ONCOGENIC STRAP REGULATES c-JUN STABILITY AND PERSISTENT MIGRATION

By

Jennifer Elisabeth Reiner

Dissertation

Submitted To the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cancer Biology

August, 2011

Nashville, TN

APPROVED:

Dr. Pran K. Datta

Dr. Ann Richmond

Dr. Pierre Massion

Dr. Brian Wadzinski

ACKNOWLEDGEMENTS

It would be impossible for me to adequately express my gratitude for all the assistance that I have received during my graduate studies at Vanderbilt. The list of people who have offered advice and support during this process is quite long but I will do my best to mention those who have had the most significant impact on my education and completion of my doctorate.

I would like to start by thanking the members of my thesis committee: Dr. Ann Richmond, Dr. Brian Wadzinski, Dr. Pierre Massion, and Dr. Pran Datta. Their suggestions and willingness to proofread several drafts of my manuscripts were immensely helpful to me. I would particularly like to thank Dr. Ann Richmond for her unwavering support and everything she has done for me. Dr. Richmond's willingness to engage and offer suggestions on my projects was instrumental to my work. I would like to sincerely thank Dr. Hal Moses for extending his lab and resources to me during difficult times. I also want to extend my gratitude to Dr. Lynn Matrisian and Dr. Roger Chalkley for their assistance. I would certainly not have reached this point without all of your strong support.

I would like to thank Dr. Jiquing Sai in the Richmond lab for his technical advice and allowing me to use his chemotaxis devices. I am grateful to Dr. Ye Fei and Greg Ayers for their assistance with the statistical analysis of my data. I would like to thank Dr. Steve Haan for generously providing the c-myc custom antibody and Dr. Bill Tansey and Dr. Steve Hanks for their willingness to share their scientific expertise on ubiquitylation and FAK signaling, respectively.

I would also like to thank Chirie Sumanasekera for her friendship, aliquots of gumbo, and helpful suggestions regarding my experiments and manuscripts. I am also

grateful to Wanguang Zhang in the Datta lab for his good humor and friendship. Of course, a big thank you goes out to all the members of the Moses Lab for their generosity and hospitality.

Apart from the Vanderbilt community, there have been a few key people who have supported me and helped me maintain some semblance of sanity through the years. I am grateful to Kathy Perkins for her friendship and support. Last but not least, I would like to thank my parents, Ivan and Jun Reiner. Since I was young, my parents have always supported me in my endeavors and have made significant sacrifices for my benefit. They have been a constant source of support and advice.

The path to completing my doctorate at Vanderbilt has been a long and sometimes rocky one. Having had the opportunity to interact with many accomplished scientists, I have come to admire those who are of good character and treat others with respect. So, I have learned that to be a success in science, it is both important to be a good scientist and a good person. I would like to express my sincere gratitude to all the people mentioned above who have offered their love, friendship, support, and advice.

Page

ACKNOWLEDGEMENTS ii

TABLE OF CONTENTS iv

LIST OF FIGURES vii

LIST OF TABLES ix

LIST OF ABBREVIATIONS x

Chapter

I. INTRODUCTION 1

STRAP 2
Characterization of STRAP 2
SMAD-dependent and -independent TGF-β signaling 6
STRAP mediated inhibition of TGF-β signaling 9
STRAP association with NM23-H1 affects the TGF-β and p53 signaling pathways 10
STRAP promotes cell survival by regulating ASK1 and PDK1 signaling 11
STRAP regulates cell growth signaling pathways 12
The role of STRAP in transcription, mRNA splicing, and cap-independent translation 14
STRAP expression is associated with mesenchymal morphology in MEFs 16
Clinical significance and targeted inhibition of STRAP 16
The c-Jun proto-oncogene 18
The ubiquitin proteasome and c-Jun stability 20
Directed Migration 24
Regulators of gradient sensing and cell polarity 24
Cell adhesion and motility 28
Focal adhesion kinase signaling pathways 32
Summary and Aims 35

II. STRAP STABILIZES C-JUN AND PROMOTES CELLULAR PROLIFERATION 37

Introduction 37
Materials and methods 39
Cell lines and plasmids 39
Reagents and antibodies 39
Generation of stable cell lines 39
Western blot analysis 40
Cell growth assays 41

Reverse transcription PCR 41
Cycloheximide assay 42
Ubiquitylation assay 42
Results 43
STRAP regulates c-Jun protein expression and activation 43
STRAP does not regulate c-Jun mRNA or JNK activation 46
STRAP inhibits ubiquitin-mediated proteolysis of c-Jun 48
STRAP prolongs the half-life of c-Jun 50
Stable reintroduction of STRAP rescues the c-Jun instability defect in STRAP knockout fibroblasts 50
STRAP promotes proliferation of MEFs 52
Discussion 52

III. STRAP PROMOTES PERSISTENT MIGRATION AND TAIL RETRACTION DURING CHEMOTAXIS 58

Introduction 58
Materials and methods 62
Cell culture and plasmids 62
Reagents and antibodies 62
Generation of stable cell lines 62
Immunofluorescence 63
Transwell migration assay 63
Wound closure assay 64
Live cell imaging 65
Uropod formation and retraction 65
AKT localization 66
Adhesion assay 66
Cell Spreading assay 67
Focal adhesion disassembly assay 67
Western blot analysis 68
Results 69
STRAP is a positive regulator of cell migration 69
Loss of STRAP increases cell adhesion and spreading 71
STRAP regulates persistent chemotactic migration 73
Loss of STRAP is associated with decreased tail retraction and altered phospho-FAK Y397 distribution 75
Ectopic expression of STRAP rescues the defect in directed migration and attenuates tail formation 79
STRAP regulates FAK subcellular localization 82
Discussion 84

IV. CONCLUSIONS AND FUTURE DIRECTIONS 88
Significance of STRAP-mediated c-Jun stabilization and future directions 88

Biological implications for STRAP-induced persistent migration and future directions 90
Concluding remarks 94

REFERENCES 97

LIST OF FIGURES

Page

Figure 1: Developmental defects associated with STRAP mutation 3

Figure 2: STRAP protein domains and structural motifs 4

Figure 3: SMAD-dependent and SMAD-independent TGF-β signaling pathways (simplified) 8

Figure 4: STRAP promotes survival by regulating PDK1 and ASK1 signaling 13

Figure 5: Functional domains in c-Jun and v-Jun 19

Figure 6: The Ubiquitin Proteasome Pathway 22

Figure 7: Mechanisms regulating gradient sensing and cell polarization 27

Figure 8: Focal adhesion architecture and interactome (simplified) 31

Figure 9: Comparison of FAK and PYK2 functional domains 34

Figure 10: STRAP expression correlates with increased activation and expression of c-Jun 44

Figure 11: Effects of STRAP on the expression of various transcription factors 45

Figure 12: STRAP does not regulate c-Jun mRNA levels or JNK activation 47

Figure 13: STRAP stabilizes c-Jun by inhibiting ubiqutin-mediated proteolysis of c-Jun 49

Figure 14: STRAP prolongs the half-life of c-Jun and increases c-Jun expression in STRAP knockout fibroblasts 51

Figure 15: STRAP expression promotes cellular proliferation 53

Figure 16: STRAP regulation of cell growth pathways promotes cell autonomous and paracrine-stimulated growth 57

Figure 17: STRAP suppresses E-cadherin expression in MEFs 59

Figure 18: STRAP expression promotes cell migration 70

Figure 19: Loss of STRAP promotes cell adhesion and spreading 72

Figure 20: STRAP is required for persistent chemotaxis 74

Figure 21: Loss of STRAP does not inhibit AKT membrane recruitment 76

Figure 22: STRAP is required for efficient tail retraction during chemotaxis 77

Figure 23: Loss of STRAP alters the distribution of phospho-FAK Y397 78

Figure 24: Reintroduction of STRAP into the STRAP null MEFs increases cell migration 79

Figure 25: Ectopic STRAP in STRAP null fibroblasts reduces tail growth 81

Figure 26: STRAP regulates phospho-FAK localization 83

Figure 27: Biological effects of STRAP on the hallmarks of cancer 96

LIST OF TABLES

Page

Table 1. Regulators of c-Jun stability 23

Table 2. Integrin pairings and cognate ligands 29

Table 3: Epithelial and mesenchymal markers expressed by wild type and STRAP null MEFs 60

Table 4: Mouse models for assessing the effects of STRAP on AP-1 induced cell autonomous and paracrine growth 91

LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
AD	Activation domain
AP-1	Activating protein-1
ARP 2/3	Actin related protein 2/3
ASK1	Apoptosis Signal-Regulating Kinase 1
ATF	Activating transcription factor
B-MYB	B-myeloblastosis
BPH	Benign prostatic hyperplasia
cAMP	cyclic AMP
CAF	Cancer-associated fibroblasts
CaN	Calcineurin
CSK	COOH-terminal SRC kinase
CDK	Cyclin-dependent kinase
COP1	Constitutively photomorphogenic protein 1
DBD	DNA-binding domain
DET1	De-etiolated-1
DOCK	Dedicator or cytokinesis
ECM	Extracellular matrix
ELMO	Engulfment of cell motility
EWS	Ewing Sarcoma Protein
EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular Signal-Regulated Kinase
FAK	Focal adhesion kinase
FRET	Fluorescence resonance energy transfer
FBW7	F-box and WD repeat domain-containing 7
GSK3-β	Glycogen synthase kinase 3-beta
GM-CSF	Granulocyte Macrophage-colony stimulating factor
GPCR	G-protein coupled receptor
HB-EGF	Heparin binding-Epithelial-like growth factor
H_2O_2	Hydrogen peroxide
HNF4	Hepatocyte nuclear factor 4
I-SMAD	Inhibitory SMAD
IRES	Internal ribosome entry site
JDP	Jun dimerization protein
JNK	c-Jun N-terminal Kinase
kDa	kiloDaltons
KGF	Keratinocyte growth factor
MAPK	Mitogen activated protein kinase
MDM2	B-myeloblastosis
MEFs	Mouse Embryonic Fibroblasts
MEK	Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase
MET	Mesenchymal to epithelial transition
MKK1	MAP kinase kinase 1
MMP28	Matrix metalloproteinase 28
NDP	Nucleotide diphosphate
NLS	Nuclear localization signal

PAK1	p-21 activated kinase 1
PARP	Poly(ADP)-ribose polymerase
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent Kinase 1
PI3K	Phosphoinoside-3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 4,5-triphosphate
PH	pleckstrin homology
PKC	Protein kinase C
PYK2	Protein tyrosine kinase 2
R-SMAD	Receptor-associated SMAD
RACO-1	RING domain AP-1 co-activator-1
RT-PCR	Reverse transcription-PCR
S6K	S6 ribosomal kinase
SCID	Severe combined immunodeficiency
SMN	Survival of motor neurons
snRNP	small nuclear ribonucleoprotein
STAT3	Signal transducer and activator of transcription 3
STRAP	Serine Threonine Kinase-Receptor Associated Protein
TBRI	TGF-β receptor I
TBRII	TGF-β receptor II
TGF-β	Transforming Growth Factor-Beta
TRE	TPA-response element
Trx	Thioredoxin
WASP	Wiskott-Aldrich syndrome protein
UNRIP	Unr-interacting protein

Chapter I

INTRODUCTION

Since its initial discovery as an inhibitor of canonical Transforming Growth Factor-β (TGF-β) signaling, the serine-threonine kinase-receptor associated protein (STRAP) has been reported to regulate a wide array of biological processes and intracellular signaling pathways. At the organismal level, STRAP appears to have an essential role in development, as homozygous deletion of STRAP has been reported to cause morphological defects and embryonic lethality (Chen et al., 2006). At the cellular level, STRAP has been shown to affect cell survival and apoptosis through distinctly different mechanisms (Seong et al., 2005; Jung et al., 2010). While these studies suggest that STRAP is important for development and cellular homeostasis, deregulation of STRAP may drive pathological conditions. When considering the many roles of STRAP, it appears that most of the signaling pathways and biological functions of STRAP favor tumor promotion rather than tumor suppression (Reiner and Datta, 2011). In fact, it has previously been shown that STRAP confers oncogenic properties to cells in anchorage-independent growth assays and xenograft models of cancer (Halder et al., 2006). At the present time, a comprehensive understanding of the mechanisms by which STRAP influences normal and pathological cellular functions is unknown. Further investigation into the biological functions of STRAP will be critical not only to understanding the complexities of STRAP-mediated signal transduction but may also play an important role in the development of targeted anti-cancer therapeutics.

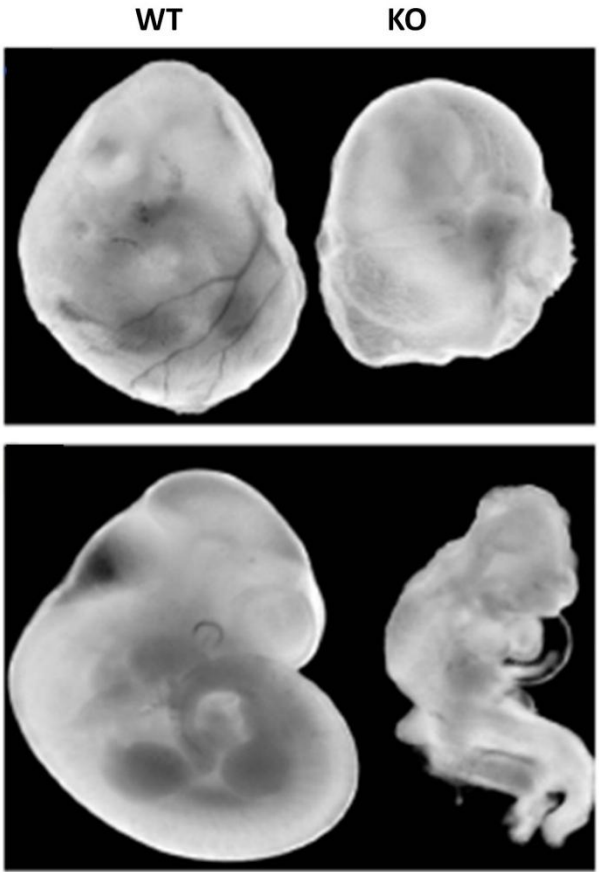
STRAP

Characterization of STRAP

STRAP is an evolutionarily conserved protein with an apparent molecular weight of 38 kilodaltons (kDa). STRAP, which was initially cloned from a mouse cDNA library, was reported to be broadly expressed in mouse (Datta et al., 1998) and human tissue (Matsuda et al., 2000). Computational analyses indicate that STRAP orthologues may be present in a wide range of species including chimpanzees, Drosophila, and Arabidopsis but functional redundancies have not been experimentally determined. The genomic loci for human STRAP has been mapped to chromosome 12p11-12 (Matsuda et al., 2000) whereas the murine orthologue is located on chromosome 6.

Although broad tissue expression is not sufficient to classify STRAP as a "housekeeping" gene, loss of STRAP has been reported to affect normal development. Homozygous deletion of murine STRAP by gene-trap mutagenesis is embryonic lethal between days E10.5 and E12.5 (Chen et al., 2004). Gross morphological examination of the STRAP knockout embryos revealed defects in angiogenesis, cardiogenesis, somitogenesis, and neural tube closure (Chen et al., 2004) (Figure 1). Additionally, overexpression of the drosophila STRAP homolog, pterodactyl, has been reported to cause defects in wing development (Khokhar et al., 2008).

At the structural level, STRAP is comprised of seven WD40 domain repeats that fold into a β-propeller structure (Figure 2). The WD40 repeat, also known as the transducin repeat, is comprised of approximately 40 amino acids with a conserved core sequence that terminates with a tryptophan-aspartic acid dipeptide. WD40 repeat proteins seem to support the formation of stable and transient protein complexes by

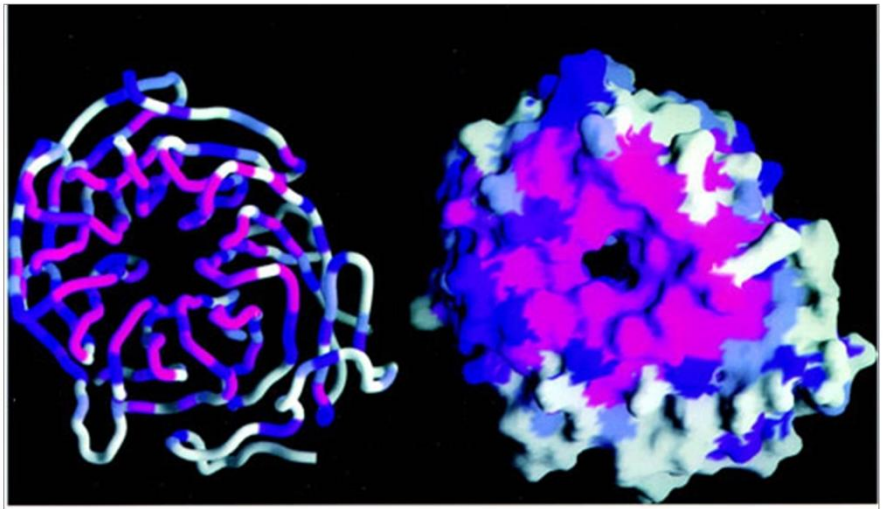


Reprinted from *Nat Genet* (2004) 36, 304-12 with permission from Nature Publishing Group

Figure 1. Developmental defects associated with STRAP mutation. A. E10.5 STRAP knockout mice exhibit defects in yolk sac vascularization (top), neural tube closure, embryonic turning, cardiogenesis, and somitogenesis (bottom). B. Homozygous insertion of a transposable element 3 bp upstream of STRAP (*pter*$^{2\text{-}4}$) causes mild wing vein defects. Excision derivatives (*exc*2-*exc*4) of the original *pter*$^{2\text{-}4}$ insertion exhibited more severe tubulogenesis defects.

A

B



Reprinted from *Embo J* (2000) 19,3016-27 with permission from Nature Publishing Group

Figure 2. STRAP protein domains and structural motifs A. The human STRAP gene encodes a 350 amino acid protein that contains 7 putative WD40 repeats. B. Four dimensional representation of a 7-bladed WD40 protein. The ribbon diagram (left) and globular structure (right) are viewed from a top-down perspective (structure shown is the WD40 domain of Tup1).

functioning as a molecular scaffold (Stirnimann et al., 2010). Apart from the WD40 repeat, STRAP does not contain any discernable structural or functional domains. Deletion of the C-terminal 56 amino acids has been reported to decrease TGF-β receptor (TBR)-dependent (TBR)-dependent phosphorylation of STRAP, but no loss of function has been associated with this truncated mutant (Datta and Moses, 2000).

Previous studies have shown that STRAP is subject to post-translational modification *in vivo*. It has been reported that the TGF-β receptors indirectly promote phosphorylation of STRAP within its C-terminus (Datta and Moses, 2000). However, STRAP C-terminal phosphorylation is dispensable to repression of Smad transcriptional activity as a STRAP C-terminal deletion mutant was still able to inhibit transactivation of a TGF-β reporter (Datta and Moses, 2000). Furthermore, phosphorylation of STRAP at T175 and S179 by ASK1 has been shown to promote complex formation with ASK1 (Jung et al., 2010). STRAP has also been reported to be a target of the small ubiquitin-related modifier 4 (SUMO4) in serum starved 293 cells (Guo et al., 2005), but the functional consequences of sumoylation have not been investigated.

Functionally, STRAP has been reported to affect multiple protein signal transduction pathways, protein stability, and gene expression. STRAP expression is required for normal developmental processes (Chen et al., 2004) and deregulation of STRAP has been implicated in tumorigenesis (Matsuda et al., 2000; Halder et al., 2006, Reiner and Datta, 2011). Although STRAP does not contain a nuclear localization signal (NLS), STRAP has been detected in the cytoplasm and nucleus by immunofluorescence (Halder et al., 2006). Taken together, these studies suggest that STRAP's biological functions and subcellular localization are dependent on its association with other cellular proteins.

SMAD-dependent and SMAD-independent TGF-β signaling

The transforming growth factor beta (TGF-β) family of proteins regulates a broad array of biological functions such as growth, differentiation, EMT, invasion, and apoptosis. The pleiotropic effects of TGF-β are achieved through activation of SMAD-dependent and SMAD-independent signaling pathways (Engel et al., 1998; Derynck and Zhang, 2003). Signaling through both pathways is initiated by an oligomeric complex comprised of the TGF-beta receptor (TBR) I and II homodimers (Figure 3). TGF-β ligand binding to a heteromeric complex comprised of TGF-β receptor I (TBRI) and receptor II (TBRII) dimers induces phosphorylation of the TBRI cytoplasmic domain. The activated TBRI/TBRII complex can then initiate SMAD-dependent signaling by phosphorylating the receptor associated SMADs (R-SMADS), SMAD-2 and SMAD-3 (Zhang et al., 1996; Nakao et al., 1997). The activated SMAD-2/3 complex then binds to SMAD-4 (Zhang et al., 1996; Nakao et al., 1997) and translocates to the nucleus where it associates with other transcriptional regulators to activate or suppress transcription from TGF-β target genes such as $p21^{Cip1}$, $p15^{INK4a}$, and PAI-1 (Engel et al., 1998). Although the specific gene expression profile induced by TGF-β/SMAD signaling varies according to cell type, the SMAD pathway is recognized as a tumor-suppressor pathway as SMAD activation is correlated with inhibition of cell cycle progression and induction of apoptosis.

