between RASSF1A and RASSF1C requires the RA domain (120-287), but is SARAH domain independent. The homodimer interaction between RASSF1C molecules is also SARAH domain independent. Data represents one independent experiment.

## 4.5 The Common Polymorphic Variant RASSF1C A63S Does Not Activate SRC

Having shown that the RASSF1A A133S common polymorphic variant of RASSF1A had decreased binding to RASSF1C we wanted to investigate whether the same polymorphism in RASSF1C, RASSF1C A63S, was able to activate SRC. The polymorphism in RASSF1A is thought to alter the structure of RASSF1A near to the RA domain and is known to inactivate the RASSF1A protein acting as a dominant negative (Yee, Grochola et al. 2012). Given that RASSF1C is can activate SRC we reasoned that if the RASSF1C A63S polymorphism was unable to activate SRC it would go some way towards explaining why mutations in the RASSF1 gene are so uncommon, with specific promoter methylation occurring preferentially.

H1299 cells stably expressing a Tetracycline inducible RASSF1C or RASSF1C A63S vector were plated and protein expression was induced by addition of doxycycline to the culture medium. As shown previously, RASSF1C expression led to a robust activation of SRC. However, expression of RASSF1C A63S failed to activate SRC indicating that the polymorphism prevents RASSF1C from activating SRC family kinases (Figure 4.10).

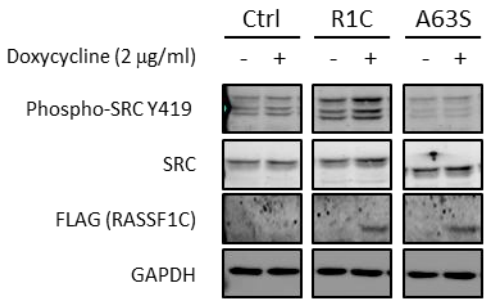


**Figure 4.10. The RASSF1C polymorphism is unable to activate SRC family kinases.** H1299 cells (2 x $10^5$ cells/plate) stably transfected with an tetracycline inducible control vector, FLAG-RASSF1C or FLAG-RASSF1C A63S were plated and doxycycline was added to the cell culture media to induce expression of RASSF1C or RASSF1C A63S. Cell were cultured for 16 hrs in DMEM supplemented with 0.1 % FBS in the presence of doxycycline prior to lysis with laemmli lysis buffer. Lysates were resolved by SDS-PAGE and analysed by western blot for RASSF1C expression and SRC activity using antibodies against FLAG-tag, phospho-SRC Y419 and total SRC. GAPDH is a loading control. Expression of RASSF1C, but not RASSF1C A63S activated SRC. Data represents one independent experiment.

## 4.6 SRC is Activated by Binding RASSF1C, an Interaction which is Lost when RASSF1A is Expressed

We have shown that exogenous expression of RASSF1C is able to activate SRC and that binding of RASSF1A to RASSF1C can inhibit this. We next wanted to investigate how RASSF1C activated SRC. SRC is held in an inactive perinuclear pool until a stimulus is applied at which point the SRC is transported to the plasma membrane by vesicular trafficking. At the membrane SRC unfolds and is able to autophosphorylate on Y419. SRC can be inactivated by phosphorylation of a second, inhibitory, site at Y530. This is carried out by the tyrosine kinase, C-terminal SRC kinase (CSK). From this there are two possible mechanisms through which RASSF1C could activate SRC. The first is to bind directly to SRC and facilitate its efficient translocation to the plasma membrane and the second is to bind and inhibit CSK. We first decided to test whether RASSF1C interacted with SRC and whether RASSF1A inhibited this interaction.

H1299 cells were transiently transfected with a control plasmid, FLAG-RASSF1A, FLAG-RASSF1C or both FLAG-RASSF1A and FLAG-RASSF1C together before endogenous SRC was immunoprecipitated. As expected, we found that SRC could immunoprecipitate RASSF1C but not RASSF1A and that the amount of RASSF1C precipitating with SRC were reduced when RASSF1C was co-expressed with FLAG-RASSF1A (Figure 4.11).

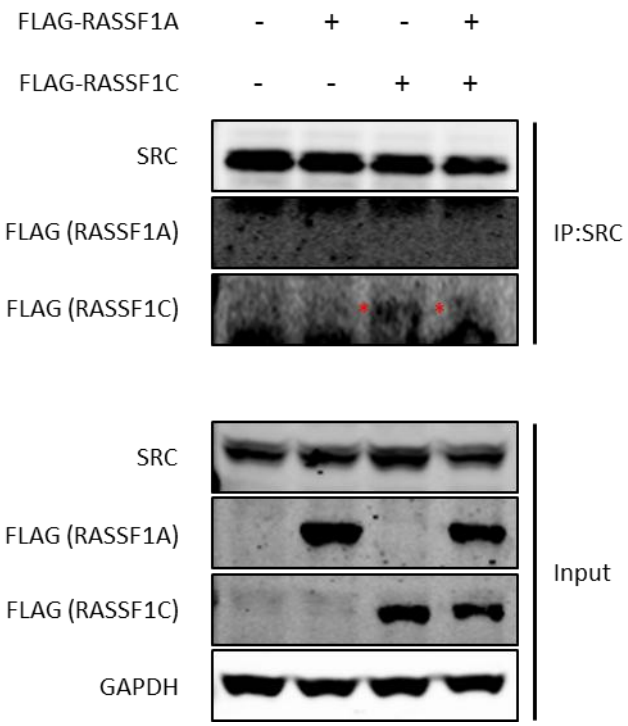


**Figure 4.11. RASSF1A Inhibits the Interaction Between RASSF1C and SRC Family Kinases.** H1299 cells (2 x $10^6$ cells/plate) were transfected with FLAG-RASSF1A and FLAG-RASSF1C, either individually or together at a level that even expression of both constructs was achieved. Cells were lysed after 48 hrs with a 0.5 % NP40, 150 mM NaCl lysis buffer. Lysates were immunoprecipitated with anti-SRC antibodies bound to protein G sepharose beads. Inputs and precipitates were analysed by SDS-PAGE and western blot for interaction between RASSF1A or RASSF1C and SRC family kinases. RASSF1C is precipitated by SRC family kinases only when expressed alone. Co-expression of RASSF1A leads to a decrease in this interaction. RASSF1A does not interact with SRC family kinases. The blot is representative of three experiments. Asterisks indicate the RASSF1C band in the IP samples.

We sought to determine whether the levels of SRC at the plasma membrane were enhanced in cells expressing RASSF1C and whether this correlated with the increased levels of activated SRC at the membrane. In order to do this MCF7 were chosen. Like H1299 cells, RASSF1C expression induced a robust activation of SRC (Figure 4.5) and MCF7 cells also form strong cell-cell junctions. This is because they are of epithelial origin and maintain E-cadherin expression. Cells were seeded onto glass cover slips before being transfected with empty vector or FLAG-RASSF1C. Cells were probed for total SRC or phospho-SRC Y419 to show increased translocation and activation of SRC in cells expressing RASSF1C. The level of total SRC in cells was enhanced, with particularly strong staining at the plasma membrane (Figure 4.12A). The stabilisation of SRC by RASSF1C was also observed by western blot (Figure 4.11). The levels of phosphorylated, active SRC may also be enhanced and associated with the plasma membrane (Figure 4.12B). Due to the poor staining of the endogenous phosphorylated SRC with the antibody used, this cannot be fully confirmed. Studies using exogenous expression of SRC family kinases may be used to confirm that the plasma membrane bound SRC is indeed activated in RASSF1C expressing cells.

Taken together these results indicate RASSF1C interacts with SRC to facilitate the translocation of SRC from the perinuclear region of the cell to the plasma membrane where it is able to be activated. Expression of RASSF1A inhibits this by binding to and sequestering RASSF1C away from SRC.

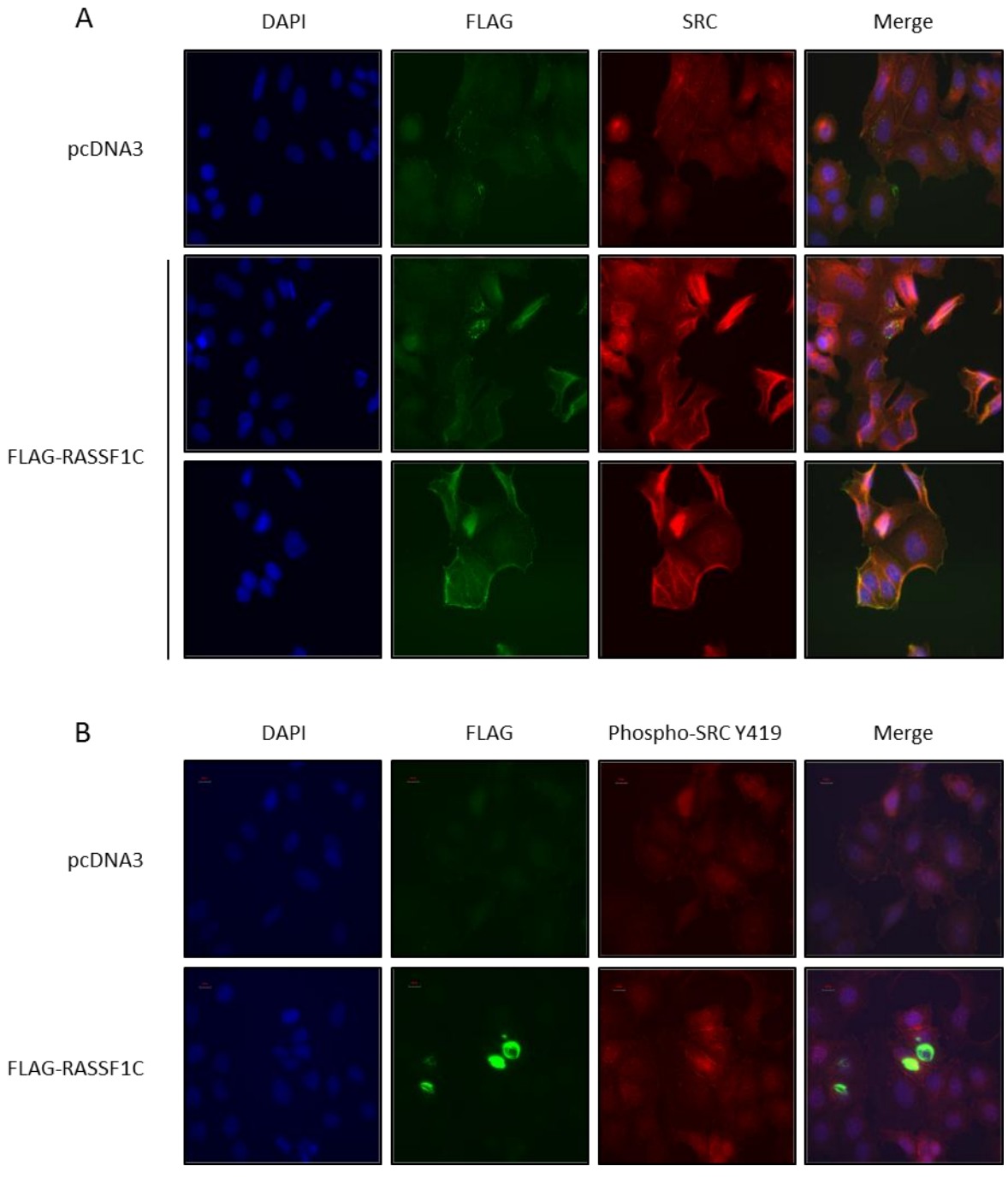


**Figure 4.12. RASSF1C Expression Promotes SRC Translocation to the Plasma Membrane Where it is Activated.** MCF7 cells (1 x $10^5$ cells/plate) were plated onto glass cover slips and transfected with 0.5 μg empty vector or FLAG-RASSF1C. 48 hrs post transfection cells were: fixed with 4 % PFA for 15 mins, permeabilised with 0.2 % triton X for 5 mins, blocked with 0.2 % Fish skin gelatin for 30 mins before being stained with anti-FLAG antibodies and either anti-SRC or anti-phospho SRC Y419 antibodies. Alexa-fluor® secondary antibodies with wavelengths 488 or 594 nm were used and DAPI staining was in the mounting medium. Images were taken with a Leica DM IRBE at 63 x magnification. RASSF1C transfected cells (green staining) have brighter staining for total SRC **(A.)** and phosphorylated SRC **(B.).** This is particularly prevalent at the plasma membrane of the cells, where active SRC is found. Data is representative of three independent experiments.

## 4.7 RASSF1A and RASSF1C Can Interact With the Negative Regulator CSK

We have seen that RASSF1C can bind to SRC promoting its translocation to the plasma membrane and its activation. We have also seen that RASSF1A is able to inhibit RASSF1C and promote the inactivation of SRC. We were interested to see whether the inhibition of SRC by RASSF1A was only through sequestration of RASSF1C or whether RASSF1A directly inhibited SRC by the recruitment of regulatory proteins. We therefore decided to investigate the role of CSK, a well characterised inhibitor of SRC. As RASSF8 has been proposed to bind CSK, we hypothesised that RASSF1A could potentially inhibit SRC by recruiting CSK to the SRC-RASSF1C complex. If this was the case then we would expect RASSF1A to interact directly with CSK. Further to this we would expect RASSF1C to interact with CSK or have an enhanced interaction with CSK when RASSF1C is co-expressed with RASSF1A. This was tested by immunoprecipitating endogenous CSK and probing for FLAG-RASSF1A and FLAG-RASSF1C when expressed alone or together. As expected RASSF1A was able to be precipitated by CSK, both when expressed alone or when co-expressed with RASSF1C. RASSF1C however, was only seen to precipitate with CSK when co-expressed with RASSF1A (Figure 4.13).

This result, together with the additional data presented in this chapter suggests a mechanism by which RASSF1C can bind to and activate SRC. RASSF1A can bind to both CSK and RASSF1C. In cells that express RASSF1A, a complex consisting of RASSF1A, RASSF1C, CSK and SRC is formed allowing CSK to phosphorylate and inactivate SRC. However, when RASSF1A is lost RASSF1C no longer interacts with CSK and therefore RASSF1C can promote the translocation of SRC to the membrane where it is able to be activated (Figure 4.14).

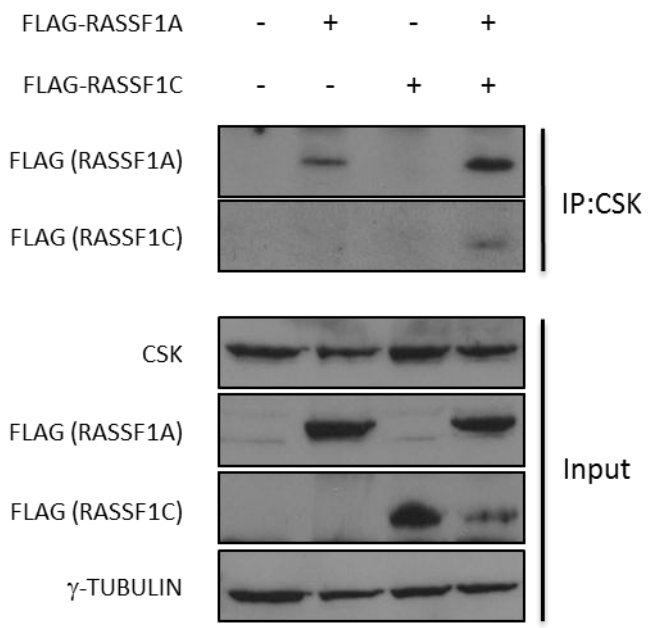


**Figure 4.13. RASSF1A Binds CSK and Brings RASSF1C to this Complex.** H1299 cells (1 x $10^6$ cells/plate) were transfected with FLAG-RASSF1A and FLAG-RASSF1C either alone or together and cultured for 48 hrs prior to lysis with a 0.5 % NP40, 150 mM NaCl lysis buffer and immunoprecipitated with an anti-CSK antibodies bound to protein G sepharose beads. Inputs and precipitates were analysed by SDS-PAGE and western blot for the presence of RASSF1A and RASSF1C using antibodies against FLAG-tag. RASSF1A immunoprecipitated with CSK. The interaction is enhanced by the presence of RASSF1C. RASSF1C only immunoprecipitated with CSK when co-expressed with RASSF1A. Data is representative of three independent experiments.

## 4.8 Discussion

In this chapter I have shown that RASSF1C is the isoform of RASSF1 that is required for SRC family kinase activation. Expression of RASSF1A is able to inhibit RASSF1C-dependent SRC activation by binding to RASSF1C. The interaction requires the RA domain, but interestingly, is independent of the SARAH domain. RASSF1C is able to interact with SRC family kinases, in the absence of RASSF1A, and promote the translocation of SRC family kinases to the plasma membrane where they are activated. RASSF1A was also found to interact with CSK and RASSF1C is recruited to the RASSF1A-CSK complex.

At the end of Chapter 3 it had been concluded that SRC could not be activated in cells lacking expression from the RASSF1 gene locus (Figure 3.13). Given that RASSF1A had been shown to bind the SRC activator, OSSA (Figure 3.11), we investigated whether expression of RASSF1A could activate SRC. We found that expression of RASSF1A was not able to activate SRC in MCF7 cells (Figure 4.2). Use of a RASSF1A isoform specific siRNA allowed us to further rule out a requirement for RASSF1A in the activation of SRC (Figure 4.3).

SRC is known to be activated by oxidative stress (Devary, Gottlieb et al. 1992; Abe, Takahashi et al. 1997; Ushio-Fukai, Griendling et al. 2001) (Figure 3.13B). However, we observed that this was not the case in MCF7 cells treated with hydrogen peroxide or exposed to IR (Figure 4.2). SRC activation by oxidative stress is thought to occur near the plasma membrane, possibly by receptor activation (Gonzalez-Rubio, Voit et al. 1996). Oxidative stress may not activate SRC in MCF7 cells potentially due to the receptor being missing or inactive.

Activation of SRC can be triggered by a number of stimuli including growth factors, oxidative stress and DNA damage and here we present evidence that these are dependent on an isoform of RASSF1 (Figure 3.13), but not RASSF1A (Figure 4.3). Of all the isoforms of RASSF1, only RASSF1C expression has been detected in the lineages of the cell lines used in this study, namely, cervical (HeLa), breast (MCF7) and lung (H1299) (Burbee, Forgacs et al. 2001). This suggests that RASSF1C is required for the essential

functions SRC has in the cells, including promotion of proliferation and migration. We were able to determine that expression of RASSF1C into H1299 cells was able to activate SRC (Figure 4.4). This is a novel role for RASSF1C, which at the time of discovery had not been described as an oncogene. Additional evidence now suggests that RASSF1C may have other oncogenic properties (Amaar, Minera et al. 2006; Reeves, Baldwin et al. 2010; Reeves, Baldwin et al. 2012).

RASSF1C activation of SRC was also shown to be independent of activation on OSSA (Figure 4.4B) fitting with the data in chapter 3 (Figure 3.14). Together these results suggest that RASSF1C can activate SRC by scaffolding a number of cofactors of which OSSA may be one. It would be interesting to see whether other factors that can activate SRC require RASSF1C. This could be addressed by repeating the experiments in figures 3.14 and 4.4B with other proteins known to be able to activate SRC for example growth factor receptor stimulation. If the hypothesis is correct then these factors will fail to activate SRC in the absence of RASSF1C, but RASSF1C expression will still be able activate SRC in the absence of any single cofactor. Our results cannot rule out the possibility that a small amount of OSSA remains within the cell transfected with siRNA targeting OSSA and that this is enough for RASSF1C to scaffold and activate SRC. Although the OSSA knock down was efficient, complete ablation will never be achieved.

We confirmed that the activation of SRC by RASSF1C was not a cell line specific phenomenon by expressing RASSF1C in a panel five commonly used cancer cell lines. We observed that RASSF1C could only activate SRC in cell lines that did not express RASSF1A. This data led us to generate the hypothesis that RASSF1C is prevented from activating SRC by RASSF1A, which represents a novel tumour suppressor role for RASSF1A. Activation of SRC family kinases by expression of RASSF1C in the RASSF1A negative cell line, RKO, and in H1299 TET ON cells only when RASSF1A expression was not induced, confirmed this hypothesis. From this we concluded that one of the tumour suppressor functions of RASSF1A was to inhibit RASSF1C to prevent aberrant activation of SRC.

We would like to have assessed how loss of RASSF1C affected SRC activity in HeLa cells in order to confirm the requirement of RASSF1C for SRC activation. In order to do this we generated two siRNA

molecules against the specific N-terminal of RASSF1C. We were unable to confirm knockdown of RASSF1C with either of these two siRNA molecules by RT-PCR (Discussed further in Chapter 6, Figure 6.4 and Figure 6.5). An alternative approach to assess the specific role of RASSF1C in SRC activation may also be achieved by using the siRNA targeting all RASSF1 isoforms in cells that are RASSF1A null, for example MCF7 or H1299 cells.

We next questioned how RASSF1A might inhibit RASSF1C from activating SRC. As scaffold proteins it is most likely that this inhibition occurs through direct binding and sequestration of RASSF1C. This mechanism of regulation is seen elsewhere in cells, for example in the Wnt signalling pathway, where β-catenin is sequestered by an Axin-Adenomatous polyposis coli (APC) complex (Clevers and Nusse 2012). RASSF1A is known to interact with a number of other isoforms of the RASSF family including RASSF5, which RASSF1C cannot (Ortiz-Vega, Khokhlatchev et al. 2002). Despite this we were able to show a robust interaction with exogenously expressed constructs of RASSF1A and RASSF1C (Figure 4.8). The RASSF1A-RASSF5 interaction occurs through the N-terminal, which is unique between RASSF1A and RASSF1C. Other interactions between RASSF1A and other SARAH domain containing proteins such as MST1/2 and Salvador have been shown to be dependent on the SARAH domain. We therefore asked whether the interaction between RASSF1A and RASSF1C occurred through the N-terminal or C-terminal domains and whether mutation of the ATM consensus sequence in RASSF1A, which is important for RASSF1A activation in response to DNA damage and induces a conformational change to RASSF1A, had any effect on RASSF1A-RASSF1C binding (Figure 4.9). The data suggested that the interaction is SARAH domain independent requiring at least the RA domain.

A second binding site in the N-terminal may also exist; however, poor expression of the N-terminal 1-119 fragment prevented us from being able to rule this out. The apparent poor expression of the N-terminal construct, seen in both Figure 3.12 and Figure 4.9, may suggest that the N-terminal fragment of RASSF1A is unstable or is rapidly degraded. This may be due to a number of reasons including, the presence of an as yet unidentified degradation sequence in the N-terminal, or an inability to fold

correctly. The degradation of RASSF1A by DDB1-Cul4A, $APC^{CDC20}$ and $SCF^{SKP2}$ require sequences that are located C-terminal to amino acid 119 and thus these proteins would not affect the degradation of the N-terminal fragment (Song, Song et al. 2008; Jiang, Rong et al. 2011; Chow, Wong et al. 2012). The N-terminal of RASSF1C is thought to be unfolded until it interacts with a target protein, for example DAXX (Escobar-Cabrera, Lau et al. 2010). The N-terminal of RASSF1A is both larger and more structured that that of RASSF1C; however, it may still require the RA domain, to which it is known to bind (Harjes, Harjes et al. 2006) in order to fold and be stabilised. The low molecular weight of the construct may also make it unstable. Use of a larger tag for example, a GFP tag, which is 27 kDa, may help to stabilise the construct leading to more efficient expression.

Although apparently unaffected by mutation of the phosphorylation site, S131A, the interaction was disrupted by mutation of A133S, a naturally occurring polymorphism. This can be explained by the fact that the A133S polymorphism is just N-terminal to the RA domain and is thought to unfold a proportion of the protein and the resulting structural change may affect binding (Yee, Grochola et al. 2012).

RASSF1C was also observed to form homodimers (Figure 4.9). The interaction was again SARAH domain independent, probably occurring somewhere in the N-terminal, either in the unique N-terminal domain or the RA domain. A RASSF1C homophilic interaction through the N-terminal would be interesting because it would provide opportunity for one RASSF1C molecule to interact with another RASSF1C and a RASSF1A molecule at the same time, just like RASSF1A is able to interact with both WW45 and MST2 at the same time through two different binding sites in the SARAH domain (Donninger, Allen et al. 2011). Another point to note from this experiment is that both RASSF1A and RASSF1C isoforms lacking their C-terminal end, containing the SARAH domain, appear to be expressed better than their full length counterparts, suggesting that they are more stable and that there is a potential degron sequence held within this region.

Both RASSF1A and RASSF1C have been shown to be degraded in a ubiquitin-dependent manner. RASSF1A has been shown to be targeted for degradation at the G1/S transition by $SCF^{SKP2}$ and during M-

phase by APC/$C^{CDC20}$ and the Cullin-4A-DDB1 E3 ligase complex (Song, Song et al. 2008; Song, Song et al. 2009; Jiang, Rong et al. 2011; Chow, Wong et al. 2012). The degradation by $SCF^{SKP2}$ and APC/$C^{CDC20}$ is dependent on phosphorylation at the S203 site by CDK4-cyclin D1 and Aurora A kinases respectively (Song, Song et al. 2008; Song, Song et al. 2009). The degradation by the Cullin-4A-DDB1 complex requires amino acids 165-200. RASSF1C has also shown to be degraded in a phospho-specific manner by the E3 ubiquitin ligase Mule under normal conditions and by the $SCF^{\beta\text{-}TRCP}$ complex in response to DNA damage. This is dependent on GSK3 phosphorylation of S19/23 in RASSF1C. Although none of these sites fall within the amino acids 288-340 of RASSF1A or 218-270 of RASSF1C a D-box and a KEN box have been shown to reside in this region and a D-box has been shown to reside immediately N-terminal to this region (Chow, Wong et al. 2012). D-Boxes and KEN-boxes are recognition sequences for APC/C binding and ubiquitination, however these C-terminally located sites have no known function. Their existence may suggest that other, as yet unidentified, ubiquitin ligase target sequence may also exist in RASSF1A and RASSF1C which may target RASSF1A and/or RASSF1C for degradation. It would be interesting to identify the ubiquitin ligase responsible for binding these sites and under what condition this occurs. This could be done by testing binding of a set of ubiquitin ligases to the full length vs. the SARAH domain truncated RASSF1A and RASSF1C by comparative mass spectrometry as was done by Zhou et al (2011) to identify the ubiquitin ligases that bind to RASSF1C (Zhou, Li et al. 2011).

RASSF1A promoter methylation has been detected in a wide variety of cancers (Donninger, Vos et al. 2007). Mutation of RASSF1 by contrast is a rare event. One single nucleotide polymorphic variant of RASSF1A has been identified with minor allele frequency at codon 133 (A133S). This polymorphism is in a region that is shared between RASSF1A (A133S) and RASSF1C (A63S). We noticed that the polymorphism effected the interaction between RASSF1A and RASSF1C, but not RASSF1C homodimer formation (Figure 4.9). The polymorphism is thought to inactivate RASSF1A and is associated with early onset of breast and lung cancer as well as soft tissue sarcoma (Endoh, Yatabe et al. 2003; Gao, Xie et al. 2008; Yee, Grochola et al. 2012). From this we reasoned that mutations to the RASSF1 sequence that effect both RASSF1A and RASSF1C would be rare because although they inactivate the tumour

suppressor functions of RASSF1A, they may also result in cells losing RASSF1C function and therefore the ability to activate SRC. Indeed, we found that expression of RASSF1C A63S, unlike RASSF1C expression, was unable to activate SRC (Figure 4.10). This result suggests that RASSF1A is preferentially lost by promoter methylation, rather than mutated, so that cells maintain the ability to activate SRC.

The conclusions drawn from the interaction data above is that RASSF1A interacted with RASSF1C to inhibit it and to prevent it from activating SRC. In order to test this we needed to investigate how RASSF1C activated SRC. RASSF1C, like RASSF1A, is a scaffold protein and therefore it is likely that RASSF1C would directly interact with SRC to unfold it and activate it, as was suggested to be the case for the scaffold protein OSSA (Tanaka, Sasaki et al. 2009). We have shown that RASSF1A can bind to RASSF1C and that RASSF1A co-expression with RASSF1C can inhibit RASSF1C from activating SRC. Therefore, we investigated whether RASSF1A acted to sequester RASSF1C away from SRC or whether it directly bound to the RASSF1C-SRC complex. RASSF1A tends to sequester proteins away from their target, for example sequestering MDM2 away from p53 leading to complex dissociation (Song, Song et al. 2008), or binding CDC20 preventing it interacting with and activating the APC/C (Song, Song et al. 2004). Using these examples to generate our hypothesis we predicted that RASSF1C would bind directly to SRC facilitating its activation by acting as an adapter to bring cofactors to SRC. Co-expression of RASSF1A would inhibit SRC by binding to and sequestering RASSF1C away from SRC. We found that this was indeed the case (Figure 4.11). Exogenously expressed RASSF1C was precipitated by SRC and co-expression of RASSF1A decreased the amount of RASSF1C in the SRC IP. Further, RASSF1A was not seen to interact with SRC.

During activation, SRC moves from the perinuclear region, where it was inactive, to the periphery of the cell where SRC it is activated (Rohrschneider 1979; Courtneidge, Levinson et al. 1980; Welham and Wyke 1988; Kaplan, Bibbins et al. 1994). From this we hypothesised that if RASSF1C was activating SRC we should see a greater amount of SRC at the periphery of the cell and that this SRC should be phosphorylated on the autophosphorylation site, Y419. We were able to show, using

immunofluorescence (IF), that this was the case. Although, we have not investigated how RASSF1C promotes translocation of SRC to the periphery of the cell there are a number of potential interactions that provide avenues for further research in this area.

SRC trafficking to the membrane is dependent on endosomal trafficking (Sandilands and Frame 2008). Endosomal trafficking moves vesicles around the cell targeted by small GTPases such as the Rab and Arf proteins. Rab and Arf proteins functional overlap controlling the formation, trafficking and subsequent fusion of vesicles targeted to various compartments. Rab proteins are associated with distinct compartments, for example, Rab5 is associated with the early endosomes (Gorvel, Chavrier et al. 1991; Bucci, Parton et al. 1992; McLauchlan, Newell et al. 1998), whereas Rab11 is associated with recycling endosomes (Ullrich, Reinsch et al. 1996). Arf proteins are localised to a number of locations where their function is determined by associated effector proteins (Schweitzer, Sedgwick et al. 2011). Both Rab and Arf proteins represent families of the Ras superfamily (Wennerberg, Rossman et al. 2005) and therefore bind specific effectors in a GTP-dependent manner. This is controlled by GTP exchange factors (GEFs) that activate the GTPases by promoting the dissociation of GDP and the uptake of GTP and GTPase-activating proteins (GAPs), which promote GTP cleavage to GDP inactivating the GTPase (Karnoub and Weinberg 2008). The GTPases perform their physiological roles through the interaction and activation of specific effector proteins to the GTP bound, active state. One example of this is Ras-GTP binds to and activates Raf. Both RASSF1A and RASSF1C contain an RA domain. The RA domain of RASSF1A and RASSF1C is defined by sequence homologies between the Ras effectors, Ral Guanosine nucleotide-exchange factor (Ral GDS) and the ALL-1 fusion partner from chromosome 6 (AF6) (Ponting and Benjamin 1996; Yamamoto, Taya et al. 1999). Studies of the RA domain of RASSF1A have revealed that it is able to interact with K-Ras (Matallanas, Romano et al. 2011). It is therefore possible that RASSF1A and RASSF1C could interact with the GTPases involved in endosomal trafficking such as the Rab or Arf family and this could help RASSF1C to recruit SRC to the plasma membrane in order to become activated.

The fusion of vesicles to their target membrane is regulated by SNARE proteins. The C-terminal of RASSF1A is also known to interact with the t-SNARE, syntaxin 16. This interaction targets syntaxin 16 to the midbody membrane and is essential to complete abscission at the end of mitosis. The interaction is driven by Aurora B, which phosphorylates the T202/S203 site on RASSF1A (Song, Kim et al. 2009; Song, Song et al. 2009). Both the phosphorylation site and the C-terminal t-SNARE interaction region are present in RASSF1C so it can be hypothesised that RASSF1C may interact with and bring SRC to the endosomal vesicles aiding the trafficking of SRC to the membrane. SRC trafficking to the periphery has been shown to be dependent on the SNARE protein SNAP23. It would be interesting to see if RASSF1C could interact with this protein.