In addition to activation of SMAD signaling, the TGF-β receptors can initiate Ras, p38 MAPK, JNK, RhoA, and PI3K signaling pathways. However, activation of the SMAD-independent pathways have been associated with early stage tumor progression, particularly in cells that harbour inactivating mutations in the TGF-β receptors, R-SMADs, and SMAD-4 (Elliott and Blobe, 2005). Although the mechanism by which the TGF-β receptors activate SMAD-independent signaling have not been well characterized, activation of these pathways has been associated with proliferation,

Figure 3. SMAD-dependent and SMAD-independent TGF-β signaling pathways (simplified). Smad-dependent and Smad-independent TGF-β signaling is initiated through a ligand-bound oligomeric complex of TBRI and TBRII receptors. Phosphorylation of the SMAD-2/-3 complex by the activated TGF-β receptors promotes SMAD-4 binding and nuclear translocation. The SMAD-2/-3/-4 complex then binds to SMAD-binding elements within the promoter regions of TGF-β target genes and functions in concert with other transcription factors to activate or inhibit gene expression. In addition to SMAD phosphorylation, the activated TGF-β receptors can activate RhoA as well as the JNK, p38 MAPK, and RAS MAP Kinase signaling pathways. Generally, activation of the SMAD pathway is associated with growth inhibition and tumor supression whereas SMAD-independent signaling is correlated with increased tumorigenicity.

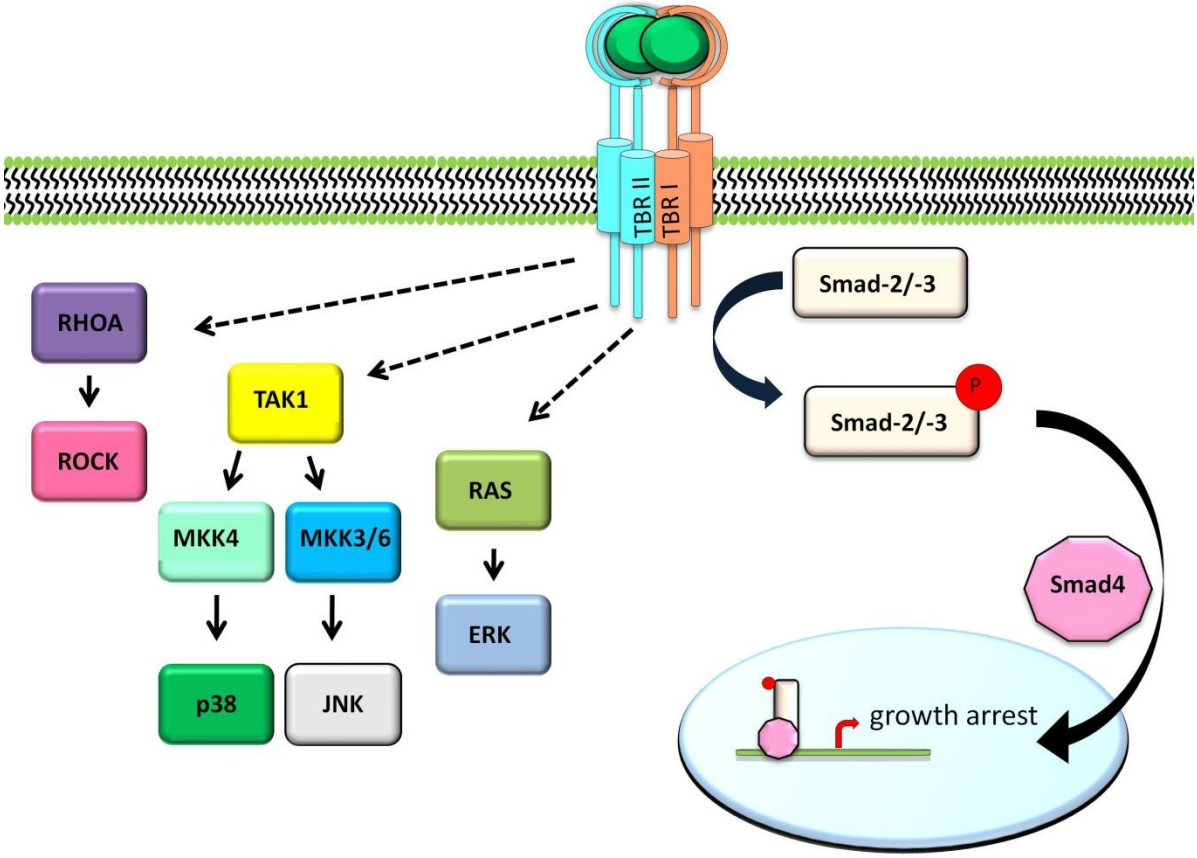


Figure 3. SMAD-dependent and SMAD-independent TGF-β signaling pathways (simplified).

epithelial to mesenchymal transition (EMT), cellular migration, and survival (Yan et al., 2002; Xie et al., 2004; Tavares et al., 2006; Wang et al., 2008; Kattla et al, 2008). Cooperation between the non-SMAD pathways has also been reported to induce malignant transformation in some cell lines (Erdogan et al, 2008; Fleming et al., 2009).

STRAP-mediated inhibition of TGF-β-signaling

STRAP was initially identified in a yeast two-hybrid screen for proteins that interact with TBRI (Datta et al, 1998). Overexpression of STRAP was shown to decrease TGF-β mediated transcription in reporter assays using constructs containing SMAD-binding elements in the promoter region. It was subsequently shown that STRAP forms a ternary complex with TBRI and SMAD-7, suggesting that the STRAP/SMAD-7 complex blocks receptor mediated phosphorylation of the R-SMADs (Datta and Moses, 2000).

Given that TGF-β SMAD signaling induces cell cycle arrest in normal cells, STRAP over-expression would be expected to confer resistance to the anti-proliferative effects of TGF-β. Indeed, wild type mouse embryonic fibroblasts (MEFs) exhibit a greater capacity for proliferation in the presence of TGF-β compared to STRAP null fibroblasts (Halder et al., 2006). Overexpression of STRAP in the lung adenocarcinoma cell line, A549, also results in a modest decrease in TGF-β induced growth inhibition relative to controls (Halder et al, 2006). At the present time, STRAP involvement in TGF-β SMAD-independent signaling remains unclear. It has been shown that overexpression of SMAD-7 in FET cells promotes activation of c-Jun in response to TGF-beta (Halder et al., 2005). Likewise, loss of STRAP appears to inhibit c-Jun phosphorylation and expression following TGF-β treatment (unpublished data). These findings may suggest that STRAP cooperates with SMAD-7 to promote c-Jun activation.

Interestingly, TGF-beta mediated activation of p38 MAPK is inhibited by STRAP (unpublished data), suggesting that STRAP employs different mechanisms for regulating TGF-β SMAD-independent signaling.

STRAP association with NM23-H1 affects the TGF-β and p53 signaling pathways

The NM23-H1 tumor suppressor belongs to the DNA-binding nucleotide di-phosphate (NDP) kinase family of proteins. NM23-H1 is regarded as a favorable prognostic indicator of poorly metastatic tumors due to its reduced expression in aggressive late stage tumors (Rosengard et al., 1989; Leone et al., 1991; Hennessy et al., 1991). In addition to its role as a metastasis inhibitor, NM23-H1 has also been reported to affect proliferation and differentiation of some cell lines (Lombardi et al, 2001; Yang et al., 2009). Currently, the mechanisms whereby NM23-H1 affects these biological pathways are unknown but efforts to identify NM23-H1 binding partners may explain the diverse functions of this protein.

With respect to the TGF-beta signaling, previous studies have shown that NM23H-1 can antagonize TGF-beta induced anchorage independent growth (Leone et al., 1991; Leone et al., 1993). However, data describing the effects of NM23-H1 expression on TGF-beta mediated growth suppression are contradictory. Early studies suggest that NM23-H1 potentiates SMAD-dependent signaling in HT29 colon cancer cells as antisense NM23 blocks TGF-beta induced growth arrest (Hsu et al., 1994). Contrary to these findings, a recent study reports that NM23-H1 association with STRAP reduces transactivation of SMAD-dependent reporter genes and attenuates TGF-beta mediated apoptosis and growth arrest (Seong et al., 2007). Subsequently, the NM23-H1/STRAP complex was shown to directly bind and stabilize p53 by dissociating murine double minute 2 (MDM2) (Jung et al., 2007). Although transactivation of some TGF-beta

responsive genes is dependent on p53 (Cordenonsi et al., 2003), the dual functions of STRAP/NM23-H1 appear to have conflicting effects on the canonical TGF-beta pathway. Like TGF-beta signaling, STRAP/NM23-H1 complex formation may yield different biological outcomes depending on the experimental context. Further investigation will be required to resolve these discrepancies.

STRAP promotes cell survival by regulating ASK1 and PDK1 signaling

The apoptosis signal-regulating kinase 1 (ASK1) is a MAPKK protein that functions as an upstream activator of the stress kinases, p38 and JNK. Under normal physiological conditions, ASK1 is maintained in an inactive state through its association with thioredoxin (Trx) or the adaptor protein, 14-3-3. However, exposure to various chemicals and oxidative stress leads to its phosphorylation, dissociation from Trx and 14-3-3, and apoptosis. It has been reported that STRAP associates with ASK1 and stabilizes the interaction between ASK1 and Trx or 14-3-3 (Jung et al., 2010). This interaction was shown to decrease hydrogen peroxide (H_20_2)-dependent ASK1 activation and apoptosis in HEK293 cells although H_20_2 treatment decreased physical association between STRAP and ASK1 (Jung et al., 2010). In addition to STRAP, PDK1 has recently been shown to negatively regulate ASK1-induced apoptosis (Seong et al., 2010). PDK1 is a serine-threonine kinase that phosphorylates a wide array of signal transduction proteins including protein kinase C (PKC), S6 ribosomal kinase (S6K), p21-activated kinase 1 (PAK1), and AKT. Activation of PDK1 signaling has been implicated in cellular proliferation, survival, migration, and invasion of tumor cells as well as breast cancer resistance to tamoxifen (Choi et al., 2008; Liu et al., 2009; Peifer et al., 2009; Westmoreland et al., 2009). Interestingly, STRAP has been reported to associate with PDK1 and promote phosphorylation of the PDK1 substrates S6K, AKT, and BAD (Seong

et. al, 2005). Reciprocally, PDK1 binding to STRAP augmented STRAP mediated inhibition of TGF-β signaling but exogenous TGF-β attenuates PDK1/STRAP complex formation (Seong et al., 2005). PDK1 was subsequently reported to inhibit TGF-β signaling through direct association with SMADs-2, -3, -4, and -7 in the absence of TGF-β (Seong et al., 2007). Furthermore, STRAP overexpression was shown to promote PDK1 complex formation with the SMAD proteins (Seong et al., 2007). These findings may suggest that negative regulation of TGF-β/SMAD signaling is achieved through STRAP's ability to function as a scaffold and recruit proteins into a macromolecular complex at the TGF-β receptors.

Taken together, these findings suggest that STRAP promotes viability through induction of the pro-survival PDK1 pathway and inhibition of pro-apoptotic ASK1 signaling (Figure 4). Although STRAP has been shown to interact with ASK1 and PDK1 separately, it has not been reported whether these three proteins can form a ternary complex. Further work will be required to fully elucidate the complex mechanisms by which these proteins coordinately regulate survival and apoptosis.

STRAP regulates cell growth signaling pathways

Overexpression of STRAP has been reported to induce extracellular signal-regulated kinase (ERK) activation in various cell lines (Matsuda et al., 2000; Halder et al., 2006). Increased activation of ERK in Mv1Lu cells was coincident with increased pRb phosphorylation and decreased $p21^{CIP1}$ expression (Halder et al., 2006). Importantly, STRAP regulation of these signaling proteins was independent of exogenous TGF-β treatment. Although ERK activation was not shown to directly affect pRb phosphorylation, these findings may suggest that STRAP can affect cellular proliferation signaling pathways in a TGF-β-independent manner.

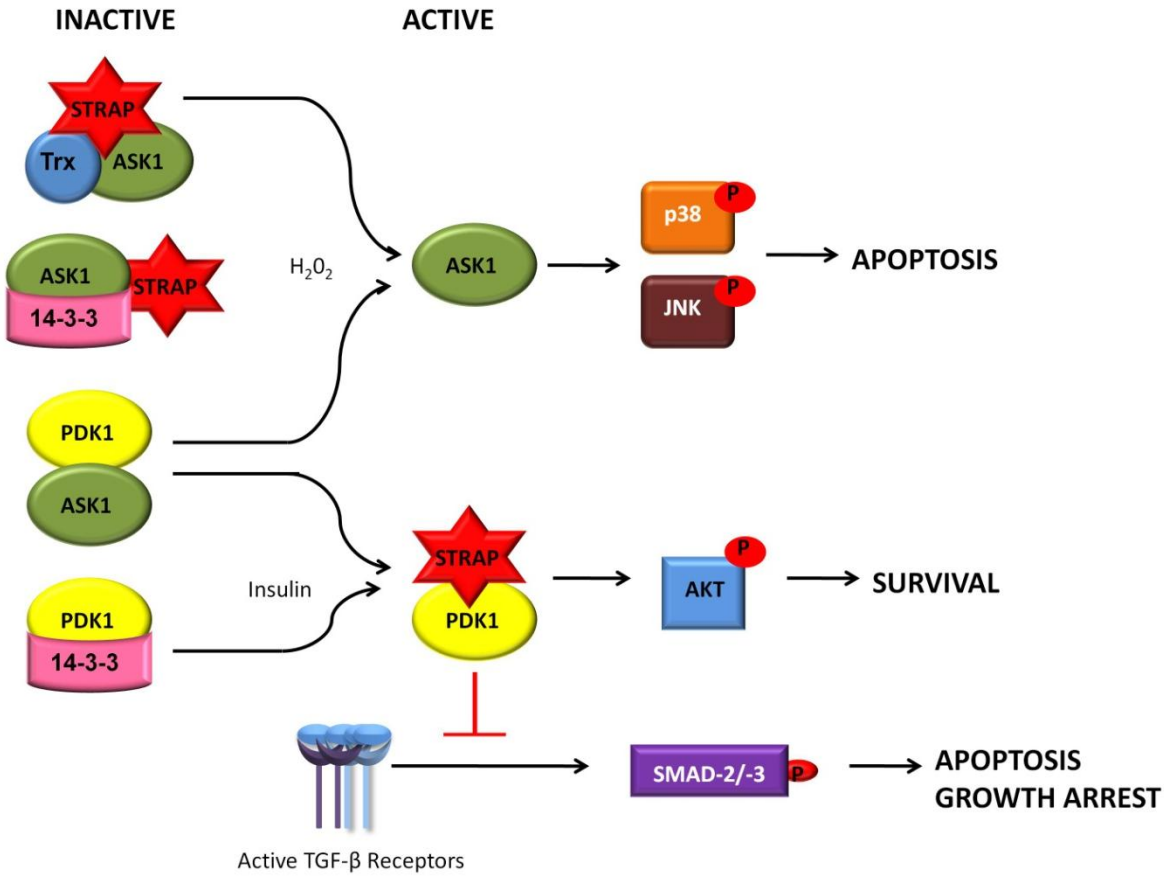


Figure 4. STRAP promotes survival by regulating PDK1 and ASK1 signaling. PDK1 is maintained in an inactive conformation through association with 14-3-3 or ASK1. Insulin treatment promotes PDK1 dissociation from its negative regulators and association with STRAP. The STRAP-PDK1 complex promotes cell survival through phosphorylation of PDK1 substrates and inhibition of SMAD activation. ASK induces apoptosis in response to oxidative stress by phosphorylating the stress-induced kinases, p38 MAPK, and JNK. ASK1 binding to PDK1, Trx, and 14-3-3 inhibits phosphorylation of ASK-1 substrates, thereby promoting cell survival. STRAP inhibits ASK1-mediated apoptosis by stabilizing complex formation between ASK1 and Trx or 14-3-3.

. The role of STRAP in transcription, mRNA splicing, and cap-independent translation

Apart from its role in the regulation of protein signaling pathways, several studies have reported a role for STRAP in the regulation of gene expression. STRAP has been shown to associate with the Ewing Sarcoma protein, EWS, and attenuate EWS/p300 dependent transcription of hepatocyte nuclear factor 4 (HNF4) reporter constructs (Anumanthan et al., 2006). HNF4 is a nuclear receptor that regulates tissue-specific differentiation and proliferation. Stable expression of HNF4 can restore a differentiated epithelial phenotype to hepatoma cells through induction of cytokeratins and E-cadherin (Spath et al., 1998). Furthermore, HNF4-alpha expression can decrease proliferation and alter cellular morphology of 293T cells (Lucas et al., 2005). These findings may suggest that STRAP can confer oncogenic properties to cells that require HNF activity for the maintenance of cellular differentiation and cell cycle progression. A recent study reported that STRAP functions as a transcriptional cofactor for Sp1-mediated activation of the matrix metalloproteinase 28 (MMP28) (Swingler et al., 2010). This finding is particularly interesting because this study provides the first functional account of STRAP activity within the nuclear compartment and may point to a role for STRAP in tumor metastasis. In addition to EWS and Sp1, it has recently been shown that STRAP binds to the B-myeloblastosis (B-MYB) transcription factor (Seong et al., 2010). B-MYB has been implicated in cell cycle progression, inhibition of apoptosis, and tumorigenesis (Sala 2005). While the scope of this study was limited to the effects of B-MYB on STRAP-mediated inhibition of SMAD activation (Seong et al. 2010), STRAP associations generally exhibit reciprocity so it is plausible that STRAP can affect transactivation of B-MYB target genes.

STRAP may also regulate gene expression by affecting pre-mRNA splicing and cap-independent translation. Pre-mRNA splicing is catalyzed by small nuclear ribonucleoproteins (snRNPs), which are assembled into a spliceosome by a multi-protein complex called the survival of motor neuron (SMN) complex (Pellizzoni et al., 1998). It has been shown that STRAP is a component of the SMN complex and that immunodepletion of STRAP markedly reduces the assembly of snRNPs (Carissimi et al., 2005). Furthermore, nuclear accumulation of the SMN complex was observed following STRAP knockdown, suggesting that incorporation of STRAP is necessary for cytosolic localization (Grimmler et al., 2005). Although there is no direct link between proper SMN complex function and cancer, patients carrying homozygous mutations in the SMN1 gene develop the progressive neuromuscular degenerative disease, spinal muscular atrophy (Rodrigues et al., 1995; Cobben et al., 1995). With respect to protein translation, previous studies aimed at identifying cellular components that initiate internal translation of rhinoviral RNA led to the discovery of a 38 kDa WD40 repeat protein complexed with UNR (Hunt et al., 1999), a cytoplasmic RNA-binding protein that has been implicated in the cap-independent translation of various proteins. This protein, termed UNRIP (UNR- interacting protein), is an alias for STRAP. Although STRAP did not appear to play a functional role in the initiation of viral translation, it has been reported that UNRIP/STRAP can function in concert with other cellular proteins to increase c-MYC translation *in vitro* through utilization of an internal ribosomal entry site (IRES) (Evans et al., 2003). Because c-MYC is a recognized oncogene, it is possible that overexpression of STRAP can promote tumor growth by up-regulating c-MYC when the necessary translational cofactors are present.

STRAP expression is associated with mesenchymal morphology in MEFs

The epithelial-to-mesenchymal transition (EMT) refers to a process by which normal and neoplastic cells down-regulate expression of junctional epithelial markers and upregulate expression of mesenchymal genes. EMT is often gauged by a morphological switch that suggests an increased capacity for cellular migration. Recently, it has been shown that STRAP knockout in mouse embryonic fibroblasts causes cells to adopt a metastable phenotype characterized by the expression of both mesenchymal and epithelial lineage markers, such as E-cadherin (Kashikar et al., 2010). Importantly, enforced expression of STRAP in knockout MEFs abrogated E-cadherin expression and restored the mesenchymal morphology to the fibroblasts (Kashikar et al., 2010). Although this study suggests that STRAP is involved in the specification of a mesenchymal cell fate, the relationship between morphology and motility remains to be determined.

Clinical significance and targeted inhibition of STRAP

Preliminary studies suggest that STRAP overexpression may be relevant to the development of various cancers. The up-regulation of STRAP has been reported in lung and colorectal tumor tissue samples analyzed by western blot and immunohistochemical staining (Matsuda et al., 2000, Halder et al., 2006). In a much larger clinical study of colorectal cancer specimens, STRAP overexpression was detected in 70.7% of specimens analyzed (Kim et al., 2007). Although there was no clinical evidence to suggest that STRAP was correlated with disease stage or survival in this study, it has been reported that STRAP amplification predicts disease outcome in response to chemotherapeutic regimens. Specifically, colorectal cancer patients whose tumors contained increased STRAP copy numbers exhibited decreased overall survival when

adjuvant 5-Fluorouracil (5-FU) therapy was administered whereas patients without STRAP amplification benefited from 5-FU treatment (Buess et al., 2004). It is not yet clear why STRAP copy number affects chemotherapeutic response and survival but it is important to note that amplification of other genes on chromosome 12p was not reported in this study. Gains in chromosome regions proximal to the STRAP locus has been reported in teratomas and basal-like breast cancer so it's possible that amplification of nearby genes may also account for the observed differences in these patients (Henegariu et al., 1998; Poulos et al., 2006; Han et. al, 2008; Natrajan et al., 2009).

Further work will be needed to clarify the role of STRAP in carcinogenesis, but clinical data reported thus far indicate that there is a strong association between STRAP overexpression and cancer. In vitro studies on the biological functions of STRAP suggest that it can modulate various oncogenic signaling pathways while in vivo animal studies indicate that STRAP expression promotes tumor formation (Halder et al., 2006). Taken together, it is likely that STRAP influences the pathways and processes that drive cancer progression and should not simply be regarded as a biomarker. As such, STRAP may be relevant target for the development of anti-cancer therapeutics. At the present time, there are no reports of any STRAP inhibitors in clinical use or development. However, it has recently been shown that Pateamine A, a product of marine sponges, can bind to STRAP and the eukaryotic translation initiation factor eIF4A (Low et al., 2005; Low et al., 2007). Although the effects of Pateamine A association with STRAP have not been characterized, Pateamine A and its analogs have already been shown to block eukaryotic translation (Low et al., 2007) and proliferation of cancer cells (Kuznetsov et al., 2009).

The c-JUN proto-oncogene

c-Jun, a component of the activating protein-1 (AP-1) transcription factor, is recognized as an immediate early gene due to its rapid induction in response to various growth factors (Lamph et al., 1998). c-Jun predominantly localizes to the nucleus and exhibits a high degree of conservation among chordates. Structurally, the c-Jun protein contains multiple functional domains (Figure 5). c-Jun contains two activation domains (AD) that facilitate transcription of AP-1 target genes and an N-terminal delta domain (δ), which interacts with the c-Jun activating protein, c-Jun N-terminal kinase (JNK). At the C-terminus, c-Jun contains a DNA-binding domain (DBD) and a leucine zipper dimerization domain. The viral counterpart to c-Jun, v-Jun, lacks the N-terminal δ domain which renders v-Jun expressing cells unresponsive to regulation by JNK. In addition to the deletion of the δ domain, v-Jun contains many amino acid substitutions and deletions that promote v-Jun protein stability.

As a constituent of AP-1, c-Jun has been reported to heterodimerize with Fos, activating transcription factor (ATF), or the Jun dimerization protein (JDP) family of proteins. Interestingly, c-Jun homodimers can also form low affinity DNA binding complexes (Ryseck and Bravo, 1991; Carrillo et al., 2009), although heterodimers are preferentially formed under physiological conditions (Carrillo et al., 2009). Depending on the specific c-Jun binding partner, AP-1 can induce or inhibit expression of genes containing the TPA-DNA response element (TRE; 5'-TGAG/CTCA), accounting for the diverse biological effects of AP-1 signaling.

Among the best characterized AP-1 complexes is the c-Jun/c-Fos heterodimer. c-Jun/c-Fos has been shown to regulate cellular proliferation and survival through

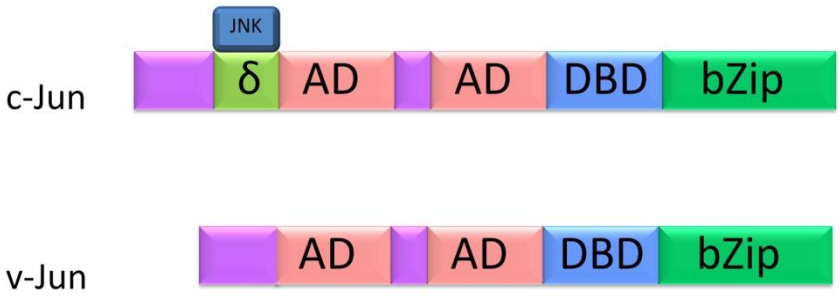


Figure 5. Functional domains in c-Jun and v-Jun. c-Jun, a component of the AP-1 transcription factor, is comprised of multiple functional domains. The C-terminus of c-Jun contains a DNA-binding domain (DBD) that directly interacts with DNA regions containing the TPA-response element. The basic leuicine zipper (bZIP) domain mediates dimerization with FOS, ATF, or JDP proteins and facilitates interactions with DNA. The activation domains (AD) in c-Jun are required for transcription of AP-1 target genes. The delta (δ) domain recruits the serine/threonine kinase, JNK, which promotes AP-1 activity and c-Jun stabilization through phosphorylation of S63 and S73 in the N-terminus of c-Jun. The viral counterpart to c-Jun, v-Jun, contains many genetic alterations that confer oncogenic properties to the protein. Loss of the δ domain renders v-Jun exempt from JNK-mediated activation. Additional point mutations and interstitial deletions within the v-Jun coding sequence have also been reported to increase protein stability.

transactivation of a wide array of genes including cyclin D1, KGF, HB-EGF and suppression of p53, $p21^{CIP1}$, and $p16^{INK4A}$ (Shaulian and Karin, 2002). Although c-Jun expression is important for normal cell growth, aberrant c-Jun activity has been implicated in oncogenesis (Zhang et al., 2007; Synder et al., 2009; Mishra et al., 2010). As such, c-Jun activity and expression are subject to stringent regulation through control of transcription, protein synthesis and post-translational modification. Expression of c-Jun is induced in the G1 phase of the cell cycle and undergoes rapid decline after entry into S phase (Rysek et al., 1988; Carter et al., 1991). At the post-transcriptional level, it has been reported that c-Jun must be phosphorylated at S63 and S73 by JNK in order to form transcriptionally active AP-1 heterodimers (Derijard et al, 1994; Minden et al.,

1994). It has also been shown that MEK/ERK signaling promotes AP-1 activation indirectly through induction of c-Jun (Lopez-Bergami et al., 2007), but JNK-independent mechanisms for c-Jun activation were not reported. However, a recent study by Davies et al. indicates that MEK1 stabilization of RING-domain containing protein-1 (RACO-1) promotes RACO-1 association with c-Jun and AP-1 activity independent of JNK-mediated phosphorylation (Davies et al., 2010). Taken together, these studies may suggest that deregulation of MAPK signaling pathways promotes tumorgenesis through convergence on AP-1 activitation.

The ubiquitin proteasome and c-Jun stability

In addition to regulation of c-Jun induction and activity, c-Jun expression is regulated through protein degradation. Protein turnover in the cytosol and nucleus of most eukaryotic cells is regulated by a large multi-protein complex called the proteasome. Proteins targeted for destruction by the proteasome undergo a post-translational modification in which a small protein, ubiquitin, is covalenty attached to lysine residues within the substrate. The process of ubiquitylation involves multiple steps, each executed by three separate enzymes (Figure 6). First, a ubiquitin molecule is activated by an E1 ubiquitin-activating protein and transferred to an active site within the E1 in an ATP-dependent manner. Next, the ubiquitin is transferred to an E2 ubiquitin conjugating enzyme. The E3 ubiquitin ligase protein can then directly mediate transfer of the ubiquitin from the E2 to the substrate or functions as an intermediate, transferring the ubiquitin to itself before modifying the target protein.

Several studies have experimentally determined that the half-life of c-Jun is approximately 1.6 hours (Wei et al., 2005; Gao et al., 2006). c-Jun protein stability can be directly regulated by multiple classes of proteins, including kinases, phosphatases, and ubiquitin E3 ligases (Table 1). Phosphorylation of c-Jun by JNK at S63 and S73 has

been reported to increase the stability of c-Jun (Fuchs et al., 1994; Sabapathy et al., 2004). Calcineurin (CaN)-mediated dephosphorylation of c-Jun at S243 has also been reported to stabilize c-Jun (Huang et al., 2008). Conversely, post-translational modification of other residues in c-Jun has been reported to destabilize c-Jun. The COOH-terminal SRC kinase (CSK) promotes c-Jun degradation through phosphorylation of c-Jun at Y26 and Y170 (Zhu et al., 2006). However, phosphorylation of Y170 by the tyrosine kinase c-ABL decreases ubiquitylation and degradation of c-Jun by inhibiting ITCH binding (Gao et al., 2006). These conflicting reports may suggest that some E3 ligases require phosphorylation of multiple residues within the target protein for substrate recognition and binding. It has also been shown that glycogen synthase kinase 3-beta (GSK3β) -dependent phosphorylation of c-Jun at T242 is essential for F-box and WD repeat domain-containing 7 (FBW7) mediated ubiquitylation of c-Jun (Wei et al., 2005). In addition to the FBW7 and ITCH, both map kinase kinase 1 (MKK1) and the De-etiolated-1 (DET1)/constitutively photomorphogenic 1 (COP1) complex have also been reported to exhibit E3 ligase activity towards c-Jun (Wertz et al., 2004; Xia et al., 2007). Although each ligase recognizes a unique sequence in c-Jun, the apparent redundancy in target selection underscores the importance of regulating c-Jun expression.

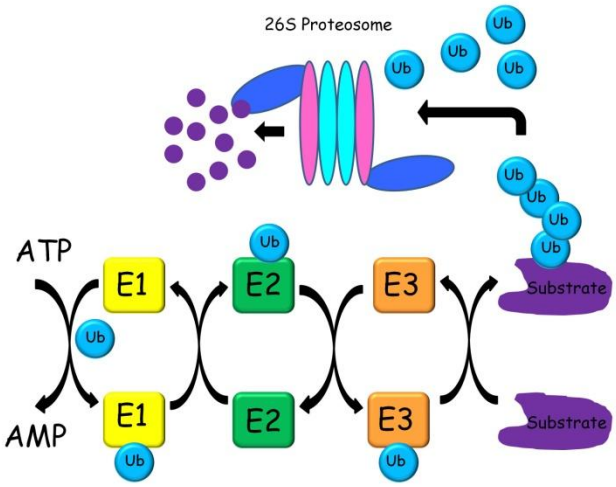


Figure 6. The Ubiquitin-Proteasome Pathway (simplified). Proteins that are marked for destruction by the 26S Proteasome are post-translationally modified through the covalent attachment of ubiquitin molecules. The process of attachment involves three distinct steps in which a ubiquitin molecule is shuttled between a ubiquitin-activating protein (E1) and a ubiquitin-conjugating protein (E2) before a ubiquitin ligase (E3) mediates transfer of the ubiquitin molecule to a lysine residue within the target protein. After attachment of one ubiquitin molecule, addition ubiquitins are attached to the preceding ubiquitin molecule to form a poly-ubiquitin chain. The ubiquitylated substrate can then enter the proteasome where it undergoes degradation.

Table 1. Regulators of c-Jun stability

PROTEIN	ACTIVITY	PROTEIN RESIDUES	c-Jun STABILITY	REFERENCE
JNK1	Kinase	S63, S73 (m,h)	increases	*Oncogene* (1996) **13**, 1531-1535 Mol Cell (2004) **15**, 713-725
CSK	Kinase	Y26, Y170 (m,h)	decreases	*Cancer Res* (2006) **66**, 5729-36
c-Abl	Kinase	Y170 (m,h)	increases	*J Biol Chem* (2006) **281**, 29711-29718
CaN	Phosphatase	S243 (h), S246 (m)	increases	*Oncogene* (2008) **27**, 2422-2429
FBW7	E3 ligase	T239/S243 (h), T242/S246 (m)	decreases	*Cancer Cell* (2005) **8**, 25-33
ITCH	E3 ligase	Y170 (m,h)	decreases	*J Biol Chem* (2006) **281**, 29711-29718
MKK1	E3 ligase	NR	decreases	*Mol Cell Biol* (2007) **27**, 510-517.
COP1/DET 1	E3 ligase	227 D/E-E-x-x-x-V-P 233 (h) 230 D/E-E-x-x-x-V-P 236 (m)	decreases	*Science* (2004) **303**, 1371-1374

Directed Migration

Directional migration is a complex process by which a cell migrates towards or away from a stimulus. Thus far, different types of directed migration have been described. Chemotaxis is a form of motility induced by a soluble chemical gradient whereas haptotaxis involves migration up a gradient of substratum-immobilized ligand. In adherent cell types, the process of directed migration has been divided into several distinct steps. Initiation of cell motility involves actin-driven membrane protrusion at the leading edge and the formation of nascent attachments to the underlying matrix proteins (Sheetz et al., 1999). Actin-myosin contractility and adhesion disassembly at the lagging edge of the cell facilitate forward movement by permitting retraction of the cell body and tail (Lauffenberger and Horowitz, 1996; Cheresh et al., 1999). Furthermore, endocytic recycling of adhesion receptors from the back to the front of the cell support continued movement (Pierini et al, 2000).

At the molecular level, cell migration requires the coordinate activity of multiple signaling pathways including focal adhesion kinase (FAK), SRC, phosphoinositide-3 kinase (PI3K), and the Rho GTPase family. Importantly, activation of these signaling pathways are spatially restricted within the cell to permit the requisite polarization that generates the leading edge during motility.

Regulators of gradient sensing and cell polarity

Cell polarization is a complex process whereby cells undergo changes to their cell shape through rearrangement of their actin cytoskeleton. The process of polarization establishes a physical asymmetry with a defined leading and lagging edge. In addition to the morphology changes, polarized cells asymmetrically redistribute

phospholipid signaling molecules in order to recruit the actin polymerization machinery to the leading edge.

Studies on the social amoebae, *Dictyostelium discoideum*, and neutrophils have significantly informed our current understanding of the mechanisms regulating both gradient sensing and polarization. In the presence of a chemoattractant, *Dictyostelium* becomes polarized with a pseudopod extending in the direction of the highest concentration of gradient. *Dictyostelium* also exhibit the capacity to reorient in response to changes in the position of the gradient. The "local excitation-global inhibition" model of gradient sensing suggests that local changes in G-protein coupled receptor (GPCR) ligand occupancy provide the upstream signal that regulates directionality of movement (Parent and Devreotes, 1999) (Figure 7A). In *Dictyostelium* and mammalian cells, GPCRs are uniformly distributed around the cell perimeter. In the absence of a stimulus, there is limited steady-state activation of GPCRs. Fluorescence resonance energy transfer (FRET) analysis of GPCR α and βγ binding has shown comparable levels of bound and unbound complexes in *Dictyostelium* before exposure to cyclic AMP (cAMP) (Xu et al., 2005). However, application of a cAMP gradient leads to increased activation of receptors on the side of the cell facing the gradient source, thus generating the localized excitatory signal that initiates downstream cellular events, including polarization and directed migration (Xu et al., 2005).

GPCR activation has been reported to promote cell polarization through Ras-mediated activation of PI3K signaling (Li et al., 2000; Funamoto et al., 2001, Funamoto et al., 2002). At the leading edge, PI3K converts the membrane phospholipid PIP2 to PIP3, which induces actin cytoskeleton remodeling through activation of AKT-RAC/CDC42 signaling. In both *Dictyostelium* and neutrophils, PI3K has been reported to localize to the leading edge of polarized cells, whereas PTEN is distributed along the

lateral and posterior axes of the cells (Funamoto et al., 2002) (Figure 7B). PI3K recruitment to the membrane occurs through direct interaction with the p101 non-catalytic subunit of $G_{\beta\gamma}$ (Brock et al., 2002).

Recruitment of AKT to the leading edge is not dependent on polarization as inhibition of actin polymerization does not prevent AKT membrane localization (Servant et al., 2000). However, PIP3/AKT signaling is dependent on GPCR signaling as inhibition of G-proteins with pertussis toxin abrogates AKT-PH membrane recruitment (Servant et al., 2000). These studies suggest that the redistribution of phospholipid signaling proteins is an intermediate step between GPCR activation and cell polarization. However, some studies suggest that the requirement of PI3K for chemotaxis is not absolute. The PI3K 1/2/3 triple knockout *Dictyostelium* are less polarized but can migrate with a reduced speed towards steep gradients of cAMP (Takeda et al., 2007). Furthermore, engulfment of cell motility engulfment of cell motility (ELMO)-dedicator of cytokinesis (DOCK) mediated activation of RAC can promote cell polarization and chemotaxis of HL-60 cells following PI3K inhibition *in vitro* (Sai et al., 2008). Interestingly, neutrophil migration to laser-induced wounds in zebrafish is PI3Kγ-dependent as activation of RAC is insufficient to rescue the anterior polarization and motility defects observed in these cells (Yoo et al., 2010). This study may suggest that PI3K-induced AKT recruitment is necessary for proper cell polarization and chemotaxis *in vivo* but further work will be needed to address the discrepancies observed in different model systems and cell types.

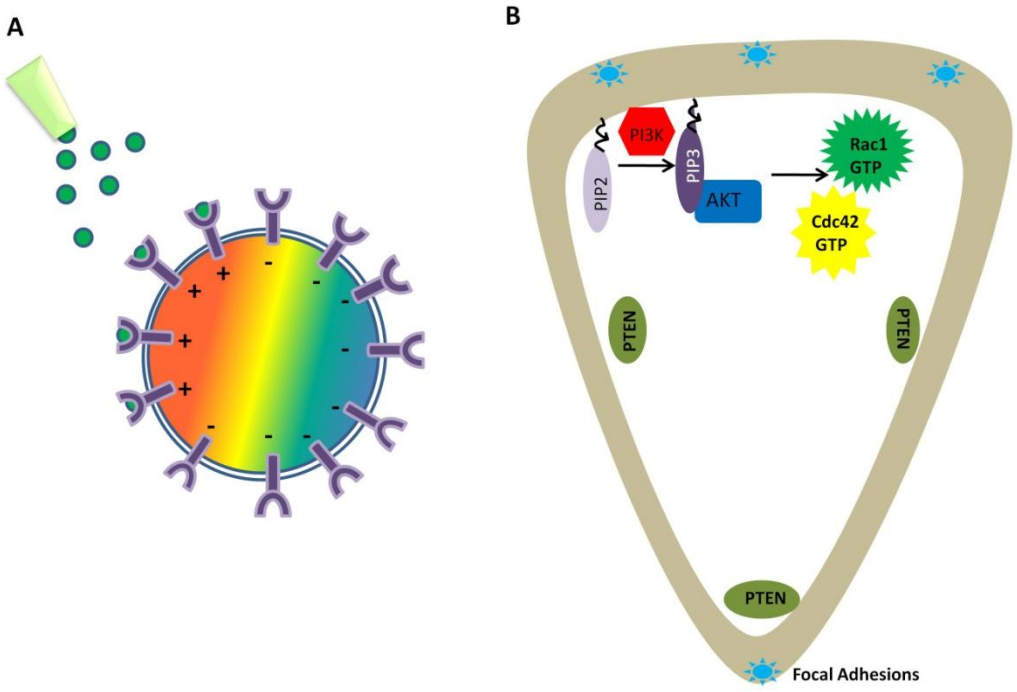


Figure 7. Mechanisms regulating gradient sensing and cell polarization. A. Gradient sensing in eukaryotes is dependent on localized activation of G protein-coupled receptors. Application of a gradient increases receptor occupancy on the side of the cell closest to the gradient source, leading to localized signal transduction events proximal to the activated GPCRs. This spatially restricted signaling initiates an asymmetric redistribution of proteins within the cell such that regulators of cell polarization and actin polymerization are concentrated at sites of GPCR activation. B. Polarized cells are characterized by the asymmetric distribution of signaling molecules. GPCR activation promotes PI3K recruitment to the leading edge through direct interaction with the $G_{\beta\gamma}$ subunit. PI3K subsequently converts the PIP2 phospholipid to PIP3, which recruits AKT to the leading edge by associating with the AKT pleckstrin homology domain. PTEN phosphatase is spatially restricted to the lateral sides and lagging edge of polarized cells to inhibit dephosphorylation of PIP3. AKT induces membrane protrusions at the leading edge by activating the actin cytoskeleton remodeling proteins, RAC1 and CDC42.

Cell adhesion and motility

Cell adhesion to extracellular matrix proteins is regulated by a large multi-protein complex that anchors the actin cytoskeleton to the underlying substratum. Nascent attachments to the ECM are generally referred to as focal complexes. In migrating cells, focal complexes are formed at the edges of the lamellapodia and have been reported to contain integrins, talin, vinculin, FAK, tensin, and α-actinin (Laukaitis et al., 2001; Zaidel-Bar et al., 2003). The resident integrin receptor, comprised of a pairing between various alpha and beta subunits, varies according to the protein composition of the matrix (Table 2). These focal complexes either disassemble or undergo a maturation process involving the incorporation of additional proteins to form focal adhesions (Zaidel-Bar et al., 2003) (Figure 8A).

Proteomic analysis of purified focal adhesions has recently identified over 900 different focal adhesion proteins (Kuo et al., 2011). Although protein-protein interactions within the focal adhesion have been identified for some proteins (Figure 8B), the complex network comprising the focal adhesion interactome and the signaling modules within the adhesions have yet to be fully realized. Early investigators discovered that focal adhesion formation occurs through bidirectional "signaling" through integrin receptors. Integrin binding to ECM enables cells to remodel their actin cytoskeleton in response to mechanical cues from the underlying matrix. The mechano-transduction represents "outside-in" signaling and can cause adaptive response due to changes in substratum rigidity. Conversely, talin association with the integrin cytoplasmic tail promotes "inside-out" signaling by inducing a conformational switch in the integrin receptor that renders the integrin permissive for ligand binding (Anthis et al., 2009). Apart from integrin activation, talin directly associates with the adaptor protein vinculin, which functionally links the focal adhesion to the actin cytoskeleton (Humphries et al.,

β subunit	α subunit	Ligand
β1	α1	collagen
β1	α2	collagen
β1	α3	laminin
β1	α4	fibronectin
β1	α5	fibronectin
β1	α6	laminin
β1	α7	laminin
β1	α8	fibronectin, vitronectin
β1	α9	tenascin-C
β1	α10	collagen
β1	α11	collagen
β1	αv	fibronectin, vitronectin
β3	αII	fibrinogen, fibronectin
β3	αv	vitronectin, fibronectin
β4	α6	laminin
β5	αv	vitronectin
β6	αv	fibronectin
β8	αv	vitronectin

β2 & β7 =leukocyte specific

Adapted from Cell Tiss Res (2010) 339, 267-80

Table 2. Integrin pairings and cognate ligands

Figure 8. Focal adhesion architecture and interactome (simplified). A. Focal adhesion complexes are formed at sites of integrin binding to extracellular matrix. Active integrin receptors recruit FAK through direct association with talin and paxillin. Autophosphorylation of FAK leads to SRC recruitment and full activation of FAK. FAK/SRC signaling regulates focal adhesion dynamics and membrane protrusions through phosphorylation of a wide array of effector proteins. B. Interactome map of focal adhesion components. Stable components within focal adhesions are contained within the shaded green box while non-resident modulators of focal adhesion proteins are placed outside the box.