Also, the screen for RASSF1A binding partners brought up a number of interesting hits. Given the shared domains between RASSF1A and RASSF1C, for example, the SARAH domain, through which they can interact with MST2 and the RA domain, through which they can interact with microtubules, there is the potential that some of the identified proteins may interact with RASSF1C. In the screen a large number of cytoskeletal proteins, particularly those linked with Actin stability were found (Table 8.1). SRC translocation to the periphery is Actin dependent and so it is possible that RASSF1C may interact with these Actin components to help drive this translocation (Kaplan, Swedlow et al. 1992; Kaplan, Bibbins et al. 1994; Gasman, Kalaidzidis et al. 2003; Sandilands, Cans et al. 2004). Immunoprecipitation of RASSF1C with Actin or one of the protein's associated with the SRC-associated Actin polymerisation, such as mDia or ARP2/3, may help shed light on RASSF1C involvement in this aspect of SRC activation. Like the trafficking of endosomal vesicles the organisation of the Actin cytoskeleton is controlled by small GTPases. In the context of SRC activation, the Rho proteins, RhoB and RhoD are known to colocalise with SRC containing vesicles and are responsible for promoting the translocation of SRC to the membrane (Sandilands, Cans et al. 2004). Both RhoD and RhoB containing vesicles require Actin polymerisation to be transported around the cell. mDia, a RhoD binding partner was shown to modulate cytoskeleton organisation, recruit Actin and activate c-SRC in HeLa cells (Gasman, Kalaidzidis et al. 2003). RhoB vesicles have been found to be associated with newly formed short-bundled Actin filaments, or Actin

clouds, thought to be produced by SCAR1, a component of the actin polymerisation machinery (Sandilands, Cans et al. 2004). RhoA, Rac1 and Cdc42 also affect SRC localisation, targeting SRC to focal adhesions, lamellipodia and filopodia respectively (Timpson, Jones et al. 2001). One obvious experiment leading on from these studies is to look for an association between RASSF1C and these GTPases using immunoprecipitation. This would highlight where future research into the activation of SRC by RASSF1C should focus.

One thing that the data from Figures 4.11 and 4.12 highlighted was that the level of SRC in the cells only expressing RASSF1C was slightly higher. This suggests that RASSF1C can stabilise SRC as well as activate it, or RASSF1C prevents the binding of proteins that lead to the degradation of SRC for example Cbl, an E3 ubiquitin ligase, or Cullin 5, which target active SRC for degradation (Hakak and Martin 1999; Ingley 2008; Laszlo and Cooper 2009). The activity of SRC leads to the recruitment of ubiquitin ligases by a mechanism that appears, at least in part, to be dependent on the suppressor of cytokine signalling (SOCS) proteins (Ingley 2008). It would be interesting to see whether expression of RASSF1C affects the interaction between active SRC and these factors that lead to its degradation.

SRC is also regulated by CSK, which phosphorylates SRC on Y530 causing SRC to fold up forming the closed, inactive, conformation (Okada and Nakagawa 1989; Xu, Harrison et al. 1997). The data discussed so far shows that RASSF1A titrates RASSF1C from SRC. However, we wished to investigate whether it also plays a more active role in the regulation of SRC by bringing CSK to SRC, such that CSK would be able to phosphorylate and inactivate SRC. If this was the case, we hypothesised that RASSF1A would be able to interact with CSK and that RASSF1C would only bind CSK in the presence of RASSF1A and bring CSK to a RASSF1C-SRC complex. We found this to be the case (Figure 4.13). We have still to discover whether this complex also contains SRC and whether SRC is being inactivated by CSK-dependent phosphorylation of Y530 under these conditions. We have failed to detect SRC precipitating with CSK in our experiments. However, we may not see the interaction because CSK and SRC as they do not need to form a stable complex in order for phosphorylation to occur. Analysis of Y530 phosphorylation and the

corresponding decrease in kinase activity of SRC would answer this question. We would predict that SRC kinase activity would be elevated with RASSF1C expression but decreased by co-expression with RASSF1A due to increased CSK phosphorylation of Y530.

One regulatory mechanism that we have not addressed in this work is the role of phosphatases. Dephosphorylation of the Y530 site is crucial to the activation of SRC. A number of phosphatases have been shown to be able to dephosphorylate SRC including: PTP1B (Bjorge, Pang et al. 2000), SHP1 (Somani, Bignon et al. 1997), SHP2 (Zhang, Yang et al. 2004), CD45 (Huntington and Tarlinton 2004) PTP1$\alpha$ (Zheng, Wang et al. 1992; Su, Muranjan et al. 1999), PTP1$\varepsilon$ (Granot-Attas and Elson 2004), and PTP1$\delta$ (Fang, Sabe et al. 1994). The phosphatases appear to have tissue specificity (Roskoski 2005). SHP1 can also dephosphorylate SRC targets (Frank, Burkhardt et al. 2004) and SHP2 maintains SRC activity by dephosphorylating PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains) which when phosphorylated recruits CSK to SRC (Zhang, Yang et al. 2004). Therefore RASSF1C may activate SRC family kinases by recruiting one of these phosphatases to SRC promoting the dephosphorylation of the Y530 site. It would be interesting to see whether RASSF1C is associated with any of these phosphatases and whether RASSF1C can enhance their activity towards SRC family kinases. No tyrosine phosphatases were identified in the RASSF1A screen, however, a serine threonine-protein phosphatase, PP5, was identified. As well as phosphorylation of Y530, Y419 must also be dephosphorylated to fully inactivate SRC family kinases. This dephosphorylation has been attributed to PTP-BAS in humans, which colocalises with SRC in membrane fractions. The mouse homolog of PTP-BAS, PTP-BL was shown to specifically dephosphorylate Y419 and this was associated with inactivation of SRC (Palmer, Zimmer et al. 2002). This therefore provides another level of regulation of SRC. It would be interesting to test whether RASSF1A recruits PTP-BAS to SRC as we predict it does for CSK.

These data led us to create a working model (Figure 4.14) in which RASSF1A and CSK form one stable complex and RASSF1C and SRC form a stable complex. Under normal conditions RASSF1A brings CSK to RASSF1C and SRC allowing CSK to inactivate SRC and dissociating RASSF1C from the RASSF1C-SRC

complex. In cancer when RASSF1A is lost, CSK is no longer brought into proximity with SRC by RASSF1A and so is unable to phosphorylate and inactivate SRC. Titration of RASSF1C from SRC also fails to occur leading to RASSF1C binding to SRC and promoting its translocation to the plasma membrane where it is activated. This model suggests that in tumours that lack RASSF1A, SRC activity is likely to be upregulated.

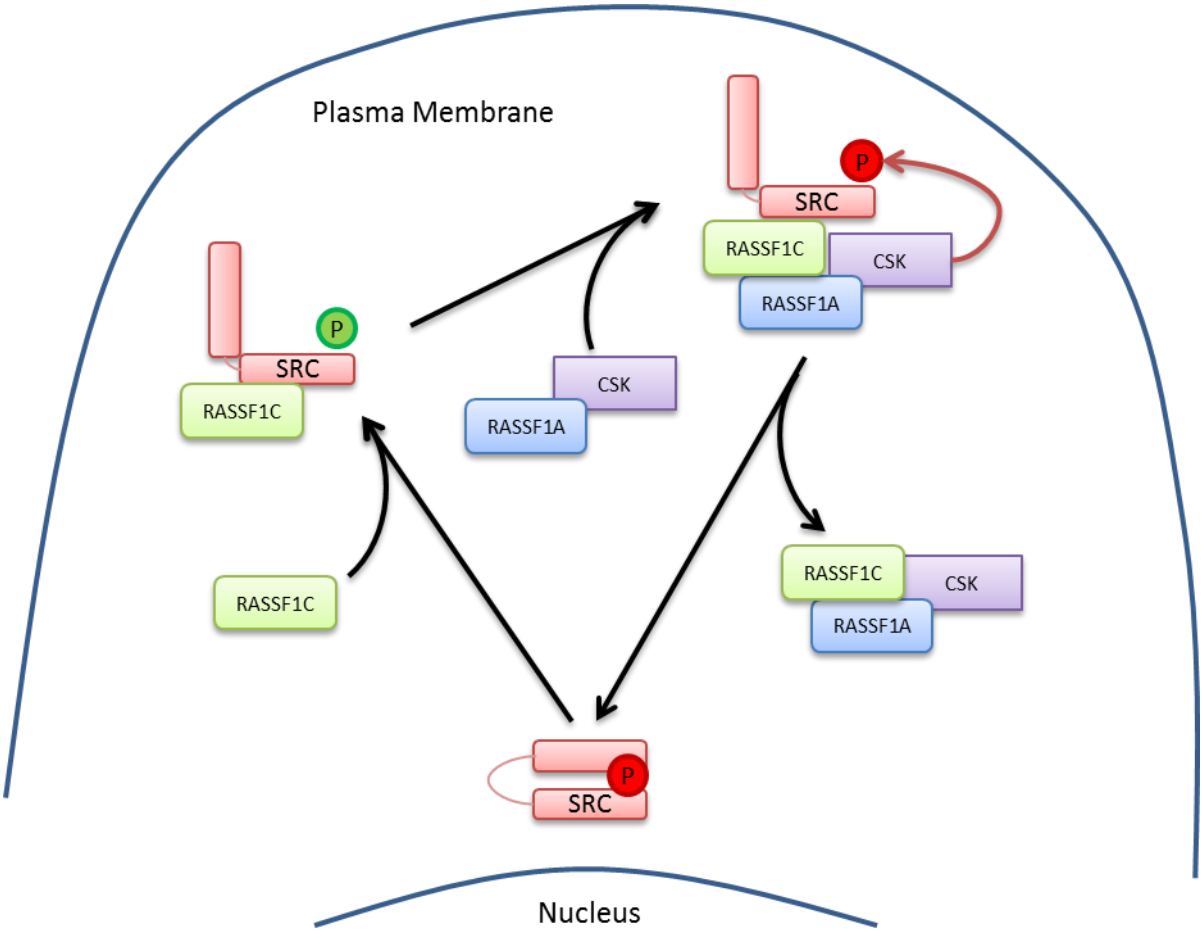


**Figure 4.14. Model Depicting Hypothesis Developed From the Work in Chapter 4.** SRC family kinases are held in an inactive pool in the perinuclear region. RASSF1C binds SRC family kinases, promoting their translocation to the plasma membrane and subsequent activation by phosphorylation of the Y419 site. RASSF1A interacts with CSK and recruits CSK to SRC by binding RASSF1C. SRC is inactivated by CSK phosphorylation of Y530. The interaction between RASSF1A and RASSF1C sequesters RASSF1C away from SRC and maintains RASSF1C in a complex with RASSF1A and CSK.

# Chapter 5 RASSF1C Expression Leads to Loss of Cell-Cell Junctions and Polarity

## 5.1 Introduction

In chapter 4 the role of RASSF1 isoforms in SRC activation was analysed. It was found that RASSF1C, and not RASSF1A, was responsible for the activation of SRC. RASSF1C was shown to bind directly to SRC and promote its translocation to the membrane. RASSF1A was shown to inhibit the activation of SRC by RASSF1C via recruitment of CSK to the RASSF1C-SRC complex.

### 5.1.1 The Role of SRC in Cell-Cell Adhesion

Epithelial cell-cell contacts include: adherens junctions (AJ), tight junctions (TJ), desmosomes and hemidesmosomes, and gap junctions. These junctions act together to maintain normal tissue architecture and are reinforced by the actin cytoskeleton. They are tightly regulated by signalling networks which act both directly and indirectly on the junctional components (Swaminathan and Cartwright 2012). Loss of E-cadherin-dependent cell-cell adhesions and acquisition of enhanced integrin-dependent cell-ECM contacts, such as focal adhesions is associated with a process known as Epithelial-Mesenchymal transition (EMT) (Thiery 2002). In general, cancers that have undergone EMT are considered to be potentially more migratory, invasive and metastatic with SRC being believed to be a major regulator of this switch (Frame 2004).

The cadherins are a family of structurally and functionally related molecules that mediate calcium-dependent homophilic cell-cell contacts (Takeichi 1988; Gumbiner 2005). E-cadherin is expressed in epithelial cells and plays a crucial role in the establishment and maintenance of cell-cell adhesion and cell polarity. The intracellular domain of E-cadherin is associated with $\alpha$, $\beta$ and p120-catenin. These

proteins regulate: attachment of the cadherin complex to the Actin cytoskeleton, complex stability, and the rate of recycling of E-cadherin at the membrane (Ozawa and Kemler 1992; Rimm, Koslov et al. 1995). SRC has been shown to phosphorylate both E-cadherin and the associated catenin proteins, destabilising this complex and targeting E-cadherin for ubiquitin mediated degradation (Gumbiner 2005; Hartsock and Nelson 2008; Jeanes, Gottardi et al. 2008; Berx and van Roy 2009). E-cadherin is normally recycled via endosomes in a calcium-dependent manner (Kartenbeck, Schmelz et al. 1991; Le, Yap et al. 1999). SRC phosphorylation tips the balance between recycling and degradation of E-cadherin in favour of degradation, limiting the ability of E-cadherin to translocate to the membrane in response to calcium stimulation (Behrens, Vakaet et al. 1993; Avizienyte, Wyke et al. 2002; Fujita, Krause et al. 2002; Palacios, Tushir et al. 2005). Tyrosine phosphorylation of E-cadherin by SRC recruits the E3 ubiquitin ligase HAKAI to the E-cadherin complex promoting ubiquitination of E-cadherin and leading to its endocytosis and degradation (Fujita, Krause et al. 2002). Therefore SRC promotes EMT in epithelial cells by regulating HAKAI-dependent destruction of E-cadherin. The adaptor protein Rack1 restricts the ubiquitin-mediated degradation of E-cadherin by inhibiting SRC and preventing the recruitment of HAKAI, stabilising E-cadherin at cell-cell adhesions (Swaminathan and Cartwright 2012).

Interestingly, SRC also appears to be required for the creation of adherens junctions. Deletion of *fyn* and *src* genes in mice causes skin architecture abnormalities that are consistent with impaired formation of adherens junctions (Calautti, Cabodi et al. 1998). Promotion of adherens junctions involves protein-protein interactions made by the SH2 and SH3 domains, as inhibition of SRC activity is conversely associated with loss of cell-cell junctions in Keratinocytes (Owens, McLean et al. 2000; Yin, Getsios et al. 2005).

### 5.1.2 Aims

In chapter 4 it was shown that RASSF1C was able to active SRC by directly binding and that RASSF1A inhibits SRC activation and translocation to the plasma membrane. One of the major biological roles of

SRC at the plasma membrane is the formation and destruction of cell-cell junctions, with particular reference to the E-cadherin-mediated adherens junctions. The destruction of these junctions is important in EMT, where epithelial cells lose epithelial morphology and become more mesenchymal-like. Therefore, in this chapter we decided to study the role of RASSF1C in SRC regulation of cell-cell contacts and cell polarity.

## 5.2 RASSF1C Leads to Increased Phosphorylation SRC targets E-cadherin and β-catenin.

It has been shown that SRC binds and phosphorylates the E-cadherin, β-catenin, p120-catenin complex (Behrens, Vakaet et al. 1993). The phosphorylation of E-cadherin forms a binding site for the E3 ubiquitin ligase HAKAI, which in turn, targets E-cadherin for degradation (Fujita, Krause et al. 2002). This phosphorylation has been shown to be modulated by scaffold proteins that bind to SRC, such as RACK1 (Swaminathan and Cartwright 2012). Given that RASSF1C is a scaffold protein that we have shown can bind directly to SRC (Figure 4.11), we wanted to investigate whether expression of RASSF1C could affect the phosphorylation and targeted degradation of E-cadherin. MCF7 cells were chosen because they are methylated for RASSF1A, but maintain an E-cadherin expression, thus allowing verification of potential regulatory effect of RASSF1C and RASSF1A.

We first analysed whether RASSF1C expression led to an increase in tyrosine phosphorylated E-cadherin and associated catenin proteins. MCF7 cells were transfected with FLAG-tagged RASSF1A and RASSF1C, either individually or together, and phospho-tyrosine proteins immunoprecipitated with an anti-phospho-tyrosine antibody. Samples were then analysed by SDS-PAGE gel and western blot for E-cadherin, β-catenin and p120-catenin. We found that an increased proportion of E-cadherin and β-catenin protein were precipitated from cells transfected with RASSF1C and that this was reduced in cells co-transfected with RASSF1A. Minimal effects were observed with p120-catenin or FAK, a target of SRC

at focal adhesions (Figure 5.1). This result suggests that more of the E-cadherin-catenin complex was phosphorylated by SRC on tyrosine residues when RASSF1C was expressed and able to activate SRC. No increase or decrease in SRC levels was detected at this time point.

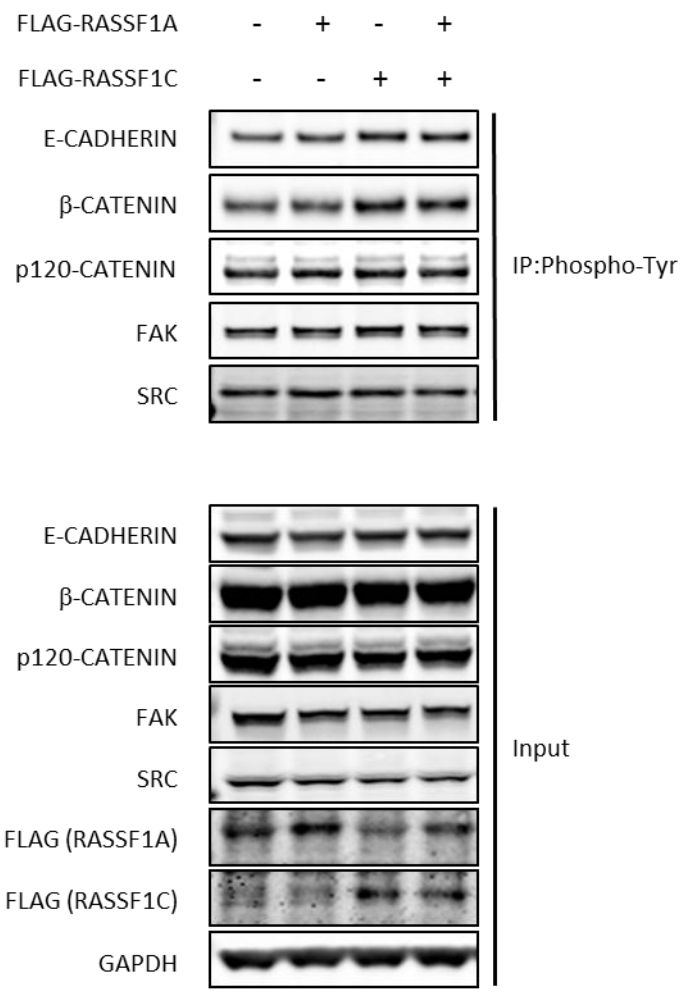


**Figure 5.1. RASSF1C Activation of SRC Leads to Tyrosine Phosphorylation of SRC Targets.** MCF7 cells (1 x $10^6$ cells/plate) were transfected with FLAG-RASSF1A and FLAG-RASSF1C, either alone or together, at a ratio such that equal expression of each construct was achieved. Cells were lysed by scraping into 0.5 % NP40, 150 mM NaCl lysis buffer supplemented with protease and phosphatase inhibitors. Cells were immunoprecipitated for 6 hrs with anti-pY 4G10 antibody. Protein was recovered from the beads by boiling into 1 x loading buffer for 5 mins. Samples were analysed by SDS-PAGE and western blot for SRC targets: E-cadherin, β-catenin, p120-catenin, FAK and SRC. Inputs were also probed with an anti-FLAG antibody to confirm RASSF1A and RASSF1C expression. Tyrosine phosphorylation of E-cadherin and β-catenin was elevated in cells expressing RASSF1C (E-cadherin: control to RASSF1C $\rho = 0.04$, RASSF1A to RASSF1C $\rho = 0.05$. β-catenin: control to RASSF1C $\rho = 0.01$, RASSF1A to RASSF1C $\rho = 0.03$). This level was decreased by co-expression of RASSF1C with RASSF1A (E-cadherin: RASSF1C to Both $\rho = 0.03$. β-catenin: RASSF1C to Both $\rho = 0.02$). FAK and p120-catenin phosphorylation was less clear. Blot is representative of three independent experiments which have been quantified using Licor Odyssey software.

## 5.3 RASSF1C Expression Promotes the E-cadherin Interaction with HAKAI

Having shown that RASSF1C expression leads to increased tyrosine phosphorylation of E-cadherin, we next asked whether this phosphorylated E-cadherin was being targeted for degradation by HAKAI. MCF7 cells were transfected with FLAG-RASSF1A, FLAG-RASSF1C, or FLAG-RASSF1A and FLAG-RASSF1C and lysates were immunoprecipitated with anti-E-cadherin antibodies. An increase in HAKAI immunoprecipitating with E-cadherin was detected in cells expressing RASSF1C (Figure 5.2A). When RASSF1A was co-expressed the amount of HAKAI precipitated by E-cadherin was reduced (Figure 5.2A) in line with the decrease in E-cadherin tyrosine phosphorylation (Figure 5.1).

The direct interaction of SRC with E-cadherin and HAKAI is inhibited by association with RACK1 (Swaminathan and Cartwright 2012). Given its activatory role towards SRC, we predicted that RASSF1C expression would drive a greater amount of E-cadherin and HAKAI to co-immunoprecipitate with SRC. MCF7 cells were transfected with FLAG-RASSF1A and FLAG-RASSF1C either alone or together and lysates were immunoprecipitated with anti-SRC antibodies. Western blot analysis showed that RASSF1C expression promoted the formation of a SRC-E-cadherin-HAKAI complex and that, similar to RACK1, RASSF1A co-expression could inhibit the formation of this complex (Figure 5.2B). RASSF1A, therefore, stabilises adherens junctions by inhibiting SRC phosphorylation and HAKAI-mediated ubiquitination of E-cadherin.

Together these three experiments show that expression of RASSF1C in the epithelial-like cancer cell line MCF7 results in an increase in SRC-mediated phosphorylation of the E-cadherin-catenin complex, leading to increased binding of HAKAI to E-cadherin. This will initially drive the internalisation of E-cadherin on endosomes which are subsequently targeted it for degradation by trafficking to the lysosomal compartment, promoting to a loss of adhesion between cells.

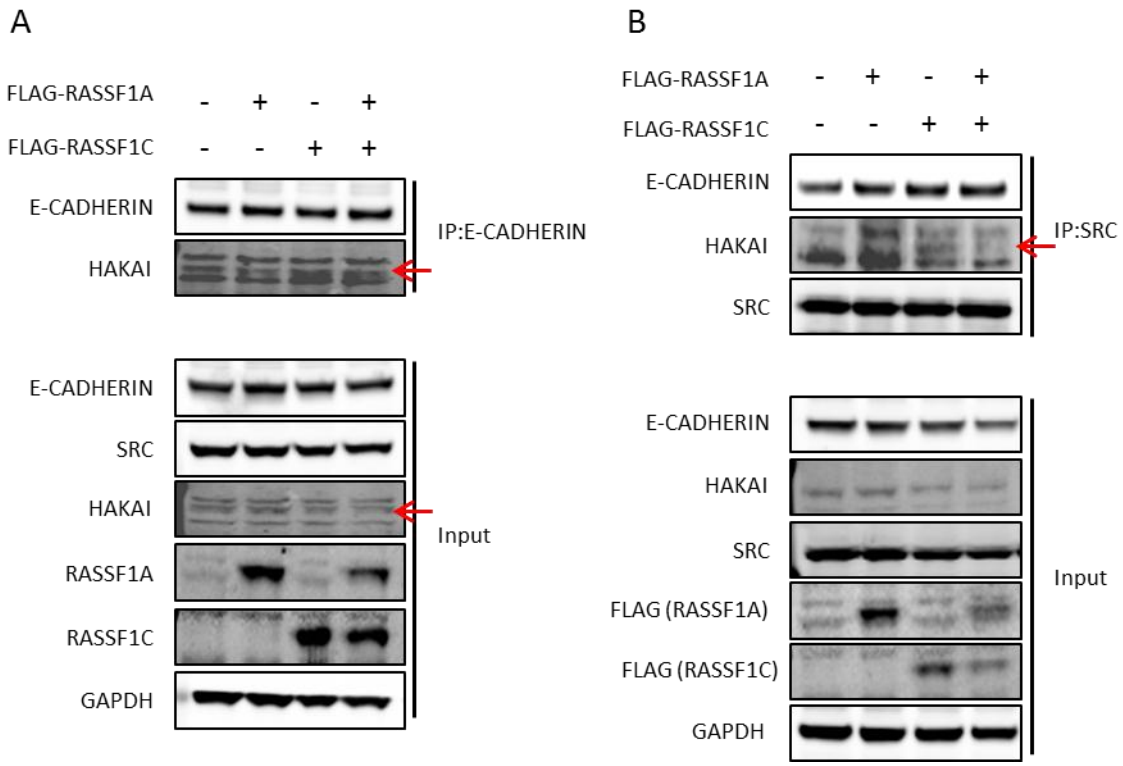


**Figure 5.2. RASSF1C Expression Targets E-cadherin for Degradation Through the E3-Ubiquitin Ligase HAKAI.** MCF7 cells (1 x $10^6$ cells/plate) were transfected with FLAG-RASSF1A and FLAG-RASSF1C, either alone or together such that equal expression of each construct was achieved. Cells were lysed by scraping into 0.5 % NP40, 150 mM NaCl lysis buffer supplemented with protease and phosphatase inhibitors. **A**. Lysates were immunoprecipitated for 6 hrs with anti-E-cadherin antibodies. Proteins were recovered from the beads by boiling for 5 mins in 1 x loading buffer and samples were analysed by SDS-PAGE and western blot for E-cadherin and HAKAI. Inputs were also probed with anti-FLAG antibodies to confirm expression of RASSF1A and RASSF1C. The interaction between E-cadherin and HAKAI is enhanced in cells expressing RASSF1C and this is reversed by co-expression of RASSF1A. **B.** Samples were treated the same as in **A.** expect that they were immunoprecipitated with anti-SRC antibodies. Samples were analysed by SDS-PAGE and western blot for E-cadherin, SRC and HAKAI. More E-cadherin and HAKAI are bound to SRC in cells expressing RASSF1C. This is decreased by co-expression of RASSF1C with RASSF1A. Red arrows indicate the HAKAI band. Data

## 5.4 RASSF1C Expression Leads to Loss of Adherens Junction Components

Having shown that RASSF1C expression drives the formation of a complex known to promote E-cadherin internalisation and degradation we wished to investigate whether we could detect loss of adherens junctions in cells expressing RASSF1C by IF. MCF7 cells were plated onto glass cover slips, transfected with FLAG-RASSF1C or control plasmid. Cells were probed with antibodies against the adherens junction components E-cadherin and p120-catenin. RASSF1C expression was visualised with an anti-FLAG antibody. Cells staining positively for FLAG-RASSF1C showed a minor decrease in E-cadherin staining at the sites of cell-cell contacts. However, a perinuclear pool of E-cadherin stained strongly in the RASSF1C expressing cells (Figure 5.3A). From the results in Figure 5.1 and Figure 5.2 we would predict that this pool represents E-cadherin targeted for degradation and therefore it would be interesting to see if it correlated with lysosome staining. Interestingly, cells surrounding those expressing RASSF1C also had high levels of internalised E-cadherin, suggesting that either low levels of RASSF1C, below detection, can promote E-cadherin internalisation or a potential role for paracrine signalling.

E-cadherin and p120-catenin form a complex at the plasma membrane and therefore internalisation of E-cadherin is likely to result in loss of p120-catenin from these sites as well (Reynolds and Carnahan 2004). As expected controls cells displayed p120-catenin staining as thin strongly staining bands around the outside of the cell as observed above for E-cadherin. Expression of RASSF1C led to p120-catenin staining becoming more diffuse at the membrane (Figure 5.3B). Taken together the data suggests that RASSF1C expression disrupts E-cadherin and consequently cells fail to form junctions.

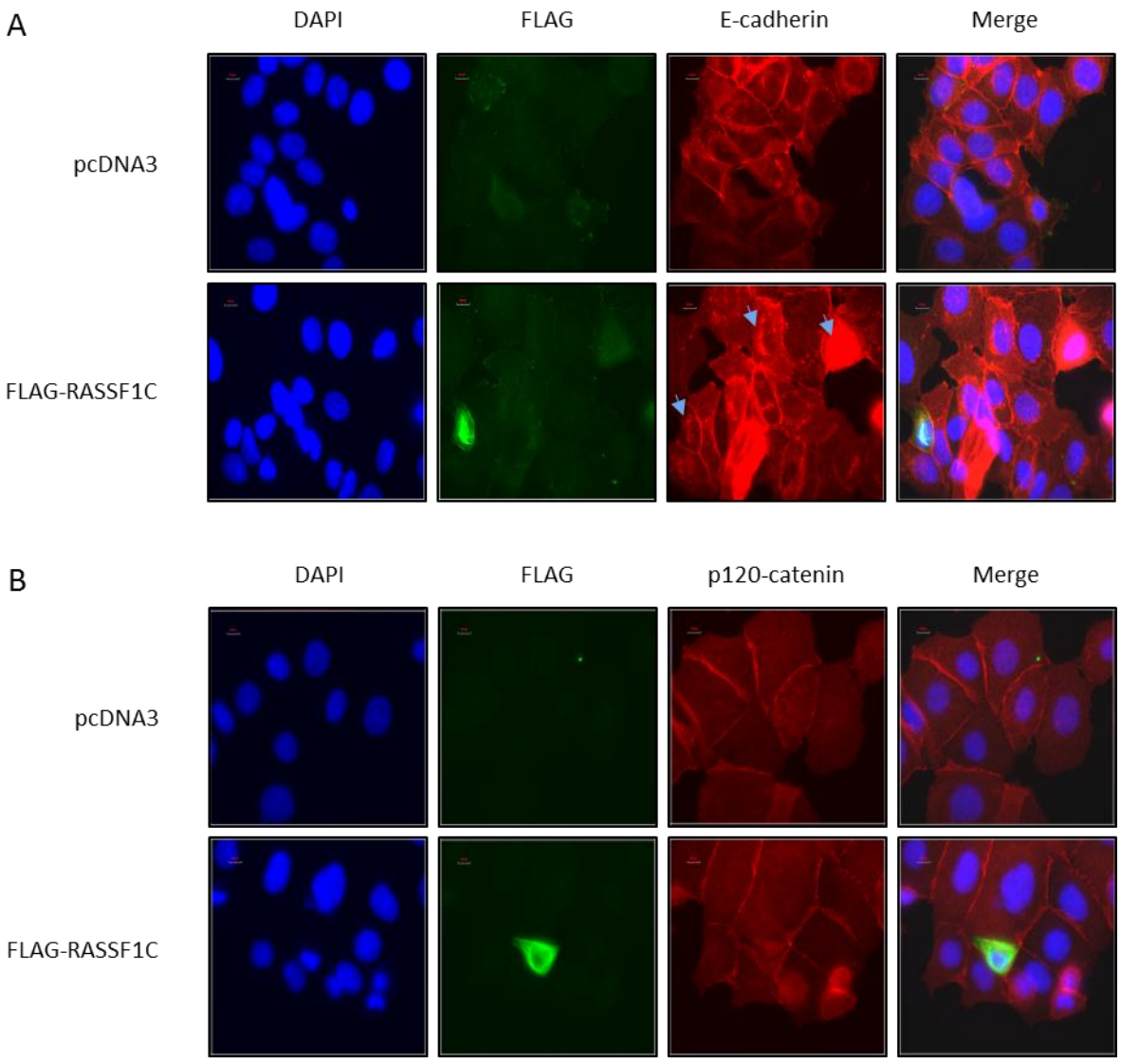


**Figure 5.3. RASSF1C Expression Promotes E-cadherin Internalisation and p120-catenin Mislocalisation. A.** MCF7 cells (1 x $10^5$cells/plate) were plated onto glass cover slips and transfected with either pcDNA3 or FLAG-RASSF1C. Cells were fixed with 4 % paraformaldehyde (PFA) for 15 mins, permeabilized with 0.2 % Triton X solution for 5 mins and blocked with 0.2 % Fish skin gelatin (FSG) for 30 mins. Cells were then probed with the anti-FLAG antibodies to detect RASSF1C and anti-E-cadherin antibodies. Secondary antibodies were Alexa-fluor 488 nm for anti-FLAG and Alexa-fluor 594 nm for anti-E-cadherin. Cells were mounted onto slides using mounting media containing DAPI. Images were taken with a Leica DM IRBE at 63 x magnification. Cells expressing RASSF1C and surrounding cells have increased perinuclear E-cadherin (blue arrows in E-cadherin image). **B.** MCF7 cells were treated as in **A.** except they were probed with anti-p120-catenin antibodies instead of anti-E-cadherin antibodies. Cells expressing RASSF1C fail to correctly localise p120 catenin to sites of cell-cell contact as shown by reduced and more diffuse p120-catenin staining at these sites. RASSF1C expressing cells also appear to have greater overlap of cell membranes suggesting they fail to form a proper monolayer. Data is representative of three independent experiments.