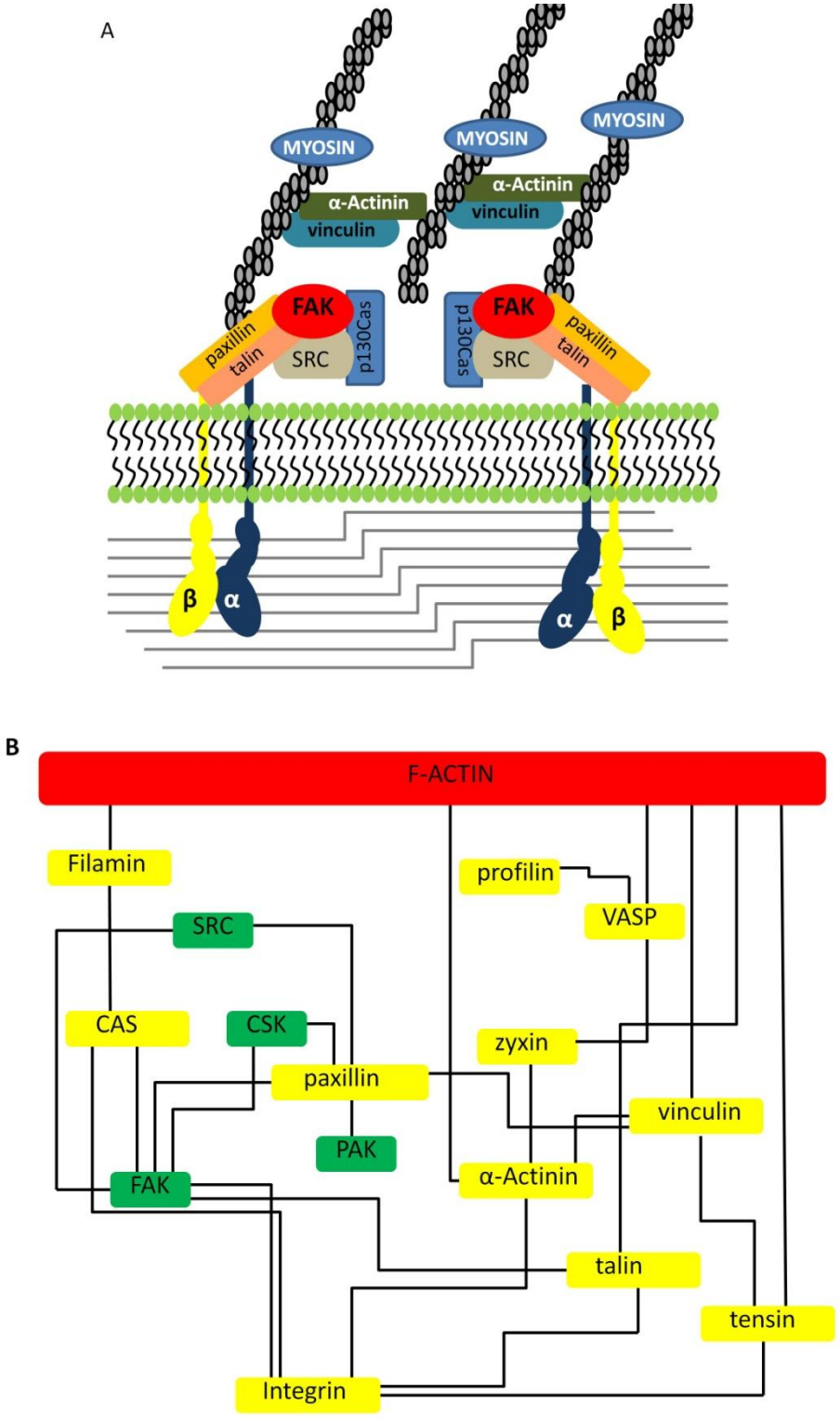


Figure 8. Focal adhesion architecture and interactome (simplified).

2007). Vinculin has also been reported to bind paxillin (Wood et al., 1994), which supports focal adhesion growth through recruitment of actin-binding proteins and various enzymes (Schaller, 2001). The tyrosine kinase, FAK, is recruited to cell-matrix adhesions through association with paxillin (Hildebrand et al., 1995; Tachibana et al., 1995; Hayashi et al., 2002) and talin (Chen et al., 1995). FAK then promotes focal adhesion enlargement by recruiting additional proteins such as SRC and p130Cas to the focal adhesion complex to generate the central signaling module of focal adhesions. Taken together, these studies highlight the complex interactions that support focal adhesion assembly. These interactions generate a stable link between the integrin-ECM complex and the actin cytoskeleton, and serve as a large signaling conduit that allows cells to respond to mechanical and chemical stimuli in the extracellular environment

Focal adhesion kinase signaling pathways

FAK is a cytoplasmic protein tyrosine kinase that mediates cell adhesion and cell motility through activation of actin cytoskeleton remodeling proteins. FAK enzymatic activity and interactions with its various effectors are modulated through phosphorylation of key residues within FAK as well as through discrete functional domains within FAK (Figure 9) (Mitra et al., 2005). After recruitment to the focal complexes, activation of FAK is initiated by autophophorylation of Y397, which creates a docking site for c-SRC (Schaller et al., 1994). SRC fully activates FAK through phosphorylation of Y596, Y597, Y861, and Y925 residues (Mitra et al., 2005).

The biological significance of FAK signaling was initially gleened from *in vitro* studies using fibroblasts isolated from FAK knockout embryos. Loss of FAK expression caused fibroblasts to adopt a round morphology characterized by an increased number of small focal adhesions and decreased haptotaxis towards fibronectin (LLić et al.,

1995). Reintroduction of full length FAK rescued the morphology and motility defects in fibroblasts (Sieg et al, 1999), supporting the initial finding that FAK signaling promotes motility. The FAK-related kinase, protein tyrosine kinase 2 (PYK2), exhibits >60% sequence homology to FAK and is predicted to contain identical functional domains (Figure 9). However, endogenous PYK2 does not localize to focal adhesions (Sieg et al., 1998). As such, overexpression of PYK2 fails to rescue the motility defect in FAK -/- fibroblasts (Sieg et al., 1998), whereas a PYK2 chimera containing the FAK C-terminal FAT domain rescues both the fibroblastoid morphology and haptotaxis defect (Klingbeil et al., 2001). Collectively, these studies suggest that FAK signaling within the focal adhesion complex is necessary for motility.

The effects of FAK on cell adhesion, spreading, and motility are achieved through its interactions with a wide array of effector proteins. FAK recruitment and phosphorylation of p130Cas promotes membrane protrusions and motility through downstream activation of Rac1. In addition to p130CAS, FAK has been reported to promote Rac1-mediated cell spreading through activation of the RAC/CDC guanine nucleotide exchange factor (GEF), βPIX (Chang et al., 2007). Previous studies suggest that FAK promotes actin related protein 2/3 (ARP 2/3) activation and membrane protrusions through phosphorylation-dependent regulation of neuronal Wiskott-Aldrich syndrome protein (N-WASP) subcellular localization (Wu et al., 2004). Interestingly, FAK has also been reported to promote cell spreading by direct association with ARP3 during the early phases of cell attachment to fibronectin (Serrels et al., 2007). FAK mediated inhibition of RhoA has been reported to promote focal adhesion formation and cell spreading (Ren et al., 2000). Subsequently, it was shown that FAK forms a complex with p120Ras GTPase-activating protein (GAP) and p190RhoGAP and that p190RhoGAP localization to membrane protrusions was dependent on FAK expression

in MEFs (Tomar et al., 2009). These findings suggest that leading edge ruffling and cell polarization are achieved through FAK-mediated RhoA inhibition in membrane

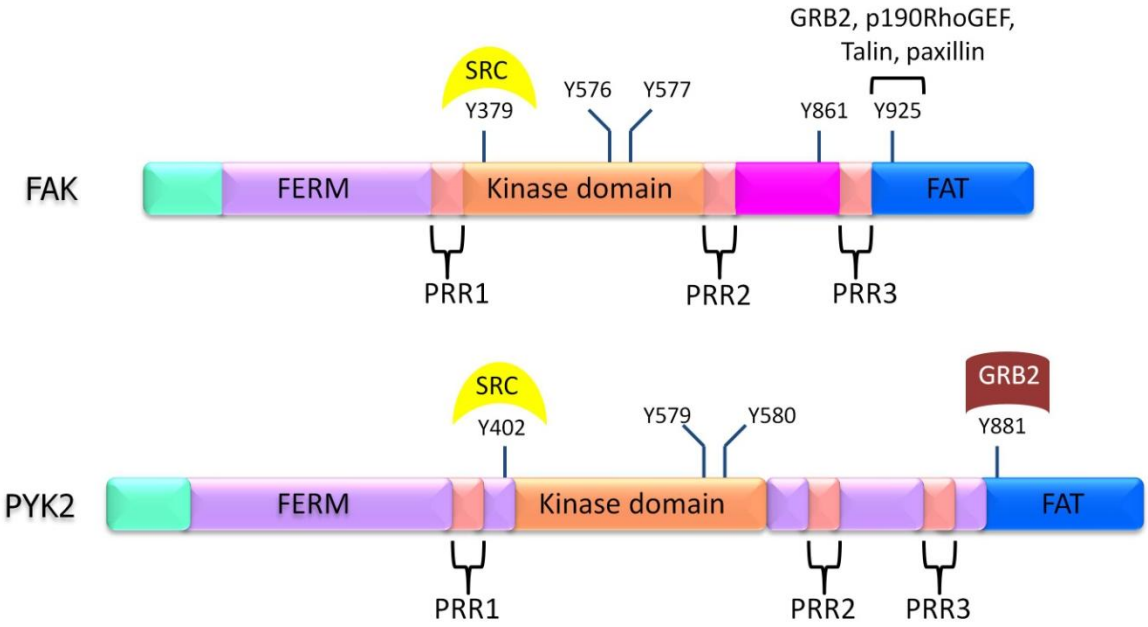


Figure 9. Comparison of FAK and PYK2 functional domains. FAK and the related PYK2 kinase share similar functional domains but are not functionally redundant. The FAK FERM domain mediates interactions with PDGFR, EGFR, Ezrin, and ETK while the PYK2 FERM promotes self oligomerization and binding to NIR proteins (Lipinski and Loftus, 2010). The kinase domains of FAK and PYK2 exhibit the greatest degree of sequence identity. Activation of FAK and PYK2 depends on recruitment of the Src family of protein kinases to phospho-tyrosine residues within the FAK and PYK2 kinase domains (Y397 and Y402, respectively). The Src kinases phosphorylate key tyrosine residues within both proteins (Y576, Y577, Y861, and Y925 of FAK vs. Y579, Y580, and Y881 of PYK2). The proline-rich regions (PPRs) of FAK and PYK2 mediate binding with p130Cas, ASAP1, and GRAF. The FAT domain of FAK associates with GRB2, p190RhoGEF, talin, and paxillin. The FAK FAT domain is required for FAK targeting to focal adhesion complexes. The FAT domain of PYK2 has also been reported to promote protein interactions with GRB2 and paxillin but does not direct PYK2 localization to focal adhesions. PYK2 is primarily concentrated in the perinuclear region of cells.

In addition to regulation of focal adhesion assembly and membrane protrusion, FAK-SRC signaling has also been reported to regulate focal adhesion disassembly.

Loss of FAK and SRC expression significantly slow the rate of focal adhesion turnover (Webb et al., 2004). Disassembly of focal adhesions is dependent on the activation of FAK as mutation of the FAK autophosphorylation site, inhibits adhesion turnover (Hamadi et al., 2005). Chemical inhibition or mutation of phospho-acceptor sites within the FAK targets, ERK and paxillin, inhibited focal adhesion disassembly (Webb et al., 2004). Furthermore, mutation of SRC phosphorylation sites on FAK has also been reported to inhibit adhesion turnover by disrupting complex formation between FAK, ERK, and calpain 2 (Westhoff et al., 2004). As calpains have been implicated in adhesion turnover through cleavage of focal adhesion components such as FAK and talin (Carragher et al., 1999; Westhoff et al., 2004), recruitment of calpains by FAK to the focal adhesion suggests that FAK-SRC scaffolding functions also regulates adhesion dynamics.

Summary and Aims

Based on the literature published thus far, it appears that many of STRAP's signal transduction activities are context-dependent as the specific protein-protein interactions are dependent on agonist treatment. Furthermore, STRAP's binding partners have also been reported to bind to each other, which may indicate that there is considerable and complex cross-talk between the signaling pathways regulated by STRAP. Much of the literature on STRAP has been published in recent years so it is likely that many novel interactions and biological roles for STRAP will continue to be discovered.

In consideration of the biological functions of STRAP, it's becoming increasingly apparent that deregulation of STRAP expression can contribute to the development of cancer through activation of mitogenic and survival signaling pathways. Furthermore,

the role of STRAP in cell morphology determination and MMP28 transcription suggests that STRAP may also be involved in EMT and tumor metastasis. The overall aim of this dissertation work was to further explore the oncogenic characteristics of STRAP. Immortalized fibroblasts isolated from wild type and STRAP knockout embryos were selected as a model system for these studies in order to mitigate the confounding effects associated with incomplete or off-target gene knockdown by RNA interference. Although the majority of cancers arise from epithelial cells, STRAP has been reported to regulate ERK and TGF-β/SMAD signaling in both fibroblasts and epithelial cells (Halder et al., 2006; Datta and Moses, 2000), suggesting that the effects of STRAP on oncogenic signaling pathways may affect parallel processes in different cell types. Furthermore, stromal fibroblasts have been shown to promote epithelial tumor progression by affecting tumor growth, vascularization, and metastasis (Kalluri and Zeisberg, 2006). As such, STRAP signaling in fibroblasts may provide useful insights into how stromal fibroblasts affect epithelial-derived cancers.

Herein, I present data on the role of STRAP in mitogenic signaling and cellular motility. In the second chapter, I will show that STRAP inhibits ubiquitin-mediated proteolysis of the c-Jun proto-oncogene and promotes cell autonomous growth of fibroblasts. In the third chapter, I provide evidence that STRAP expression is required for persistent migration in serum gradients and uropod retraction during chemotaxis. The significance of this work in the broad context of cancer and the role of fibroblasts in cancer are discussed in the last chapter.

Chapter II

STRAP STABILIZES c-JUN AND PROMOTES CELLULAR PROLIFERATION

Introduction

There is growing evidence to suggest that STRAP over-expression exerts a largely tumorigenic effect on cells. STRAP over-expression has been detected in human lung, colon, and breast cancer (Matsuda et al., 2000; Halder et al., 2006). Furthermore, STRAP expression promotes anchorage-independent growth of various cell lines *in vitro* as well as tumor formation in nude mice (Halder et al., 2006). At the cellular level, STRAP over-expression may support tumor growth through inhibition of TGF-β-induced growth inhibition. However, STRAP overexpression has been correlated with ERK activation and pRb phosphorylation in the absence of exogenous TGF-β (Matsuda et al., 2000; Halder et al., 2006).

In light of the well known mitogenic and oncogenic effects of ERK activation, it is plausible that overexpression of STRAP can drive proliferation of neoplastic cells. In some experimental contexts, activation of ERK has been shown to promote S phase entry through induction of cyclin D1 (Zhang et al., 1999; Teixeira et al., 2000). Cyclin D1 associates with cyclin-dependent kinase 4/6 (CDK 4/6) to form a functional enzymatic complex that directly phosphorylates the tumor suppressor protein, pRb. Hyper-phosphorylation of pRb then promotes dissociation from the E2F transcription factor, which translocates to the nucleus and induces expression of genes required for cell cycle progression. However, pRb phosphorylation may not be dependent on ERK activation as previous studies have shown that activation of the p38 and JNK MAPK pathways can also induce cyclin D1 expression (Papassava et al., 2004; Slisz et al.,

2008). As such, we hypothesized that STRAP regulates signaling by affecting components of the MAPK signaling pathways. Here, we will show that STRAP expression promotes c-Jun activation and expression in fibroblasts by inhibiting c-Jun ubiquitylation and degradation. The increased stability of c-Jun is correlated with increased expression of the AP-1 target gene, cyclin D1, and increased proliferation. These findings may suggest a novel mechanism by which STRAP can promote tumorigenesis by stimulating cell autonomous growth.

Materials and methods

Cell lines and plasmids

Immortalized STRAP knockout and wild type fibroblasts were generously donated by Dr. Philippe Soriano (Mount Sinai Medical Center, New York, NY). MEFs and 293T cultures were maintained in DMEM supplemented with 7% FBS. pcDNA3-c-Jun was kindly provided by Dr. Mike Engel (Vanderbilt University, Nashville, TN). The His6-Ubiquitin expression construct was a gift from Dr. Christoph Eglert (Leibniz Institute for Age Research, Jena, Germany). Construction of the pcDNA3.1-STRAP-HA and pBABE puro-STRAP-HA expression vectors has been described elsewhere (Datta et al., 1998).

Reagents and antibodies

MG132, cyclohexamide, and sodium butyrate were purchased from Sigma (St. Louis, MO). Lithium chloride was obtained from Calbiochem (La Jolla, CA). The STRAP monoclonal antibody was purchased from BD Transduction Labs (San Jose, CA). PARP, c-Jun, cyclin D1, p53, and JNK antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho c-Jun, JNK/SAPK, c-fos, and FosB antibodies were from Cell Signaling Technology (Beverly, MA). The cleaved caspase 3 antibody was purchased from Trevigen, Inc (Gaithersburg, MD). The c-myc custom antibody was provided by Dr. Steve Hann (Vanderbilt University, Nashville, TN).

Generation of stable cell lines

For the preparation of retroviral particles, 2.5 x 10^6 Phoenix cells were seeded into 10 cm tissue culture dishes. The following day, the cultures were transfected with 10 µgs of pBABE puro control vector or pBABE puro-STRAP-HA vector using lipofectamine LTX.

Forty eight hours post-transfection, the viral supernatant was centrifuged at 1,000 rpm and passed through a .45 µM syringe filter. The clarified virus was then pipetted onto early passage STRAP null fibroblasts. Approximately fifteen hours later, the viral supernatant was aspirated off the cells and the cells were fed with DMEM containing 10% serum. The following day, the cultures were selected with 0.75 µg/ml of puromycin to isolate polyclonal vector control and STRAP-HA-expressing cultures.

Western blot analysis

For terminal experiments requiring treatments, 10 mM sodium butyrate (24 hours), 10 mM LiCl (16 hours) and 50 µM MG132 (4 hours) was added directly to the growth medium before cell lysis. Subconfluent cultures were incubated in mammalian lysis buffer [50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, 5 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1.0 mM phenylmethylsulfonyl fluoride, 3 µg/ml each of leupeptin, pepstatin, and aprotinin] for 15 minutes on ice. The cells were then scraped, sonicated, and centrifuged at 13,000 rpm for 15 minutes at 4°C. The protein concentration of the clarified lysates was measured using Bradford reagent (Bio-Rad, Hercules, CA). Equal concentrations of protein extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were then immunoblotted with the indicated antibodies. Quantitation of protein bands was performed using NIH Image J software. The relative densities for each sample were calculated by normalizing against the corresponding actin densities. The average relative density from at least three independent experiments is shown.

Cell growth Assays

For the cell counting assays, 3 x 10^3 cells were seeded in triplicate wells of 12-well plates. Forty eight hours after seeding, cells were trypsinized daily and counted in a hemocytometer. The total number of cells was calculated for each well. Results are expressed as the log of the total cell number $\pm$ SEM for six independent experiments. For the thymidine incorporation assays, 2 x 10^4 cells were seeded in triplicate wells of 12-well plates. After 44 hours, the cultures were labeled with 4 µCi of [^{3}H]-thymidine (NEN, Boston, MA) for two hours at 37°C. The cells were fixed with an ice cold 10% trichloroacetic acid solution for 30 minutes and washed with the same solution two times for ten minutes. The fixed cultures were then lysed in 300 µl of 0.2 N NaOH. One hundred microliters of the lysate was added to four milliliters of scintillation fluid and the incorporated [^{3}H]-thymidine was measured in a scintillation counter. Results are expressed as the log of the counts per minute (CPM) $\pm$ SEM for six independent experiments. Statistical analyses of cell counting and thymidine incorporation assays were performed using mixed-model ANOVA.

Reverse transcription-PCR

Total RNA was extracted from sub-confluent MEF cultures using TRIzol reagent. Two micrograms of total RNA was reverse transcribed at 37°C for one hour using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Two microliters of the resulting cDNA was PCR amplified for 25 cycles using primers specific for c-Jun and glyceraldehyde-3-phosphate (GAPDH). The PCR reaction was carried out using the following conditions: denaturation at 95°C for one minute, annealing at 54°C for one minute, and elongation at 72°C for one minute. The specific primer sequences are as

follows: c-Jun 5'-GACTGCAAAGATGGAAACGA and 5'-GGGTTGAAGTTGCTGAGGTT; GAPDH 5'-ACCACAGTCCATGCCATCAC, and 5'-TCCACCACCCTGTTGCTGTA.

Cycloheximide assays

Sub-confluent wild type and STRAP knockout fibroblasts were treated with 100 µg/ml of cyclohexamide diluted in DMEM containing 7% serum. The cultures were harvested at 0, 0.5, 1, 2, and 4 hours after treatment. The cells were lysed in mammalian lysis buffer and c-jun expression was determined by immunoblot analysis.

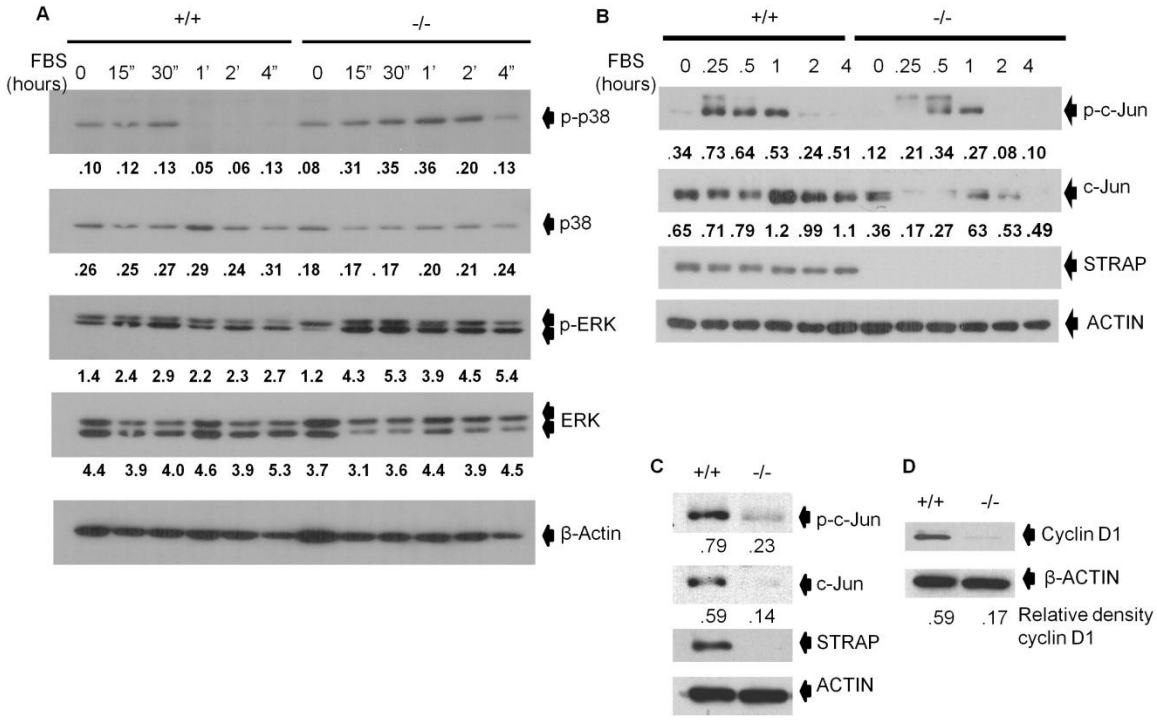
Ubiquitylation assay. 293T cells were transiently transfected with c-Jun, His6-Ubiquitin, and STRAP-HA expression plasmids using lipofectamine 2000 (Invitrogen, Calsbad, CA). Empty pcDNA3 vector was used as a filler to ensure that each culture was transfected with an equal concentration of DNA. Two days post-transfection, the cells were collected by scraping and washed with PBS. One tenth of the cell suspension was boiled in 5X Laemmli buffer for immunoblot analysis of protein expression. The remaining cell suspension was lysed in Buffer A pH 8.0 (6 M guanidine hydrochloride, 0.1M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole). Ubiquitylated proteins were pulled down from the lysates with Ni-NTA resin for three hours at 4ºC and washed two times in Buffer A. The beads were then washed two times in Buffer A/T1 pH 6.8 (6 M guanidine hydrochloride, 0.1M Na_2HPO_4/NaH_2PO_4, 25 mM Tris-Cl, 15 mM imidazole) followed by one wash in Buffer T1 pH 6.8 (25 mM Tris-Cl, 20 mM imidazole). The bound proteins were eluted by boiling in 3X Laemmli buffer containing 350 mM imidazole. Equal volumes of eluate and whole cell extract were separated by SDS-PAGE and electro-blotted onto PVDF membranes. Ubiquitylated c-Jun was detected by immunoblot analysis.

Results

STRAP regulates c-Jun protein expression and activation

In order to examine the role of STRAP in the regulation of MAPK signaling, the kinetics of p38 MAPK, ERK, and c-Jun activation was assessed in serum re-stimulated wild type and STRAP knockout cultures. STRAP expression was not associated with increased ERK or p38 MAPK activation after the addition of serum (Figure 10A). However, serum induced c-Jun phosphorylation in wild type MEFs (Figure 10B). Activated c-Jun has been reported to positively auto-regulate its expression through binding to AP-1 sites on its own promoter (Angel et al., 1988). Given that c-Jun activation levels peak more rapidly than c-Jun total protein for both cultures, it is possible that the phosphorylated protein is promoting c-Jun transcription. The activation and expression of c-Jun was also examined in asynchronous cultures without re-stimulation. STRAP knockout significantly diminished c-Jun phosphorylation and expression, supporting our finding that STRAP regulates c-Jun activation and expression (Figure 10C).

Activated c-Jun promotes cell cycle progression by transcriptionally regulating genes that are necessary for the G1 to S transition, including many growth factors and cyclin D1. In accordance with the reduced phospho-c-Jun levels, STRAP deletion decreased cyclin D1 expresion by western blot (Figure 10D) as well as cyclin D1 mRNA levels in a microarray study (unpublished data) . To determine whether STRAP generally upregulates expression of other transcription factors, MEF lysates were immunoblotted for p53, c-Myc, c-Fos, and FosB (Figure 11). p53 expression was used as positive control as STRAP has previously been shown to stabilize p53 through direct association (Jung et al., 2007). As expected, p53 expression was significantly reduced



Reprinted from *Biochem Biophys Res Comm* (2011) 407, 372-7 with permission from Elsevier

Figure 10. STRAP expression correlates with increased activation and expression of c-Jun. A. Serum-starved wild type and STRAP null fibroblasts were stimulated with 10% FBS for the indicated times and immunoblotted for the expression of phospho-p38 MAPK, p38 MAPK, phospho-ERK, and ERK expression. Loss of STRAP expression in MEFs increased p38 MAPK and ERK phosphorylation in response to serum. Protein densities were normalized against actin and the mean density from three independent experiments is shown. B. Wild type and STRAP knockout MEFs were stimulated with serum after overnight serum withdrawal. Protein lysates were prepared at various times post-treatment with 10% serum. Wild type MEFs exhibited increased phosphorylation and activation of c-Jun in response to serum. The mean relative phospho-c-Jun and total c-Jun densities are shown. C. Phospho-c-Jun and total c-Jun expression was detected by western blot analysis of lysates prepared from wild type and STRAP null MEFs in logarithmic growth phase. Wild type MEFs have increased levels of phosphorylated and total c-Jun protein. The mean relative densities from three independent experiments are shown. D. Immunoblot analysis of the AP-1 target gene, cyclin D1, in wild type and STRAP null MEFs. Cyclin D1 is down-regulated in STRAP knockout MEFs. The mean relative density of cyclin D1 from three independent experiments is shown.

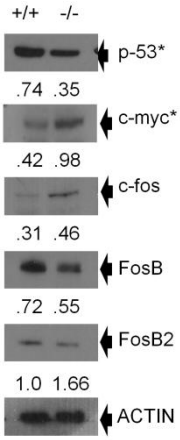
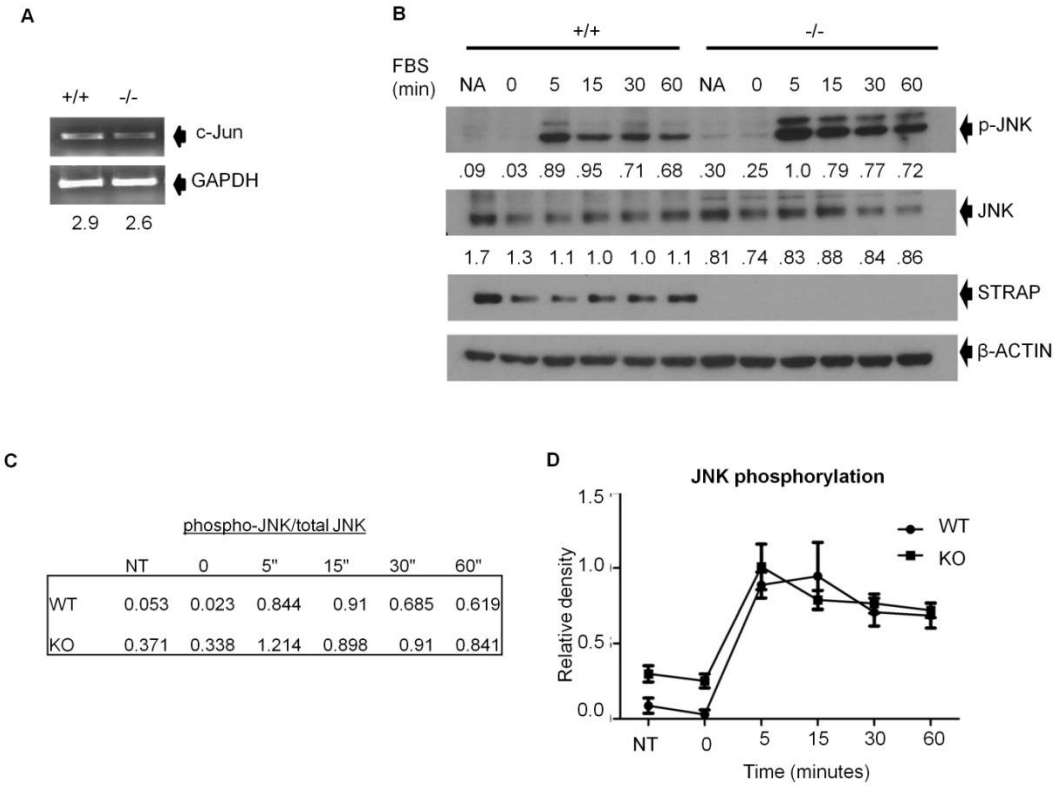


Figure 11. Effects of STRAP on the expression of various transcription factors. Sub-confluent wild type and STRAP knockout MEFs were immunoblotted with the indicated antibodies. The density of the individual transcription factors was normalized to actin and the mean relative densities from at least four experiments are shown. Statistical significance was determined with the Student's t-test. Transcription factors that exhibit significant differences ($p<0.05$) between the means are denoted with an asterisk (*)

in STRAP null fibroblasts ($p<0.05$). However, c-Myc was significantly up-regulated in the STRAP knockout cultures. No significant differences in c-Fos, FosB, or FosB2 expression were detected between wild type and STRAP knockout MEFs. These findings suggest that STRAP exhibits specificity towards c-Jun rather than indiscriminately up-regulating the expression of all transcription factors.

STRAP does not regulate c-Jun mRNA or JNK activation

Expression of c-Jun can be regulated by transcription, post-transcriptional modification, and degradation. In order to determine whether STRAP transcriptionally regulates c-Jun expression, we examined c-Jun mRNA levels by RT-PCR (Figure 12A). No apparent differences in steady state c-Jun mRNA were detected, suggesting that STRAP does not affect transactivation or degradation of c-Jun mRNA. To determine whether STRAP can promote c-Jun activation and expression through regulation of JNK activity, the kinetics of JNK activation in response to serum stimulation were examined in wild type and STRAP null MEFs. Western blot analysis revealed marginal differences in the relative amounts of phosphorylated JNK and total JNK between wild type and STRAP MEFs (Figure 12B). The ratio of phospho-JNK to total JNK was slightly higher in STRAP null MEFs (Figure 12C). However, the ratio of phosphorylated to total protein may not an appropriate metric for determining JNK activity since the biological outcome is likely to depend on the active fraction rather than total protein pool. The normalized p-JNK/ACTIN levels were not statistically significant by ANOVA suggesting that STRAP does not promote c-Jun expression through activation of JNK (Figure 12D).

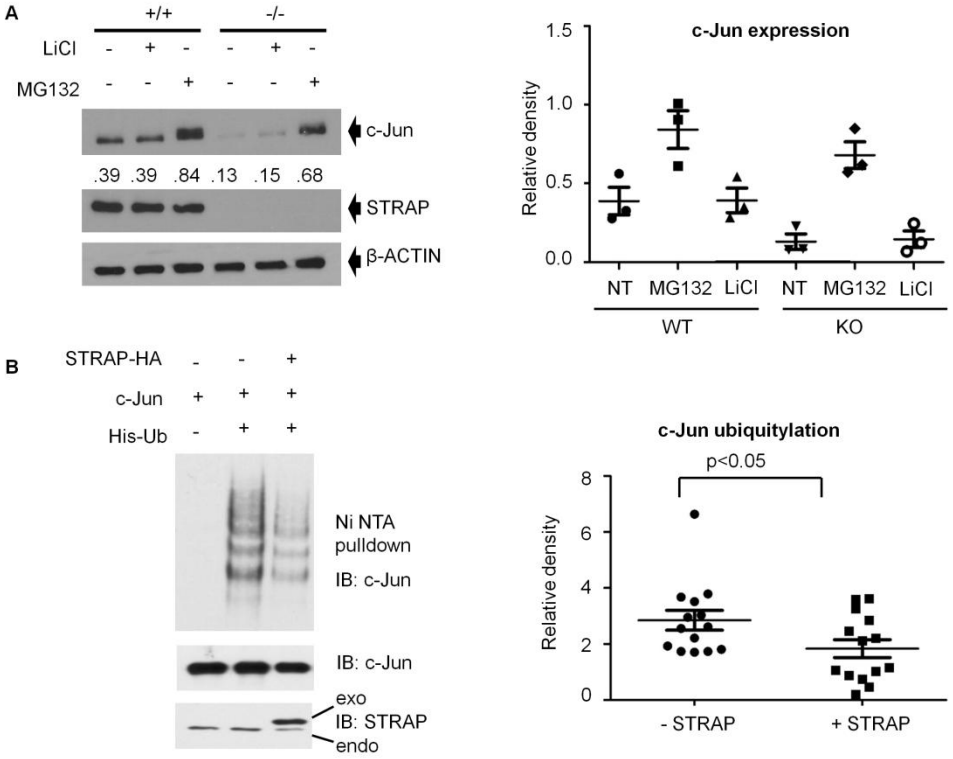


Reprinted from *Biochem Biophys Res Comm* (2011) 407, 372-7 with permission from Elsevier

Figure 12. STRAP does not regulate c-Jun mRNA levels or JNK activation. A. RT-PCR analysis of c-Jun mRNA levels in wild type and STRAP null MEFs. The c-Jun mRNA was normalized against GAPDH. The mean relative density from three independent experiments is shown. B. Wild type MEFs were serum-starved and re-stimulated with 10% serum for the indicated time points. "NA" samples represent cultures in normal log growth phase. The lysates were immunoblotted for phospho-JNK and total JNK (top). p-JNK and JNK were normalized against actin and the mean densities from four independent experiments are shown. C. The ratio of the mean phospho-JNK density to the mean total JNK density from four independent experiments is shown. D. Graph of the mean relative density $\pm$ SEM of p-JNK/Actin.

STRAP inhibits ubiquitin-mediated proteolysis of c-Jun

In order to determine whether c-Jun expression levels in MEFs are regulated through proteasomal degradation, wild type and STRAP null MEFs were treated with the proteasome inhibitor, MG132, and the GSK3β inhibitor, LiCl. As shown in Figure 13A (left), 50 μM MG132 treatment markedly increased Jun expression in both wild type and STRAP null MEFs, suggesting that proteasomal degradation modulates c-Jun protein levels in these cells. The mean relative density ± SEM from three independent experiments was plotted to show the considerable differences between the untreated and MG132 treated cultures (Figure 13A, right). Interestingly, c-Jun protein levels in wild type and STRAP knockout MEFs were unaffected by treatment with 10 mM LiCl (Figure 13A) or another GSK3β inhibitor, SB415286 (unpublished data). These findings suggest that GSK3β/Fbw7 mediated degradation of c-Jun may not be necessary for c-Jun turnover in MEFs. Next, we tested the effect of STRAP on c-Jun ubiquitylation in 293T cells (Figure 13B, left). Co-expression of c-Jun and His-Ubiquitin produced a ladder comprised of mono- and poly-ubiquitylated Jun protein. Over-expression of STRAP markedly decreased the ubiquitylation of c-Jun. Importantly, western blot analysis of the corresponding lysates showed approximately equal amounts of poly-ubiquitylated proteins (unpublished data), indicating that STRAP expression does not result in a global decrease in ubiquitylation. Mann Whitney analysis of fourteen independent experiments indicated that the effect of STRAP on c-Jun ubiquitylation is statistically significant, $p<0.05$ (Figure 13B, right).



Reprinted from *Biochem Biophys Res Comm* (2011) 407, 372-7 with permission from Elsevier

Figure 13. STRAP stabilizes c-Jun by inhibiting ubiquitin-mediated proteolysis of c-Jun. A. Wild type and STRAP null fibroblasts were treated with MG132 and LiCl. The effect of treatment on total c-Jun protein levels was analyzed by immunoblot analysis. Total c-Jun levels were normalized to actin and the mean relative densities from three independent experiments are shown (left). The mean relative densities $\pm$ SEM for each treatment were plotted (right). B. The ubiquitylation of c-Jun in the presence and absence of STRAP expression was assessed by transiently transfecting 293T cells with the indicated expression constructs. Ubiquitylated proteins were pulled down from cell lysates using Ni^{2+}-NTA resin and analyzed for c-Jun by immunoblot analysis. The relative expression of c-Jun and STRAP was determined by immunoblot analysis of whole cell extracts (left). Ubiquitylated c-Jun was normalized to total Jun levels in the whole cell extracts. The mean relative density $\pm$ SEM of ubiquitylated c-Jun from 14 experiments is shown. Statistical significance was determined using the Mann Whitney test (right).

STRAP prolongs the half-life of c-Jun

In order to determine whether STRAP expression affects the half-life of c-Jun, wild type and STRAP null MEFs were treated with cycloheximide and the kinetics of c-Jun degradation were examined by western blot analysis (Figure 14A, left). In STRAP knockout MEFs, c-Jun protein levels experienced a sustained and rapid decline over the four hour time course. However, c-Jun levels in wild type appeared to gradually decline and stabilize at four hours, suggesting that STRAP inhibits c-Jun degradation. The relative densities of c-Jun at various time points after cycloheximide addition were normalized to the initial density of c-Jun and plotted to show the approximate rate of c-Jun decay (Figure 14A, right). Based on nonlinear regression analysis, the half-life of c-Jun in STRAP null fibroblasts can be estimated at one hour. While the half-life of c-Jun in wild type MEFs can't be interpolated from the normalized c-Jun values, the smaller slope indicates that the rate of c-Jun decay is markedly slower in these cells.

Stable reintroduction of STRAP rescues the c-Jun instability defect in STRAP knockout fibroblasts

To confirm that STRAP regulates c-Jun expression, STRAP null MEFs were transduced with retrovirus harboring empty vector or STRAP-HA and the effect of STRAP overexpression on c-Jun protein levels was examined by western blot (Figure 14B, left). Relative to the wild type, STRAP knockout and vector control fibroblasts exhibited a statistically significant reduction in c-Jun protein levels (Figure 14B, right). Ectopic expression of STRAP-HA increased c-Jun protein levels although endogenous STRAP expression levels were markedly higher in wild type MEFs. These data suggest that STRAP can rescue the c-Jun instability defect in the knockout cell line.

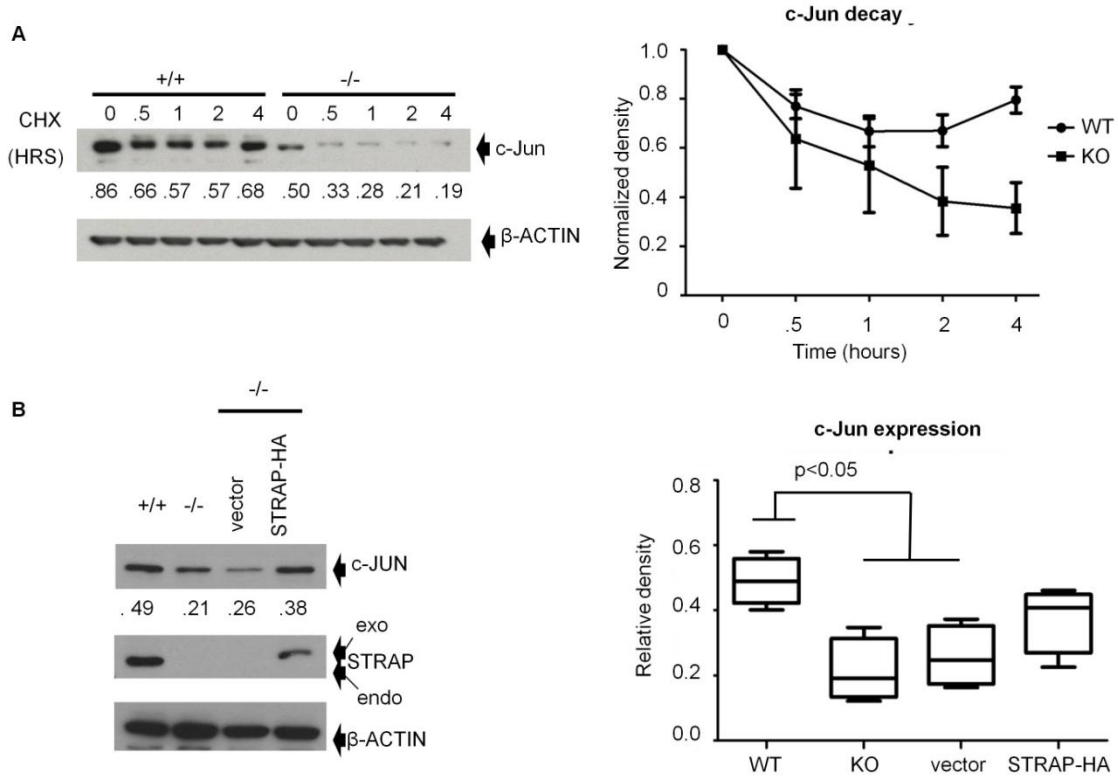


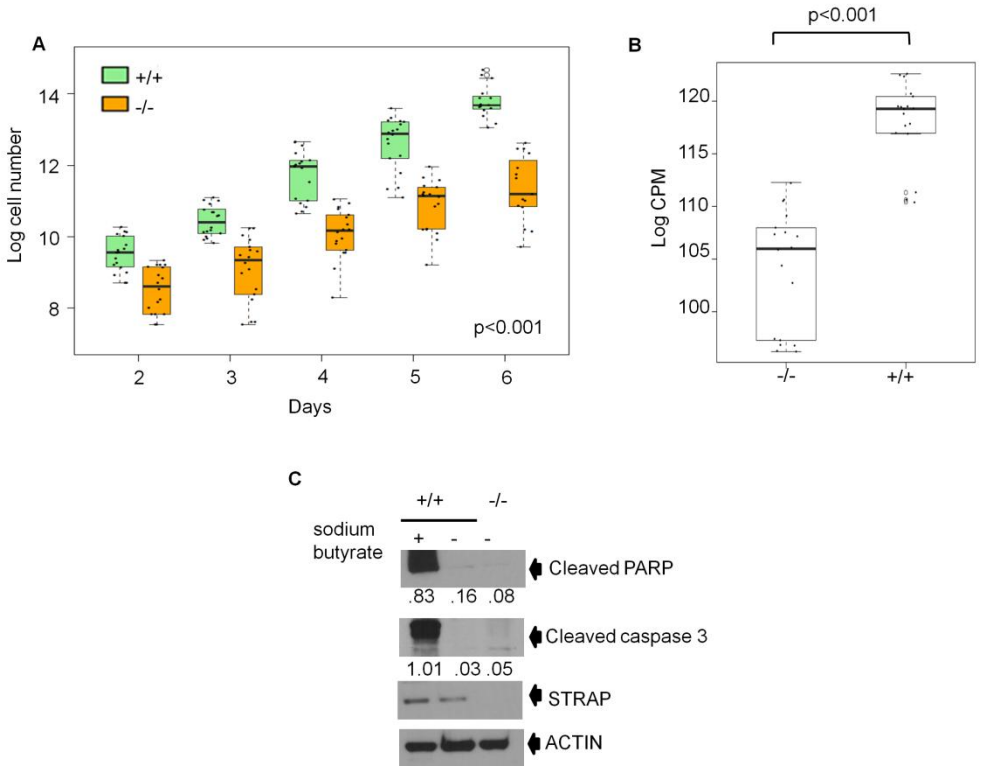
Figure 14. STRAP prolongs the half-life of c-Jun and increases c-Jun expression in STRAP knockout fibroblasts. A. Wild type and STRAP null fibroblasts were treated with cycloheximide (CHX) and lysed after 0, 0.5, 1, 2, and 4 hours of treatment. c-Jun protein levels were analyzed by immunoblot analysis. The c-Jun density was normalized against actin and the mean relative density of three independent experiments is shown (left). To show c-Jun decay over time, the relative densities (c-Jun density/Actin density) were normalized to the baseline c-Jun values for each culture. The adjusted c-Jun values are plotted $\pm$ SEM for three experiments (right). B. STRAP null fibroblasts were transduced with retrovirus to obtain polyclonal cultures expressing either an empty vector or a STRAP-HA construct. The effects of enforced STRAP expression on c-Jun protein levels were determined by immunoblot analysis (left). The c-Jun density was normalized against actin and the mean relative density from four independent experiments is shown. Statistical significance was determined using the Mann Whitney test (right).