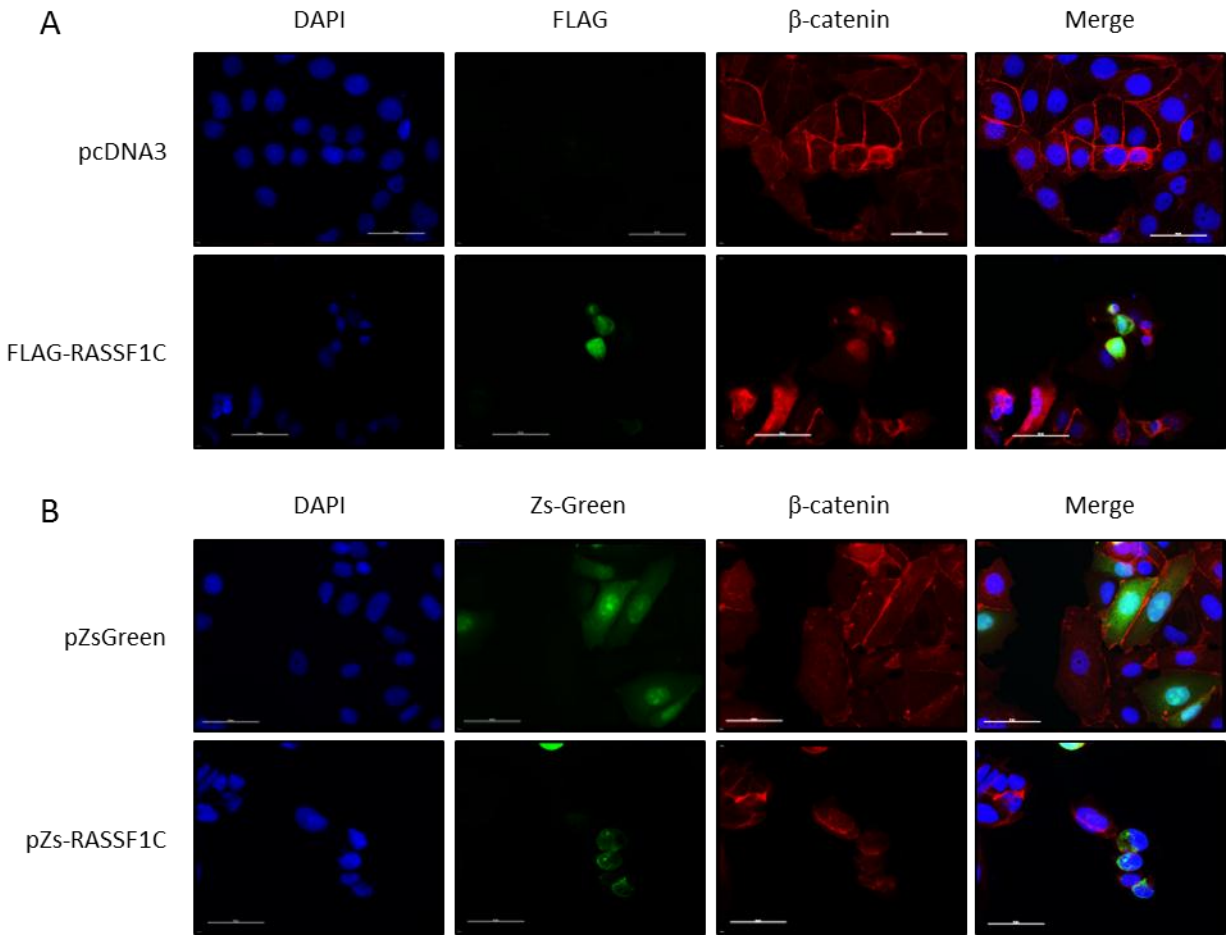


**Figure 5.4. β-catenin is Internalised in RASSF1C Expressing Cells.** MCF7 cells ($1x10^5$ cells/plate) were plated onto glass cover slips and transfected with (**A**) pcDNA3 or FLAG-RASSF1C or (**B**) pZsGreen empty vector or pZsGreen-RASSF1C. Cells were fixed for 15 mins with 4 % PFA, permeabilised for 5 mins with 0.2 % Triton X and blocked for 30 mins with 0.2 % FSG before being probed with anti-FLAG (**A.** only) and anti-β-catenin antibodies. Secondary antibodies were Alexa-fluor 488 nm for FLAG and 594 nm for β-catenin. Cover slips were mounted onto slides using mounting media containing DAPI. Images were taken using a Nikon 90i microscope at 40 x magnification. Scale bar is 50 mm. The β-catenin is distributed throughout the cell in cells expressing RASSF1C and staining at sites of cell-cell contact is reduced. RASSF1C expressing cells also appear smaller and rounded. Data is representative of three independent experiments.

Loss of E-cadherin from cell-cell junctions is associated with translocation of β-catenin from sites of cell-cell contact, where it plays a structural role, to the cytoplasm, where β-catenin acts as a signalling molecule responsible for transducing Wnt signalling. Cytoplasmic β-catenin is able to enter the nucleus where it upregulates pro-proliferative genes (Behrens, von Kries et al. 1996; Molenaar, van de Wetering et al. 1996). Phosphorylation of β-catenin by SRC also reduces β-catenin affinity for E-cadherin, promoting dissociation (Roura, Miravet et al. 1999; Coluccia, Benati et al. 2006). Next we were interested to determine whether RASSF1C-dependent phosphorylation of E-cadherin and β-catenin, and subsequent internalisation of E-cadherin, would promote β-catenin translocation to the cytoplasm and nucleus. Expression of either FLAG-tagged or ZS-green-tagged RASSF1C in MCF7 cells led to a loss of β-catenin from the sites of cell-cell contact, redistributing it throughout the cell (Figures 5.4A and B). We also observed that cells had adopted a rounded phenotype. As in Figure 5.3B, cells also appeared to overlap potentially breaking contact with the substratum in addition to cell-cell contacts. Together these data suggest that RASSF1C activation of SRC leads to loss of adherens junctions from cell-cell junctions. Consequently, β-catenin is redistributed and appears to enter the nucleus where it may act as a pro-proliferative transcription factor (Coluccia, Benati et al. 2006; Lee, Dedhar et al. 2006).

## 5.5 RASSF1C Expression Promotes the Loss of Tight Junction and Cell Polarity Components from Sites of Cell-Cell Contact

Given that adherens junctions stabilise the tight junctions and together they provide binding sites for cell polarity complexes (Adams, Nelson et al. 1996; Pokutta and Weis 2002; Gumbiner 2005), we wanted to investigate whether RASSF1C-mediated internalisation of adherens junctions also promoted destabilisation of tight junctions and mislocalisation of cell polarity factors. MCF7 cells were plated onto glass cover slips and transfected with FLAG-RASSF1C or control plasmid. Cells were then stained for tight junctions and cell polarity with anti-ZO-1 (Figure 5.5) or anti-Par-3 (Figure 5.6) antibodies respectively. In

control cells, ZO-1 forms a continuous band along the edges of cells that are in contact with other cells. In cells expressing RASSF1C the ZO-1 staining at the cell periphery was disrupted, shown by punctate staining at sites of cell-cell contact. In some cases ZO-1 can be seen to be internalised forming loop like structures within the cell (Figure 5.5). Par3 co-localises with ZO-1 at tight junctions where it enforces apical membrane polarity (Izumi, Hirose et al. 1998). We predicted that if ZO-1 was internalised then Par3 distribution would also be affected. This was indeed the case. In control cells, Par-3 stains strongly at the sites of cell-cell contact. In RASSF1C expressing cells however, Par-3 does not (Figure 5.6). It can be seen in the image of cells transfected with RASSF1C (Figure 5.6) that two cells (top of the image) that do not stain positively for RASSF1C, stain strongly for Par3 at the point of contact. However, when one of the cells stains positively for RASSF1C (bottom of the image) no contact is made, instead the RASSF1C positive cell appears to be moving over the RASSF1C negative cell. This suggests that EMT may be promoted through the loss of cell-cell junctions.

Taken together, the results from the IF experiments confirm the effects on cell-cell junctions predicted by the physical binding detected in Figure 5.1 and Figure 5.2. RASSF1C expression activates SRC, promoting the phosphorylation of E-cadherin and the associated catenin proteins. Subsequently, these proteins are redistributed away from the membrane. The tight junction component ZO-1, normally stabilised by the E-cadherin containing adherens junctions, is lost resulting in the mislocalisation of the polarity protein Par-3.

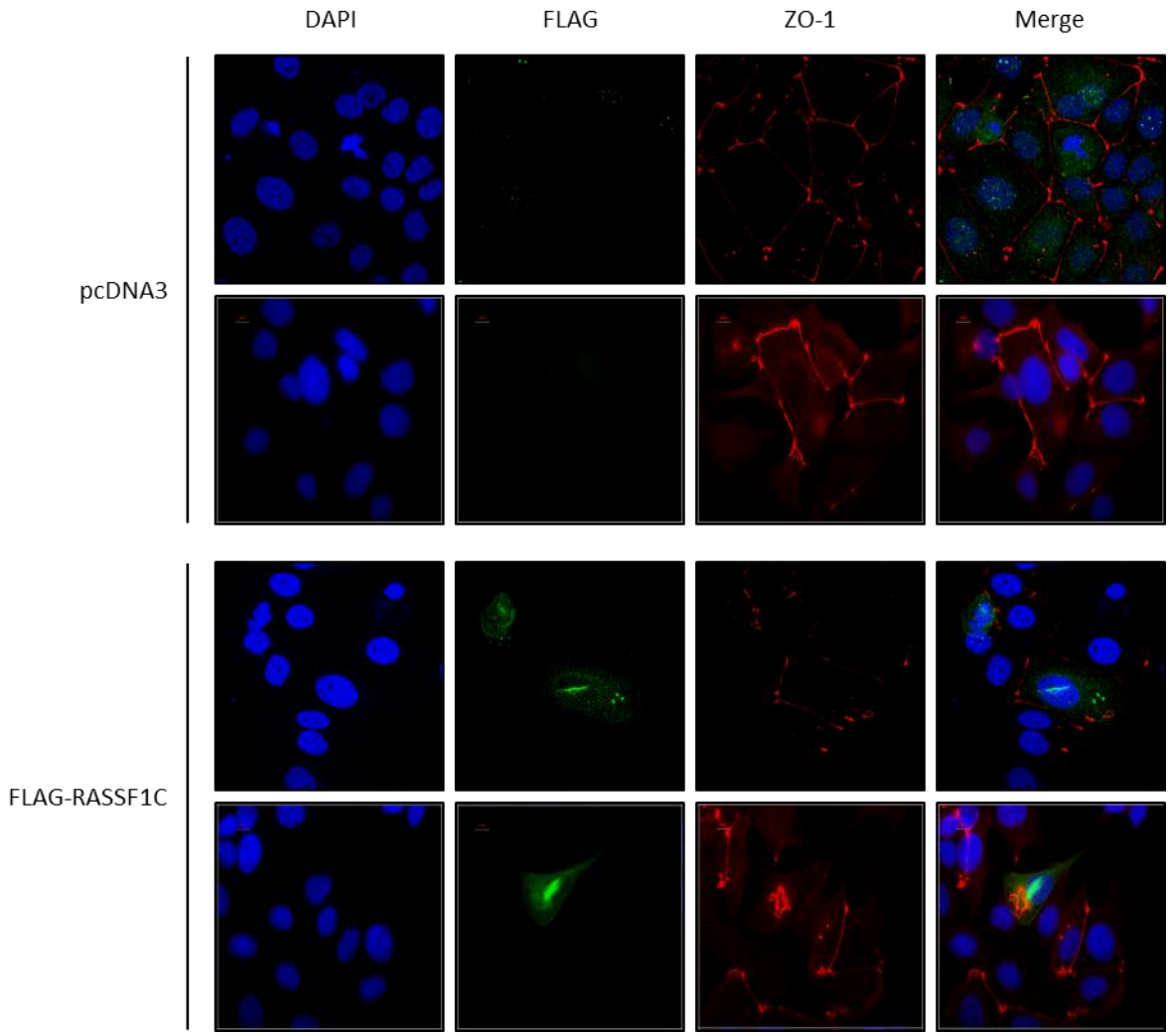


**Figure 5.5. RASSF1C Expression Leads to a Disruption of the Tight Junction Protein ZO-1**. MCF7 cells (1 x $10^5$ cells/plate) were plated onto glass cover slips and transfected with either pcDNA3 or FLAG-RASSF1C. Cells were fixed for 15 mins with 4 % PFA, permeabilized for 5 mins with 0.2 % Triton X solution, and blocked for 30 mins with 0.2 % FSG. Cells were probed with the anti-FLAG and anti-ZO-1 antibodies. Secondary antibodies were Alexa-fluor 488 nm for FLAG and 594 nm for ZO-1. Cover slips were mounted onto slides using mounting media containing DAPI. Images were taken using a Leica DM IRBE microscope at 63 x magnification. ZO-1 is located at cell-cell junctions in control cells (top panels). In RASSF1C expressing cells ZO-1 staining at the cell-cell junction is reduced. ZO-1 is also seen to be completely internalised in some cells. Data is representative of three independent experiments.

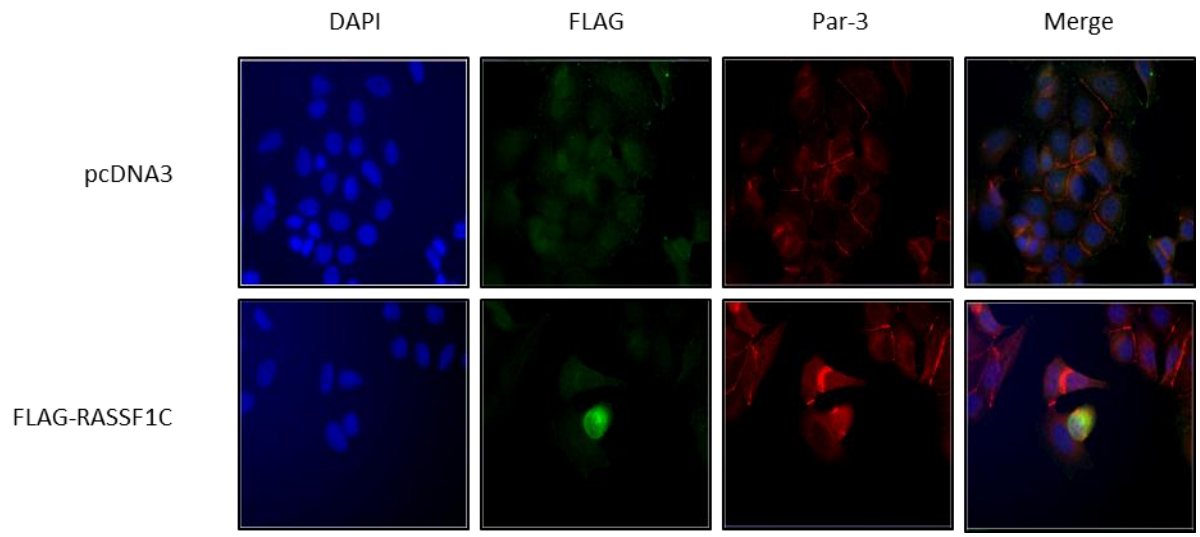


**Figure 5.6. Expression of RASSF1C Leads to Loss of the Polarity Marker Par-3 from Sites of Cell-Cell Contact.** MCF7 cells (1 x $10^5$ cells/plate) were plated onto glass cover slips and transfected with either pcDNA3 or FLAG-RASSF1C. Cells were fixed for 15 mins with 4 % PFA, permeabilized for 5 mins with 0.2 % Triton X solution and blocked for 30 mins with 0.2 % FSG. Cells were probed with the anti-FLAG and anti-Par-3 antibodies. Secondary antibodies were Alexa-fluor 488 nm for FLAG and 594 nm for Par-3. Cover slips were mounted onto slides using mounting media containing DAPI. Images were taken using a Leica DM IRBE microscope at 63 x magnification. Cells expressing RASSF1C have reduced staining for Par-3 at cell-cell junctions. Data is representative of three independent experiments.

## 5.6 RASSF1C Expression Affects the Kinetics of Adherens Junction Formation

Having shown that cells expressing RASSF1C have increased internalised E-cadherin and appear more rounded, and overlapping with neighbouring cells, we wished to investigate whether RASSF1C could also be involved in the formation of junctions. E-cadherin is constantly trafficked between the plasma membrane and the endosomes. We observed increased binding of E-cadherin to HAKAI in cells expressing RASSF1C, which is known to target E-cadherin to the lysosome. This would decrease the amount of E-cadherin available to be recycled back to the membrane, decreasing the ability of cells to form stable junctions. Formation of cadherin-dependent cell-cell contacts is dependent on calcium. Removal of calcium leads to a loss of junctions and re-addition of calcium to the cell culture media then allows the junctions to reform. By measuring the repopulation of E-cadherin to the sites of cell-cell

contact following re-addition of calcium the rate at which cells can form junctions can be estimated. MCF7 cells were transfected with GFP empty vector or GFP-RASSF1C before putting the cells into calcium free media. Calcium was then re-added to the media and cells were fixed every 30 min for 150 min. From the previous data (Figure 5.3-5.6) we predicted that RASSF1C expressing cells would be slower at forming junctions after re-addition of calcium. We saw that cells transfected with the control plasmid, E-cadherin was trafficked back to the areas of cell-cell contacts within 30 min and appeared to have formed strong adherens (E-cadherin) and tight (ZO-1) junctions by 90 min (Figure 5.7). By contrast RASSF1C expressing cells appeared to have only just begun for form these junctions by 150 min (Figure 5.8). It is interesting to note that a significant proportion of the ZO-1 appeared to remain in the perinuclear region of the cells even at this time point. This experiment was carried out by Nikola Vlahov, a D.Phil student in the laboratory.

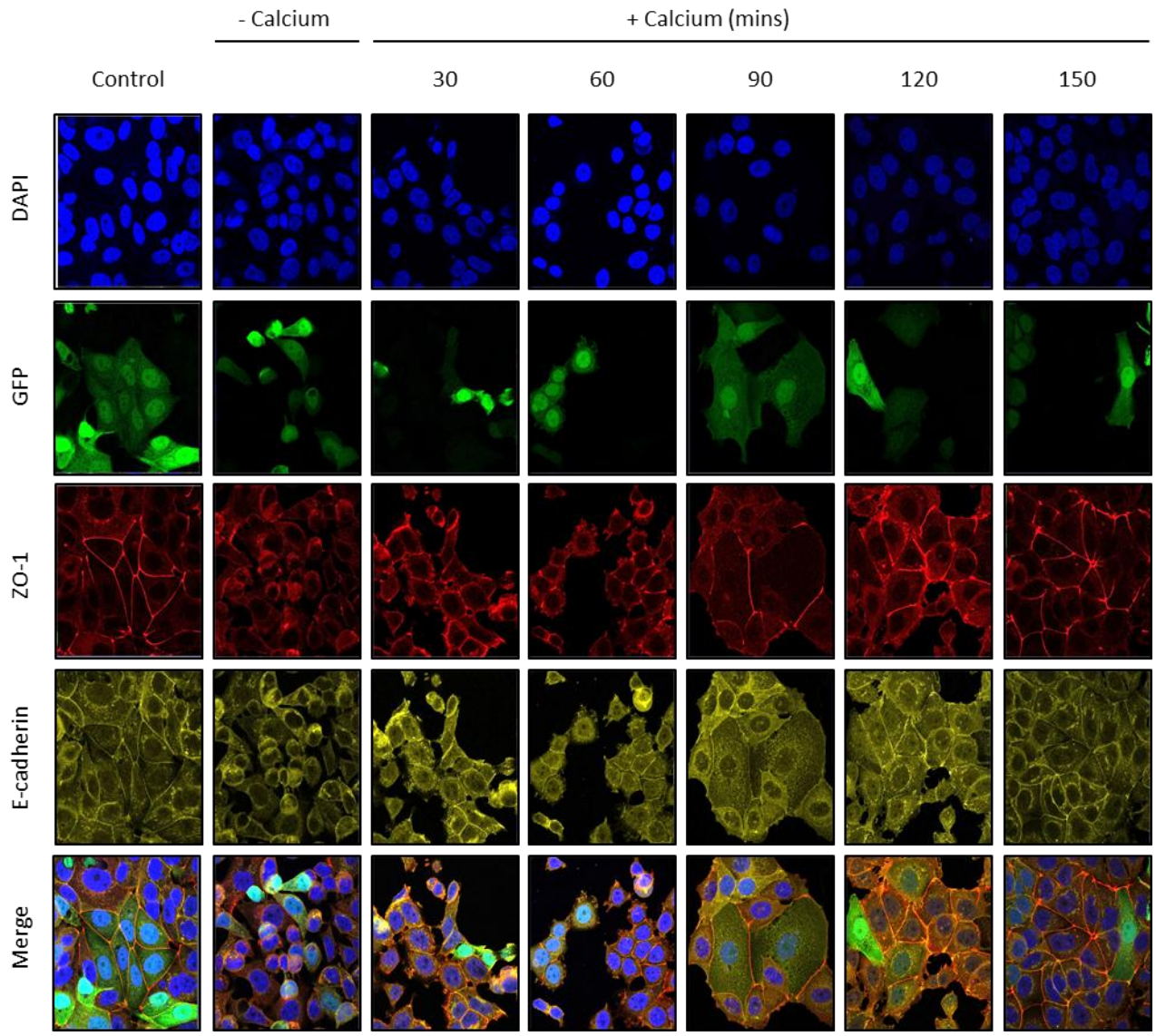


**Figure 5.7. Calcium-Dependent Cell-Cell Contact Formation Occurs Within 90 Mins of Calcium Introduction in MCF7 Cells.** MCF7 cells (3 x $10^5$ cells/condition) were plated onto glass cover slips and transfected with GFP empty vector. Cells were incubated for 48 hrs before the media was changed to calcium free media for 16 hrs to disrupt calcium-dependent cell-cell junctions (with the exception of the slides for the images marked 'control' that were maintained in calcium containing media). Calcium was then re-added to the media and cells were fixed for 15 mins with 4 % PFA every 30 mins for 150 mins. Cells were permeabilised for 5 mins with 0.2 % Triton X solution and blocked for 30 mins with 0.2 % fish skin gelatin before being probed with anti-ZO-1 and anti-E-cadherin antibodies. Secondary antibodies were Alexa-fluor 566 nm for E-cadherin and Alexa-fluor 633 nm for ZO-1. Cover slips were mounted onto slides using mounting medium containing DAPI. Images were taken with a Zeiss LSM710 confocal microscope at 63 x magnification. Cell junctions are lost and cells have a rounded phenotype upon withdrawal of calcium. Cell-cell junctions begin to reform 30 mins after re-addition of calcium forming strong mature junctions by 90 mins. Data is representative two independent experiments.

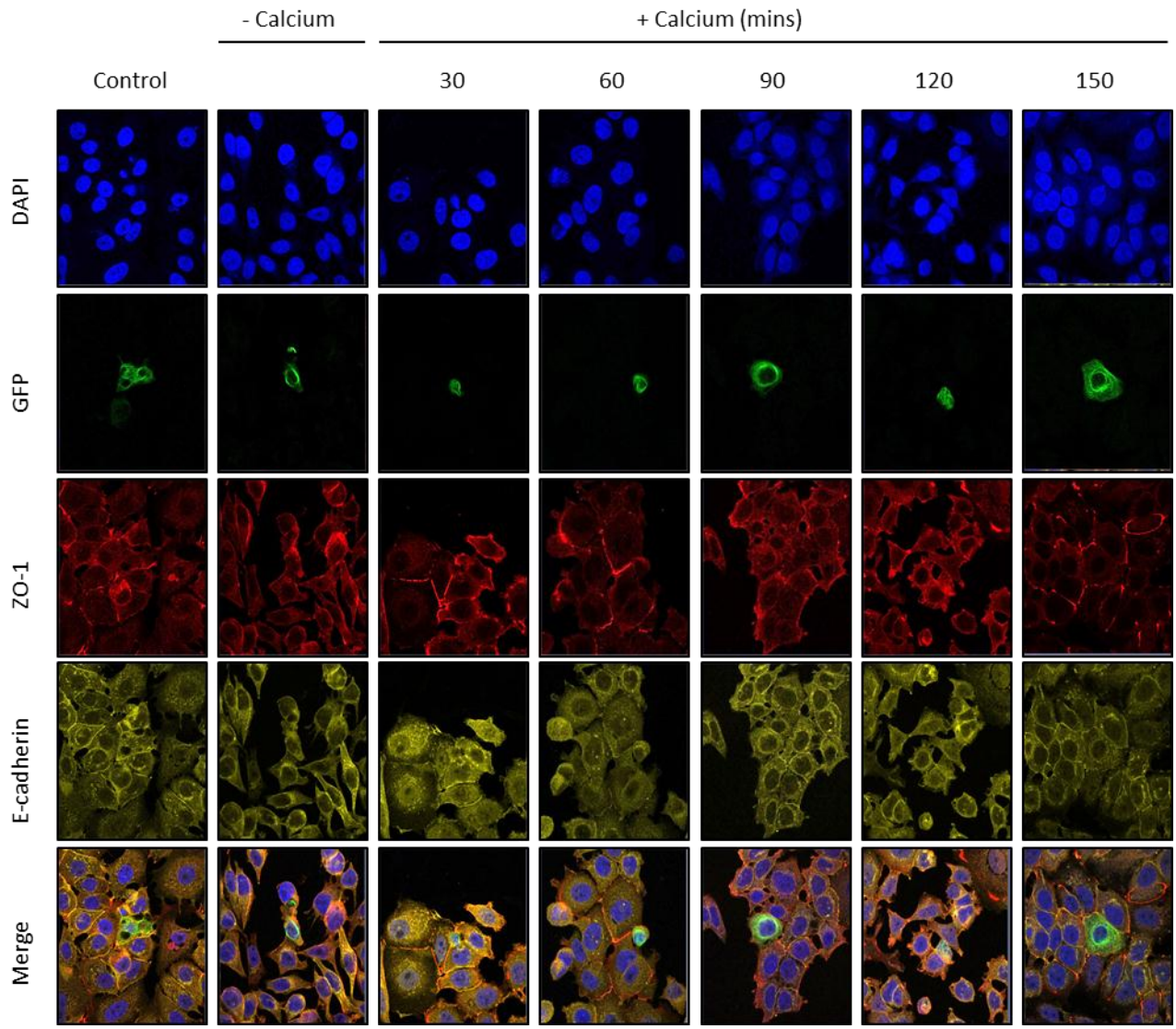


**Figure 5.8. Calcium-Dependent Cell-Cell Contact Formation is Disrupted by RASSF1C Expression.** MCF7 cells (3 x $10^5$ cells/condition) were plated onto glass cover slips and transfected with GFP-RASSF1C. Cells were incubated for 48 hrs before the media was changed to calcium free media for 16 hrs to disrupt calcium-dependent cell-cell junctions (with the exception of the slides for the images marked 'control' that were maintained in calcium containing media). Calcium was then re-added to the media and cells were fixed for 15 mins with 4 % PFA every 30 mins for 150 mins. Cells were permeabilised for 5 mins with 0.2 % Triton X solution and blocked for 30 mins with 0.2 % fish skin gelatin before being probed with anti-ZO-1 and anti-E-cadherin antibodies. Secondary antibodies were Alexa-fluor 566 nm for E-cadherin and Alexa-fluor 633 nm for ZO-1. Cover slips were mounted onto slides using mounting medium containing DAPI. Images were taken with a Zeiss LSM710 confocal microscope at 63 x magnification. It can be seen that the junctions are already weakened in the RASSF1C expressing cells before the removal of calcium, but like the control cells junctions are completely lost and the cells form a rounded phenotype with the removal of calcium from the media. Unlike control cells, RASSF1C expressing cells do not form strong, mature junctions by 90 mins. This is delayed and can be seen to begin to occur at only 150 mins post re-addition of calcium. Data is representative of two independent experiments.

## 5.7 Discussion

In this chapter I have shown that expression of RASSF1C drives tyrosine phosphorylation of E-cadherin and β-catenin. The increase in tyrosine phosphorylation of E-cadherin correlated with increased association with SRC and the E3 ubiquitin ligase HAKAI. The tyrosine phosphorylation and the complex formation were inhibited by co-expression of RASSF1A with RASSF1C. Immunofluorescence experiments demonstrated that RASSF1C expression promotes E-cadherin internalisation and the formation of a perinuclear pool of E-cadherin. RASSF1C expression also promoted the loss of the tight junction protein, ZO-1 and the polarity protein, Par3 from sites of cell-cell contact. Formation of calcium-dependent cell-cell contacts following reintroduction of calcium to cell culture media was also found to be retarded in RASSF1C expressing cells.

E-cadherin is a tumour suppressor that mediates homophilic cell-cell adhesions and along with its binding partners, the catenin proteins, regulates contact inhibition through the activation of the hippo pathway (Kim, Koh et al. 2011). The E-cadherin-catenin complex functions to block epithelial tumour progression and consequently, in tumours there is often loss of expression, mislocalisation or mutation of E-cadherin resulting in destabilisation of cell-cell adhesion, promoting EMT (Gumbiner 2005; Hartsock and Nelson 2008; Jeanes, Gottardi et al. 2008; Berx and van Roy 2009). SRC family kinases can phosphorylate both E-cadherin and catenin proteins on tyrosine residues. This leads to destabilisation of the cadherin-catenin complex and ubiquitination of E-cadherin by the E3 ubiquitin ligase HAKAI mediating the internalisation and degradation of E-cadherin in the lysosomes (Behrens, Vakaet et al. 1993; Fujita, Krause et al. 2002; Palacios, Tushir et al. 2005). We have shown that RASSF1C is an adapter protein that can activate SRC. This is opposite to the SRC adaptor, RACK1, which is known to stabilise E-cadherin at cell-cell junctions by inhibiting SRC (Chang, Conroy et al. 1998; Swaminathan and Cartwright 2012). We reasoned that expression of RASSF1C would lead to increased SRC kinase activity towards its target proteins and were able to show the tyrosine phosphorylation of SRC targets, E-cadherin and β-catenin was upregulated in cells expressing RASSF1C (Figure 5.1). Further to this we also showed that

the association between E-cadherin, SRC and HAKAI was enhanced by RASSF1C expression (Figure 5.2). Together, these results suggest that RASSF1C can activate SRC and target it towards cell-cell junctions where it is able to phosphorylate target proteins, such as E-cadherin, which would subsequently be ubiquinated by HAKAI and targeted for degradation. In agreement with this data we observed that E-cadherin staining was enriched in a perinuclear pool in cells expressing RASSF1C. Interestingly, internalisation of E-cadherin also appeared in the cells surrounding those expressing high levels of RASSF1C (Figure 5.3A).

One unanswered question is whether RASSF1C is promoting degradation of E-cadherin or simply affecting the trafficking of E-cadherin. We observe complex formation between E-cadherin and HAKAI and increased perinuclear staining for E-cadherin, which are indicative of E-cadherin degradation (Fujita, Krause et al. 2002; Palacios, Tushir et al. 2005). However, at the time points where cell-cell contacts were lost we did not observe any change in the total levels of E-cadherin (Figure 5.1 and Figure 5.2). E-cadherin coupling to p120-catenin stabilises the cadherin complex at the membrane and regulates E-cadherin recycling via the endosome recycling pathway (Yap, Niessen et al. 1998; Miyashita and Ozawa 2007). Uncoupling of E-cadherin from p120-catenin leads to E-cadherin accumulation in the early endosomes, as shown by co-localisation with Early Endosomes Antigen 1 (EEA1) (Miyashita and Ozawa 2007). E-cadherin targeting to the late endosomes and lysosomes is thought to be dependent on a conserved dileucine motif at 587-588 in the juxtamembrane cytoplasmic domain (Bonifacino and Traub 2003). Mono-ubiquitination is also an essential step for shuttling E-cadherin to the lysosomes (Fujita, Krause et al. 2002; Palacios, Tushir et al. 2005). It would be interesting to see whether the localisation of E-cadherin in RASSF1C expressing cells changed with alteration of the dileucine sequence. To confirm that RASSF1C was targeting E-cadherin to the lysosome and not just affecting the endocytic recycling pathways, cells expressing RASSF1C could be co-stained with E-cadherin and either LysoTracker or EEA1 to indicate where E-cadherin was being targeted. Rab proteins are associated with specific membrane compartments, for example Rab5 indicates early endosomes whereas Rab11 indicates recycling endosomes. Therefore these could also be used to determine the location of E-cadherin. Biochemically,

sucrose gradients could be used to detect the density of the compartment containing E-cadherin, thereby indicating where E-cadherin was being targeted.

Further to this, E-cadherin degradation could be detected through the use of lysosome inhibitors, for example $NH_4Cl$ or leupeptin. This would allow the accumulation of E-cadherin degradation products, which could potentially be visualised by western blot. We would expect that in cells expressing RASSF1C the accumulation of these products would increase. This may occur at a later time point, because we observe phenotypic changes associated with SRC activation without reduction in total E-cadherin.

The lack of E-cadherin degradation (Figures 5.1 and 5.2), despite the increased internalised pool of E-cadherin (Figure 5.3), suggests that RASSF1C promotes phospho-tyrosine mediated E-cadherin internalisation, which subsequently may become a target for ubiquitination by HAKAI and eventual degradation or lysosomal sorting. As well as the direct regulation of E-cadherin through HAKAI, described above, SRC has been shown to downregulate E-cadherin through other cofactors including: Focal Adhesion Kinase (FAK) and Arf6 GTPase. Arf6 has been shown to regulate the recycling of E-cadherin between the plasma membrane and the early endosomes in MDCK cells (Palacios, Price et al. 2001). This internalised E-cadherin is then targeted to the lysosome for degradation by SRC phosphorylation and the recruitment of HAKAI (Fujita, Krause et al. 2002; Palacios, Tushir et al. 2005). Activation of Arf6 by the Arf-GEF, EFA6 has also been shown to regulate tight junction formation, which we have shown RASSF1C can also regulate (Figure 5.5) (Luton, Klein et al. 2004).

We also examined the SRC target FAK in the phospho-tyrosine immunoprecipitation. We detected a minor induction of phosphorylation of FAK with RASSF1C expression. Given that we only expect FAK to be activated at the leading edge of cells expressing RASSF1C it may be hard to detect. Particularly in epithelial cells such as MCF7 that primarily form cell-cell, rather than cell-ECM interactions. However, we did observe cell rounding in the IF experiments which would require loss of adhesion to the substratum. Use of mesenchymal-like cells, for example MDA-MD-231 cells may show more effect on FAK. In addition, IF techniques such as a co-localisation studies may reveal more light on the role

RASSF1C plays in the activation of FAK. FAK also plays a role in the SRC-dependent downregulation of E-cadherin, however, the precise mechanism of this remains unclear but is known to require integrin signalling (Avizienyte, Wyke et al. 2002). FAK also plays a significant role in regulating SRC-mediated cell motility and invasion, which we shall cover in Chapter 6, and so although not covered in this thesis, it would be interesting to examine the role of RASSF1C in SRC-dependent activation of FAK.

Rac1 activity is essential for cadherin and catenin translocation to sites of cell-cell contact, Actin filament recruitment and maintenance of adherens junctions (Hordijk, ten Klooster et al. 1997). A Rac1-GEF, Tiam1 is responsible for activating Rac1 to stimulate cadherin adhesion (Hordijk, ten Klooster et al. 1997; Sander, ten Klooster et al. 1999). Phosphorylation of Tiam1 by SRC on Y384 creates a binding site for Grb2, which through SOS can activate ERK1/2 leading to the destruction of Tiam1 by the protease Calpain (Woodcock, Rooney et al. 2009). RASSF1C has previously been shown to activate ERK1/2, but a direct link between RASSF1C and ERK1/2 was not shown (Reeves, Baldwin et al. 2010). Our model therefore provides a potential link between RASSF1C and ERK. Here we suggest that RASSF1C activation of SRC may promote ERK1/2 activation by phosphorylating Tiam1. It would be interesting to ascertain whether Tiam1 was phosphorylated and cleaved in cells expressing RASSF1C. The phosphatase tumour suppressor protein, PTEN, has also been shown to negatively regulate E-cadherin destruction. PTEN stabilises adherens junctions in cells expressing v-SRC, suggesting a role for PI3K in this process as well (Kotelevets, van Hengel et al. 2001). RASSF1A has previously been shown to inhibit PI3K and Rac (Dallol, Agathanggelou et al. 2005). Given that RASSF1C can only activate SRC in cells lacking RASSF1A expression and SRC can activate PI3K, this may provide a link between loss of RASSF1A and PI3K regulation.

SRC phosphorylation on Y419 was detected at 48 hrs post transfection with RASSF1C by western blot (Figure 4.4). However, we did not observed any increase in the amount of SRC precipitated in the phospho-tyrosine IP at this time point (Figure 5.1). This anomaly can be explained by the fact that SRC has two tyrosine phosphorylation sites, an inhibitory site at Y530 and an activation site at Y419. The

phosphorylation of these two sites is mutually exclusive and so any increase in Y419 phosphorylation would be countered by a decrease in Y530 phosphorylation. Since the anti-phospho-tyrosine antibodies do not distinguish between these two sites, both active and inactive SRC would be precipitated. We would expect that repeating the IP with anti-phospho-SRC Y419 antibodies would show an increase in active SRC with RASSF1C expression at this time point.

An interesting consequence of the re-disruption of E-cadherin is the translocation of β-catenin away from the cell-cell junctions to the cytoplasm where it is able to enter the nucleus, something we detected with RASSF1C expression (Figure 5.4). The re-distribution of β-catenin (Figure 5.4) was much more dramatic than for p120-catenin (Figure 5.3B) fitting with the biochemical data (Figure 5.1), which showed the phosphorylation of β-catenin was more intense in the RASSF1C expressing cells than it was for p120-catenin. RASSF1C expression promoted p120-catenin staining to become more diffuse, however this staining was still located in the juxtamembrane region (Figure 5.3B).

The β-catenin staining was completely lost from the membrane in cells expressing RASSF1C, forming a diffuse localisation throughout the cells that is both cytoplasmic and nuclear. As nuclear β-catenin upregulates target genes such as C-*MYC* and *CYCLIN D1* to promote EMT (Klaus and Birchmeier 2008) it would be interesting to see whether RASSF1C expression, or loss of RASSF1A expression, regulated β-catenin target gene expression and EMT.

The re-localisation of β-catenin and ZO-1 away from the cell-cell junctions in cells expressing RASSF1C was much more extensive than for E-cadherin. This could be explained by the fact that the recycling pool of E-cadherin is constantly replenished from newly translated protein. Therefore, an interesting approach to visualise the loss of E-cadherin from cell-cell junctions in RASSF1C expressing cells would be inhibition of translation using cycloheximide. Downregulation of E-cadherin could also be taking place as RASSF1C promoted nuclear β-catenin which is able to interact with and upregulate SNAIL leading to transcriptional repression of the E-cadherin gene (Cano, Perez-Moreno et al. 2000; Stemmer, de Craene et al. 2008). This can occur through binding of β-catenin to SNAIL, promoting SNAIL activation (Stemmer,

de Craene et al. 2008), or by promoting the nuclear localisation of SNAIL through the upregulation of the Wnt target gene Axin2 which inhibits the negative regulator of β-catenin and SNAIL, GSK3β (Zhou, Deng et al. 2004; Bachelder, Yoon et al. 2005; Yook, Li et al. 2006). Stable expression of RASSF1C in MCF7 cells would allow us to monitor the downregulation of E-cadherin and other epithelial markers and the upregulation of mesenchymal markers over time. We would predict that this process would be controlled by β-catenin. Interestingly, RASSF1C has been shown to bind to and inhibit the F-box protein β-TRCP, which is known to drive ubiquitin-mediated degradation of β-catenin, leading to accumulation of oncogenic β-catenin in the nucleus (Estrabaud, Lassot et al. 2007). Therefore, RASSF1C expression promotes the re-localisation of β-catenin away from the cell-cell junctions and removes its negative regulation by inhibiting β-TRCP. Loss of RASSF1A leads to the accumulation of β-catenin, in agreement with a role for RASSF1A in restraining the oncogenic activities of RASSF1C (Chapter 4) (Estrabaud, Lassot et al. 2007).

It is known that adherens junctions are the first of the junctions to form upon cell-cell contact. Their formation enables the formation and enhances the stability of the other junctions, for example the tight junctions (Adams, Nelson et al. 1996; Pokutta and Weis 2002; Gumbiner 2005). Both adherens junctions and tights junctions are associated with polarity proteins, for example Lgl is known to colocalise with adherens junctions upon formation of E-cadherin-dependent cell-cell contacts (Reuver and Garner 1998) and Par-3 colocalises with ZO-1 at tight junctions (Izumi, Hirose et al. 1998; Joberty, Petersen et al. 2000). The association of polarity complexes with the junctional components is essential for them to function to promote either apical (at tight junctions) or basolateral (at adherens junctions) polarity. Therefore, we predicted that loss of E-cadherin would destabilise tight junctions and result in loss of apical-basolateral cell polarity. This is exactly what we see. In control cells ZO-1 forms a solid, uninterrupted band at points where cells are in contact. In the RASSF1C expressing cells this line is thinner and often interrupted by regions that show no staining (Figure 5.5). Further to this, large sections of ZO-1 are seen within the cell cytoplasm (Figure 5.5 bottom image). Par3 also fails to stain at

the sites of cell-cell contact. Interestingly, the image in Figure 5.6 shows the overlapping of the cells that is also observed in Figures 5.3A and B and 5.4, which is indicative of cells failing recognise neighbouring cells and form junctional contacts.

Three studies have examined the *Drosophila* isoforms of RASSF (dRASSF and dRASSF8) and the mammalian RASSF8, in the context of cell polarity and cell-cell junction regulation (Langton, Colombani et al. 2009; Grzeschik, Parsons et al. 2010; Lock, Underhill-Day et al. 2010). Grzeschik et al (2010) showed that dRASSF and hippo are mislocalised to the basolateral region of photoreceptor cells in $lgl^-$ or aPKC overexpressing cells (Grzeschik, Parsons et al. 2010). This suggests that dRASSF, which is orthologous to the mammalian classical RASSF proteins (RASSF1-6) is apically localised along with hippo pathway components and that this localisation is dependent on the polarity machinery. The other two studies looked at the N-terminal RASSF isoforms dRASSF8 and human RASSF8. Langton et al. (2009) showed that dRASSF8 was able to bind dASPP and direct it towards dCsk which negatively regulates Src. This was thought to stabilise adherens junctions which Src normally destabilises (Langton, Colombani et al. 2009). Lock et al (2010) showed that RASSF8 was a tumour suppressor that acts to stabilise adherens junctions. This was thought to be through stabilisation of F-actin stress fibres, which would be used to translocate E-cadherin to the membrane, because loss of E-cadherin from cell-cell junctions in cells depleted of RASSF8 correlated with these cells also having fewer stress fibres (Lock, Underhill-Day et al. 2010). Another consequence of the loss of E-cadherin was that β-catenin re-localised from the junctions to form a cytoplasmic pool which was able to enter the nucleus where it presumably was able to upregulate target genes (Lock, Underhill-Day et al. 2010). It would be interesting, in light of these studies, to investigate whether RASSF1C expression has an effect on Actin stabilisation. This is particularly relevant given the motility phenotype that is discussed in Chapter 6.

The homophilic interaction between E-cadherin molecules are calcium dependent (Takeichi 1977; van Roy and Berx 2008). Removal of calcium results in destabilisation and internalisation of E-cadherin leading to the loss of contacts between cells. Re-introduction of calcium stimulates the cells to recycle

the E-cadherin back to the plasma membrane where it can reform cell-cell junctions. By following the reformation of these junctions in control and RASSF1C expressing cells we were able to see that cells expressing RASSF1C were less efficient at forming the cell junctions (both adherens and tight). This data suggests that RASSF1C expression inhibits the recycling of E-cadherin back to the membrane upon calcium stimulation (Figure 5.7 and 5.8). Data from Figures 5.1 and 5.2 would suggest that this is due to enhanced targeting of E-cadherin to internal endosomes. If RASSF1C-mediated internalisation of E-cadherin solely promoted internalisation and not destruction, we might expect to see calcium-mediated recycling of E-cadherin unaffected because the internalised E-cadherin would be held in a recyclable pool. One way that this could be tested is by blocking the trafficking to the late endosomes and therefore, the lysosomes. This can be done by expressing a dominant negative Rab7 protein. Vitelli et al (1997) showed that expression of a dominant negative Rab7 resulted in a marked inhibition of $^{125}$I-LDL without affecting the initial internalisation rate (Vitelli, Santillo et al. 1997). They further showed that expression of mutant Rab7 had no effect on the internalisation or recycling of Transferrin or horseradish peroxidase. These earlier effects are controlled by Rab5 (Barbieri, Li et al. 1994; Stenmark, Parton et al. 1994).

We observed that in cells cultured in calcium free media E-cadherin is completely lost from the cell periphery. Cells become also rounded up and can be seen to overlap one another (Figure 5.7 and 5.8). Cells expressing RASSF1C show a similar phenotype, rounding up, moving over one another and failing to form a monolayer when cultured in the presence of calcium (Figure 5.3-5.6). This data suggests that it is the targeted internalisation of junctional proteins by RASSF1C that is promoting these EMT like phenotypes.

Together the work presented in this chapter (summarised in Figure 5.9) shows that in cancer, where RASSF1A is lost due to promoter methylation, RASSF1C is able to activate SRC, targeting it to the membrane where it is able to phosphorylate target proteins including E-cadherin and β-catenin. Investigation of E-cadherin, because of its crucial role in inhibition of tumourigenesis and EMT, revealed

that RASSF1C expression promotes E-cadherin internalisation potentially targeted E-cadherin for HAKAI-mediated ubiquitination and degradation. Use of immunofluorescence further showed internalisation of other SRC targets, such as β-catenin and ZO-1. Loss of β-catenin from cell-cell junctions potentially allows it to enter the nucleus where it is able to upregulate pro-proliferative genes, such as *C-MYC* and *CYCLIN D1* (Ahmed, Hayashi et al. 1998; Gat, DasGupta et al. 1998; He, Sparks et al. 1998; Wong, Rubinfeld et al. 1998; Tetsu and McCormick 1999). Loss of the tight junction protein ZO-1 was also associated with loss of polarity marker Par3 from the sites of cell-cell contacts. Finally, we showed that RASSF1C-mediated targeting of E-cadherin for destruction depleted the pool of recycling E-cadherin resulting in a reduced ability to recycle E-cadherin back to junctions upon calcium stimulation.

Following on from this we aim to look at another of the roles of SRC, cell motility and we shall examine the role of RASSF1C in promoting EMT and tumour invasiveness.

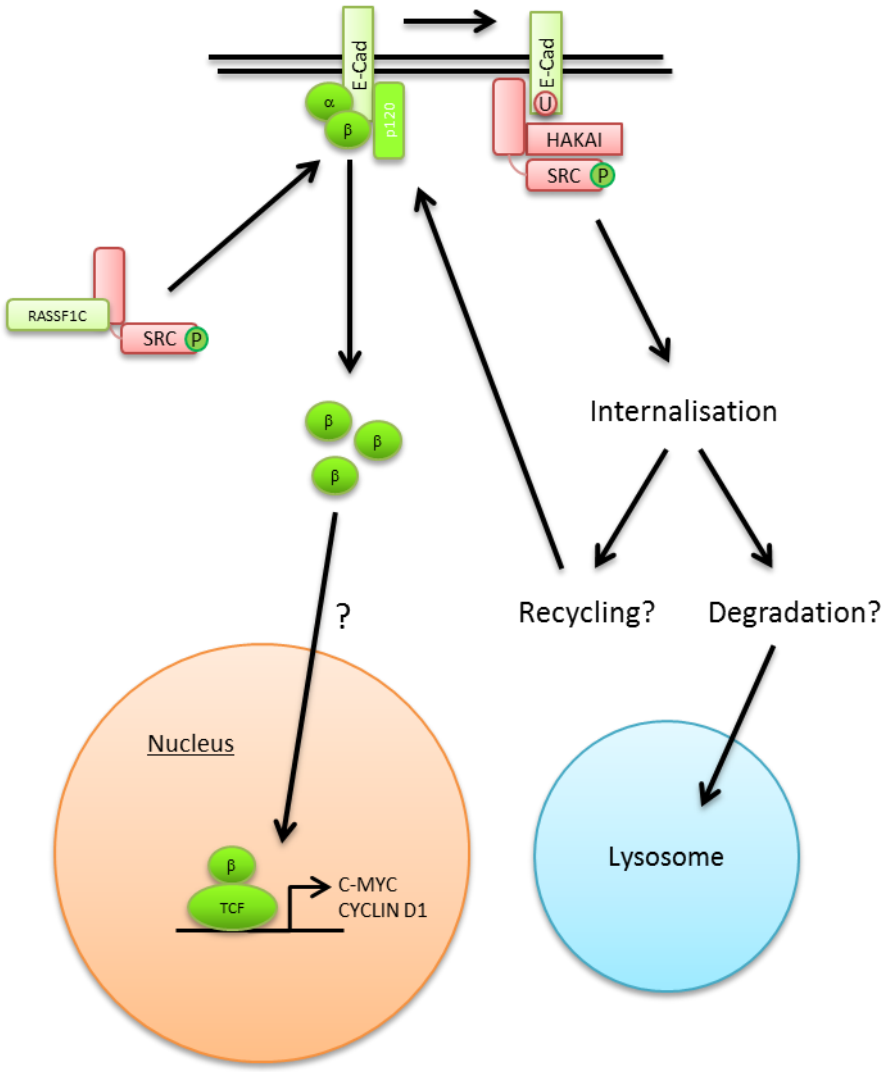


**Figure 5.9. Cartoon Depicting the Hypothesis Generated from the Results in Chapters 5.** When RASSF1A is lost, RASSF1C can activate SRC promoting translocation of SRC to the plasma membrane. At the plasma membrane SRC phosphorylates target proteins such as, E-cadherin (E-cad) and β-catenin (β). This leads to the formation of an E-cadherin-HAKAI complex, which may target E-cadherin for degradation in the lysosome. E-cadherin may also be recycled back to the membrane. β-catenin release from the membrane allows it to potentially enter the nucleus, upregulating pro-survival genes such as *C-MYC* and *CYCLIN D1*.

# Chapter 6 RASSF1C Activation of SRC Leads to Increased Cell Motility

## 6.1 Introduction

In chapter 5, the role of SRC at the cell-cell junctions was investigated. RASSF1C activation of SRC was shown to have a destabilising effect on cell-cell adhesion, a key step in EMT.

### 6.1.1 The Role of SRC in Migration

In addition to the regulation of cell-cell contacts, SRC plays an important role in cell motility, which is regulated through binding to and phosphorylation of FAK. The promotion of a more migratory phenotype by active SRC is dependent on the deregulation of E-cadherin and requires FAK phosphorylation at $\beta_1$ integrin-dependent protrusions (Avizienyte, Wyke et al. 2002). FAK-dependent SRC signalling results in a change in the predominant cell adhesion type from cadherin-based to integrin-based, promoting a more motile phenotype. This occurs through the regulation of the actin cytoskeleton.

At the leading edge of a migrating cell SRC activates Rac1 through the recruitment of Rac-GEFs, for example: β-PIX (Pak-interacting exchange factor-beta) (ten Klooster, Jaffer et al. 2006), Dock180 (Kiyokawa, Hashimoto et al. 1998; Brugnera, Haney et al. 2002), and Vav2 (Marignani and Carpenter 2001; Marcoux and Vuori 2003). Rac1 activity promotes membrane protrusion through the rearrangement of Actin (Ridley, Paterson et al. 1992; Machesky and Hall 1997). RhoA-ROCK activity promotes actomyosin contractility at the back of a migrating cell, but is suppressed at the front of the cell by mutual inhibition by Rac1 which upregulates the Rho-GAP p190RhoGAP. The RhoA effector protein mDia1 is however active at the leading edge of the cell. mDia1 is responsible for the production

of long straight Actin fibres, which are required for membrane protrusions (Huveneers and Danen 2009). Therefore the differential activation of Rac and Rho promotes directionality of movement.

### 6.1.2 SRC and Invasion

A prerequisite for the development of distant metastatic sites is the invasion of cells through matrix barriers, such as basement membranes and also tissue compartments, vessels, perineural spaces and organ boundaries (Guarino 2010). There are two types of invasion; Rac-mediated mesenchymal-type, which combines migration and pericellular ECM breakdown effected by matrix-degrading proteases at the leading edge, creating a path through which the cell can move; and amoeboid-type, where rounded cells move through the matrix by a Rho-ROCK-mediated actomyosin-dependent mechanism (Price, Hajdo-Milasinovic et al. 2004; Carragher, Walker et al. 2006). SRC is thought to regulate mesenchymal invasion. SRC can activate Rac, through the mechanisms described above, to drive mesenchymal invasion (Hsia, Mitra et al. 2003; Van Slambrouck, Jenkins et al. 2009). In addition, SRC phosphorylation of Caspase 8 switches its function from pro-apoptotic to pro-migratory. This dual function protects cells from apoptosis by inhibiting Caspase 8 activation and enables Caspase 8 to interact with PI3K thereby stimulating the subsequent activation of Rac1, ERK and Calpain (Frisch 2008).

### 6.1.3 Microtubule Polarisation and Migration

In addition to Actin cytoskeleton polarisation, the microtubules and golgi are also polarised in a migrating cell. They orientate towards the leading edge of the cell in a manner dependent on the Rho family member Cdc42 (Etienne-Manneville and Hall 2002; Rodriguez, Schaefer et al. 2003). Cdc42 regulates the MTOC position through the Cdc42 effector proteins Par3, Par6 and aPKC (Etienne-Manneville and Hall 2003). Although the mechanism is not completely understood, it has recently been suggested to occur by local capture of microtubule ends at the leading edge by APC, which binds to

microtubules via CLIP170 and IQGAP (Rodriguez, Schaefer et al. 2003), and/or via a microtubule based dynein/dynactin motor protein complex (Etienne-Manneville and Hall 2003). The polarised localisation and activation of Cdc42 is dependent on Arf6 vesicular trafficking and SRC phosphorylation and activation of β-PIX (Osmani, Vitale et al. 2006; Osmani, Peglion et al. 2010). Other reports however, have concluded that the MTOC is immobile and that polarisation occurs by reorienting the nucleus. This has been proposed to be through Myosin Light Chain Kinase (MLCK) regulation of Myosin Light Chain (MLC) (Gomes, Jani et al. 2005). The polarisation of the golgi has recently been linked to vesicular trafficking. It was shown that SRC accumulated in early endosomes in cells depleted of ESCRT proteins preventing it from activating MLCK and therefore inhibiting several aspects of cell migration, namely, cell spreading, turnover of focal adhesions and cell polarisation in fibroblasts (Lobert and Stenmark 2012).

### 6.1.4 RASSF1 and Migration

Both RASSF1A and RASSF1C have previously been linked to cell migration. Dallol et al (2005) suggested that RASSF1A could inhibit cell migration by preventing the activation of Rac1 by PI3K (Dallol, Agathanggelou et al. 2005). RASSF1C has been proposed to upregulate the chemokine receptor CXCR4 through the activation of the Ras-Raf-MEK-ERK1/2 pathway. It was shown that the elevated CXCR4 expressed by RASSF1C expressing breast cancer cells enabled more efficient migration towards a CXCL12 (CXRC4 ligand) gradient (Reeves, Baldwin et al. 2010). In cancer cells the hippo pathway members MST1/2 and LATS1/2 have been shown to inhibit YAP and TAZ which can promote migration (Overholtzer, Zhang et al. 2006; Lei, Zhang et al. 2008; Zhang, Liu et al. 2009; Mizuno, Murakami et al. 2012). However, the effect of regulating the levels of these proteins has not directly been assessed in this system. Both MST and LATS kinases have been linked to Actin cytoskeleton organisation, a process which I have already highlighted as important for regulating migration. (Yang, Yu et al. 2004; Densham, O'Neill et al. 2009). YAP regulation by the hippo pathway was also shown to be regulated by F-actin stress fibre formation (Wada, Itoga et al. 2011). Interestingly, YAP is activated by increased Rho-GTPase-

dependent actomyosin tension, although this was shown to be hippo pathway independent (Dupont, Morsut et al. 2011).

In contrast to the anti-migratory regulation of YAP by MST1/2 in cancer cells, MST1 has been shown to promote T-cell egress from the thymus and induction of rolling attachment in the blood stream. In T-cells MST1 is found in a complex with RASSF5B/RAPL. This complex was shown to bind and cluster LFA-1 at the leading edge of migrating T-cells in response to T-cell receptor activation. Loss of MST1 prevented the clustering of LFA-1 and decreased attachment to endothelial venules and entry into peripheral lymph nodes (Katagiri, Katakai et al. 2009). MST1/2 also binds to and phosphorylates Mob1A/B. In T-cells Mob1A/B can bind and activate the Rho-GEF Dock8. Knockout of MST1 and MST2 therefore inhibits the activation of Rho-GTPases, inhibiting T-cell migration out of the thymus (Mou, Praskova et al. 2012).

### 6.1.5 Aims

In this chapter the role of RASSF1C in motility shall be assessed. SRC activity promotes actin reorganisation and integrin turnover, which promote cell migration (Frame, Fincham et al. 2002; Huveneers and Danen 2009). Work in this chapter aims to uncover whether RASSF1C behaves as an oncogene that can drive EMT through the activation of SRC family kinases in cancers where RASSF1A has been lost, and potentially lead to more invasive, aggressive tumours.

## 6.2 Loss of RASSF1 Leads to a Decrease in Motility

In chapter 4 it was shown that RASSF1C expression was required for the activation of SRC and that loss of RASSF1, but not RASSF1A, led to a complete ablation of SRC activity. In chapter 5, one of the consequences of this activation was shown to be the internalisation of cell-cell junctions. As a major activity of SRC is the promotion of directional movement, we decided to examine whether RASSF1A and

RASSF1C regulation of SRC affected cell motility. We started with the hypothesis that loss of RASSF1, which correlates with loss of SRC activity should lead to a decrease in motility in a scratch wound assay. HeLa cells were plated at high density and transfected with non-targeting siRNA, siRNA targeting RASSF1 or siRNA targeting the SRC activator, OSSA. We found that loss of expression of RASSF1 led to a dramatic decrease in motility (Figure 6.1A). From this we concluded that RASSF1 promotes motility through activation of SRC.

Treatment of cells with siRNA targeting OSSA had no effect on cell motility (Figure 6.1B). This was not surprising because loss of OSSA had had little effect on the levels of phospho-SRC (Figure 3.13) and therefore would not be expected to affect the motility of cells.

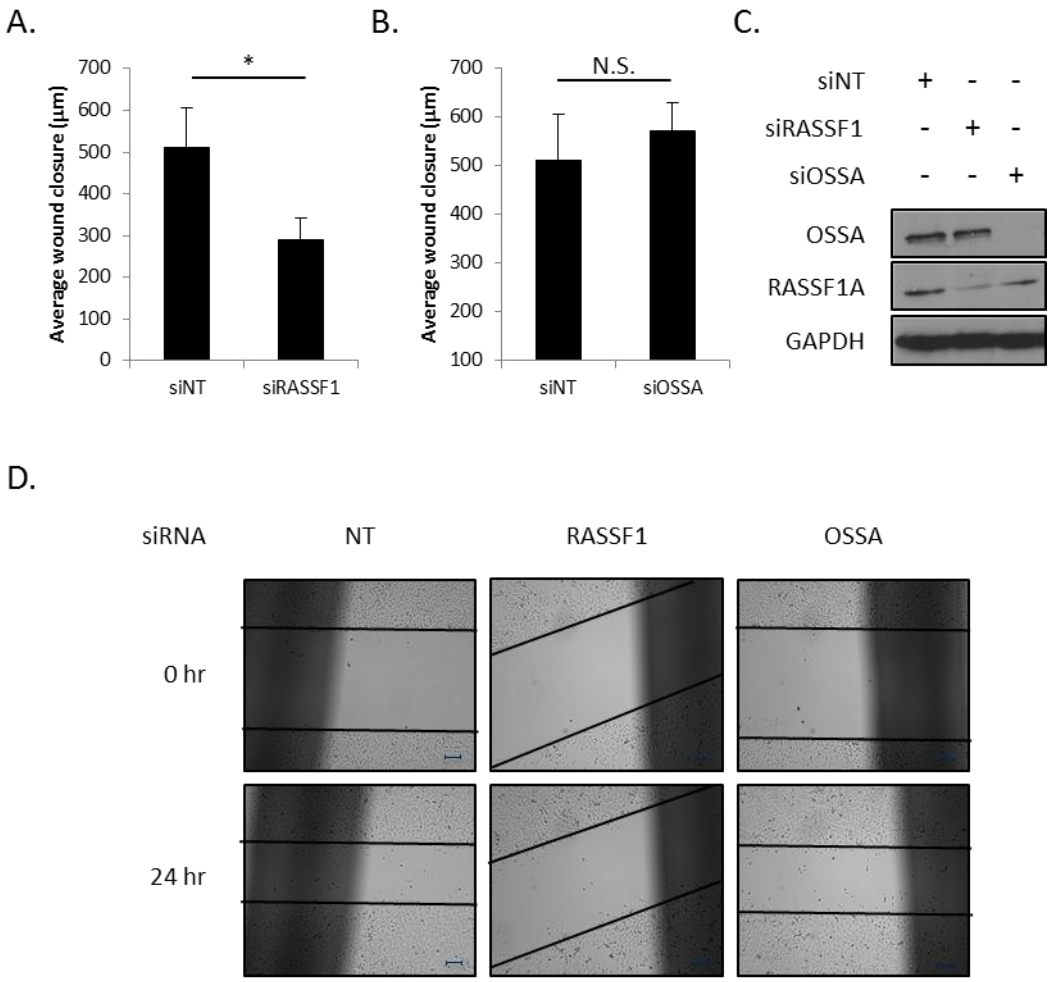


**Figure 6.1. Expression from the RASSF1 Locus is Required for Cells to Migrate into a Wound.** HeLa cells (2 x $10^5$ cells/plate) were plated and transfected with 50 nM non-targeting siRNA, siRNA targeting RASSF1 or siRNA targeting OSSA. When cells had reached confluency, a wound in the monolayer was made using a 200 µl pipette tip. Images were taken at 0 hr and 24 hr at the same position in the well. The size of the wound was then measured and the distance the cells had migrated into the wound determined. **A.** Graph of average distance moved for cells treated with non-targeting vs. RASSF1 targeting siRNA. Cells transfected with siRASSF1 have a reduced ability to migrate into the wound ($\rho$ = 2.6 x $10^{-4}$) Error bars represent 1 x SEM **B.** Graph of average distance moved for cells treated with non-targeting vs. OSSA targeting siRNA. Knockdown of OSSA has no effect on the ability of cells to migrate into the wound ($\rho$ = 0.16). Error bars indicate 1 x SEM **C.** Western blot to show knockdown of RASSF1A and OSSA expression. **D.** Representative images of the wound at 0 hr and 24 hr. Black lines mark the edge of the wound. Results are representative of 16 wounds from 4 independent plates. Bar on images indicates 100 µm. Asterisks represent a $\rho$-value of less than 0.05. N.S. represents not significant result.

## 6.3 Cells Depleted of RASSF1 Fail to Polarise Towards the Wound Edge

Having shown that loss of RASSF1 expression led to a decrease in motility we wished to investigate whether we were altering the ability of cells to polarise towards the wound. RASSF1C expression promotes the internalisation of cell junction proteins and mislocalisation of the polarity protein Par3 (Figure 5.6). We reasoned that as overexpression of RASSF1C appears to affect the homeostasis of polarity proteins by regulating endosomal trafficking, then loss of RASSF1 may similarly be required to maintain normal endosomal mediated, cell polarisation. In the context of cell migration, cells must reorientate their Golgi to face the leading edge of the cell. This process is thought to be dependent on endosome trafficking as Arf6 has been associated with polarised localisation of Cdc42, a major regulator of cell polarity, and loss of the ESCRT proteins have been shown to prevent SRC-dependent cell polarisation (Osmani, Peglion et al. 2010; Lobert and Stenmark 2012). We therefore hypothesised that loss of RASSF1 may inhibit Golgi polarisation and that this could contribute to the decrease in motility seen in Figure 6.1.

HeLa cells were plated onto glass cover slips, transfected with non-targeting siRNA or siRNA targeting RASSF1 and allowed to reach confluency before wounds were made to the cell monolayer. Cells were fixed one hour after the wound was made and stained with the Golgi marker GM130, and DAPI. Cells were classified as polarised if the majority of the Golgi was positioned wound side of the nucleus and lay within the edges of an equilateral triangle with its base at the non-wound side of the nucleus (Figure 6.2A). Approximately 65 % of HeLa cells transfected with non-targeting siRNA polarised towards the wound within one hour, however, less 30 % of the cells transfected with siRNA targeting RASSF1 were able to polarise during this time (Figure 6.2B). The number of cells at the 0 hr time point that were facing the wound was equal between the two conditions. This further implicates a role for endosome recycling in RASSF1C-mediated SRC activation and maintenance of polarity.