STRAP promotes proliferation of MEFs

A role for STRAP as an oncogene has been proposed due to its ability to antagonize TGF-β mediated growth inhibition (Halder et al., 2006). However, STRAP over-expression has been correlated with tumorigenicity in the absence of exogenous TGF-β (Halder et al., 2006). In cell counting assays, STRAP knockout MEFs exhibited a marked defect in proliferation relative to the wild type cultures (Figure 15A). The STRAP knockout MEFs also exhibited a significant decrease in thymidine incorporation, suggesting that STRAP promotes cellular proliferation (Figure 15B). Because decreased cell numbers can be attributed to increased cell death, markers for apoptosis were examined by western blot analysis (Figure 15C). As a positive control, wild-type MEFs were treated with 10 mM sodium butyrate for 24 hours to induce apoptosis. Minimal cleaved caspase-3 and PARP were detected in the untreated cultures, whereas both markers were present in the positive control. Furthermore, visual examination of the cultures under normal growth conditions did not reveal any morphological changes associated with cell death or reduced viability (unpublished results). Collectively, our observations suggest that STRAP promotes cellular proliferation in the absence of exogenous TGF-β.

Discussion

Here, we show that STRAP regulates c-Jun expression by inhibiting the ubiquitylation and degradation of c-Jun. It has been previously reported that STRAP decreases ubiquitylation of the p53 tumor suppressor through formation of a ternary complex with NM23 and p53 (Jung et al., 2007). As expected, wild type fibroblasts expressed significantly more p53 than STRAP null fibroblasts. However, unlike p53,



Reprinted from *Biochem Biophys Res Comm* (2011) 407, 372-7 with permission from Elsevier

Figure 15. STRAP expression promotes cellular proliferation. A. Proliferation of wild type and STRAP knockout MEF cell lines was examined by counting cells over a five-day consecutive time course. The data are presented as the log of the average cell number from six independent experiments. The statistical significance between was determined using a mixed model ANOVA ($p<0.001$). B. Growth of wild type and STRAP knockout MEFs was assessed by measuring ^{3}H-thymidine incorporation 46 hours after cell culture seeding. The data is presented as the log of the counts per minute (CPM) for six independent experiments. Statistical analysis was performed using a mixed-model ANOVA ($p<0.001$). C. MEF lysates were analyzed for markers of apoptosis by western blotting with antibodies directed against cleaved caspase-3 and PARP. Wild-type MEFs were pre-treated with sodium butyrate for 24 hours as a positive control for apoptosis. The relative density of caspase-3 and PARP was determined by normalizing against the corresponding actin band. The mean relative density from three independent experiments is shown.

c-Jun does not co-immunoprecipitate with STRAP (unpublished results). This suggests that STRAP-mediated c-Jun stabilization occurs through a novel mechanism. Previous studies have shown that c-Jun is a substrate for the ubiquitin E3 ligase, FBW7 (Wei et al., 2005). FBW7-mediated degradation depends on GSK3β phosphorylation of T242 in the C-terminus of c-Jun. Because treatment with the GSK3β inhibitor, LiCl, did not alter the basal level of c-Jun protein in MEFS, we do not expect c-Jun stability to be regulated by FBW7 in these cells. In addition to FBW7, the ubiquitin E3 ligases Itch and DET/COP1 have been shown to promote c-Jun degradation. Further work will be required to determine whether STRAP can influence c-Jun association with these E3 ligases.

Previous studies have shown that c-Jun protein levels are up-regulated in squamous cell lung cancer (Woodrich and Volm, 1993) and transitional cell bladder carcinoma (Skopelitou et al., 1997) while STRAP protein overexpression has been detected in lung, colon, and breast cancers (Matsuda et al., 2000, Halder et al., 2006). Interestingly, no gain-of-function mutations in c-Jun or STRAP have been described in human cancers. However, loss of function mutations in the FBW7 c-Jun E3 ligase have been reported in leukemias (O'Neil et al., 2007;Song et al., 2008), suggesting that increased stabilization of FBW7 targets promote tumorigenesis. Although FBW7 does not promote c-Jun turnover in fibroblasts, it is possible that STRAP-mediated stabilization of c-Jun contributes to cancer development.

While very little is presently known about the cellular functions of STRAP, it appears that STRAP can regulate a wide array of signaling pathways by associating with a diverse group of cellular proteins. Although the specific biological function of STRAP varies according to its binding partner, STRAP binding to these proteins generally promotes signaling pathways and processes that are frequently associated with

tumorigenesis. Previous studies on c-Jun indicate that its expression is important for cellular proliferation as c-Jun knockout MEFs exhibit a significant delay in cell cycle progression (Smith et al., 1992; Johnson et al., 1993). Similarly, loss of STRAP significantly impairs the proliferation of MEFs suggesting that the defect in cellular growth may be in part due to increased turnover of c-Jun.

In light of the evidence that c-Jun signaling promotes tumor formation (Jin et al., 2011; Sancho et al., 2009), STRAP over-expression could support cell autonomous tumor growth through deregulation of c-Jun expression. My observation that STRAP that loss of STRAP decreases proliferation of fibroblasts raises the possibility that STRAP induction of AP-1 signaling could promote sarcoma growth. Because the majority of cancers originate from dysplastic epithelia, it will be important to examine the effects of STRAP on c-Jun stability and growth in various epithelial cancer cell lines. Our preliminary data suggests that knockdown of STRAP in the lung adenocarcinoma cell line, A549, has a modest effect on proliferation whereas STRAP knockdown in the HeLa cervical cancer cell line does not inhibit growth (data not shown). These findings highlight the contextual significance of changes in gene expression so it may be necessary to screen cell lines derived from a wide array of cancers to identify specific cancers where STRAP expression may have a significant biological effect.

In normal tissues, interactions between epithelial and stromal cells are not only important for maintenance of tissue architecture but also for regulation of normal biological processes including differentiation and growth. Activation of signaling pathways leading to biological changes within the epithelium are partially due to stromal fibroblast secretion of soluble factors such as growth factors and chemokines. It has been reported that cancer associated fibroblasts (CAFs) support tumorigenesis by modulating growth (Olumi et al., 1999; Cheng et al., 2005) and vascularization (Crawford

et al., 2009;Zhang et al., 2011). CAFs isolated from human tumor tissues exhibit increased loss of heterozygosity at genetic hotspots (Weber et al., 2006; Weber et al., 2007), suggesting that deregulated gene expression in stroma can have a significant effect on surrounding epithelial cells. With respect to c-Jun, it has reported that loss of c-Jun expression in fibroblasts inhibits proliferation and terminal differentiation of keratinocytes in organotypic co-cultures (Maas-Szabowski et al., 2001). Treatment with the AP-1 induced growth factors, GM-CSF and KGF, rescued the keratinocyte growth and differentiation defects (Maas-Szabowski et al., 2001), suggesting that stromal AP-1 activity plays a critical role in the regulation of epithelial cell behavior. Given that c-Jun ablation in stromal cells disrupts normal biological processes, the relative expression of c-Jun expression in fibroblasts may also affect growth of tumor cells. Li et al. have reported that wild type fibroblasts exhibit a greater capacity for stimulation of benign prostatic hyperplasia (BPH) proliferation through IGF-1 paracrine signaling compared to c-Jun null fibroblasts (Li et al., 2007). Collectively, these studies suggest that STRAP mediated stabilization of c-Jun in stromal cells could activate oncogenic signaling pathways in nearby epithelia through AP-1 dependent secretion of growth factors and cytokines. Our microarray analysis indicates a 35-fold increase in GM-CSF expression in wild type fibroblasts relative to STRAP null MEFs (data not shown). This may suggest that STRAP-mediated stabilization of c-Jun in the stroma can promote growth of associated epithelium through GM-CSF secretion. However, further work will be required to establish a role for STRAP in epithelial-stromal interactions.

Based upon our findings and the literature published thus far, STRAP may promote cell growth and tumorigenicity by regulating the TGF-β, ERK, and c-Jun signaling pathways (Figure 16).

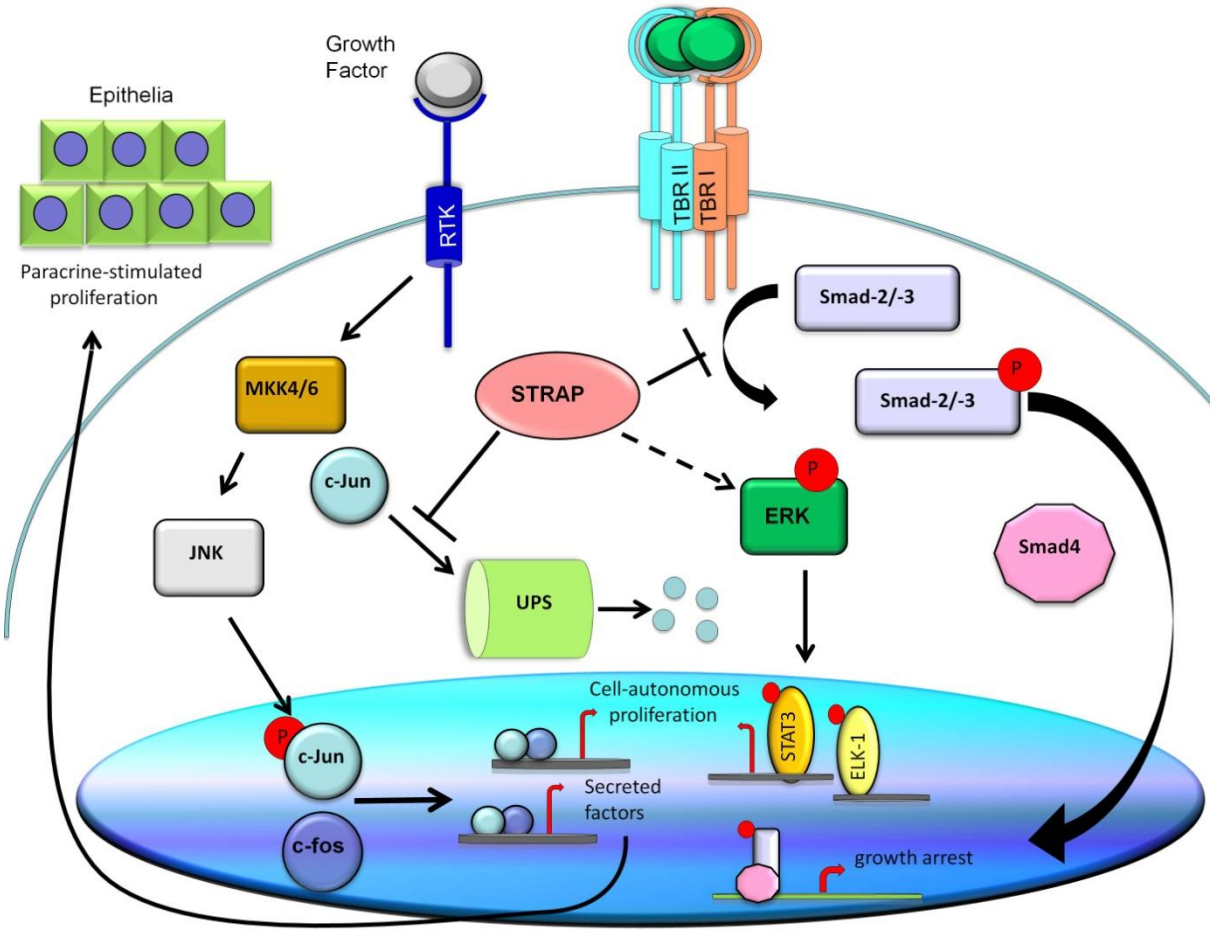


Figure 16. STRAP regulation of cell growth pathways promotes cell autonomous and paracrine-stimulated growth. STRAP regulates multiple cell signaling pathways that affect growth and tumorigenesis. Canonical TGF-β signaling inhibits cellular proliferation through SMAD-dependent transactivation of anti-proliferative genes and repression of growth-inducing genes. STRAP inhibits the anti-proliferative effects of TGF-β by sterically blocking TGF-β receptor association with SMAD-2 and -3, thereby blocking SMAD activation. STRAP over-expression induces ERK activation, which can promote cell growth through activation of the transcription factors, ELK-1 and STAT3. Growth factor binding with cognate receptor tyrosine kinases (RTKs) promotes cellular proliferation through activation of MAP kinase pathways, including the JNK/c-Jun axis. Phosphorylated c-Jun and its binding partner, c-Fos, form the AP-1 transcription factor, which promotes cell cycle progression by inducing transcription of genes necessary for the G1/S transition. STRAP mediated inhibition of c-Jun turnover increases the total cellular pool of c-Jun, thereby permitting increased activation and transcription of AP-1 targets. Increased AP-1 activity also leads to the production and secretion of soluble factors that can promote the growth of nearby cells through paracrine signaling.

Chapter III

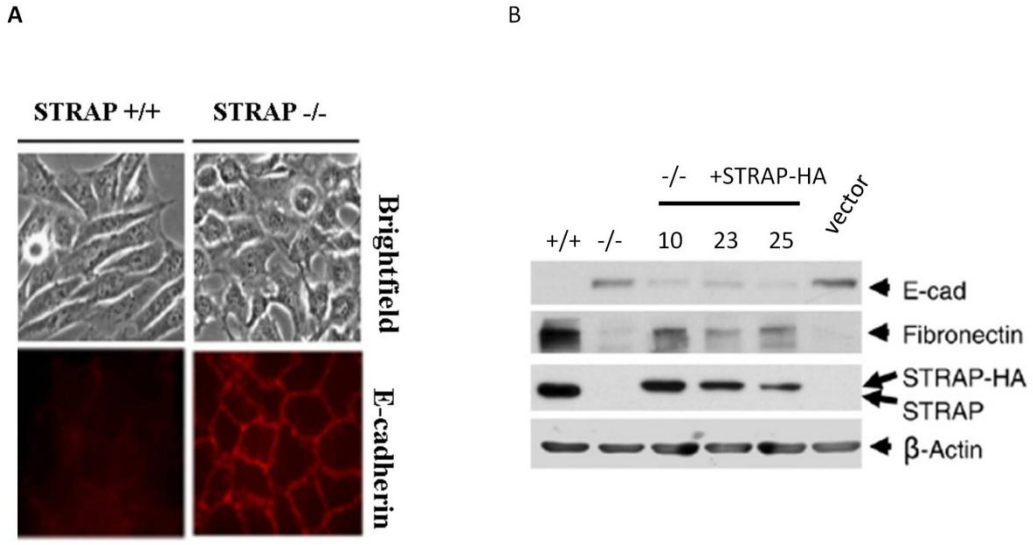
STRAP PROMOTES PERSISTENT MIGRATION AND TAIL RETRACTION DURING CHEMOTAXIS

Introduction

Loss of STRAP expression in the developing mouse causes gross morphological defects in somitogenesis, organogenesis, and neural tube closure (Chen et al., 2004). Interestingly, many of the observed defects have been associated with a failure in embryonic EMT (Zohn et al., 2006; Zwerts et al., 2007). It has recently been reported that immortalized embryonic fibroblasts isolated from the STRAP knockout mouse express E-cadherin and exhibit an epithelioid morphology (Figure 17A) (Kashikar et al., 2010). During the early stages of embryonic patterning, epithelial cells undergo an EMT, which is characterized by the down-regulation of epithelial markers, such as E-cadherin, and induction of mesenchymal genes (Duband et al., 1995; Boyer and Thiery, 1998). Embryonic EMT, referred to as Type 1 EMT involves primitive epithelial cells that undergo mesenchymal transdifferentiation and populate different organ systems (Kalluri and Weinberg, 2009). These cells can then revert to an epithelial cell type, through a process called mesenchymal-to-epithelial transition (MET), and support organ development in their resident tissue.

Given that STRAP knockout mice have defects in organogenesis and STRAP null fibroblasts do not exhibit normal fibroblastoid morphology, it is possible that loss of STRAP impairs type 1 EMT. Analysis of various epithelial and mesenchymal markers indicated that STRAP knockout MEFs express markers of both lineages (summarized in Table 3) (Kashikar et al., 2010). Furthermore, enforced expression of STRAP in the STRAP null MEFs was sufficient to abrogate E-cadherin expression, upregulate

fibronectin (Figure 17B), and induce a morphological switch to a fibroblast-like phenotype (Kashikar et al., 2010). Collectively, these findings may suggest that loss of STRAP does not inhibit all of the molecular changes associated with EMT but is sufficient to inhibit acquisition of a spindle-shaped morphology.



Reprinted from *Cell Signal* (2010) **22**, 138-49 with permission from Elsevier

Figure 17. STRAP suppresses E-cadherin expression in MEFs. A. Loss of STRAP expression in MEFs results in an epithelioid morphology coincident with up-regulation of E-cadherin. B. Stable reintroduction of STRAP into the STRAP null MEFs decreases expression of the epithelial marker, E-cadherin, and increases expression of the fibroblast marker, fibronectin.

	Western Blot		RT-PCR	
EMT markers	WT	KO	WT	KO
Acquired				
N-cadherin	+	++	ND	ND
FSP1	ND	ND	+	+
Vimentin	+	++	ND	ND
β-catenin	+	++	ND	ND
Fibronectin	++	-	ND	ND
Snail	ND	ND	+	+
Slug	ND	ND	+	+
Twist	ND	ND	+	+
LEF1	ND	ND	+	-
Attenuated				
E-cadherin	-	+++	-	+++

ND-not determined
Summarized from *Cell Signal* **22** (2010) 138-49.

Table 3. Epithelial and mesenchymal markers expressed by wild type and STRAP null MEFs

Although EMTs in the embryo and adult organisms may differ in the expression of markers, a common feature of EMT is an increased capacity for cellular motility (Bakin et al., 2000; Bakin et al., 2002; Xu et al., 2003). Embryonic EMT is required for proper developmental patterning whereas EMT in the adult regulates wound healing processes (Savagner et al., 2005; Hudson et al., 2009). EMT also plays an important role in the dissemination of cancer cells from the primary tumor (Smit and Peeper, 2011; Wendt et al., 2011). While these preliminary studies highlight the importance of STRAP in embryonic development and cell fate determination, the relationship between morphology and motility was not examined.

The aim of this project is to explore the role of STRAP in migration. Based on the morphological differences associated with STRAP expression, we hypothesized that STRAP may promote cellular motility by regulating the expression or activation of proteins involved in actin cytoskeleton remodeling. In the event that STRAP promotes cellular migration, targeted inhibition of STRAP may be a useful avenue for the treatment of metastatic cancers.

Materials and methods

Cell culture and plasmids

Immortalized wild type and STRAP knockout Mouse Embryonic Fibroblasts (MEFs) were a gift from Dr. Philippe Soriano (Mount Sinai Medical Center, New York, NY). MEF and HT29 colon adenocarcinoma cultures were maintained in DMEM medium supplemented with 7% FBS. The STRAP shRNA and GIPZ empty vector control were purchased from Open Biosystems (Huntsville, AL). The viral packaging plasmids pMD2.G and psPAX2 are available from Addgene (Cambridge, MA; deposited by Didier Trono). The Akt-PH-eGFP expression construct was generously provided by Dr. Ann Richmond.

Reagents and antibodies

Lipofectamine LTX, Phalloidin-Alexa Fluor 594, Alexa-Fluor conjugated secondary antibodies, and Prolong Gold mounting medium were purchased from Invitrogen (Carlsbad, CA). Mitomycin C, Fibronectin, nocodazole, phosphatase inhibitor cocktails, and protease inhibitor cocktails were purchased from Sigma (St. Louis, MO). Type I collagen was obtained from BD Biosciences (Sparks, MD). The pFAK and FAK antibodies are from Cell Signaling (Danvers, MA). The β-tubulin antibody was obtained from the University of Iowa Developmental Biology Hybridoma Bank (Iowa City, Iowa) and the STRAP monoclonal antibody was purchased from BD Transduction Labs (San Jose, CA).