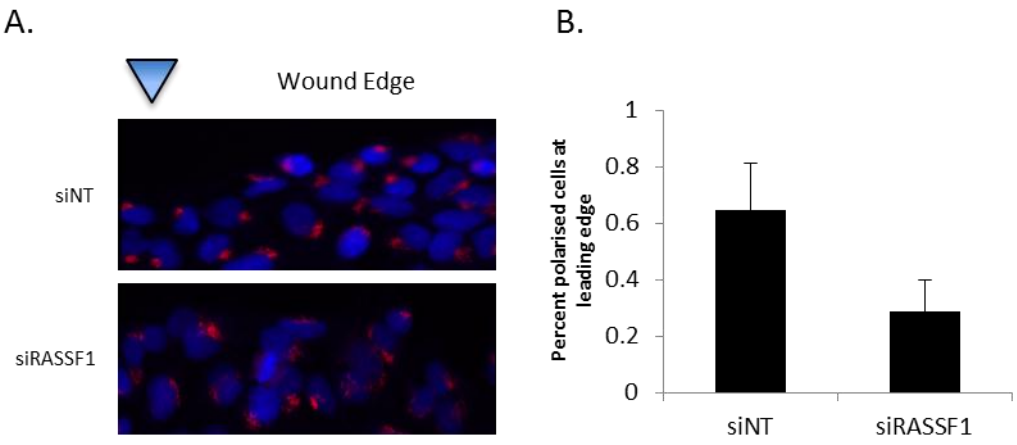


**Figure 6.2. Cells Ablated of RASSF1 Do Not Polarise Towards a Scratch Wound.** HeLa cells (1 x $10^5$ cells/plate) were plated onto glass cover slips and transfected with non-targeting siRNA or siRNA targeting RASSF1. Once cells reached confluency a wound was made using a 200 µl pipette tip. Cells were fixed at 0 hr and 1 hr. Cells were fixed for 15 mins with 4 % PFA, permeabilised for 5 mins with 0.2 % Triton X and blocked for 30 mins with 0.2 % FSG before being probed with anti-GM130 anitbody to stain the golgi. Cover slips were mounted onto slides using mounting medium containing DAPI to stain the nucleus. Cells adjacent to the wound were marked as polarised (golgi polarised towards the wound) or unpolarised (golgi dispersed around the nucleus or facing away from the wound). Data is representative of two experiments, 10 images per experiments. **A.** Example images of cells at the wound edge at 1 hr. Wound is positioned at the top of the image. The blue triangle represents the area within which the golgi must be positioned to be classified as polarised. Images were taken with a Leica DM IRBE at 40 x magnification **B.** Graph depicting the percentage of cells at wound edge with polarised golgi. Cells transfected with siRASSF1 failed to efficiently polarise towards the wound. Error bars represent 1 x SEM.

## 6.4 RASSF1 Promotion of Motility is Independent of the Hippo Pathway

RASSF1A, RASSF1C and MST1/2 have been linked to regulation of motility (Dallol, Agathanggelou et al. 2005; Katagiri, Katakai et al. 2009; Reeves, Baldwin et al. 2010; Mou, Praskova et al. 2012). Although MST1/2 have been shown to promote motility in T-cells (Katagiri, Katakai et al. 2009; Mou, Praskova et al. 2012), the role of MST1/2 in migration has not been studied in cancer cells. RASSF1C, like RASSF1A, can bind to MST1/2 (Matallanas, Romano et al. 2007). Interaction between RASSF1A and MST2 promotes apoptosis (Matallanas, Romano et al. 2007). The function of the RASSF1C-MST2 interaction is unknown, but given previous associations between MST2, Actin and Rho-GTPases (Densham, O'Neill et al. 2009; Mou, Praskova et al. 2012) there remains the possibility that it promotes motility. Therefore,

we sought to confirm whether the loss of motility associated with ablation of RASSF1 expression was through SRC and not through MST1/2. We hypothesised that if RASSF1 isoforms promote motility through MST2 then loss of MST2 would also lead to a similar decrease in motility. HeLa cells were transfected with non-targeting siRNA or siRNA targeting RASSF1 or MST2. Cells transfected with siRNA targeting RASSF1 again showed a decrease in motility, however, no significant change in motility was observed in cells transfected with siRNA to MST2 (Figure 6.3). This suggests that MST2 does not play a role in motility in cancer cells and that the decrease in the rate of motility seen in cells depleted of RASSF1 is due to the ablation of SRC activity, however, incomplete siRNA mediated knocked may also be responsible.

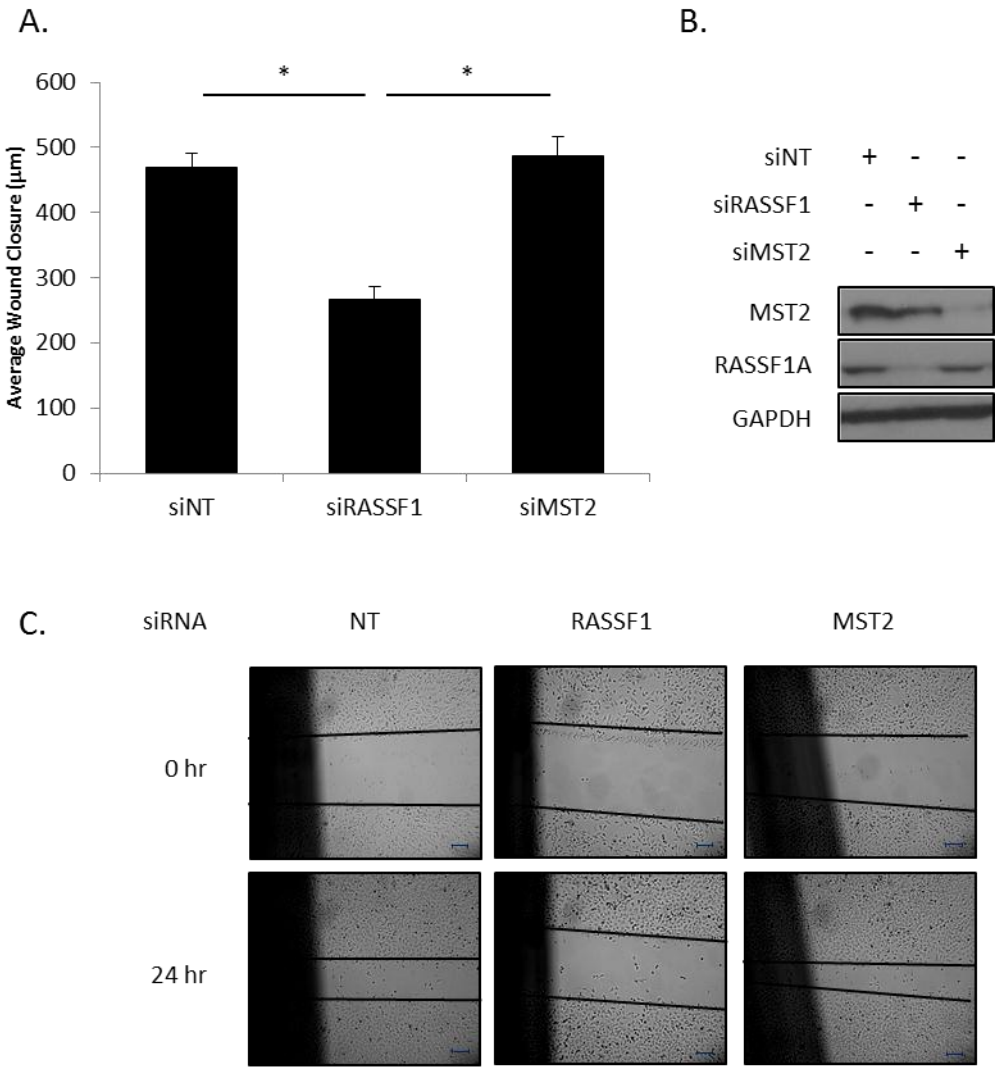


**Figure 6.3. Wound Healing is Not Dependent on the Hippo Pathway**. HeLa cells (2 x $10^5$ cells/plate) were plated and transfected with 50 nM non-targeting siRNA , siRNA targeting RASSF1 or siRNA targeting MST2. When cells had reached confluency a wound in the monolayer was made using a 200 µl pipette tip. Images were then taken at 0 hr and 24 hr at the same position in the well. The size of the wound was then measured and the distance the cells had migrated into the wound was determined. Cells transfected with siRASSF1, but not siMST2 resulted in a significant decrease in migration into the wound (siNT vs siRASSF1 $\rho = 9.2 \times 10^{-23}$, siRASSF1 vs siMST2 $\rho = 7.6 \times 10^{-24}$, siNT vs siMST2 $\rho = 0.16$). **A.** Graph of average distance moved. Asterisks represent significance. Error bars represent 1 x SEM. **B.** Western blot to show knockdown of RASSF1A and MST2 expression. **C.** Representative images of the wound at 0 hr and 24 hr. Black lines mark the edge of the wound. Results represent 16 wounds from 4 independent plates. Bar on images indicates 100 µm.

## 6.5 The Decrease in Motility Observed in Cells Depleted of RASSF1 is Due to Loss of RASSF1C Not RASSF1A

Having shown that loss of RASSF1 leads to a reduced rate of wound closure (Figure 6.1) in accordance with the loss of SRC family kinase activity (Figure 3.13) we wished to investigate the role of the RASSF1 isoforms in cell motility. We have shown that loss of RASSF1A does not affect SRC family kinase activity (Figure 4.3) and has previously been associated with increased motility (Dallol, Agathanggelou et al. 2005). Together these data suggest that RASSF1C is the isoform of RASSF1 that is responsible for driving motility through the activation of SRC family kinases. To confirm this hypothesis we designed two siRNA molecules that would specifically target the RASSF1C isoform of RASSF1, leaving RASSF1A expression unaffected. These siRNA molecules had no effect on motility in scratch wound assays (Figure 6.4A). We postulated that this is because the siRNA molecules did not effect RASSF1C expression. We confirmed that the two siRNA molecules targeting RASSF1C failed to knock down expression of endogenous or exogenous protein using RT-PCR (Figure 6.5A) and western blot analysis (Figure 6.5B, quantified in Figure 6.5C) respectively.

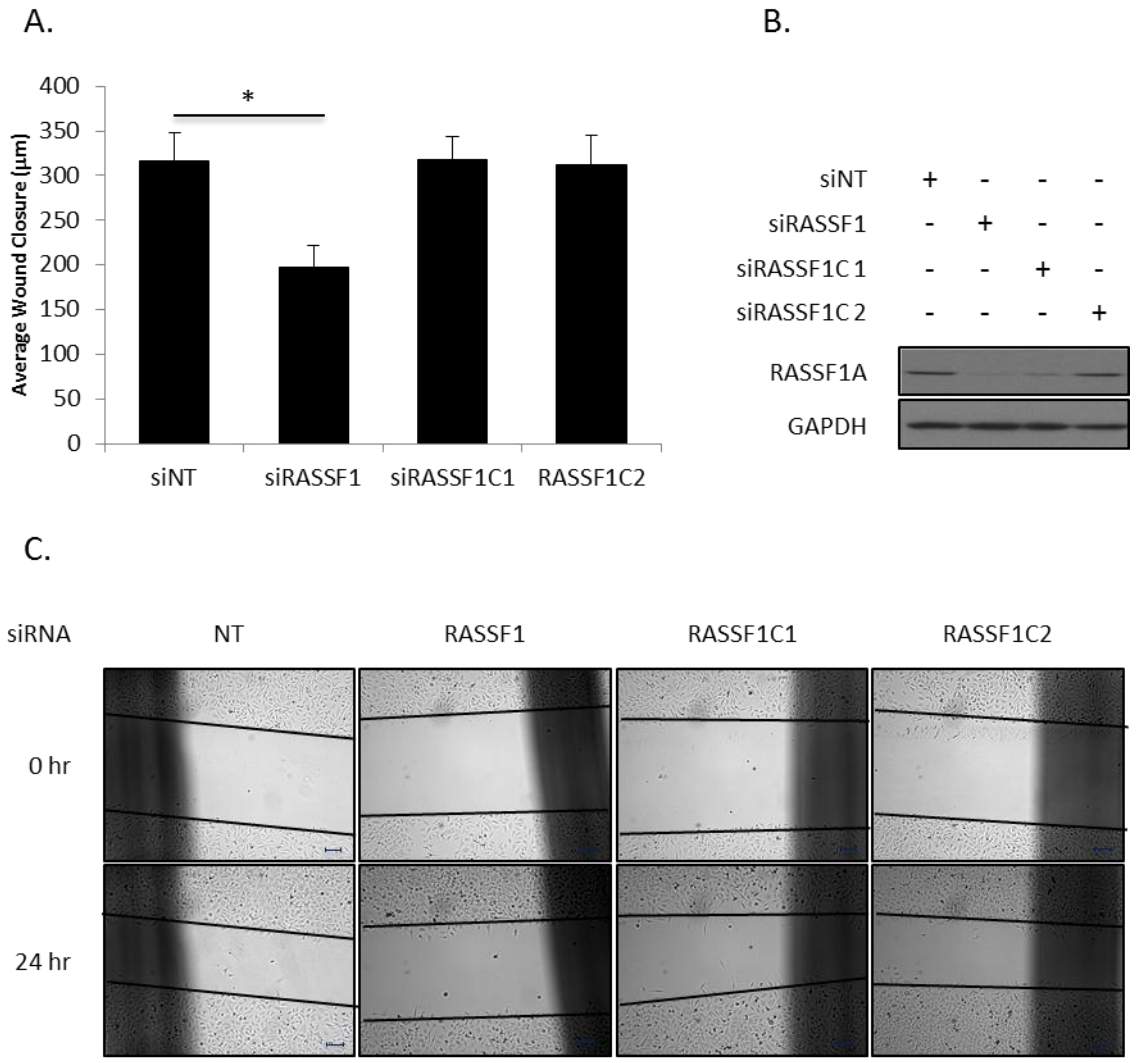


**Figure 6.4. The RASSF1C Isoform Specific siRNAs Do Not Affect Migration into the Wound.** HeLa cells (2 x $10^5$ cells/plates) were plated and transfected with non-targeting siRNA, siRNA targeting RASSF1 or isoform specific siRNA targeting RASSF1C (siRASSF1C_1 and siRASSF1C_2). When cells had reached confluency a wound in the monolayer was made using a 200 µl pipette tip. Images were then taken at 0 hr and 24 hr at the same position in the well. The size of the wound was then measured and the distance the cells had migrated into the wound was determined. Cells transfected with siRASSF1 show a decrease in migration into the wound, but cells transfected with RASSF1C specific siRNA did not affect migration into the wound (siNT vs siRASSF1 $\rho$ = 1.28 x $10^{-6}$, siNT vs siRASSF1C1 $\rho$ = 0.48, siNT vs siRASSF1C2 $\rho$ = 0.42, siRASSF1 vs siRASSF1C1 $\rho$ = 7.8 x $10^{-9}$, siRASSF1 vs siRASSF1C2 $\rho$ = 4.7 x $10^{-7}$). **A.** Graph of average distance moved. Error bars represent 1 x SEM. **B.** Western blot to show knockdown of RASSF1A expression. **C.** Representative images of the wound at 0 hr and 24 hr. Black lines mark the edge of the wound. Results represent 8 wounds from 2 independent plates. Bars on images indicates 100 µm.

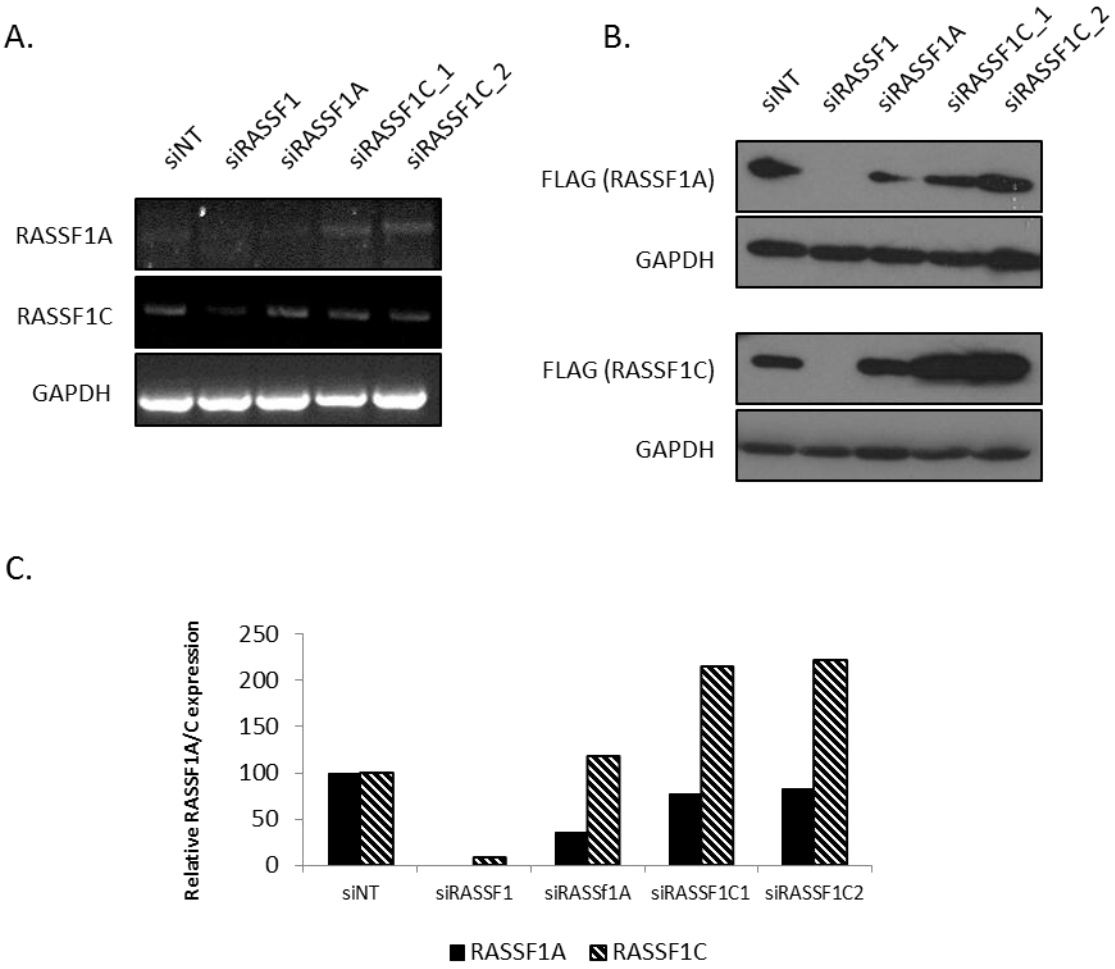


**Figure 6.5. The RASSF1A, but Not RASSF1C Isoform Specific siRNA is Successful in Knocking Down the Specific Isoform Targeted. A.** HeLa cells (2x $10^5$ cells/plate) were plated and transfected with non-targeting siRNA or siRNA targeting RASSF1, RASSF1A or RASSF1C (siRASSF1C1 and siRASSF1C2). RNA was extracted using Qiagen QIAshredder and RNeasy kit. mRNA transcript levels were analysed by RT-PCR using primers to RASSF1A, RASSF1C and GAPDH. Cells transfected with siRASSF1 show reduced RASSF1A and RASSF1C levels. Cells transfected with siRASSF1A only show reduced RASSF1A levels. Transfection of cells with siRASSF1C1 or siRASSF1C2 does not reduce the levels of RASSF1A or RASSF1C. **B.** HeLa cells (2 x $10^5$ cells/plate) were transfected with non-targeting siRNA or siRNA targeting siRASSF1, RASSF1A or RASSF1C (siRASSF1C1 and siRASSF1C2). Cells were subsequently transfected with FLAG-RASSF1A (top panel) or FLAG-RASSF1C (bottom panel). Cells were lysed with laemmli lysis buffer and analysed by SDS-PAGE and western blot with anti-FLAG antibody. RASSF1A levels are decreased by co-transfection with siRASSF1 or siRASSF1A. RASSF1C levels are only reduced by siRASSF1. **C.** Bar graph to show quantitation of western blots in **B.** Black bars are RASSF1A and striped bars are RASSF1C levels. Blots were quantitated using Image J. Relative expression is equalised to GAPDH. Data represents one independent experiment.

Following this, an alternative approach was taken to show that RASSF1C was responsible for the decrease in motility seen in cells transfected with siRNA targeting RASSF1. HeLa cells were first transfected with siRNA targeting all RASSF1 isoforms or one that specifically targets RASSF1A. Cells ablated of all RASSF1 isoforms led to a decrease in motility, but specific ablation of RASSF1A led to a significant increase in motility in agreement with Dallol et al (2005) (Dallol, Agathanggelou et al. 2005) (Figure 6.6). This data is in keeping with the idea that RASSF1A inhibits RASSF1C from activating SRC, because in cells ablated of RASSF1A, RASSF1C is able to activate SRC and therefore, promote motility.

We next generated siRNA resistant isoforms of RASSF1A and RASSF1C to test their ability to rescue the rate of motility in cells transfected with siRNA targeting RASSF1. Two siRNA resistant constructs for RASSF1A and for RASSF1C were produced, one against each of two siRNAs from the SMARTpool RASSF1 siRNA used thus far. These constructs allowed us to knockdown the expression of the endogenous RASSF1 (Figure 8.3), without affecting the expression of the exogenously expressed RASSF1A or RASSF1C. HeLa cells were transfected with non-targeting siRNA or one of the two siRNA molecules targeting RASSF1. RASSF1A and RASSF1C resistant constructs were then co-expressed and cells were grown to confluency before a wound was made. Both RASSF1 siRNA molecules decreased the rate of motility as expected. Expression of RASSF1C, but not RASSF1A, significantly rescued this decrease in motility (Figure 6.7, Figure 8.4 for example images)). Therefore, RASSF1C is responsible for the decrease in motility seen in cells transfected with siRNA targeting RASSF1. We next investigated whether exogenous expression of RASSF1C was able to promote motility, in accordance with the ability of RASSF1C expression to activate SRC (Figure 4.4 and 4.5).

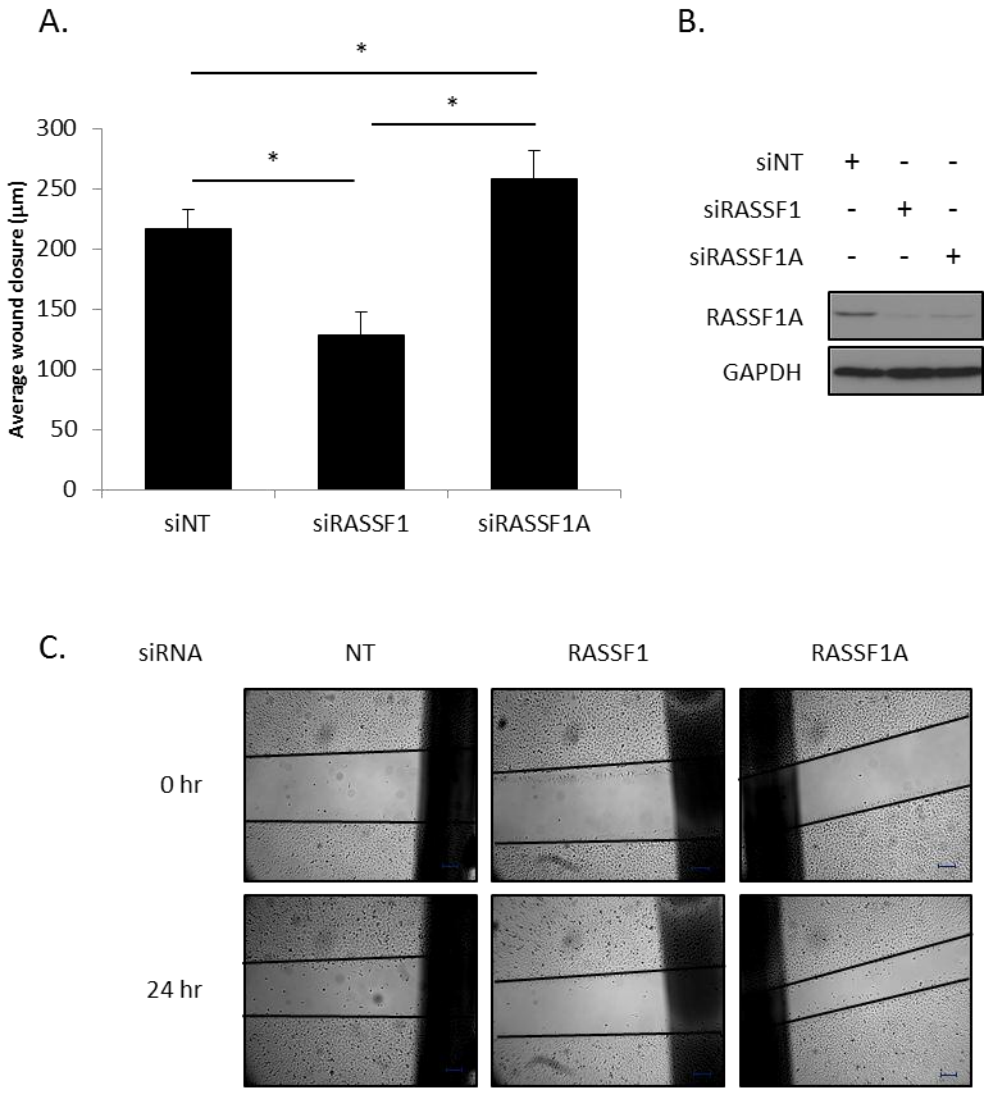


**Figure 6.6. Loss of RASSF1A Promotes Migration into the Scratch Wound.** HeLa cells (2 x $10^5$ cells/plate) were plated and transfected with either non-targeting siRNA or siRNA targeting RASSF1 or an isoform specific siRNA against RASSF1A. When cells had reached confluency a wound in the monolayer was made using a 200 µl pipette tip. Images were then taken at 0 hr and 24 hr at the same position in the well. The size of the wound was then measured and the distance the cells had migrated into the wound was determined. Cells ablated of RASSF1 again showed a decrease in motility. Loss of RASSF1A however, led to an increase in migration into the wound (siNT vs siRASSF1 $\rho = 1.8 \times 10^{-9}$, siNT vs siRASSF1A $\rho = 3.8 \times 10^{-4}$, siRASSF1 vs siRASSF1A $\rho = 1.2 \times 10^{-13}$). **A.** Graph of average distance moved. Asterisks represent significance. Error bars represent 1 x SEM. **B.** Western blot to show knockdown of RASSF1A expression. **C.** Representative images of the wound at 0 hr and 24 hr. Black lines mark the edge of the wound. Results represent 16 wounds from 4 independent plates. Bar on images indicates 100 µm.

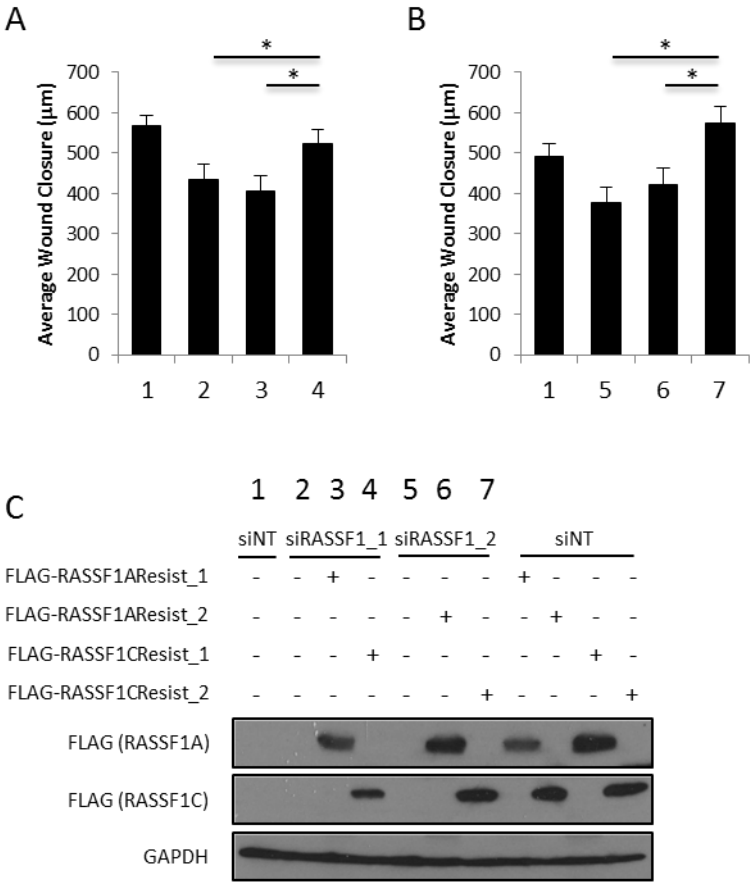


**Figure 6.7. Expression of siRNA Resistant RASSF1C, but Not RASSF1A Can Rescue Loss of Wound Healing from Ablation of RASSF1**. HeLa cells (2.5 x $10^5$ cells/plate) transfected with 50 nM non-targeting siRNA or RASSF1 siRNA (one of two individual siRNA molecules. RASSF1_1 (**A**) and RASSF1_2 (**B**). Cells were subsequently transfected with 1 µg empty vector or siRNA resistant constructs expressing RASSF1A or RASSF1C (FLAG-RASSF1AResist_1 and FLAG-RASF1CResist_1 (**A**) and FLAG-RASSF1AResist_2 and FLAG-RASSF1CResist_2 (**B**)). A wound in the monolayer was made using a 200 µl pipette tip and images were then taken at 0 hr and 24 hr at the same positions within the well. The size of the wound was then measured and the distance the cells had migrated into the wound was determined. Decreased wound healing is detected in cells transfected with either RASSF1 siRNA. This can be significantly rescued by expression of siRNA resistant RASSF1C constructs, but not siRNA resistant RASSF1A constructs (siRASSF1_1: siNT vs siRASSF1_1 $\rho = 5.7 \times 10^{-9}$, RASSF1C vs pcDNA3 $\rho = 6.2 \times 10^{-5}$, RASSF1C vs RASSF1A $\rho = 9.7 \times 10^{-10}$. siRASSF1_2: siNT vs siRASSF1_2 $\rho = 1.9 \times 10^{-6}$, RASSF1C vs pcDNA3 $\rho = 5.4 \times 10^{-12}$, RASSF1C vs RASSF1A $\rho = 1.2 \times 10^{-8}$). Graphs are representative of 16 wounds from 4 independent plates. Asterisk indicates $\rho$-value of less than 0.05. Error bars represent 1 x SEM. **C.** Cell lysates were generated using laemmli lysis buffer and analysed by SDS-PAGE and western blot for construct expression using anti-FLAG antibodies. GAPDH is a loading control. Western blot shows that construct expression is not affected by siRNA targeting RASSF1. Representative images of the scratch wound are in Figure 8.3.

## 6.6 RASSF1A Expression Can Inhibit RASSF1C-Dependent Motility

Having shown that loss of RASSF1C leads to reduced wound healing we decided to investigate whether expression of RASSF1C would promote motility. Expression of RASSF1C can activate SRC in cells that lack RASSF1A expression (Figure 4.5). We therefore reasoned that expression of RASSF1C in cells that lacked RASSF1A would be able to increase the rate of motility. To test this we used the MDA-MB-231 breast cancer cell line, which are highly motile and methylated for RASSF1A. Importantly, RASSF1C expression is able to activate SRC family kinases in this cell line (Figure 4.5). MDA-MB-231 cells were transfected with a pcDNA3, FLAG-RASSF1A, FLAG-RASSF1C or both constructs together. We found that expression of RASSF1C, but not RASSF1A, significantly increased the rate of motility when compared to control. In agreement with our previous data, cells co-expressing RASSF1A and RASSF1C migrated significantly less that cells expressing RASSF1C alone (Figure 6.8).