Generation of stable cell lines

Lentivirus for STRAP knockdown was generated by co-transfecting 293T cultures with the shRNA GIPZ vector (Open Biosystems clone ID V2LMM_14787) and the plasmids

encoding viral proteins, psPAX2 and pMD2.G. Two days post-transfection, the viral supernatant was centrifuged at 1000 rpm and passed through a 0.45 µm syringe filter. The clarified viral supernatant was then applied directly to wild-type MEF and HT29 cultures. The following morning, the viral supernatant was aspirated off the cells and the cultures were fed with DMEM containing 7% FBS. Twenty four hours later, the MEFs and HT29 cells were selected with 1 µg/ml of puromycin. After puromycin selection, single colonies were isolated from the polyclonal population of MEFs and screened for STRAP expression by immunoblot analysis using a mouse monoclonal STRAP antibody. Monoclonal cell lines were not derived for the HT29 cultures. The procedure for generation of the STRAP-HA and vector control cells has been described in the previous chapter.

Immunofluorescence

MEFS were seeded in chamber slides coated with 10 µg/ml fibronectin. The attached cells were fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 1% Triton X-100 for 8 minutes. The fixed cells were blocked with 10% BSA for 30 minutes and incubated with rabbit anti-pFAK Y397 overnight. pFAK 397 was detected by staining with a anti-rabbit secondary conjugated to Alexa Fluor 594. Coverslips were mounted with Prolong Gold containing DAPI.

Transwell migration assay

The underside of Corning/Costar 8.0 µm, 6.5 mm transwell inserts (Lowel, MA) were coated with 100 µg/ml of collagen I overnight at 4ºC. The following morning, the inserts were washed 2-3 times with serum free medium to neutralize the pH of the collagen coating. The upper surface of the insert was then blocked with 10% sterile-filtered BSA

for one hour at 37ºC. The BSA was aspirated and 4 x 10^4 MEFs or 2 x 10^4 HT29 cells, suspended in serum free media, were seeded in triplicate into the upper chamber of transwell inserts. Cells were allowed to migrate towards a gradient of 7% FBS for 5 hours before fixation with 4% paraformaldehyde. After 30 minutes of fixation, the paraformaldehyde was aspirated and the cells were stained with 1% crystal violet at least one hour. The crystal violet was aspirated and the inserts were washed with copious amounts of water. The water was aspirated out of the well and 1 ml of 1X PBS was pipetted into each well. A moistened cotton swab was used to remove cells and debris from the upper surface of the transwell inserts. Cells in five randomly selected fields were counted for each triplicate well. For each experiment, the average number of cells per culture was determined by averaging the number of cells counted per field in each replicate. The data presented represents the average number of cells from at least three independent experiments $\pm$ SEM. For comparison of the wild type and STRAP knockout MEFs, statistical significance was determined using the Wilcoxon signed rank test. Statistically significant differences in the motility of STRAP knockdown MEFs and HT29 cultures were determined using ANOVA.

Wound closure assay

Wild type and STRAP knockout MEFs were grown to confluence in 6 well plates. Prior to wounding, the cultures were pre-treated with 0.5 µg/ml mitomycin C for 3 hours. A single scratch was introduced into the cell monolayer with a 100 µl pipet tip and the dislodged cells were removed by washing the cultures in DMEM. The cultures were refed with DMEM containing 0.5 µg/ml mitomycin C and cell migration into the wound was monitored up to 24 hours post-wounding.

Live cell imaging

Wild type and STRAP knockout MEFs were seeded into petri dishes coated with 10 µg/ml of fibronectin and allowed to attach overnight. The following morning, the cultures were serum starved for seven hours. A gradient of FBS was delivered to the cells using a 0.8 µm transwell insert secured to the top of the petri dish lid. Chemotaxis was monitored for 5 hours by time lapse video microscropy. The migration of individual cells was tracked using Molecular Device's Metamorph software. A representative cell track is shown with the red dot representing the final recorded position of the tracked cell. The data from at least three independent experiments was pooled for analysis of the distance to origin (distance of the final recorded position from the first point in the track) and mean velocity. The distance to origin is displayed at the mean value $\pm$ SEM. The average velocity for each tracked cell is shown on a scatter plot along with the mean velocity $\pm$ SEM for the each population. Statistical significance was determined using the Mann Whitney test ($p<0.0001$).

Uropod formation and retraction

Images of cell tails were recorded by time lapse video microscopy as previously described. For determination of tail formation, the number of cells containing discernable tails was manually counted from images taken before chemotaxis. The percentage of cells within the field of view was calculated for three independent experiments and plotted as the average percentage $\pm$ SEM. Statistical significance was determined using a paired t-test. The length of the tails was quantified using the Metamorph region measurement tool. The tail length measurements from three independent experiments were pooled for each group. The data is presented as the

mean tail length ± SEM. Statistical significance was determined using an unpaired t-test.

Akt localization assay

Approximately 2.5 x 10^5 wild type and STRAP null MEFs were seeded into 6 cm petri dishes and cultured overnight in DMEM containing 10% FBS. The following day, the cultures were transfected with 1 µg of an Akt-PH-eGFP expression construct using Lipofectamine LTX reagent. The DNA-lipofectamine complexes were aspirated 4 hours after transfection and the cultures were fed with DMEM containing 10% FBS. The following morning, wild type and STRAP null MEFs were seeded at low density into 35 mm glass-bottom dishes coated with 10 µg/ml fibronectin. Twenty four hours later, the cultures were serum starved for 5-7 hours. A gradient of FBS was generated as previously described and GFP recruitment to the plasma membrane was recorded by time lapse video microscopy. Bright-field and GFP images were taken every minute for 30 minutes.

Adhesion assay

96-well plates were coated overnight at 4ºC with 10 µg/ml type I collagen or 5 µg/ml fibronectin. The wells were washed three times with 0.1% BSA and blocked with 0.5% BSA for one hour at 37ºC. The wells were washed again with 0.1% BSA and 3x 10^4 MEFs were seeded into triplicate wells. The cells were allowed to attach to the matrix for one hour before washing cultures 3 times with 0.1% BSA. The attached cells were then fixed with 4% paraformaldehyde for 10 minutes at room temperature and stained with 0.5% crystal violet for 10 minutes. The plates were washed with copious amounts and water and allowed to dry before solubilization of crystal violet with 1% SDS for 30

minutes. The absorbance$_{550}$ was measured in the Molecular Device's SpectroMax M5 plate reader and the average of the triplicate measurements was calculated. The data is presented as the mean absorbance of four independent experiments $\pm$ SEM.

Cell spreading assay

Chamber slides were coated overnight at 4ºC with 10 µg/ml fibronectin. 5 x 10^3 MEFs were seeded/well and allowed to attach for one hour. The cultures were then fixed with 4% paraformaldehyde for 10 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. The fixed cultures were blocked for one hour with 10% BSA and stained with phalloidin-Alexa Fluor 594 diluted 1:50 in 10% BSA for one hour. The wells were washed three times with PBS and coverslips were mounted with Prolong Gold containing DAPI. Random fields were selected for image acquisition and the total cell size/area was measured for each group ($n \leq 40$) with Metamorph's integrated morphometry analysis software. The area of each cell is displayed on a scatter plot along with the mean area $\pm$ SEM. Statistical significance was determined using the Student's t-test. The results are representative of at least three independent experiments.

Focal adhesion disassembly assay

Chamber slides were coated overnight at 4ºC with 10 µg/ml fibronectin. Approximately 5 x 10^3 wild type and STRAP null MEFs were seeded/well and allowed to attach overnight. The cultures were then treated with 10 µM nocodazole for 6 hours to dissociate microtubules. The nocodazole containing medium was aspirated and replaced with DMEM containing 10% FBS. The cultures were fixed with 4% paraformaldehyde at 0, 0.5, 1, and 2 hours after nocodazole washout and permeabilized with 0.1% Triton X-100.

After blocking with 10% BSA for 30 minutes, the cells were incubated with rabbit phospho-FAK Y397 (1:250) and mouse β-tubulin (1:250) antibodies overnight at 4ºC. Secondary anti-rabbit-Alexa Fluor 488 and anti-mouse-Alexa Fluor 594 were used for detection of p-FAK and β-tubulin by immunofluorescent microscopy.

Western blot

Cells were lysed in RIPA buffer containing 10 mM Tris [pH 7.2], 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholic acid supplemented with Sigma protease inhibitor and phosphatase inhibitor cocktails (1:100). The cells were scraped and the lysates were incubated on ice for 20 minutes before centrifugation at 13,000 rpm for 15 minutes at 4°C. The protein concentration of the clarified lysates was then measured using Bradford reagent. Equal concentrations of protein extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA). The membranes were then immunoblotted with the indicated antibodies.

Results

STRAP is a positive regulator of cell migration

We have recently reported that STRAP expression in MEFs is required for the acquisition of a mesenchymal morphology in mouse embryonic fibroblasts. Because a fibroblastoid morphology is associated with an increased capacity for migration, we directly tested the motility of wild type and STRAP null fibroblasts in Boyden chamber assays using serum as a chemoattractant. As expected, a significantly greater number of wild type MEFs migrated to the other side of the transwell insert than the epithelial-like STRAP knockout MEFs (paired t-test, $p<0.005$) (Figure 18A). To further examine the effects of STRAP expression on cell migration, STRAP was knocked down in wild-type MEFs and the colon adenocarcinoma cell line, HT29, by lentiviral transduction of shRNA. Individual clones (designed C, F, and J) were isolated from polyclonal mouse fibroblast cultures after selection and STRAP knockdown was confirmed by western blot analysis (Figure 18B). The motility of the monoclonal cultures was then tested in Boyden chamber assays. As shown in Figure 18B, STRAP knockdown MEFs exhibited a statistically significant reduction in migration towards serum compared to wild type fibroblasts (ANOVA, $p<0.005$). A Dunnett's multiple comparison post-test confirmed that the motility of wild type MEFs was significantly greater than the STRAP knockout and STRAP knockdown fibroblasts. STRAP knockdown polyclonal HT29 cultures (designated 87-1 and 87-2) also exhibited a marked reduction in cell motility relative to the parental and vector control culture (Figure 18C). Collectively, these findings suggest that STRAP expression promotes directed motility.

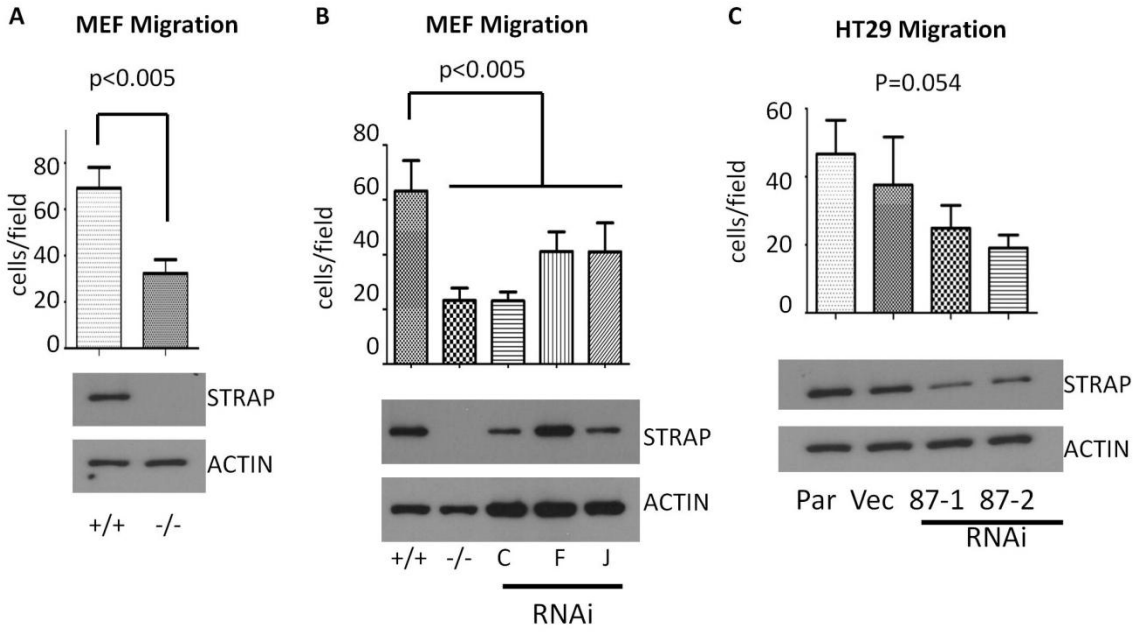


Figure 18. STRAP expression promotes cell migration. A. Boyden chamber assays were performed to assess the motility of wild type and STRAP knockout fibroblasts towards a gradient of 7% serum. The average number of cells/field ± SEM from ten independent experiments is shown. Statistical significance was determined using a paired t-test ($p<0.005$). B,C. The effects of STRAP knockdown on the motility of monoclonal MEF (B) and polyclonal HT29 cultures (C) was examined using Boyden chambers. Seven percent FBS was used as a chemoattractant. The average number of cells/field ± SEM from at least three independent experiments is shown. Statistically significant differences in the migration of STRAP knockdown MEF and HT29 cultures was determined using one-way ANOVA (MEF shRNA experiment, $p<0.005$). A Dunnett's multiple comparison post-test confirmed that there were significant differences in migration between the wild type MEFs and the other fibroblast cultures.

Loss of STRAP increases cell adhesion and spreading

Because motility defects are often associated with increased cell adhesion, the effects of STRAP on cell-matrix attachment and cell spreading was investigated. Wild type and STRAP knockout fibroblasts were seeded onto fibronectin and collagen matrices and permitted to attach for one hour. Loss of STRAP markedly increased adhesion to fibronectin (Figure 19A). Similarly, loss of STRAP increased cell attachment to type I collagen (Figure 19B). Cell spreading was examined by seeding wild type and STRAP knockout MEFs on fibronectin for one hour followed by phalloidin staining to demarcate the cell boundaries. In accordance with the increased adhesiveness, STRAP knockout MEFs generally appeared to spread more on fibronectin than wild-type fibroblasts (Figure 19C), suggesting that loss of STRAP promotes cell spreading. Metamorph software was then used to quantify cell spreading as a function of cell size. Based on the analysis of randomly selected cells, loss of STRAP expression significantly enhanced spreading on fibronectin (unpaired t-test, $p<0.001$) (Fig. 19D). Taken together, these findings may suggest that STRAP promotes motility by limiting the relative strength of cell-matrix adhesions.

FAK incorporation and subsequent activation in immature focal contacts promotes cell adhesion and spreading through activation of various effector proteins. Because loss of STRAP increased fibroblast adhesion and spreading, STRAP may be involved in the recruitment of FAK to protrusive membrane structures. To examine the effects of STRAP on the subcellular distribution of activated FAK, wild type and STRAP null MEFs were seeded onto fibronectin and allowed to spread for four hours before immunostaining for phospho-FAK Y397 (Figure 19E). Wild-type fibroblasts exhibited large peripheral phospho-FAK foci that were predominantly concentrated at protrusive regions. Conversely, STRAP null fibroblasts had small punctate peripheral pFAK foci

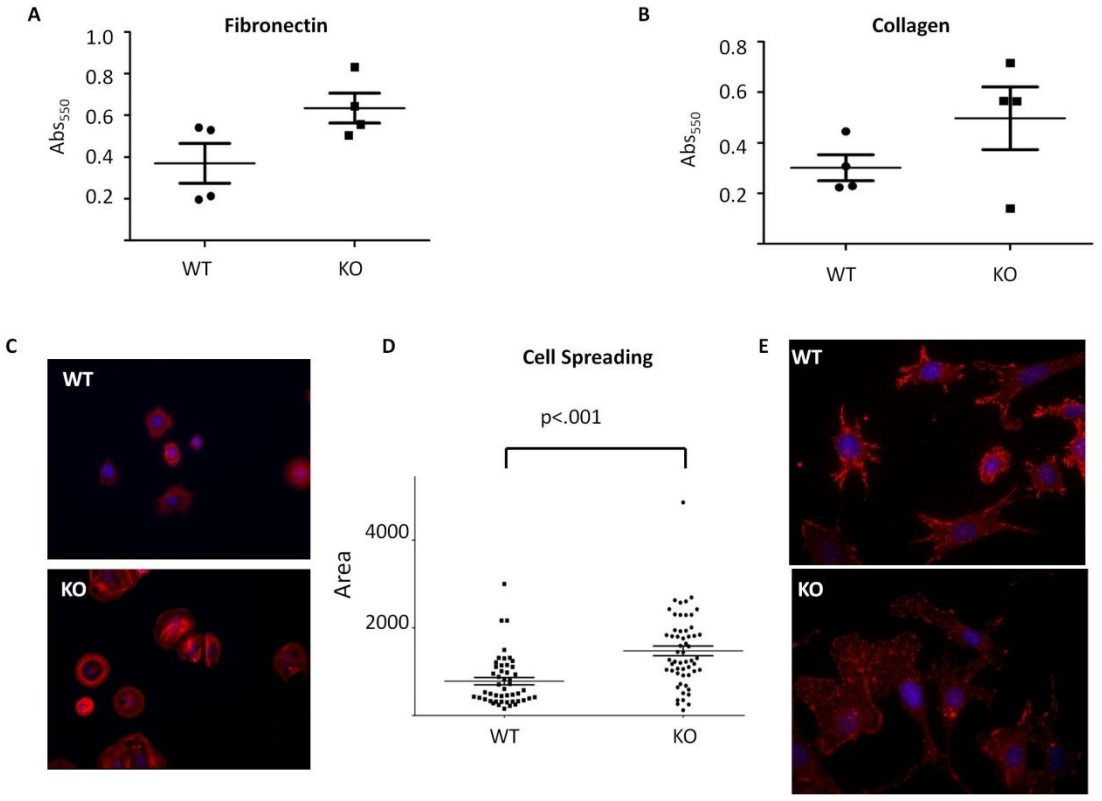


Figure 19. Loss of STRAP promotes cell adhesion and spreading. A,B. Wild type and STRAP knockout MEFs were seeded into 96 wells plates coated with 5 µg/ml fibronectin (A) and 10 µg/ml collagen (B). The cells were allowed to attach for one hour before fixation and crystal violet staining. The relative adhesion $\pm$ SEM from four independent experiments is plotted as a function of absorbance$_{550}$. C. Wild type and STRAP null fibroblasts were seeded into chamber slides coated with 10 µg/ml fibronectin. The cells were allowed to attach and spread for one hour before staining with phalloidin-Alexa Fluor 594. Images were acquired using a 63X objective. D. Cell spreading was quantified by using Metamorph software to measure the total area of randomly selected cells (N>40). The plot represents the average area $\pm$ SEM. Statistical significance was determined using an unpaired t-test ($p<0.001$). The experiment was repeated at least three times with similar results. E. The relative distribution of focal adhesions was examined by immunostaining wild type and STRAP null MEFs for activated FAK. MEFs were seeded on fibronectin plates and incubated with a phospho-specific antibody directed against Y397 four hours post-seeding. Images were taken using a 63X objective.

with numerous large foci that were distributed along the basal surface of the membrane. These findings may suggest that loss of STRAP promotes adhesion through stabilization of centrally-located focal adhesions.