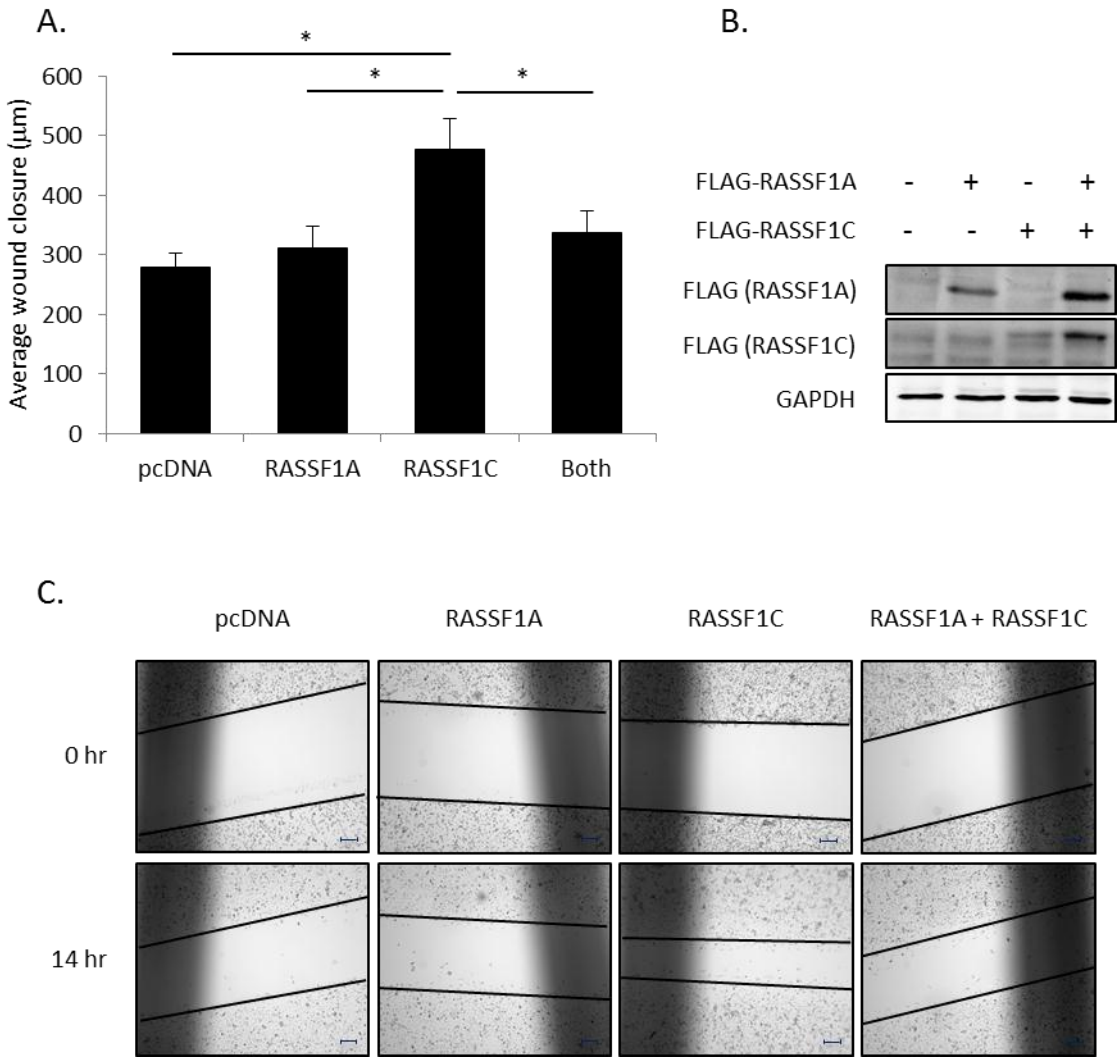


**Figure 6.8. Expression of RASSF1C Increases the Rate of Motility, but Can Be Reversed by RASSF1A Co-Expression.** MDA-MB-231 cells (2 x $10^5$ cells/plate) were plated and transfected with pcDNA3 (Control), FLAG-RASSF1A, FLAG-RASSF1C or FLAG-RASSF1A and FLAG-RASSF1C. When cells had reached confluency a wound in the monolayer was made using a 200 μl pipette tip. Images were then taken at 0 hr and 14 hr at the same position in the well. The size of the wound was then measured and the distance the cells had migrated into the wound was determined. Cells expressing RASSF1C, but not RASSF1A showed an increase in migration into the wound. Co-expression of RASSF1A with RASSF1C significantly decreased the rate of motility of RASSF1C expressing cells (pCDNA3 vs RASSF1C $\rho = 1.8 \times 10^{-7}$, RASSF1A vs RASSF1C $\rho = 9.0 \times 10^{-4}$, RASSF1C vs RASSF1A + RASSF1C $\rho = 3.3 \times 10^{-8}$). **A.** Graph of average distance moved. Asterisks represent significance. Error bars represent 1 x SEM. **B.** Western blot to show expression of FLAG-RASSF1A and FLAG-RASSF1C. **C.** Representative images of the wound at 0 hr and 14 hr. Black lines mark the edge of the wound. Results represent 16 wounds from 4 independent plates. Bar on images represents 100 μm

To confirm the results, a second 'transwell' assay was employed. In this assay, cells migrate through a membrane towards a 10 % serum containing media. We first performed this assay in MDA-MB-231 cells stably expressing RASSF1C (Figure 8.5 for clone selection and expression data). We saw that a greater number of the cells stably expressing RASSF1C migrated through the membrane than controls (Figure 6.9A). We next repeated the experiment in MDA-MB-231 transiently expressing RASSF1A, RASSF1C or both together. In agreement with the scratch wound assay, RASSF1C expression promoted cell migration towards the stimulus. Importantly, expression of FLAG-RASSF1A alone had no effect on motility, but significantly reduced the rate of motility in the cells co-transfected with FLAG-RASSF1C, again confirming the result of the scratch wound (Figure 6.9B).

Expression of RASSF1C was also shown to increase motility in H1299 Tet ON cells with inducible RASSF1C (Figure 8.6 for clone selection and expression data) using the exCELLigence analyser (Roche). The exCELLigence analyser measures migration towards a 10 % serum containing media stimulus by measuring the electrical impedance across a circuit. As cells migrate towards the stimulus they come into contact with the circuit. This creates a signal which can be detected by the analyser. Measurement of the change in the signal over time is used to determine the rate of migration. H1299 TET ON cells with empty vector or inducible RASSF1C were plated into the top chamber into media that either contained doxycycline to induce the RASSF1C expression or media that did not contain doxycycline. Cells were then allowed to migrate for 25 hours with measurements taken every 15 min. Addition of doxycycline to control cells had no effect on the rate of migration (Figure 6.9C, left panel). Addition of doxycycline to the cells containing the inducible RASSF1C, however, promoted increased migration towards the stimulus (Figure 6.9C, right panel). Taken together, these data show that RASSF1C is able to promote increased motility in a number of RASSF1A negative cell lines and that co-expression of RASSF1A with RASSF1C can decrease the motility of these cells by inhibiting the activation of SRC by RASSF1C.

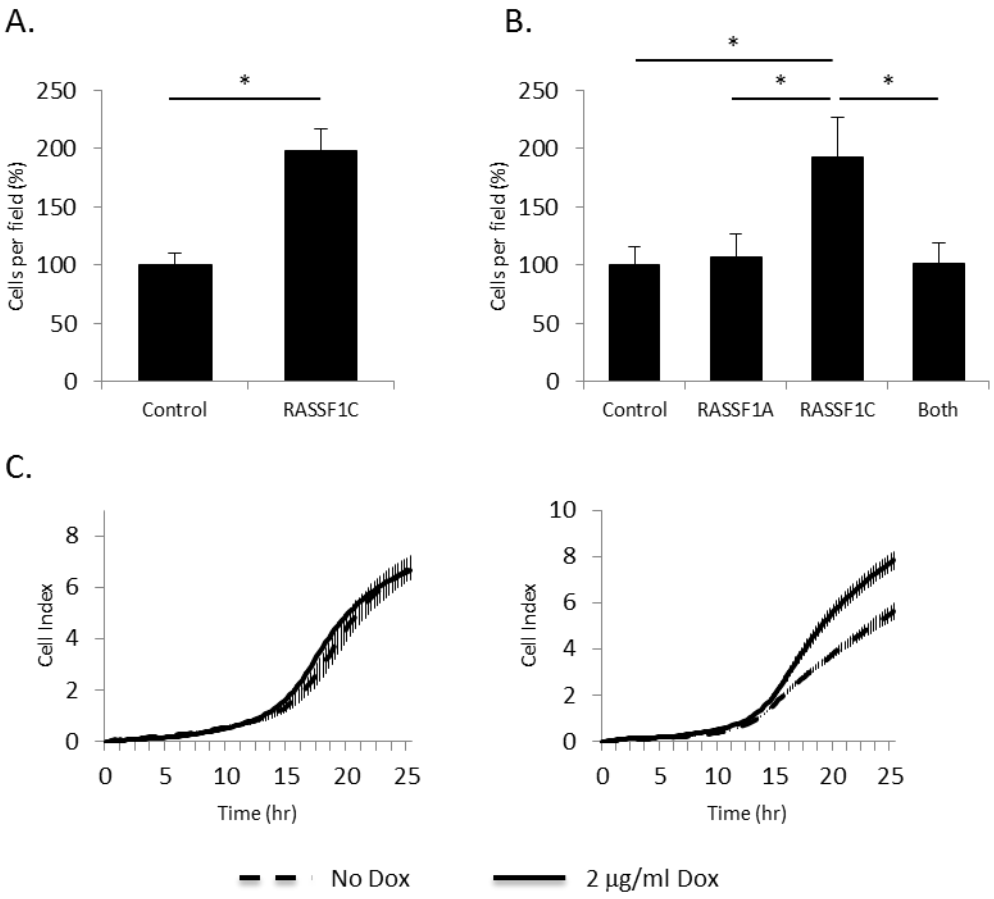


**Figure 6.9. RASSF1C Promotes Directional Motility in MDA-MB-231 and H1299 Cells. A.** Transwell motility assay using MDA-MB-231 cells stably expressing a control plasmid or MYC-RASSF1C. Cells (25,000 cells per plate) were plated into the top chamber in 0.1 % BSA containing DMEM and allowed to migrate towards a stimulus (10 % FBS) for 18 hrs. Significantly more RASSF1C expressing cells migrated through the membrane than control cells ($\rho = 5 \times 10^{-4}$). Data is representative of 3 independent plates. **B.** Transwell motility assay using MDA-MB-231 cells transiently transfected with pcDNA3, FLAG-RASSF1A, FLAG-RASSF1C or FLAG-RASSF1A and FLAG-RASSF1C. Experimental procedure the same as in **A.** In agreement with the scratch wound Figure 6.8, more RASSF1C expressing cells migrate through the membrane than control or RASSF1A expressing cells. Co-expression of RASSF1A led to a significant decrease in migration of the RASSF1C expressing cells (Control vs RASSF1C $\rho = 2.1 \times 10^{-6}$, RASSF1A vs RASSF1C $\rho = 1.2 \times 10^{-5}$, RASSF1C vs Both $\rho = 7.0 \times 10^{-7}$). Data is representative of 6 individual transwell wells from two independent experiments. Asterisks in **A** and **B** represent $\rho$-value of less than 0.05. Error bars represent 1 x SEM. **C.** Transwell migration assay using exCELLigence CIM plates. H1299 Tet ON inducible Control and RASSF1C cells (40,000 cells per plate) were plated into the top chamber of the plate in serum free DMEM either supplemented with 2 µg/ml doxycycline or without doxycycline and allowed to migrate towards a stimulus (10 % FBS). Measurements were made every 15 mins for 25 hrs. The migration of control cells was unaffected by the addition of doxycycline to the media (Left panel). The migration of RASSF1C inducible cells was significantly increased by the addition of doxycycline to the media (Right panel). Graphs are representative 9 wells from three independent experiments. Error bars represent 1 x SEM.

## 6.7 Expression of RASSF1C Increases Anchorage-Independent Growth, Proliferation Rate and Invasiveness of MDA-MB-231 Cells

In this thesis it has been shown that RASSF1C behaves as an oncogene that can activate SRC to promote cell-cell junction breakdown and cell motility. Following this, we wished to investigate the consequences of this on tumourigenesis. We hypothesised that cancer cells expressing RASSF1C would have a more aggressive phenotype. To test this, MDA-MB-231 cells expressing empty vector and transiently or stably expressing RASSF1C were plated onto matrigel and allowed to form mammospheres. Nine days after seeding the cells images were taken and scored for: number of mammospheres per field, presence of an aggressive phenotype and mammosphere size. In order to form a mammosphere, the cells must invade into the matrigel, and grow under anchorage-independent conditions. More aggressive cells will invade more and be less likely to undergo annoikis and therefore will seed more mammospheres. More aggressive cells will also proliferate faster and therefore the mammospheres will be larger. Cells stably expressing RASSF1C formed significantly more mammospheres (Figure 6.10D) and these mammospheres were significantly larger than controls (Figure 6.10F). Another measure of invasive capacity is to measure the amount of invasion from the mammospheres into the surrounding matrigel. Aggressive mammospheres will form star shaped colonies rather than a more spherical shape formed by less aggressive cells (Figure 6.10B). Mammospheres with greater than 5 projections were considered to have an aggressive phenotype. A significantly higher proportion of the mammospheres formed from cells stably expressing RASSF1C had an aggressive phenotype compared to control (Figure 6.10E). Transient expression of RASSF1C also led to a greater number of mammospheres being seeded and these were again more aggressive than the controls. However, although cells transiently expressing RASSF1C they were less aggressive than stable expressing clones, they were significantly more invasive than controls (Figure 8.7). The mammospheres seeded from the cells transiently expressing RASSF1C were not larger than the controls (Figure 8.7), suggesting that transient or stable expression of RASSF1C inhibits annoikis and promotes invasion. It was also observed that in five of the six wells containing the

RASSF1C stably expressing cells, at least one cell had invaded completely through the matrigel and formed a colony on the bottom of the dish (Figure 6.10C). This was not the case for any of the other conditions, further suggesting that RASSF1C expressing cells are more invasive during the time frame of the experiment.

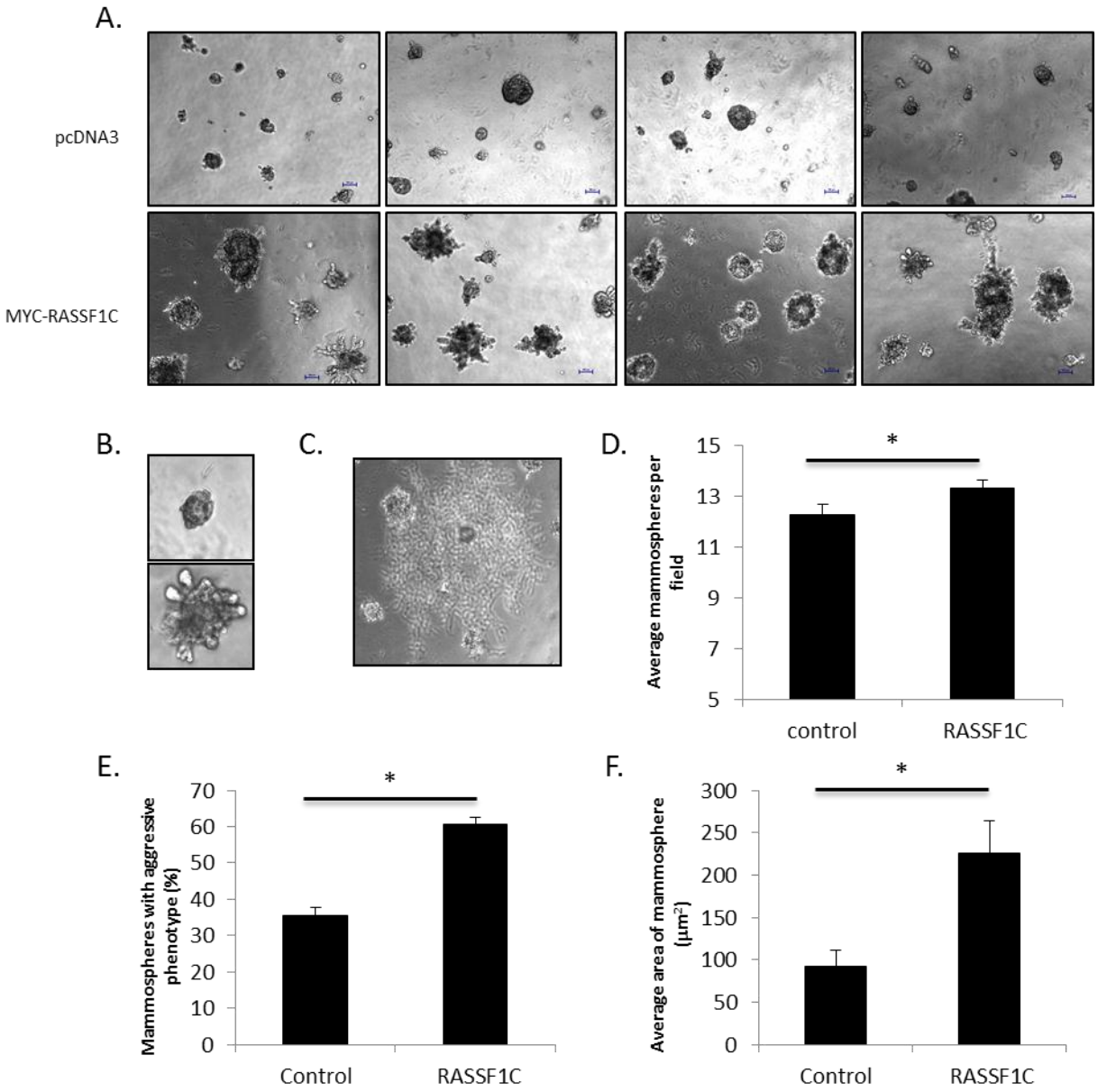


**Figure 6.10. RASSF1C Expression Drives a More Aggressive Phenotype.** MDA-MB-231 control and stably expressing RASSF1C were seeded onto matrigel and cultured for 9 days, changing media every 3 days to allow mammospheres to form before images were taken at 4 x magnification using a Nikon TE2000 Eclipse microscope. **A.** Example pictures of mammospheres of control cells (top panel) and RASSF1C cells (bottom panel). **B.** Example pictures of non-aggressive (top picture) and aggressive (bottom picture) mammospheres. **C.** Five of the 6 wells containing cells stably expressing RASSF1C invaded through the matrigel to form a colony underneath. None of the control wells invaded through the matrigel. **D.** A graph to show the average number of mammospheres counted per field. More mammospheres grew in the RASSF1C expressing cells ($\rho = 0.035$). **E.** Mammospheres were graded as aggressive if they had greater than or equal to five projections from the main mammosphere body into the surrounding matrigel. RASSF1C expressing cells formed significantly more aggressive mammospheres than the control cells ($\rho = 1.7 \times 10^{-13}$). Graph shows the percent of mammospheres with aggressive phenotype. **F.** Mammospheres from cells expressing RASSF1C grew significantly larger than control cells ($\rho = 0.001$). Graph depicts the average area of mammosphere. **D and E.** Mammospheres were counted from 60 images taken from 6 independent experiments. $\rho$-values were generated using a student's T-test. **F.** Five mammospheres were chosen at random in each of the 60 images and the area was measured using image J. $\rho$-value was generated using a student's T-test. Asterisk indicates a $\rho$-value of less than 0.05. Error bars represent 1 x SEM.

## 6.8 Discussion

In this chapter I have demonstrated that depletion of RASSF1 expression leads to decreased cell migration associated with reduced ability to polarise. RASSF1C was shown to promote motility in agreement with the SRC activation data presented in Chapter 4, in a manner that is negatively regulated by RASSF1A. Finally, RASSF1C expression was shown to lead to faster growing, more invasive and aggressive mammospheres in MDA-MB-231 cells.

As well as disruption of cell-cell junctions, EMT is characterised by increased motility. Activation of SRC is known to promote cell motility by activating a number of signalling pathways that lead to the activation of the Rho family of proteins, which promote the reorganisation of the cytoskeleton. One of the downstream proteins known to be activated by SRC is PI3K, which can activate Rac1 promoting both migration and invasion (Cantley 2002; Yip, El-Sibai et al. 2007). RASSF1A has previously been shown to decrease cell motility by inhibiting PI3K and as a consequence, reducing Rac1 activity (Dallol, Agathanggelou et al. 2005). However, a direct link between PI3K and RASSF1A was not established in the paper. Data presented in this chapter would hypothesise that loss of RASSF1A would lead to RASSF1C-mediated activation of PI3K by SRC. This could occur by recruitment of PI3K to phosphorylated FAK (Chen, Appeddu et al. 1996; Guarino 2010), or directly either by an interaction between the SH3 domain of SRC and PI3-Kinase or by SRC-mediated phosphorylation of PI3K (Fincham, Brunton et al. 2000; Cuevas, Lu et al. 2001). RASSF1A would inhibit this by restricting RASSF1C activation of SRC.

To test this hypothesis we first investigated whether loss of RASSF1 had an effect on cell motility in a scratch wound assay. We showed that loss of RASSF1, which led to complete ablation of SRC activity (Figure 3.13), resulted in decreased motility into a scratch wound (Figure 6.1). Loss of OSSA had no effect on motility, fitting well with the limited decrease in SRC activity observed (Figure 3.13).

Migrating cells, like epithelial cells, are polarised, rearranging their MTOC and Golgi such that they are between the nucleus and the leading edge of the cell. In wound healing assays, cells that fail to polarise

towards the wound edge appear to move less due to an inability to recognise the wound. In Chapter 4 we observed that expression of RASSF1C increased translocation of SRC to the plasma membrane, a process dependent on vesicular trafficking and in Chapter 5 we observed the re-localisation of junctional components from the plasma membrane to vesicular structures in RASSF1C expressing cells. From this we hypothesised that RASSF1C may affect vesicular trafficking. Polarisation of migrating cells also requires vesicular trafficking with both Arf6 and ESCRT proteins implicated in this process (Lobert and Stenmark 2012). We decided therefore to investigate whether cells ablated for RASSF1 were able to polarise towards the wound edge. We found that loss of RASSF1 expression led to a significant decrease in the ability of cells to polarise towards the wound edge.

One question arising from this data is, why does both loss and gain of RASSF1C lead to a loss of cell polarity? If RASSF1C were to increase endosomal trafficking this could enhance the translocation of SRC to the membrane where it would become activated. SRC activity would lead to an increase in E-cadherin turnover, potentially through the activation of Arf6, which would promote increased internalisation and recycling of E-cadherin and loss of polarity (Chapter 5). Also, if RASSF1C promotes vesicular trafficking, loss of RASSF1C would decrease vesicular trafficking. In agreement with this hypothesis, cells that have lost ESCRT proteins, which regulate vesicular trafficking, fail to translocate SRC to the membrane and fail to polarise towards a wound (Lobert and Stenmark 2012). Loss of RASSF1C therefore, would prevent SRC from translocating to the plasma membrane and fail to orientate the golgi, resulting in decreased polarisation.

The ESCRT machinery has been shown to be required for SRC trafficking to focal adhesions, maintenance of apical polarity and polarisation of the golgi towards the leading edge of a migrating cells (Tu, Ortega-Cava et al. 2010; Dukes, Fish et al. 2011; Lobert and Stenmark 2012). These overlapping roles for RASSF1C and ESCRT proteins may provide another link between RASSF1C and vesicular trafficking. However, loss of ESCRT protein has little effect on ZO-1 and E-cadherin, two proteins that we have seen to be affected by RASSF1C expression (Dukes, Fish et al. 2011) (Figure 5.3, Figure 5.5). Therefore, at least

part of the role that RASSF1C plays in vesicular trafficking is likely to be ESCRT independent. One protein that has been shown to regulate adherens junctions, tight junctions and cell motility in a SRC-dependent manner is Arf6. It would be interesting to see if RASSF1C affects Arf6 function or whether the internalised junctional components seen in Chapter 5 co-localise with Arf6 by immunofluorescence.

Following the confirmation that loss of RASSF1 expression led to a decrease in motility we wished to determine whether the hippo pathway contributed to this regulation. Results showed that loss of MST2 had no effect on wound healing, further suggesting that the migration was driven through activation of SRC by RASSF1C. We also confirmed the isoform specific nature of the role of RASSF1C by demonstrating that specific loss of RASSF1A led to an increase in motility (Figure 6.6), whereas only expression of siRNA resistant RASSF1C rescued motility upon ablation of RASSF1 expression (Figure 6.7).

After demonstrating that re-expression of RASSF1C was sufficient to rescue motility associated with loss of RASSF1, we investigated whether exogenous expression of RASSF1C was able to promote motility in MDA-MB-231 cells. Expression of RASSF1C, but not RASSF1A, was able to enhance motility in MDA-MB-231 cells (Figure 6.8 and Figure 6.9B) and again this is negatively regulated by RASSF1A co-expression. We also confirmed that RASSF1C could promote directional migration in H1299 Tet ON cells using the ExCELLigence analyser (Figure 6.9C). It would be interesting to determine whether re-expression of RASSF1A using demethyltransferase inhibitors such as Zebularine or the 5-aza-2'-deoxycytidine was also able to inhibit RASSF1C-induced SRC activation and promotion of motility, as this class of agents, particularly 5-aza-2'-deoxycytidine, are currently under investigation as therapeutic agents in clinical trials.

SRC promotes motility through the activation of Rac1 leading to lamellipodial formation at the front of the cell (Huveneers and Danen 2009). With regard to Rac1 activity, one interesting hit from the mass spectrometry screen is Filamin A. Filamin A is a scaffold that has been shown to bind to FilGAP, a Rac1 GTP-activating protein, in a phosphorylation dependent manner. ROCK activation by RhoA promotes the phosphorylation of FilGAP. FilGAP is then translocated to the site of membrane protrusion where it

interacts with Filamin A promoting the inactivation of Rac1, and decreasing motility (Ohta, Hartwig et al. 2006). An interaction between RASSF1A and Filamin A may provide an additional level of cell motility regulation. Potentially, RASSF1A may be recruited to the leading edge of a motile cell by Filamin A where it could interact with RASSF1C and inactivate SRC.

Dallol et al (2005) proposed that expression of RASSF1A inhibited PI3K activity and therefore reduced Rac1 activation (Dallol, Agathanggelou et al. 2005). We were unable to detect activation of Rac1 using a PAK domain-binding assay in cells expressing RASSF1C (data not shown). However, we also failed to see Rac1 stimulation by EGF in this assay, suggesting that optimisation of the protocol may have yielded a different result.

As well as activation of Rac at the front of the cell, SRC activity also leads to Rho activation, promoting actomyosin contraction at the back of the cell (Huveneers and Danen 2009). Many of the pathways that stimulate Rac and Rho activity rely on signalling through FAK and $\beta_1$-integrins (Gimond, van Der Flier et al. 1999; Avizienyte, Wyke et al. 2002). One of these pathways acts through the ERK1/2 (Avizienyte, Fincham et al. 2004). Integrin/FAK activation of MEK leads to the activation of ERK1/2. ERK1/2 is able to activate MLCK leading to the peripheral accumulation of phospho-MLC. MLCK activity can promote extension of cellular protrusions (Brahmbhatt and Klemke 2003), regulate the dynamics of focal complexes at the tips of protrusions (Webb, Donais et al. 2004) and promote efficient spreading of lamellipodia (Giannone, Dubin-Thaler et al. 2004). MLC is associated with Actin stress fibres, the formation of which is controlled by RhoA and its effector kinase ROCK (Maekawa, Ishizaki et al. 1999). RASSF1C expression has previously been shown to activate ERK (Reeves, Baldwin et al. 2010) and loss of RASSF1A has been associated with increased Actin stress fibre formation (Dallol, Agathanggelou et al. 2005). Therefore, it would be interesting to see whether RASSF1C expression effects ROCK activity and MLC phosphorylation.

Metastasis is thought to occur through a set of discrete steps often termed the invasion-metastasis cascade (Fidler 2003; Talmadge and Fidler 2010). A prerequisite for the development of distant

metastatic sites is the invasion of cells through matrix barriers, for example basement membranes (Guarino 2010). There are two types of invasion; mesenchymal-type, and amoeboid-type (Price, Hajdo-Milasinovic et al. 2004; Carragher, Walker et al. 2006).

The activation of Rac1 at the leading edge of the cell is crucial to the mesenchymal-type invasion. SRC acting through FAK, CAS or Paxillin is able to activate Rac1, which remodels the cytoskeleton and activates JNK signalling. JNK activation leads to the upregulation of matrix metalloproteases (MMPs) 2 and 9 which are responsible for the breakdown of the ECM at the leading edge (Hsia, Mitra et al. 2003; Van Slambrouck, Jenkins et al. 2009). The breakdown of the ECM creates a space into which the cell can move. SRC could also potentially promote amoeboid-type invasion through the activation of Rho. This could occur as a consequence of losing cell-cell junctions. DDR1 localises to E-cadherin and recruits Par3 and Par6 to these sites. The Par proteins activate p190RhoGAP, which together with RhoE inhibit Rho-dependent actomyosin contractility and promote collective cell migration (Hidalgo-Carcedo, Hooper et al. 2011). If E-cadherin is lost due to SRC activity then Rho activity will be able to rise potentially promoting amoeboid invasion.

As invasion and migration are intricately linked through SRC, we decided to investigate at whether RASSF1C could promote invasion and tumourigenesis. Mammosphere growth in matrigel was used to investigate this. This assay gives information regarding anchorage-independent growth, proliferation rate as well as invasive potential and tumour aggressiveness. We were able to see that stable expression of RASSF1C was able to drive all of the aspects of the mammosphere growth such that there were significantly more colonies; these colonies were larger and had significantly more invasive projections into the surrounding matrigel suggesting that constitutive RASSF1C expression drives an aggressive tumour phenotype (Figure 6.10).

Taken together these data indicate that RASSF1C expression drives SRC activity, migration and invasion, all the hallmarks of EMT. The directed migration and invasion are probably as a result of Rac1 and

CDC42 activation, which are key to cell polarisation for directed migration and invasion through the upregulation of MMPs.

Finally, conclusive proof of the oncogenic potential of RASSF1C can be investigated using mouse models. One convenient model relies on the fact that MDA-MB-231 cells preferentially develop osteolytic bone metastases (Myoui, Nishimura et al. 2003). The prediction from the data presented in this thesis is that RASSF1C expressing cells would be more metastatic as they are more invasive, motile and resistant to annoikis as shown by the mammosphere experiments.

The data presented in this chapter (Summarised in Figure 6.11) shows that loss of RASSF1 leads to a decrease in motility and failure to polarise the golgi towards a scratch wound. RASSF1C activation of SRC promotes cell motility in both scratch wound and transwell assays in cells that are RASSF1A negative. Expression of RASSF1A with RASSF1C can inhibit RASSF1C-dependent cell motility, in agreement with the novel tumour suppressor role of RASSF1A inhibiting RASSF1C (Chapter 4). The motility is probably regulated through the loss of cell-cell junctions (Chapter 5) and activation of the small GTPases Rho and Rac. Finally, we show that RASSF1C expression drives tumourigenesis by the formation of mammospheres with an invasive, aggressive phenotype. Together this data suggests that cancers that have lost RASSF1A, where RASSF1C promotes SRC activation, are more likely to be invasive and metastasise (Fendri, Masmoudi et al. 2009; Tommasi, Pinto et al. 2011).

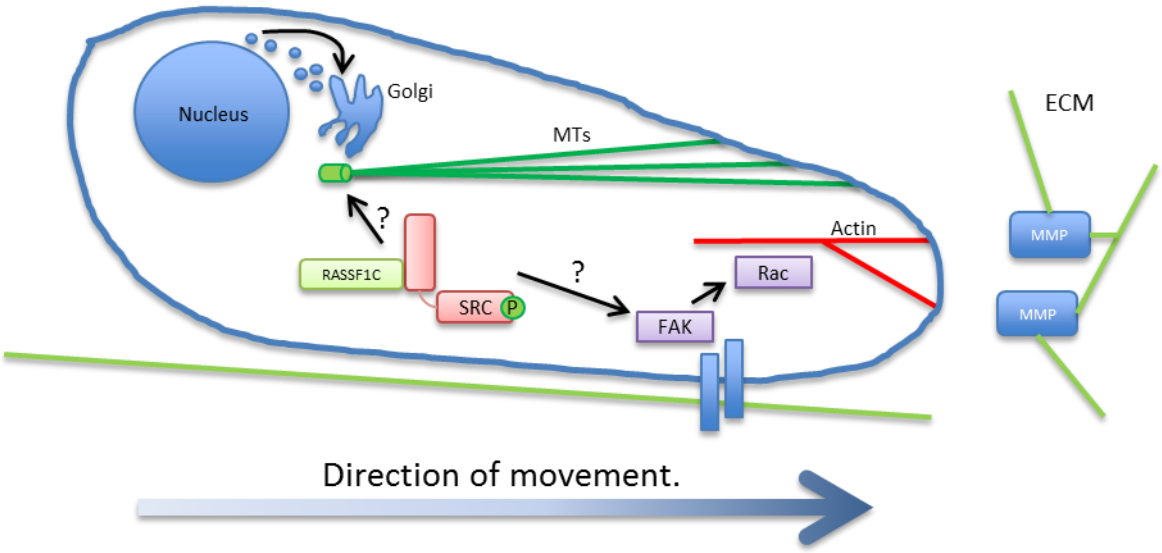


**Figure 6.11. Cartoon Depicting Model Proposed from Data in Chapter 6.** RASSF1C activation of SRC promotes cell polarisation and motility through re-orientation of the golgi and MTOC. This occurs through actin re-organisation, possibly through activation of Rac in a FAK- and Integrin b1-dependent manner. Rac activation also leads to the secretion of Matrix metalloproteases (MMP) which breakdown the surrounding ECM promoting invasion. Golgi and microtubule re-organisation occurs by an unknown mechanism, but is thought to involve endosomal trafficking and the Rho protein CDC42.

# Chapter 7 Discussion

The data presented in this thesis describes a novel regulation of SRC family kinases by RASSF1 isoforms. We have shown that RASSF1C behaves as an oncogene and that RASSF1C expression is essential for SRC activation in response to growth factors and additional stimuli. Moreover, the expression of RASSF1C (in RASSF1A negative cells) can activate SRC in the absence of extrinsic signals. RASSF1C expression promotes SRC translocation to the plasma membrane where its activity is enhanced and it can engage with and phosphorylate target proteins such as the known substrates E-cadherin and β-catenin. Further investigation has shown that RASSF1C drives the internalisation of both adherens and tight junction components and consequently, cell polarity markers are lost from these sites. Furthermore, β-catenin translocates to the cytoplasm from where it is hypothesised to be able to enter the nucleus and upregulate genes associated with EMT (Brabletz, Hlubek et al. 2005). RASSF1C has previously been implicated in promoting the internalisation of β-catenin by inhibiting the negative regulator β-TRCP (Estrabaud, Lassot et al. 2007); however, we provide evidence for a novel mechanism through which β-catenin is released from cell-cell junctions from where it can enter the nucleus. RASSF1C expression can also promote migration, shown using both scratch wound and transwell assays. Finally, breast cancer cells expressing RASSF1C appear to be more invasive, resistant to annoikis and display a more aggressive phenotype, forming large invasive mammospheres in matrigel. Each of these SRC driven activities of RASSF1C have been shown to be inhibited through a novel, SARAH domain-independent, interaction between RASSF1C and the tumour suppressor RASSF1A. This interaction not only sequesters RASSF1C away from SRC family kinases, but also brings the SRC inhibitory kinase, CSK to a RASSF1A-RASSF1C complex where we propose it is able to phosphorylate and inactivate SRC family kinases. Taken together this data suggests why RASSF1C expression is retained in tumours while RASSF1A expression is lost. It may also suggest why RASSF1A activity is preferentially downregulated by promoter methylation and not mutation, which given the sequence similarity between RASSF1A and RASSF1C would potentially inactivate RASSF1C as well.

Transcriptional silencing of RASSF1A by inappropriate promoter methylation has been extensively studied and has been found in a vast number of human cancers including: lung, breast, colorectal, gastric and cervical amongst others (Donninger, Vos et al. 2007). Recent studies have shown that RASSF1A promoter methylation is associated with tumour stage in non-muscle invasive bladder cancer (Kim, Chae et al. 2012), lymph node metastasis in nasopharyngeal carcinoma (Fendri, Masmoudi et al. 2009), and distant metastasis to the liver in colorectal cancers (Tommasi, Pinto et al. 2011). Meta-analysis of eight breast cancer studies involving 1795 patients and 19 non-small cell lung carcinoma studies involving 2802 patients further emphasises the importance of RASSF1A promoter methylation as a prognostic marker showing significant correlation between RASSF1A methylation and both overall survival and relapse-free survival (Wang, Wang et al. 2011; Jiang, Cui et al. 2012). Clearly, RASSF1A promoter methylation is associated with more aggressive, metastatic cancers. The data presented in this thesis would predict that this is due to the activation of SRC by RASSF1C. We suggest that one of the major tumour suppressor roles for RASSF1A is to modulate the activity of SRC family kinases through an interaction with RASSF1C. RASSF1A prevents RASSF1C-dependent translocation of SRC family kinases to the plasma membrane. In cancers that have lost RASSF1A, RASSF1C is able to activate SRC family kinases promoting EMT through the destruction of cell-cell junctions and cell polarity, and by promoting migration and invasion (Frame 2004).

In some cancers promoter methylation of the RASSF1 gene extends beyond the upstream CpG island containing the 1$\alpha$ promoter (RASSF1A) persisting into the second downstream CpG island containing the 2$\gamma$ promoter from with RASSF1C is transcribed (Figure 1.1). In these cases all isoforms of the RASSF1 gene are silenced (Vos, Ellis et al. 2000; Dreijerink, Braga et al. 2001). It would be interesting, given the oncogenic role for RASSF1C described here, to determine the metastatic potential of these tumours. It would also be interesting to see whether the prognostic power of RASSF1A promoter methylation could be improved by measuring promoter methylation across both promoters. We would predict that patients with DNA methylation only at the 1$\alpha$ promoter, which retain RASSF1C expression, would have a

worse prognosis than those who are methylated at both the 1α and 2γ promoters, where both RASSF1A and RASSF1C are lost.

SRC is often overexpressed or activated in tumours, including those derived from breast, lung and colorectal cancers (Cartwright, Kamps et al. 1989; Mazurenko, Kogan et al. 1992; Verbeek, Vroom et al. 1996), cancers also heavily methylated for RASSF1A. SRC has only been found to be mutated in a subset of advanced metastatic colon cancers (Irby, Mao et al. 1999). An activating truncation mutation at codon 531 in the C-terminal regulatory domain prevents SRC regulation by CSK resulting in a constitutively active SRC protein (Irby, Mao et al. 1999). RASSF1A has already been shown to be important in promoting senescence and apoptosis in response to oncogenic K-Ras signalling (Thaler, Hahnel et al. 2009; Matallanas, Romano et al. 2011). However, RASSF1A methylation correlates poorly with *KRAS* or *BRAF* mutations. Instead MST2 downregulation through promoter methylation is favoured in these cells (Matallanas, Romano et al. 2011). Given the novel link between RASSF1 and SRC activation it would be interesting to see whether RASSF1A methylation correlated firstly with SRC activity and secondly with activating mutations of SRC. We would predict that increasing levels of RASSF1A methylation would positively correlate with increasing SRC activity, because we determined that RASSF1C expression was only able to activate SRC in the absence of RASSF1A. We might expect from this that SRC inhibitors, such as dasatinib, bosutinib and saracatinib, maybe an effective treatment in cells in which RASSF1A has been silenced. We might also expect that *SRC* mutations, like *KRAS* mutations would not correlate with RASSF1A methylation because mutation of SRC would negate the requirement for SRC activation by RASSF1C and therefore decrease the selection pressure on cells to downregulate RASSF1A.

Unlike promoter methylation, mutation of RASSF1A is rarely observed in tumours. However, a number of mutations have been identified that impair RASSF1A functionality (van der Weyden and Adams 2007). One of the most notable sequence alterations observed is a germline SNP with minor allele frequency located close to the ATM phosphorylation site (S131). This SNP, which converts an alanine to a serine at

codon 133 (A133S), has been associated with an increased risk in breast cancer (Schagdarsurengin, Seidel et al. 2005), and lung cancer (Kanzaki, Hanafusa et al. 2006; Xiao, Zhang et al. 2009). It has also been associated with early onset of breast cancer in BRCA1/2 mutation carriers (Gao, Xie et al. 2008) and in males with soft tissue sarcoma (Yee, Grochola et al. 2012). The A133S polymorphism was shown to inactivate RASSF1A preventing it from activating p53 and p73 (Yee, Grochola et al. 2012). Fitting with this, soft tissue sarcoma patients with the A133S polymorphism have a worse prognosis, as do patients with RASSF1A promoter methylation (Seidel, Bartel et al. 2005; Yee, Grochola et al. 2012). Our model would predict that the inactivation of RASSF1A by the A133S polymorphism would prevent RASSF1A from inhibiting RASSF1C. Indeed, in Figure 4.9 we see that the interaction between RASSF1A A133S and RASSF1C was inhibited. Secondary structure prediction using "scratch protein predictor" analysis by Yee et al (2012) suggested that the A133S polymorphism disrupted an alpha helical structure in the region which we found to be important for the RASSF1A-RASSF1C interaction (Figure 4.9) (Yee, Grochola et al. 2012). However, given that the polymorphism would also be present in RASSF1C, occurring at codon 63 (A63S), it may inactivate RASSF1C as well. Indeed preliminary evidence (Figure 4.10) indicates that RASSF1C A63S is unable to activate SRC in H1299 cells and fails to promote the initial stages of EMT, namely loss of cell junctions and cell polarity (data not shown). It would be interesting to investigate how the presence of the polymorphism would affect prognosis. We might expect that primary tumours with the polymorphism would be refractory to treatment due to the inactivation of RASSF1A, which activates apoptotic pathways in response to cellular stress, but are less likely to metastasise because RASSF1C is unable to activate SRC.

In keeping with recent reports, our data shows that RASSF1C is an oncogene (Amaar, Minera et al. 2006; Reeves, Baldwin et al. 2010). We also show that RASSF1A inhibits RASSF1C mediated activation of SRC. In agreement with this work, both homozygous and heterozygous Rassf1A knockout mice show increased incidence of spontaneous tumourigenesis and decreased survival rate compared to wildtype mice (Tommasi, Dammann et al. 2005; van der Weyden, Tachibana et al. 2005). Rassf1A null mice also show a more severe tumour susceptibility phenotype when exposed to physical (irradiation) or chemical

(benzo[a]pyrene and urethane) mutagens (Tommasi, Dammann et al. 2005; van der Weyden, Tachibana et al. 2005). However, heterozygous mice with a minimal deletion region encompassing the 12 genes located at Chromosome 3p21.3 (the Rassf1 locus) show no increase in spontaneous tumourigenesis (Smith, Xian et al. 2002). Homozygous mice with this deletion were embryonic lethal. This suggests that one of the proteins produced from the 12 genes lost in this mouse promotes tumourigenesis in the absence of Rassf1A. Our work suggests that this may potentially be due to Rassf1C expression.

Rassf1A knockout mice had significantly more tumours than the wildtype mice, however, only 13 tumours were found in the 41 Rassf1A null mice used in the study. This suggests that loss of Rassf1A, alone is not enough to promote tumourigenesis. RASSF1C is an unstable protein and is readily degraded by the E3 ubiquitin ligase Mule under normal conditions and is further degraded by $SCF^{\beta\text{-}TRCP}$ in response to UV radiation (Zhou, Li et al. 2011). We have observed that RASSF1A is able to bind to RASSF1C and therefore could sequester it and target it for degradation. In support of this idea, we identified Mule in our screen of RASSF1A binding proteins. Although we did not confirm Mule binding to RASSF1A in independent experiments we might suggest a model whereby RASSF1A binds Mule and RASSF1C, targeting RASSF1C for ubiquitin-mediated degradation. This method of regulation is similar to regulation of another important oncogene, β-catenin by the APC-containing complex. The APC-containing complex also sequesters β-catenin and targets it for ubiquitin-mediated degradation (Clevers and Nusse 2012). One question that this data raises is what is the signal to release RASSF1C that would allow it to accumulate and activate SRC family kinases in non-transformed cells? One trigger could be the downregulation of RASSF1A, which occurs in cancers. RASSF1A is also degraded at various stages of the cells cycle and so it would be interesting to investigate whether the levels of RASSF1C increase at these times (Song, Song et al. 2008; Song, Song et al. 2009; Jiang, Rong et al. 2011; Chow, Wong et al. 2012). Other triggers that would warrant investigation are the role of novel E3 ubiquitin ligases and post translational modifications, such as phosphorylation of RASSF1A and RASSF1C.

We have identified a role for RASSF1C in promoting SRC translocation to the membrane. However, we do not know how RASSF1C does this or whether RASSF1C is also translocated to the membrane. The RASSF family of proteins are defined by the presence of an RA domain. RASSF1A and RASSF5A have been shown to interact with K-Ras-GTP to activate proapoptotic signals through MST1/2 (Khokhlatchev, Rabizadeh et al. 2002; Ortiz-Vega, Khokhlatchev et al. 2002). The RA domain of RASSF1C is identical to that of RASSF1A and has also previously been shown to interact with K-Ras to promote apoptosis (Vos, Ellis et al. 2000). Ras is activated by growth factor receptors, for example EGFR. It is therefore plausible that in non-transformed cells RASSF1C interacts with receptor stimulated Ras-GTP leading to RASSF1C stabilisation and activation. Stabilised RASSF1C would then able to promote the translocation of SRC to the membrane where it is activated and able to phosphorylate target proteins. When RASSF1A is expressed this signal can be turned off by RASSF1A recruitment to RASSF1C or to Ras. RASSF1A could also promote the inactivation of SRC family kinases through recruitment of CSK.

Given that RASSF1C binds to SRC family kinases, RASSF1C may activate SRC in a manner similar to that proposed for OSSA; binding to SRC and causing a conformational change. This could be tested using an *in vitro* kinase assay for SRC activity. If RASSF1C bound directly to SRC, changing its conformation and activating it, then we would expect to see SRC activity increase proportionally with RASSF1C concentration. Alternatively, RASSF1C may recruit co-factors, such as OSSA, FAK or CAS to SRC to promote its activation. We identified OSSA in our screen for RASSF1A binding proteins; however, we were unable to confirm a role for OSSA in RASSF1C-dependent activation of SRC.

In Chapter 5 it was shown that RASSF1C expression promoted SRC phosphorylation of target genes including E-cadherin and β-catenin. Activation of SRC is known to promote E-cadherin internalisation through Arf6 GTPase (Palacios, Price et al. 2001) and E-cadherin degradation by recruiting the E3 ubiquitin ligase HAKAI, which targets E-cadherin to the lysosomes (Behrens, Vakaet et al. 1993; Fujita, Krause et al. 2002; Palacios, Tushir et al. 2005). We were able to show by immunofluorescence that RASSF1C expression promoted the internalisation of E-cadherin and the associated catenin proteins. We

also observed an induction of the HAKAI binding to E-cadherin and SRC. However, we did not observe any decrease in the total levels of E-cadherin in cells expressing RASSF1C by western blot. These data therefore suggest that RASSF1C promotes activation of SRC and E-cadherin internalisation as an early event and that HAKAI-mediated E-cadherin degradation occurs as a late event, due to elevated SRC activity. Investigation of E-cadherin internalisation and trafficking to the lysosomes using immunofluorescence could be used to confirm the fate of the internalised E-cadherin in cells expressing RASSF1C.

Another observation made from the immunofluorescence was that cells expressing high levels of RASSF1C were often small and rounded and also were relatively infrequent despite efficient transfection observed in the control cells. This observation deserves some discussion as they highlight potentially interesting avenues of future research.

E-cadherin stability at adherens junctions is controlled by the Rho family of GTPases (Menke and Giehl 2012). Rac activation by TIAM1 has been shown to stabilise junctions (Hordijk, ten Klooster et al. 1997; Sander, ten Klooster et al. 1999). Rho activity has been shown to both stabilise and destabilise junctions depending on the cell system examined (Etienne-Manneville and Hall 2002; Bruewer, Hopkins et al. 2004; Popoff and Geny 2009). Both Rho and Rac activity can be controlled by SRC recruitment of GEFs and GAPs (Huveneers and Danen 2009). The activation of Rho and Rac is spatially regulated such that both are not active in the same part of the cell. This is further controlled by antagonistic activity of Rac and Rho against each other (Nimnual, Taylor et al. 2003; Ohta, Hartwig et al. 2006). The most visual demonstration of the activities of Rho and Rac is seen in PC-3 cells that have lost either Rho or Rac. Cells that have lost Rho become elongated, whereas cells that have lost Rac become rounded (Sequeira, Dubyk et al. 2008). This shows that Rho activity promotes cell rounding. This rounding is caused by the promotion of ROCK-driven actomyosin contractility (Kimura, Ito et al. 1996; Amano, Chihara et al. 1997). The cells that express RASSF1C are small and rounded, but do not have condensed nuclei suggesting that they are not undergoing apoptosis or mitosis. Indeed, Matallanas et al. (2007) showed RASSF1C

expression did not promote apoptosis (Matallanas, Romano et al. 2007). Given that RASSF1C expression activates SRC and promotes E-cadherin internalisation, RASSF1C may promote Rho activation through SRC recruitment of a Rho-GEF. This could be tested by measuring Rho activity in cells expressing RASSF1C using a Rho-GTPase activation assay precipitating active Rho using GST-Rhotekin conjugated beads (Pellegrin and Mellor 2008). Rho also promotes Actin stress fibre formation (Ridley and Hall 1992). Increased Actin stress fibre formation has been associated with loss of RASSF1A expression (Dallol, Agathanggelou et al. 2005), conditions under which RASSF1C is able to activate SRC family kinases. This hypothesis is relatively easy to test. Rho-mediated contractility is mediated through ROCK, which promotes myosin contractility through upregulation of MLC phosphorylation. This is achieved by directly phosphorylating MLC and by phosphorylation and inactivation of myosin phosphatase (MYPT) (Amano, Ito et al. 1996; Kimura, Ito et al. 1996; Kureishi, Kobayashi et al. 1997). Therefore, by inhibiting ROCK using Y27632, we should see decreased myosin light chain phosphorylation and that the cells expressing RASSF1C become less rounded.

The importance of Rho signalling in cancer cell invasion and metastasis cannot be underestimated. In melanoma, cells were found to have a rounded phenotype at the invasive edge of the tumour. Rounded cells, migrating in a Rho-dependent manner, were found to migrate 10-40 times faster than cells migrating through Rac-mediated single cell or collective cell migration (Friedl, Zanker et al. 1998; Friedl 2004; Sanz-Moreno, Gadea et al. 2008). Rounded cells are also thought to be more resistant to shear forces following entry into the blood, potentially improving the chance that they will be able to metastasise to a distant site (Pinner and Sahai 2008; Sanz-Moreno, Gadea et al. 2008). An experiment in which cells migrate through a collagen matrix could be used to determine the mechanism through which RASSF1C cells migrate by determining their shape in this 3D matrix. Identification of RASSF1C driving invasion through a matrix in a Rho-dependent fashion may provide an interesting treatment option for patients with methylated RASSF1A. Treatment with a ROCK inhibitor or an inhibitor of contraction, such as blebbistatin may prevent tumour metastasis. It would be interesting to investigate this in a mouse metastasis model.

YAP and TAZ were recently shown to be activated by Rho-mediated actomyosin contractility (Dupont, Morsut et al. 2011). Therefore, one consequence of RASSF1C activation of Rho is that YAP/TAZ may be activated and able to promote the aggressive phenotypes seen in the mammospheres growth assay (Figure 6.10). Therefore inhibition of Rho signalling may not only inhibit metastasis, but may also promote YAP/TAZ translocation from the nucleus to the cytoplasm inhibiting transcription of pro-proliferative genes and EMT.

EMT is characterised by the loss of epithelial characteristics such as formation of cell-cell junctions and the acquisition of mesenchymal characteristics such as motility, invasiveness and self-renewal (Mani, Guo et al. 2008; Morel, Lievre et al. 2008; Thiery, Acloque et al. 2009). The acquisition of self-renewal properties has led people to propose that cancer cells that have undergone EMT have acquired the properties of stem cells (Mani, Guo et al. 2008). This process is controlled by the induction of transcription factors such as Twist, Snail, Slug, ZEB1 and ZEB2 which orchestrate EMT programs. We observed a number of traits associated with EMT in RASSF1C expressing cells, including the loss of cell-cell junctions and the acquisition of a more motile, invasive phenotype. The model proposed at the end of Chapter 5 was that the loss of cell-cell junctions would lead to the re-localisation of β-catenin from the membrane to the cytoplasm where it would be able to translocate to the nucleus and upregulating the expression of genes that promote EMT. In embryonic stem cells, β-catenin is thought to be required to maintain properties of self-renewal as β-catenin overexpression or inhibition of the upstream negative regulator GSK3β is known to upregulate NANOG, a stem cell marker, through an interaction with Oct3/4. Also, β-catenin has been shown to upregulate SNAIL signalling, the expression of which induces stem cell formation (Mani, Guo et al. 2008; Stemmer, de Craene et al. 2008).

Another potential consequence of loss of cell-cell junctions is the deregulation of YAP/TAZ. Contact inhibition induced by E-cadherin outside-in signalling has been shown to be mediated by the inhibition of YAP by the hippo pathway (Kim, Koh et al. 2011) and YAP/TAZ are known to be sequestered to the sights of cell-cell junctions by α-catenin and Angiomotin (Chan, Lim et al. 2011; Schlegelmilch, Mohseni

et al. 2011; Zhao, Li et al. 2011). Therefore we might expect that in cells that have lost E-cadherin, and associated catenin proteins, that YAP/TAZ would not be sequestered to the plasma membrane instead remaining nuclear and active. TAZ expression has been shown to induce stem cell markers in breast cancer cells (Cordenonsi, Zanconato et al. 2011) and YAP has been shown to promote self-renewal in mouse embryonic stem cells in response to LIF signalling (Tamm, Bower et al. 2011), suggesting that activation of YAP/TAZ may lead to cancer cells gaining stem-like properties. Interestingly, RASSF1C expression in H1299 cells also appeared to upregulate the expression of LIF and the stem cell marker PIWIL (Reeves, Baldwin et al. 2012).

LIF is a member of the IL-6 family of cytokines that was discovered to be essential for stem cell pluripotency and inhibition of leukemic cell differentiation (Gough, Gearing et al. 1988; Smith, Nichols et al. 1992). LIF binding to receptors that contain the gp130 subunit activates pathways that lead to the upregulation of NANOG through Sox2 and Oct4 (Gearing 1993). NANOG is a transcription factor that directly regulates the expression of a number of other factors including KLF4, which RASSF1C can also potentially upregulate (Reeves, Baldwin et al. 2012). KLF4, like LIF is a member of the Yamanaka cocktail which is used to re-programme somatic cells into induced pluripotent stem cells (iPSC). KLF4 also regulates NANOG by direct interaction with Oct4 creating a positive feedback loop and maintaining pluripotency (Bourillot, Aksoy et al. 2009; Hall, Guo et al. 2009; Niwa, Ogawa et al. 2009; Zhang, Andrianakos et al. 2010). Therefore, RASSF1C expression may promote EMT and stem-like cells through direct activation of SRC leading to loss of cell-cell junctions and subsequent internalisation of β-catenin and YAP/TAZ. These factors further promote EMT through upregulation of factors such as SNAIL, which represses the transcription of E-cadherin (Cano, Perez-Moreno et al. 2000). Activation of these transcription factors may lead to the upregulation of genes such as LIF and FGF2, which are also thought to be upregulated by RASSF1C expression (Reeves, Baldwin et al. 2012). These cytokines can be exocytosed from the cell and signal to the surrounding cells through paracrine signalling. Certainly, we see that junctional components are lost throughout the population of cells even when expression of

RASSF1C is only seen in a few cells in that population. This appears to occur in a density dependent manner.

YAP and TAZ drive EMT through an interaction with TEAD proteins (Ota and Sasaki 2008). They also play a role in β-catenin signalling. YAP has been shown to be upregulated by β-catenin and YAP-TEAD complexes have also been shown to interact with β-catenin-TCF4 complexes on shared target genes in colorectal carcinoma, promoting their upregulation (Heallen, Zhang et al. 2011; Imajo, Miyatake et al. 2012; Konsavage, Kyler et al. 2012). This pool of YAP associated with β-catenin also appeared insensitive to hippo signalling (Konsavage, Kyler et al. 2012). Phosphorylated, inactive, TAZ however, has been shown to inhibit Wnt signalling to β-catenin by binding to and stabilising DVL2 promoting β-catenin degradation (Varelas, Miller et al. 2010). We have seen that expression of RASSF1C can drive the re-localisation of β-catenin away from the membrane (Figure 5.4). It would be interesting to see whether loss of RASSF1A or expression of RASSF1C affected YAP/TAZ localisation and the crosstalk between the hippo pathway and the Wnt pathway. If so, inhibition of these two pathways, for example by inhibiting the YAP-TEAD complex (Liu-Chittenden, Huang et al. 2012), may be able to reverse the RASSF1C-driven EMT phenotype presented in this thesis.

Much of our work has focussed on the phenotypes of cells expressing RASSF1C in RASSF1A negative cells. However, our model predicts that in cancer, loss of RASSF1A alone will lead these EMT phenotypes. We have shown that cells depleted of RASSF1A have greater mobility, but we might also predict that they will also acquire stem-like properties. Indeed, loss of RASSF1A has been linked to decreased cell differentiation. In a recent paper by van der Weyden and Papaspyropoulos et al (2012) tumour formation in RASSF1A null mice was statistically associated with loss of the differentiation factor RUNX2. Further to this, loss of RASSF1A promoted significantly faster tumour development, specifically of poorly differentiated lymphomas or CD3-positive T-cell lymphomas (van der Weyden, Papaspyropoulos et al. 2012). It was shown that loss of RASSF1A and RUNX2 led to a loss of the

association of YAP and p73, promoting enhanced YAP-TEAD binding (van der Weyden, Papaspyropoulos et al. 2012) which promotes EMT and inhibits differentiation (Zhang, Liu et al. 2009).

Our model predicts that loss of RASSF1A would promote the enrichment of a tumour with stem-like cells through the release of RASSF1C. Indeed, RASSF1C expression in breast and lung cancer cell lines is thought to upregulate stem cell markers through an unknown mechanism (Amaar, Minera et al. 2006; Reeves, Baldwin et al. 2010; Reeves, Baldwin et al. 2012). Our model would propose that this may occur through RASSF1C activation of SRC promoting the re-localisation of β-catenin to the nucleus. This could be investigated by analysing the cell population for expression of stem cell markers by western blot, for example NANOG expression; using FACS, for example breast cancer stem cells exhibit a distinctive CD44 high, CD24 low cell surface marker expression (Al-Hajj, Wicha et al. 2003); and using mammosphere culture. It would also be interesting to investigate the stem cell population of xenografts with or without RASSF1A expression. We would predict that a tumour formed form RASSF1A expressing cells would have a smaller stem cell population.

Cancer stem cells are proposed to be more chemo and radio-resistant (Bao, Wu et al. 2006; Diehn, Cho et al. 2009; Krause, Yaromina et al. 2011). Therefore tumours that have lost RASSF1A would be more resistant to therapy. This is indeed the case. RASSF1A methylation correlates with decreased responsiveness to DNA damaging therapies (Catto, Azzouzi et al. 2005; Dote, Cerna et al. 2005; Honda, Haruta et al. 2008). One potential consequence of this is that tumours that have lost RASSF1A may be re-sensitised to therapeutic regimes through the re-expression of RASSF1A using DNMT inhibitors, inhibition of SRC or a combination of both together.

Re-expression of RASSF1A using DNMT inhibitors in cell lines and mouse models have shown increased sensitivity to DNA damage induced by radiation and cisplatin, and have also shown increased sensitivity to other chemotherapeutics such as, fluorouracil and mitomycin (Balch, Yan et al. 2005; Dote, Cerna et al. 2005; Zhao and Dou 2007). In the clinic, 5-aza-2'-deoxycytidine (known as decitabine), has been used to enhance the sensitivity of testicular germ cell tumours to cisplatin (Beyrouthy, Garner et al. 2009).

The effectiveness of this treatment correlated with DNMT3B expression and therefore DNA methylation. One of the most significant targets for DNMT3B has been shown to be RASSF1A (Palakurthy, Wajapeyee et al. 2009) and therefore treatment effectiveness is dependent on re-expression of RASSF1A. Finally, a phase 2 clinical trial used low dose decitabine, restoring carboplatin sensitivity in heavily pre-treated ovarian cancer patients resulting in high response rates and prolonged progression free survival (Matei, Fang et al. 2012). Expression of RASSF1A will lead to an increase in radiosensitivity due to the induction of stress response pathways by RASSF1A. However, the role of RASSF1C in cells treated with DNMT inhibitors may be examined by investigating stem cell markers. We would predict that treatment of tumours methylated for RASSF1A with DNMT inhibitors would lead to a tumour with a smaller stem cell population that is more sensitive to radiotherapy.

Tumours methylated for RASSF1A may also be effectively treated with SRC inhibitors. There are numerous SRC inhibitors in development of which the three best studied are dasatinib, bosutinib and saracatinib. All three of these drugs have been shown to have robust antitumour and antiproliferative effects, blocking cell growth, invasion and angiogenesis in preclinical trials (Aleshin and Finn 2010). Our data would suggest that Inhibition of SRC activity would prevent the internalisation of β-catenin inhibiting its promotion of EMT. Indeed, the effects of bosutinib were shown to be through inhibition of SRC-dependent β-catenin activation (Coluccia, Benati et al. 2006). In colon carcinoma cells, dasatinib was shown to act synergistically with oxaliplatin (Kopetz, Lesslie et al. 2009) and increase the sensitivity of lung cancer cell lines to cisplatin (Ceppi, Papotti et al. 2009). There are numerous phase 1 and 2 clinical trials progressing with these drugs either as single agents or in combination with other therapeutic agents. Although none of these appear to include radiotherapy, trials using DNA damaging agents such as carboplatin and gemcitabine are on-going.

Taking the data presented in this thesis in context with the literature a final testable model can be proposed (Figure 7.1). In normal cells, RASSF1A regulates the activity of SRC family kinases through the binding of RASSF1C. This interaction recruits CSK to SRC allowing CSK to phosphorylate and inactivate

SRC. RASSF1A also inhibits the interaction between RASSF1C and SRC. In cancers, when RASSF1A is lost, RASSF1C levels become elevated in a small subset of cells, where it is able to bind to and activate SRC, through an unknown mechanism that may involve direct alteration of the conformation of SRC, recruitment of co-factors, such as OSSA, or promotion of the translocation of SRC to the membrane. Once active RASSF1C induced SRC activity is targeted towards the membrane where it promotes EMT through the internalisation of E-cadherin and ZO-1 and subsequently cells lose polarity markers from these sites. Cells also become rounded suggesting a role for Rho activity. The loss of the cell-cell junctions is also associated with the internalisation of β-catenin and potentially also the hippo pathway members YAP and TAZ. We propose that EMT is further promoted by the translocation of β-catenin and YAP/TAZ to the nucleus, where they upregulate genes for cell proliferation, motility and self-renewal. Cytokines including LIF and FGF2 are also upregulated and potentially exocytosed from the RASSF1C expressing cells where they act on the cell population via paracrine signalling pathways, promoting EMT in the cells in which RASSF1C is not stabilised. The proposed model predicts that the resulting population of cells will be enriched for cancer-stem cells that will be more aggressive and invasive as well as less sensitive to radio- and chemotherapies. Re-sensitisation of this cell population may occur through treatment with DMNT inhibitors which re-express RASSF1A or by direct inhibition of SRC family kinases.

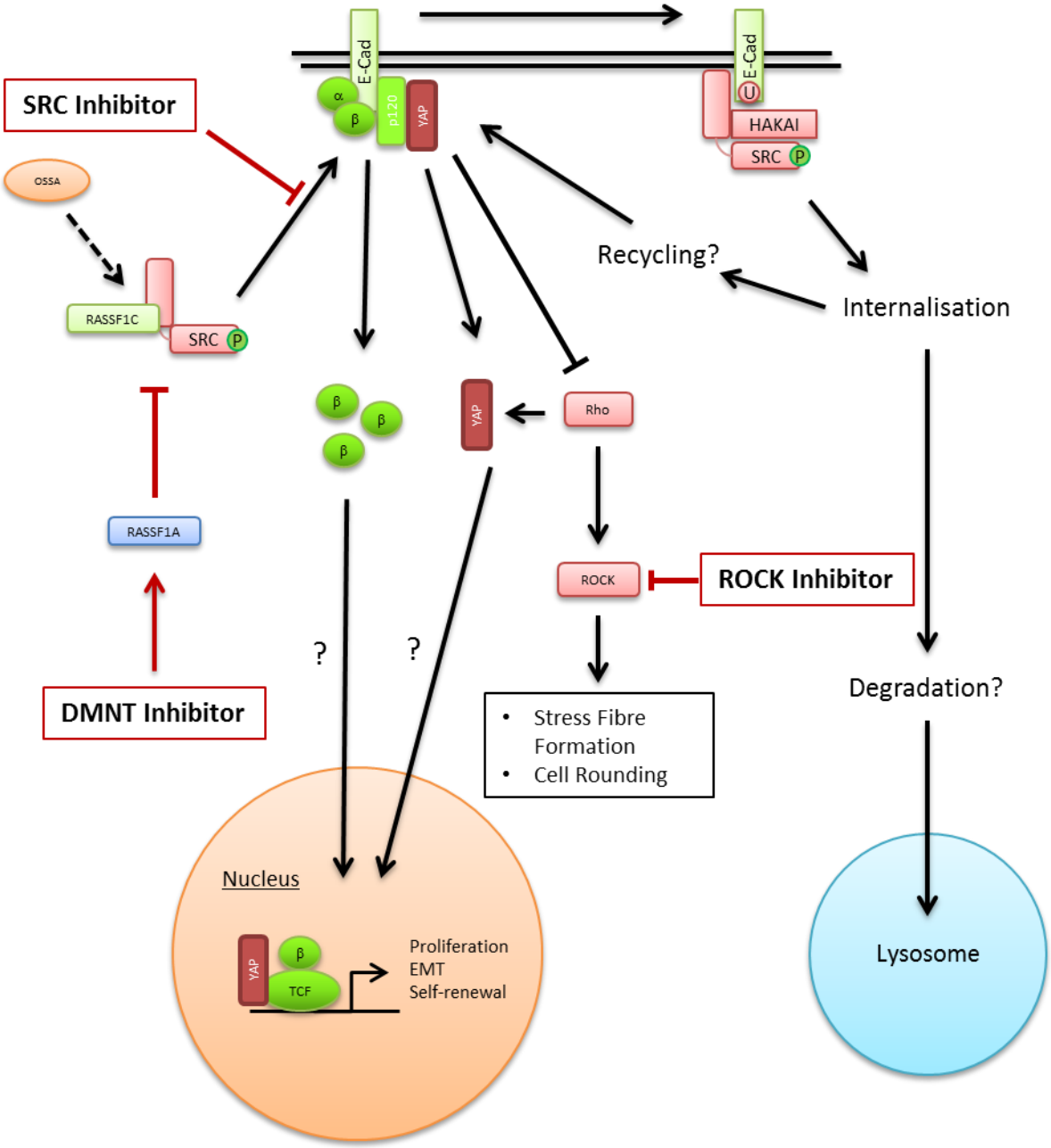


**Figure 7.1. Cartoon Depicting the Model Generated from the Results in this Thesis.** When RASSF1A is lost, RASSF1C can activate SRC, by a mechanism that may be dependent on the recruitment of co-factors, such as OSSA, promoting translocation of SRC to the plasma membrane. At the plasma membrane SRC phosphorylates target proteins such as, E-cadherin (E-cad) and β-catenin (β). This leads to the formation of an E-cadherin-HAKAI complex, which may target E-cadherin for degradation in the lysosome. E-cadherin may also be recycled back to the membrane. β-catenin and YAP, which are normally sequestered by p120-catenin, are released from the membrane potentially allowing them to enter the nucleus, where they are able to promote proliferation, EMT and self-renewal. YAP may also be activated by Rho activity. Re-expression of RASSF1A, using DNMT inhibitor, may inhibit SRC activation by binding to RASSF1C. SRC may also be targeted directly using a SRC inhibitor. Invasion and metastasis maybe inhibited using a ROCK inhibitor, which may have a secondary effect of preventing YAP activation.

# Chapter 8 Appendix

## 8.1 Identified Proteins

**Table 8.1 Selected RASSF1A Interacting Proteins From Mass Spectrometry Screen Described in Chapter 3.**

| Band | Protein Identified | Role/why interesting |
|---|---|---|
| **Known Binding Partners** | | |
| 17 | 14-3-3 protein epsilon | Has been shown to bind GST-RASSF1A. RASSF1A was shown to be released with TNFα stimulation (Ghazaleh, Chow et al. 2010). |
| 26 | DNA Damage Binding protein 1 | Targets both RASSF1A and RASSF1C for destruction. RASSF1A is targeted during late mitosis and RASSF1C after DNA damage. (Jiang, Rong et al. 2011; Zhou, Li et al. 2011). Both of these articles have been published since this screen was done. |
| 17 | DNA repair protein complementing XPA | With binding partner RPA forms part of the pre-incision complex during nucleotide excision repair. RASSF1A was first identified as an interaction partner with XPA by yeast-2-hybrid (Dammann, Li et al. 2000). |
| 10, 30 | Mammalian Sterile-like Kinase 2 (MST2) | Member of the hippo pathway and confirmed RASSF1A interacting partner. |
| 24 | Microtubule associate protein 1S | Also known as C19ORF5 and RABP1. Shown to associate with RASSF1A to induce RASSF1A binding to microtubules and to enhance binding of RASSF1A to CDC20 to regulate the APC/C (Dallol, Agathanggelou et al. 2004; Song, Chang et al. 2005). |
| **5,6,13,14,22,** | **Ras association domain-containing** | Confirmation that RASSF1A is indeed present in the |

| | | |
|---|---|---|
| **34** | **protein 1** | immunoprecipitate. |
| 4 | Serine/threonine-protein kinase 6 | Aurora kinase A. Shown to bind and phosphorylate RASSF1A (Rong, Jiang et al. 2007; Song, Song et al. 2009). |
| 12 | Tubulin (alpha 1D chain, alpha 1A chain, alpha 1B chain, beta 2C chain, and beta 5 chain). | RASSF1A has been shown to bind and stabilise microtubules. Binding microtubules appears key to RASSF1A tumour suppressor function (Liu, Tommasi et al. 2003). |
| | | |
| **Related to the Hippo pathway** | | |
| 1 | Adenomatous polyposis coli protein | Tumour suppressor involved in Wnt signalling which is known to regulate YAP and TAZ and be regulated by the hippo pathway (Varelas, Miller et al. 2010; Heallen, Zhang et al. 2011; Imajo, Miyatake et al. 2012; Konsavage, Kyler et al. 2012). Also, known to bind and stabilise microtubules as RASSF1A does (Deka, Kuhlmann et al. 1998). |
| 15 | Casein kinase isoform 1 alpha | Multi-specificity kinase. Known to be located at mitotic spindles as RASSF1A is (Brockman, Gross et al. 1992). Also other isoforms are known to phosphorylate YAP leading its degradation (Zhao, Li et al. 2010). |
| 35 | Peroxiredoxin-1 and 2 | MST1 has been shown to interact with and be activated by Peroxiredoxin 1 to cause cell death in a p53 dependent manner in response to $H_2O_2$ and cisplatin induced oxidative stress (Morinaka, Funato et al. 2011) |
| 11 | Serine/Threonine-protein kinase 38 (NDR1) | Along with NDR2 (STK38L), closely related to the AGC kinase LATS1/2 (Pearce, Komander et al. 2010). NDR kinase has been shown to be activated by a |

| | | |
|---|---|---|
| | | RASSF1A/MST1 complex in response to TNFα (Vichalkovski, Gresko et al. 2008). |
| | | |
| **Cytoskeletal** | | |
| 31 | Actin-related protein 3 (ARP3) | ATP binding protein in ARP2/3 complex that regulates actin polymerisation (Mullins, Heuser et al. 1998). |
| 22 | Beta actin like protein 2 (Kappa actin) | Highly conserved actin isoform. Cytoskeletal protein involved in motility. |
| | Cytoplasmic actin 1 (beta actin) | Cytoskeletal protein involved in motility. |
| 9 | Cytoskeleton-associated protein 4 | Mediates anchorage of endoplasmic reticulum to microtubules |
| 1 | Dynein heavy chain 17 | Protein that generates force towards the minus end of microtubules. |
| 7 | Epiplakin | Very large cytoskeletal protein. |
| 24 | Filamin A | Large cytoskeleton associated scaffold protein that links actin to the membrane and to membrane proteins. Also, scaffold many signalling events at the membrane (Razinia, Makela et al. 2012). Localises FilGAP a Rac1 GTPase activating protein to in activate Rac in response to Rho-ROCK activity (Ohta, Hartwig et al. 2006). |
| 3 | Myosin 9 | Cellular, heavy chain of myosin. Plays a role in cell shape and cytokinesis. RASSF1A has been linked to a role in cytokinesis, as have other hippo pathway members (Song, Kim et al. 2009). |
| 23 | Plectin-1 | Large scaffold protein that links intermediate filaments to microtubules and intermediate filaments to desmosomes. This hit along with epiplakin was the |

| | | |
|---|---|---|
| | | most obviously upregulated binder of RASSF1A on the gel. |
| | | |
| **Novel Proteins** | | |
| 30 | 52kDa Ro protein | Is a ubiquitin ligase that forms part of the SCF complex. Interacts with SKP2 which is known to be involved in the degradation of RASSF1A (Song, Song et al. 2008). Also known to be involved in the degradation of $p27^{Kip1}$ in a phosphorylation dependent manner (Sabile, Meyer et al. 2006). |
| 15 | Apoptosis-stimulating of p53 protein 2 | ASPP2 is linked to the hippo pathway as it enhances the dephosphorylation of TAZ by PP1 (Liu, Lv et al. 2011). ASPP1 is linked to LATS kinase. Cytoplasmic ASPP1 inhibits LATS1 inhibition of YAP and LATS2 activates p53 via nuclear accumulation of ASPP1 (Aylon, Ofir-Rosenfeld et al. 2010; Vigneron, Ludwig et al. 2010). Drosophila ASPP interacts with dRASSF8 to regulate Csk at adherens junctions (Langton, Colombani et al. 2009). |
| 1 | E3 ubiquitin-protein ligase HUWE1 (Mule, ARF-BP1) | Ubiquitin ligase acting on a number of targets including MCL-1, polymerase beta, p53 etc. Is bound to and inhibited by p14 ARF. It therefore plays a role in the DNA damage response and regulating p53. |
| 3 | Endoribonuclease Dicer | RASSF1A has been linked to regulation of proteins at the levels of mRNA (Shivakumar, Minna et al. 2002). Dicer, as a member of the miRNA synthesis machinery may provide some a mechanism by which this is occurring. |
| 9, 10, 18 29 | Heat shock 70 kDa protein 1, Heat | A number of heat shock proteins were found in the |

| | | |
|---|---|---|
| 30 | shock 70 kDa protein 1B, Heat shock 70 kDa protein 6, Heat shock cognate 71 kDa protein 2 | hits. Act as chaperones to stabilise proteins. MST has been shown to be downregulated by Heat shock protein 70 in response to cisplatin (Ren, Yan et al. 2008) LATS1/2 have been shown to be stabilised by HSP90 (Huntoon, Nye et al. 2010). |
| 28 | Heterogeneous nuclear ribonucleoprotein M | Involved in splicing. Also, may initiate a cascade that results in the induction of IL-1α, IL-6 and TNF cytokines. RASSF1A is involved in TNFα signalling (Foley, Freedman et al. 2008). |
| 33 | Nuclease sensitive element binding protein 1 | Regulates splicing and stability of cytoplasmic mRNA. As with Dicer, may play a role in how RASSF1A regulates cyclin D1 levels. |
| 16 | Nucleophosmin | Regulates a vast number of signalling pathways including p53 and centrosome duplication. RASSF1A also plays a role in centrosome duplication and activation of p53 (Liu, Guo et al. 2008; Song, Song et al. 2008). |
| 34 | Serine/threonine-protein phosphatase 5 (PP5) | Interacts with HSP90 with which it is thought to regulate glucocorticoid receptor signalling. Key to activation of signalling pathways including MAPK (Hinds and Sanchez 2008). |
| 25 | Oxidative stress-associated SRC activator (OSSA) | Scaffold protein that activates SRC in response to DNA damage and regulates IGF-II mRNA and promotes the extracellular secretion of IGF-II (Tanaka, Sasaki et al. 2009). |
| 27 | Transcription factor E2F7 | Inhibitory transcription factor that directly inhibits E2F1 target genes. RASSF1A inhibits E2F1 by maintaining the pRb checkpoint at the G1/S transition. |

## 8.2 H1299 TET ON Cell Expression

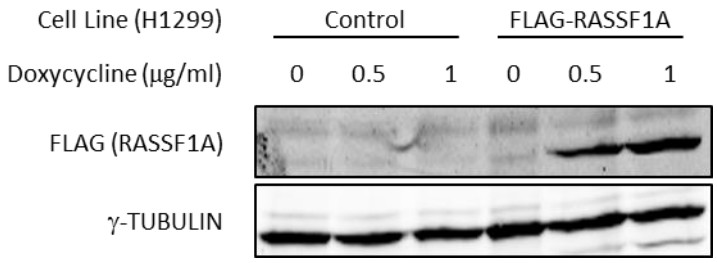


**Figure 8.1. Induction of RASSF1A Expression in H1299 Tetracycline Inducible (TET ON) Cell Lines.** H1299 cells containing empty vector or TET ON inducible RASSF1A vector were plated (2 x $10^5$ cells/plate) before being cultured in media containing 0, 0.5 or 1 μg/ml doxycycline for 24 hrs. Cells were lysed with Laemmli lysis buffer and analysed by SDS-PAGE and western blot for RASSF1A expression using anti-FLAG-tag antibodies. γ-TUBULIN is a loading control. Dose dependent induction of RASSF1A is only seen in the RASSF1A-inducible cell line and not in the control cell line. Data represents one independent experiment.

## 8.3 Vector Competition Analysis

We noticed that expression of the MYC-tag constructs reduced the expression of the HA-RASSF1C in Figure 4.9. We designed a series of experiments to test whether this decrease in HA-RASSF1C expression was due to vector competition or due to targeted degradation of the HA-RASSF1C construct. We found that MYC-RASSF1C expression decreased both FLAG-RASSF1A and HA-RASSF1A expression in a dose dependent manner (Figures 8.2A and 8.2B), which was not the case when FLAG-RASSF1C was coexpressed with FLAG-RASSF1A (Figure 8.2C). The expression of these construct was also not affected by addition of the proteasome inhibitor MG132. Expression of FLAG-RASSF1A (Figure 8.2D) and HA-RASSF1A (Figure 8.2E) was also decreased when coexpressed with MYC-OSSA. This was to a less severe than when coexpressed with MYC-RASSF1C. Taken together these data indicate that the MYC-tag constructs out compete the FLAG-tag and HA-tag constructs leading to decreased expression of the FLAG-tag and HA-tag protein. However, Figures 8.2D and 8.2E may suggest that RASSF1C and RASSF1A do influence the stability of each other, in keeping with the inhibition of RASSF1C by RASSF1A.

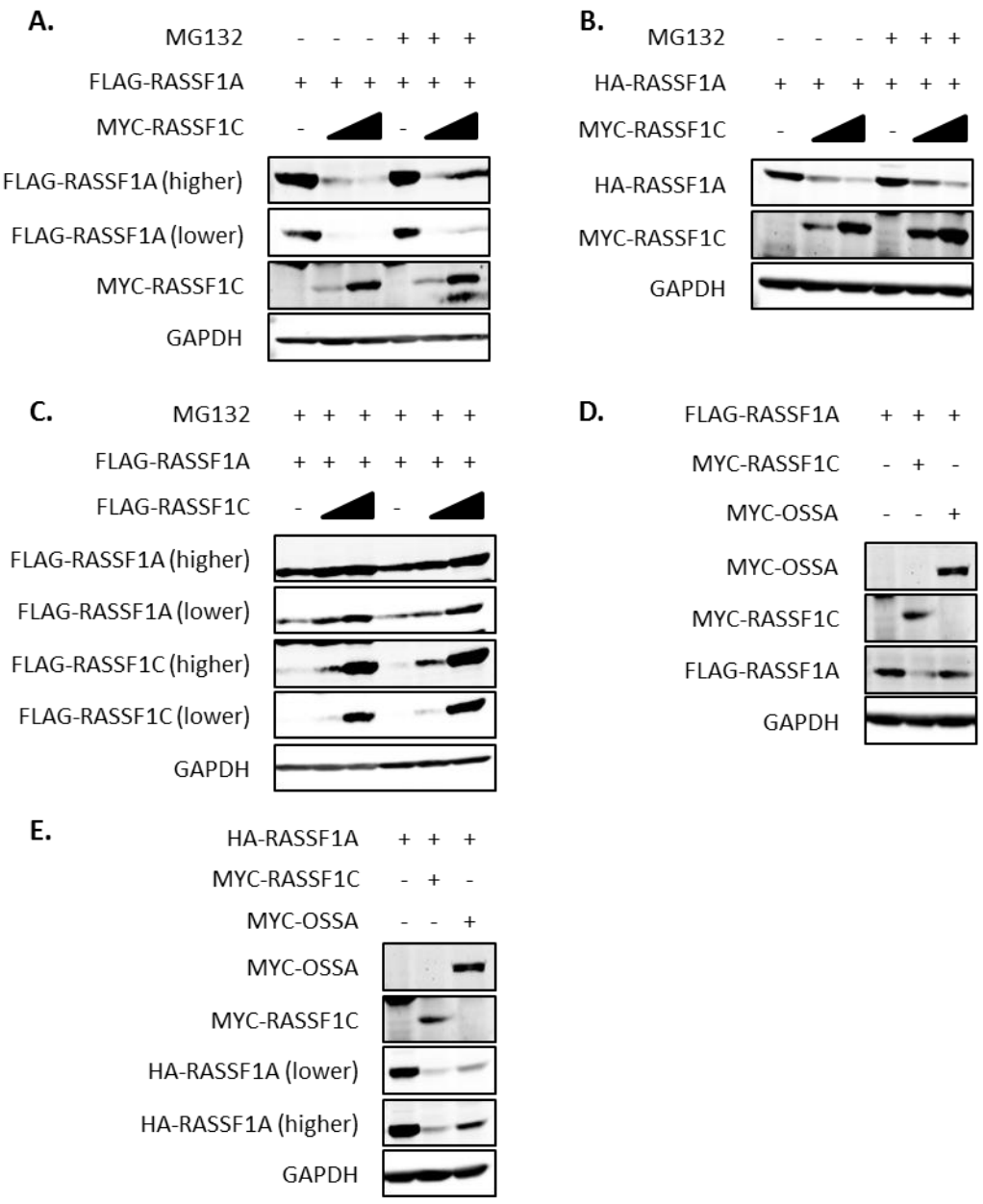


**Figure 8.2. MYC-Tagged Constructs Out-Compete HA- or FLAG-Tagged Constructs. A and B.** H1299 cells (2 x $10^5$ cells/plate) were co-transfected with 1 μg of FLAG-RASSF1A (**A.**), or HA-RASSF1A (**B.**) and 0, 0.5 or 1 μg MYC-RASSF1C and cultured for 48 hrs before being incubated with 10 μM MG132 for 6 hrs. Cells were lysed with Laemmli lysis buffer and analysed by SDS-PAGE and western blot for levels of RASSF1A using anti-FLAG-tag (**A.**) or anti-HA-tag (**B.**) antibodies and RASSF1C using anti-MYC-tag antibodies. Expression of MYC-RASSF1C decreases the expression of FLAG-RASSF1A and HA-RASSF1A in a dose-dependent manner. **C.** H1299 cells (2 x $10^5$ cells/plate) were transfected with 1 μg FLAG-RASSF1A and 0, 0.5 and 1 μg FLAG-RASSF1C before being lysed with Laemmli lysis buffer and analysed by SDS-PAGE and western blot for levels of RASSF1A and RASSF1C using anti-FLAG-tag antibodies. FLAG-RASSF1C has a dose-dependent stabilising effect on FLAG-RASSF1A**. D and E.** H1299 cells (2x$10^5$ cells/plate) were co-transfected with 1 μg FLAG-RASSF1A (**D.**), or HA-RASSF1A (**E.**) and 1 μg empty vector, MYC-RASSF1C or MYC-OSSA. Cells were cultured for 48 hrs before lysis with Laemmli lysis buffer and analysis by SDS-PAGE and western blot for RASSF1A expression using anti-FLAG (**D.**) or anti-HA (**E.**) antibodies and RASSF1C and OSSA expression using anti-MYC-tag antibodies. Expression of MYC-tag constructs decreases FLAG-RASSF1A and HA-RASSF1A expression, however, the effect of MYC-RASSF1C is greater than for MYC-OSSA. Data represents one independent experiment.

## 8.4 RASSF1 siRNA Knock Down Confirmation

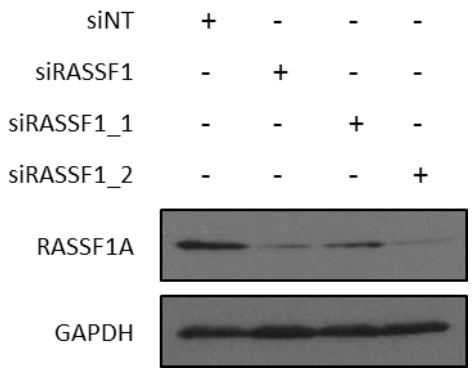


**Figure 8.3. RASSF1A Expression Can be Knocked Down by Individual siRNA Molecules form the SMARTpool siRNA Targeting RASSF1.** HeLa cells (2.5 x $10^5$ cells/plate) were transfected with 50 nM non-targeting siRNA or siRNAs targeting RASSF1. The three siRNAs targeting RASSF1 were a SMARTpool composed of 4 individual siRNA molecules, siRNA 1 from the SMARTpool (siRASSF1_1) and siRNA 2 from the SMARTpool (siRASSF1_2). Cells were cultured for 48 hrs before lysis with Laemmli lysis buffer and analysis by SDS-PAGE and western blot for RASSF1A expression using anti-RASSF1A antibodies. GAPDH represents a loading control. RASSF1A expression is decreased in cells transfected with all three RASSF1 siRNA molecules. Data is representative of 5 wells from 5 independent experiments.

## 8.5 Example Images for siRESIST Scratch Wound Assay (Figure 6.7)

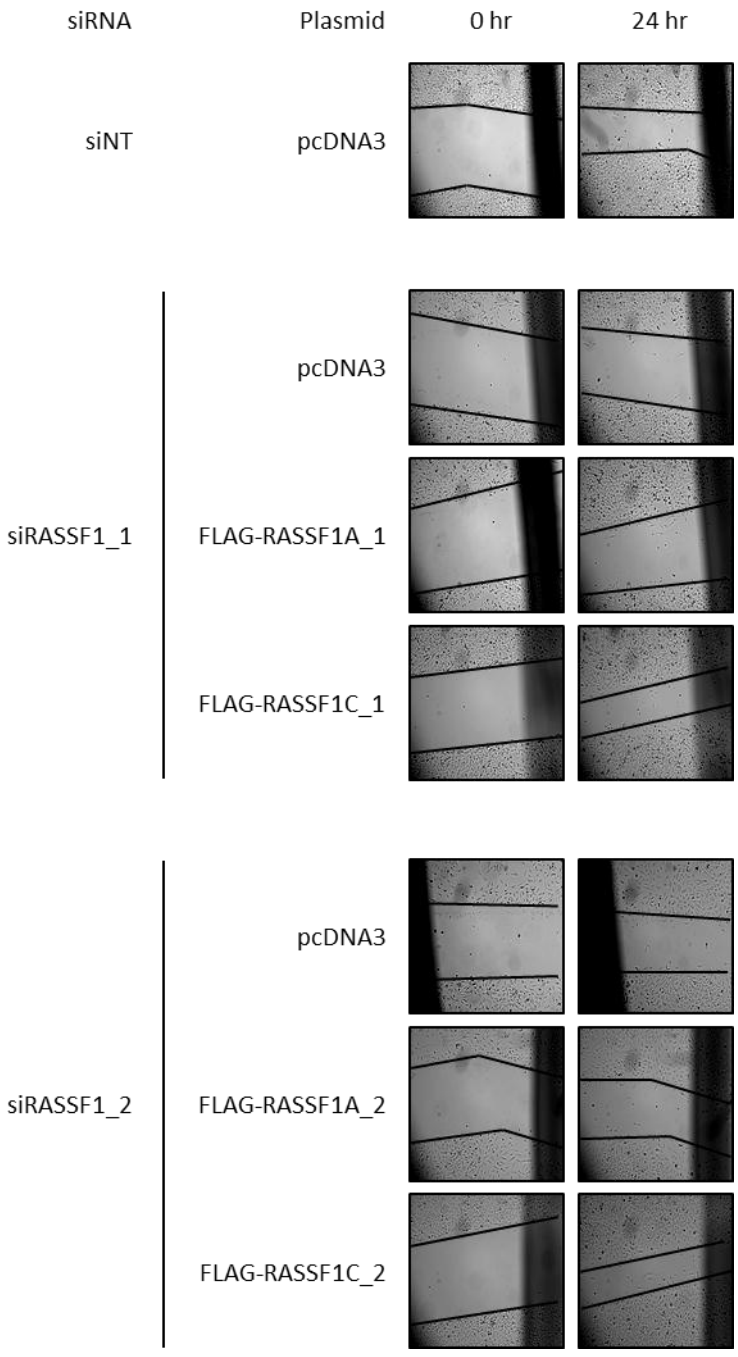


**Figure 8.4. Example Images of the Scratch Wound from Figure 6.7.** Black lines denote the edge of the Scratch. Images are representative of 16 scratches from 4 independent experiments.

## 8.6 MDA-MB-231 and H1299 RASSF1C TET ON Clone Selection

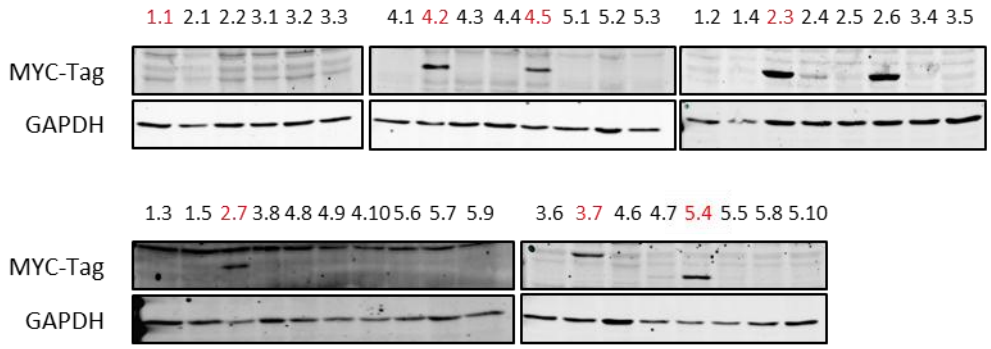


**Figure 8.5. MDA-MB-231 Stable Clones.** MDA-MB-231 cells were stability transfected with empty vector (clones #1), MYC-RASSF1A (clones #2), MYC-RASSF1A A133S (clones #3), MYC-RASSF1C (clones #4) or MYC-RASSF1C A63S (clones #5). Ten clones per condition were picked and expanded. Lysates were analysed by SDS-PAGE and western blot for expression with anti-MYC-tag antibodies. Positives highlighted in red. Clone 4.2 was used in Figure 6.9A and 6.10. Data represents one independent experiment. Expression of chosen clones was independently confirmed in subsequent experiments.

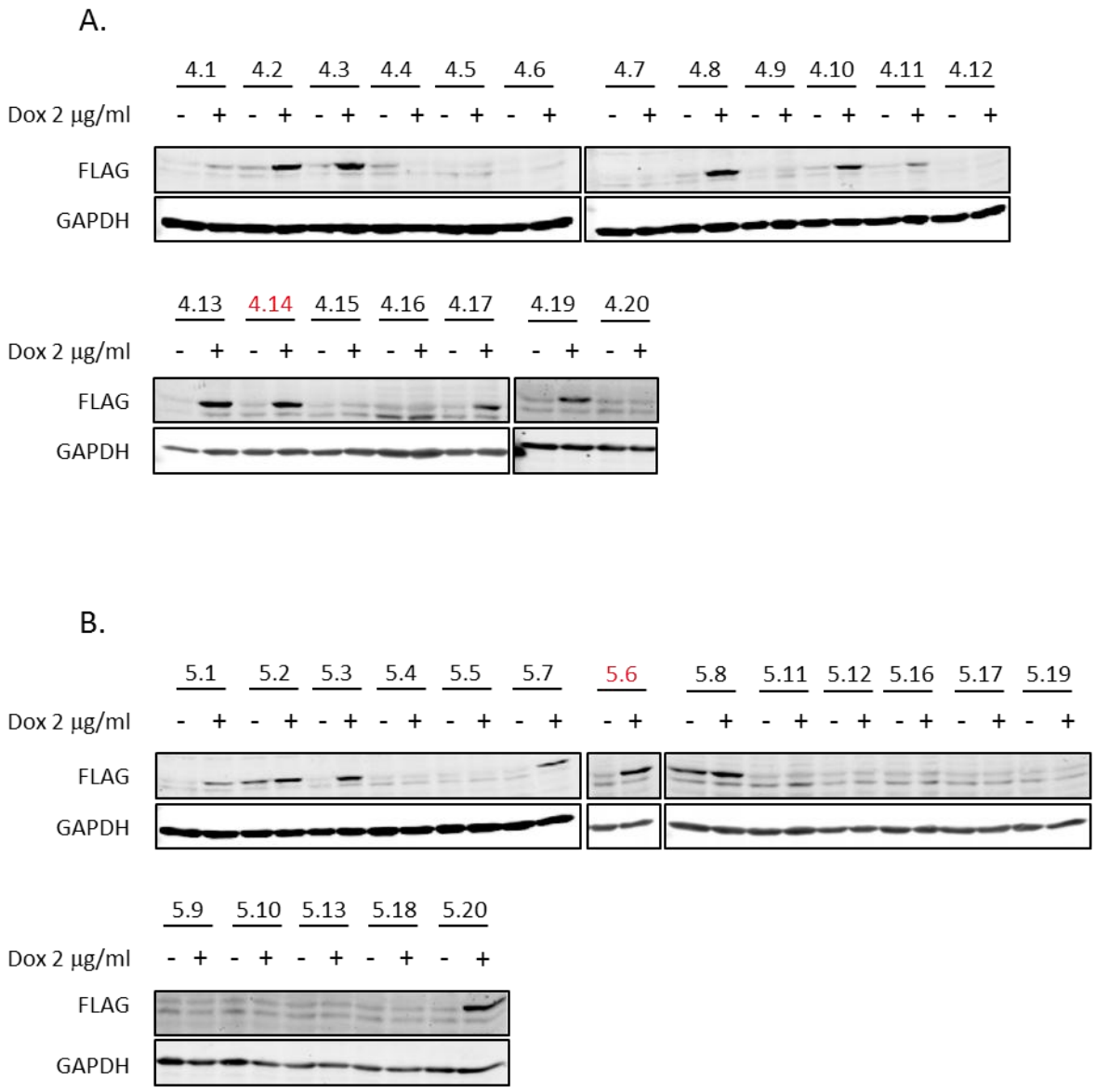


**Figure 8.6. H1299 Tetracycline Inducible Clones.** H1299 Tet ON cells were stably transfected with pTre-Tight-FLAG-RASSF1C (**A**, clone #4) or pTre-Tight-FLAG-RASSF1C A63S (**B**, clone #5). Twenty clones transfected with each construct were picked and expanded. Cells were then plated and cultured in the presence or absence of 2 µg/ml doxycycline before lysis with laemmli lysis buffer. Lysates were analysed by SDS-PAGE and western blot for expression of FLAG-RASSF1C or FLAG-RASSF1C A63S in the presence of doxycycline, but not in the absence of doxycycline using anti-FLAG antibodies. GAPDH is a loading control. Clones chosen for further experiments are highlighted in red. Data represents one independent experiment. Expression of chosen clones was independently confirmed in subsequent experiments.

## 8.7 Mammospheres from MDA-MB-231 Cells Transiently Transfected with RASSF1C

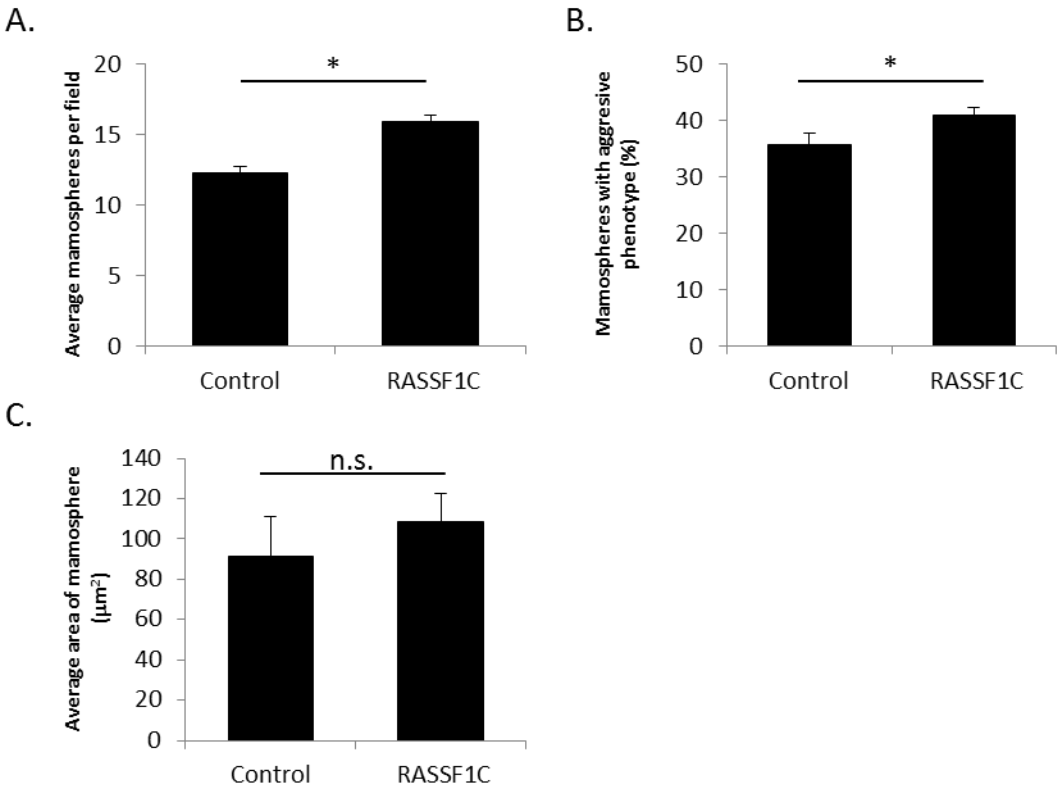


**Figure 8.7. Transient Expression of RASSF1C in MDA-MB-231 Cells Promotes Mammosphere Formation and Aggressiveness.** MCF7 cells transfected with control plasmid or FLAG-RASSF1C before being plated onto matrigel and allowed to grow for 9 days, changing the media every 3 days. Images were then acquired (10 per well, 6 wells per condition) using a Nikon TE2000 Eclipse microscope. Mammospheres were analysed using Image J software to measure the number of mammospheres formed per image, the percent of mammospheres with an aggressive phenotype and the area of each mammosphere (5 mammosphere measured per image). **A.** Transient transfection with RASSF1C promoted a greater number of mammospheres to form ($\rho$ = 1.1 x $10^{-6}$) and **B.** promoted a more aggressive phenotype in those that did form ($\rho$ = 0.026). **C.** The mammospheres were not significantly larger ($\rho$ = 0.19). Asterisk represent $\rho$-value less than 0.05. n.s. shows data that is not significant. Error bars represent 1 x SEM. Data represents 6 independent experiments.

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