STRAP regulates persistent chemotactic migration

In order to investigate the importance of cell-matrix adhesions with respect to the mechanics of motility, the motility of single cells was observed by time lapse video microscopy. Serum-starved wild type and STRAP null MEFs were exposed to a gradient of fetal bovine serum and cell migration was recorded for 5 hours. Metamorph software was then used to track the trajectory of individual cells during the elapsed time. As shown by the cell tracks (Figure 20A), wild type MEFs generally maintained an orientation and migratory path towards the gradient source whereas the STRAP null fibroblasts failed to initiate and/or maintain migration in the direction of the gradient. This suggests that STRAP is necessary for persistent migration. Although STRAP appears to be required for persistence during chemotactic migration, loss of STRAP surprisingly increased the rate of single cell migration (Figure 20B). Furthermore, the distance between the cell's point of origin and final destination was significantly higher in STRAP null fibroblasts, despite the tendency to change course (Figure 20C). Given that loss of STRAP expression disrupts directed migration in live cell chemotaxis and Boyden chamber assays, the effects of STRAP on the motility of mitomycin C-treated wild type and STRAP null fibroblasts was examined in a wound closure assay (Figure 20D). STRAP knockout fibroblasts were able to efficiently close wounds at comparable or faster rates than wild-type MEFs. This finding suggests that loss of STRAP does not

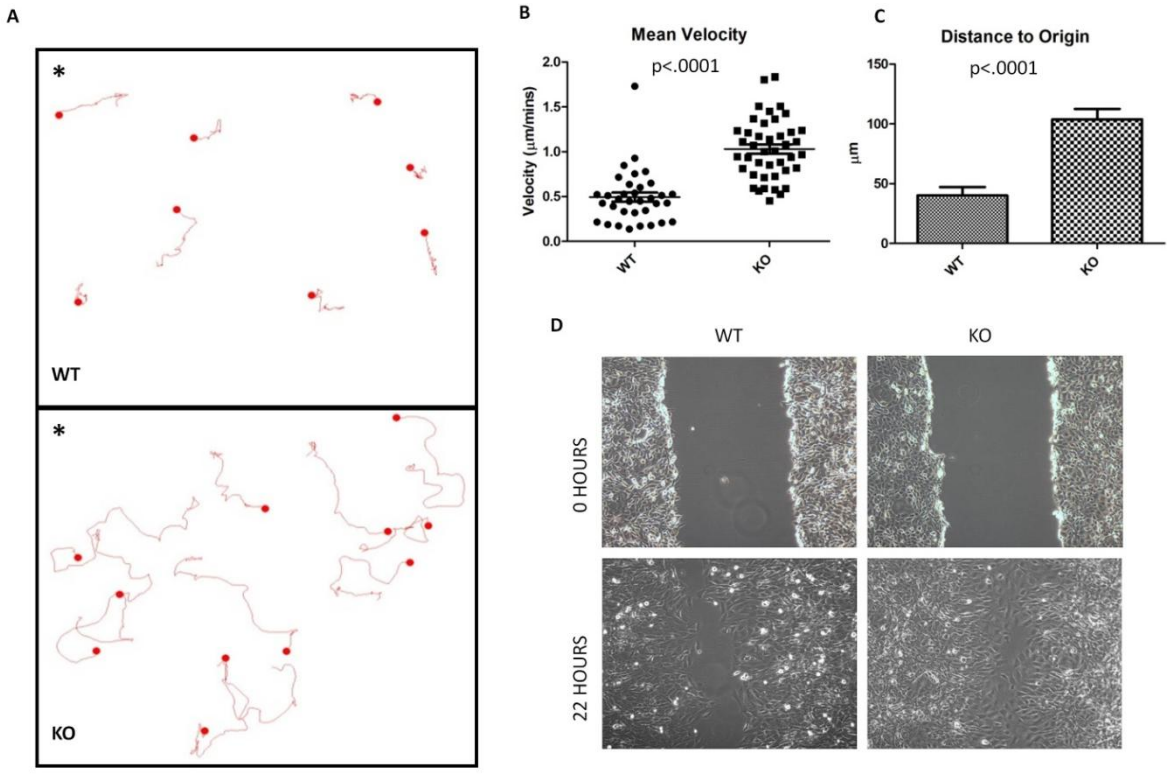


Figure 20. STRAP is required for persistent chemotaxis. A. Chemotaxis of wild type and STRAP knockout MEFs towards a serum gradient was examined by time-lapse video microscopy. Cell motility was recorded for 5 hours and the trajectory of individual cells within the field was tracked using Metamorph software. The final position of each cell is shown as a red circle. The asterik (*) indicates the direction of the gradient source. B. The velocity of wild type and STRAP null MEFs from at least three independent experiments are shown. The average velocity (µm/minute) of individual wild type and STRAP null MEFs was plotted with the mean velocity $\pm$ SEM shown for each group. Statistical significance was determined using the Mann Whitney test ($p<0.0001$). C. The mean distance from the origin $\pm$ SEM is shown. Statistical significance was determined using the Mann Whitney test ($p<0.0001$) D. The motility of wild type and STRAP knockdown MEFs was examined by wound healing assays. Migration into the wound was documented at 0 and 22 hours post-wounding.

diminish the capacity for migration but inhibits the ability to persistently migrate towards a chemical gradient.

The mechanisms that regulate gradient sensing and persistent migration are poorly understood. It has been reported that phosphoinositide-3-phosphate (PI3K) mediated recruitment of AKT to the plasma membrane is an essential step in chemotaxis (Yoo et al., 2010). Since STRAP knockout disrupts fibroblast chemotaxis, the effects of STRAP on the AKT membrane recruitment was investigated using a fusion protein comprised of the pleckstrin homology (PH) domain of AKT fused to enhanced green fluorescent protein (AKT-PH-eGFP). The pleckstrin homology domain targets AKT to the plasma membrane through direct association with the membrane phospholipid, PIP_3. As such, exposure to a gradient of chemoattractant would result in a cycle of AKT membrane recruitment followed by diffusion. Changes in AKT-PH-eGFP localization in wild type and STRAP null MEFs was monitored by time-lapse video microscopy after exposure to a gradient of serum (Figure 21). GFP was recruited to membrane ruffles and protrusions in both cell lines, suggesting that the persistent motility of wild type MEFs can not be attributed to enhanced AKT recruitment to the leading edge.

Loss of STRAP is associated with decreased tail retraction and altered phospho-FAK Y397 distribution

We have shown that loss of STRAP is correlated with increased cell adhesion to ECM proteins and large centrally-located focal adhesions, but these attachments do not inhibit cell motility. During cell migration, nascent cell attachments to the underlying matrix are formed beneath the leading edge and attachments at the rear of the cell must be disassembled to facilitate forward movement. Time lapse video microscopy of migrating cells revealed defective uropod retraction in STRAP null MEFs. Loss of STRAP expression resulted in long tails that often grew as the leading edge and cell

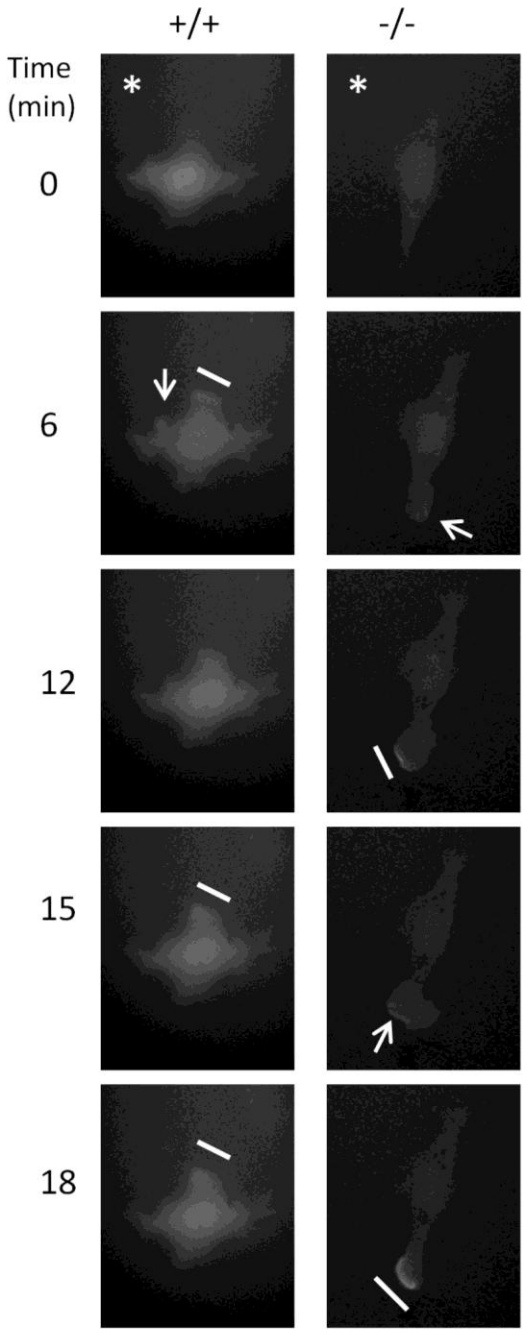


Figure 21. Loss of STRAP does not inhibit AKT membrane recruitment. Wild type and STRAP knockout fibroblasts were transiently transfected with AKT-PH-eGFP and seeded onto fibronectin. Time lapse images of AKT recruitment were recorded for 30 minutes after application of a gradient of serum. The location of the gradient source is indicated with an asterik. Arrows and bars indicate regions of AKT membrane recruitment.

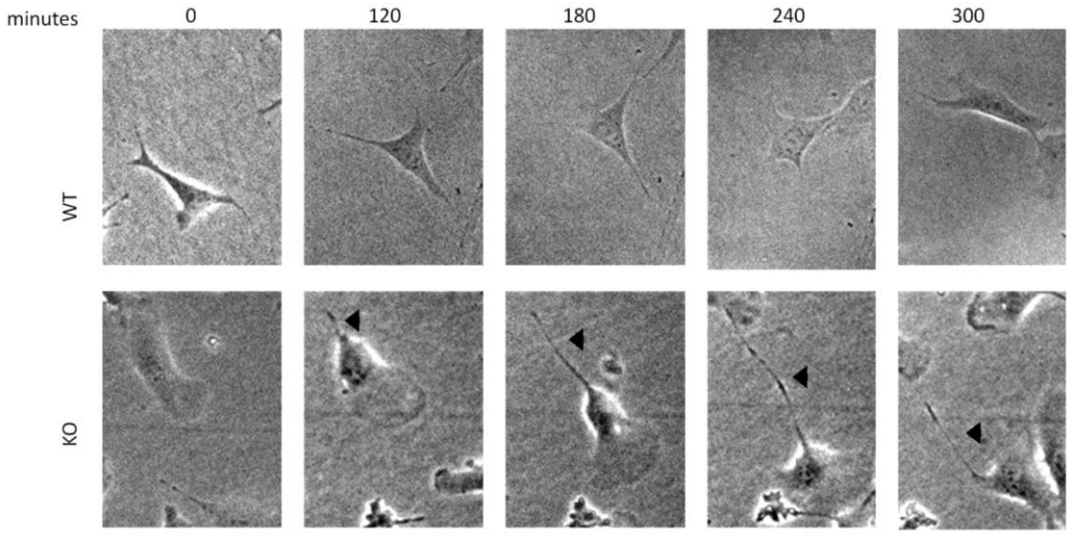


Figure 22. STRAP is required for efficient tail retraction during chemotaxis. Time-lapse images of cells undergoing chemotaxis were recorded for five hours. The morphology of a single wild type and STRAP knockout fibroblast is shown at 0, 120, 180, 240, and 300 minutes. Cell tails are denoted with arrowheads.

body moved forward whereas persistent tails were not observed on wild-type MEFs (Figure 22).

The defective tail retraction in STRAP null MEFs suggests that STRAP may regulate dissolution of cell-matrix adhesions. To investigate the role of STRAP in focal adhesion turnover, focal adhesion disassembly assays were performed using wild type and STRAP null MEFs (Figure 23). Treatment with 10 µM nocodazole effectively inhibited microtubule assembly and induced cell retraction in both wild type and STRAP null fibroblasts. After nocodazole washout, wild type fibroblasts exhibited consistent peripheral FAK staining during cell spreading suggesting that microtubule-mediated disassembly of older focal adhesions occurs at a comparable rate as the assembly of new peripheral adhesions. Conversely, STRAP null MEFs initially show increased

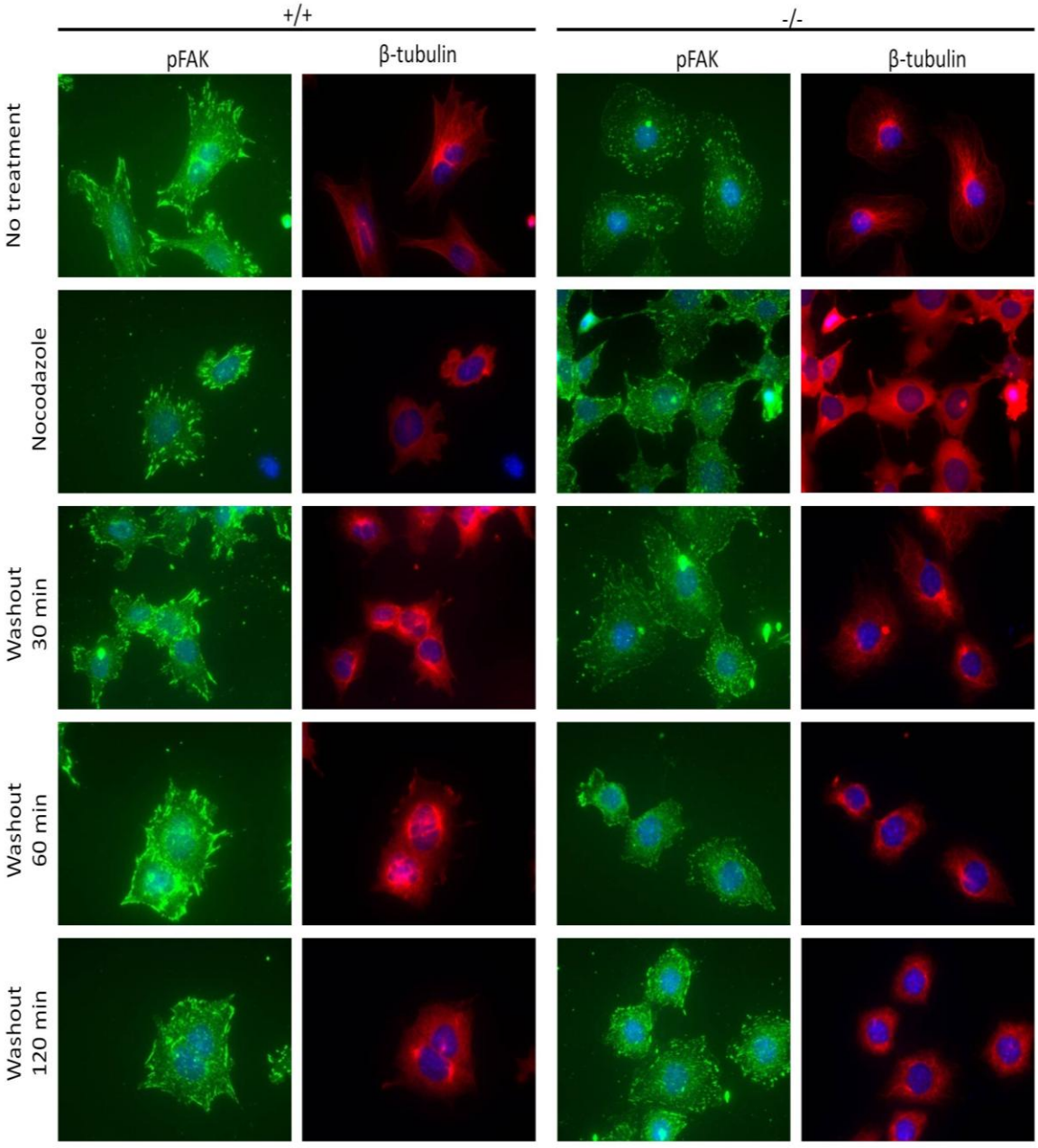


Figure 23. Loss of STRAP alters the distribution of phospho-FAK Y397.

peripheral FAK activation but foci containing active FAK appeared more punctate and centrally located as cell spreading progressed. This suggests that nascent attachments are formed as the STRAP null MEFs spread but the older adhesions are not disassembled.

Ectopic expression of STRAP rescues the defect in directed migration and attenuates tail formation

In order to determine whether the effects of STRAP expression on fibroblast motility and tail retraction are specific, HA-tagged STRAP or empty vector was stably reintroduced into STRAP null MEFs by retroviral transduction. After positive selection with puromycin, individual clones were isolated from the polyclonal populations of

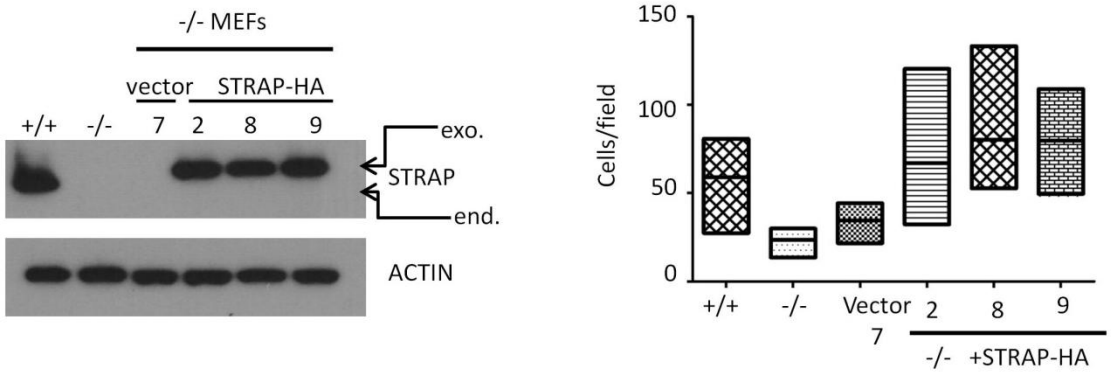


Figure 24. Reintroduction of STRAP into the STRAP null MEFs increases cell migration. A. Western blot analysis for STRAP expression in STRAP knockout MEF clones stably transduced with pBABE empty vector or pBABE-STRAP-HA. The lower molecular weight band is the endogenous ("end.") form of STRAP while the higher molecular weight band is the epitope-tagged exogenous ("exo.") STRAP. B. The motility of the stable vector control and STRAP-HA clones was examined in a Boyden chamber assay. Seven percent fetal bovine serum was used as a chemoattractant.

pBABE vector and pBABE-STRAP-HA cultures. Expression of exogenous STRAP-HA was confirmed by western blot analysis (Figure 24A) and the motility of the monoclonal cell lines was then examined in Boyden chamber assays using serum as a chemoattractant (Figure 24B). Ectopic expression of STRAP-HA in the null background markedly increased migration compared to the parental STRAP null fibroblasts and pBABE vector control, suggesting that STRAP is sufficient to rescue the persistent motility defect.

The effects of STRAP expression on tail retraction was then examined by time lapse video microscopy. Vector control clone 7 and STRAP-HA clone 8 were seeded onto fibronectin and serum starved before application of a gradient of serum. During chemotaxis, wild type cells generally exhibited a pattern of limited tail growth followed by retraction (Figure 25A). Conversely, a markedly greater number of vector control cells showed considerable defects in tail retraction during motility. The failure to retract singular tails was often accompanied by branching or formation of additional uropods (Figure 25A). Interestingly, the vector control cultures showed increased tail formation prior to initiation of chemotaxis, possibly due to retraction defects during random migration on fibronectin (Figure 25B). To quantitatively show differences in tail formation between vector control and STRAP-HA expressing cultures, the percentage of cells within the field of view that exhibited distinctive tails was calculated from three independent experiments. Compared to the STRAP-HA cultures, the vector control cultures had a significantly higher percentage of cells with tails (Figure 25C). The tail lengths were measured using Metamorph software and the individual values from three separate experiments were pooled for statistical analysis. As shown in Figure 25D, vector control cells had significantly longer tails than the STRAP-HA expressing cells.

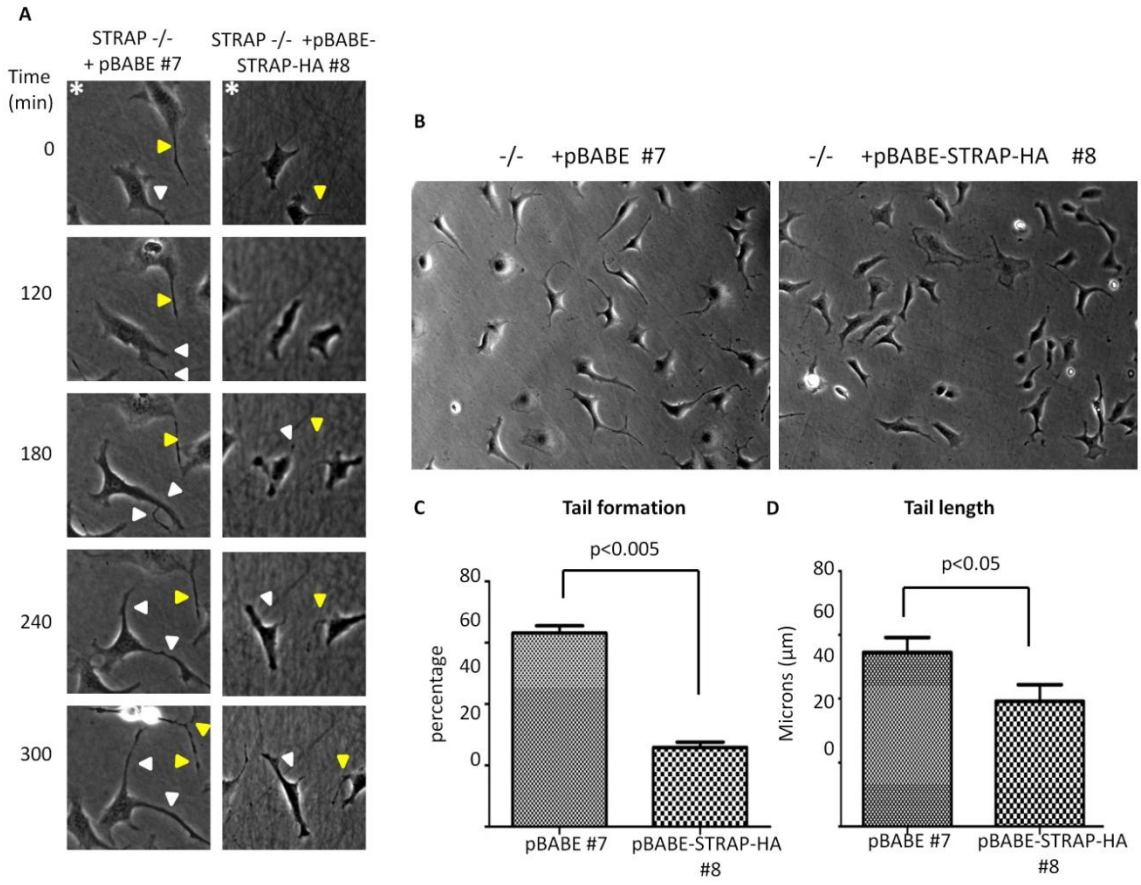


Figure 25. Ectopic STRAP in STRAP null fibroblasts reduces tail growth. A. Time-lapse images of cells undergoing chemotaxis were recorded for five hours. The morphology of a single cell from a STRAP knockout vector control clone 7 and STRAP-HA stable clone 8 is shown at 0, 120, 180 240, and 300 minutes. Asteriks (*) indicate the location of the gradient source. Cell tails are denoted with white arrowheads. B. Images of STRAP knockout vector control and STRAP-HA clones before (time=0) and after chemotaxis (time=5 hours). 20X images show increased presence of singular and branched tails in the vector control population. C. Tail formation prior to chemotaxis was quantified by determining the percentage of cells within a field that exhibited definitive uropods. Data from three independent experiments are shown. Statistical significance was determined with a paired t-test ($p<.005$). D. Tail lengths of vector control and STRAP-HA stable clones was measured using Metamorph software. The average length of cells was calculated by pooling the tail measurements from three independent experiments. Statistical significance was determined using an unpaired t-test ($p<.05$).

Collectively, these data may suggest that ectopic STRAP expression in the STRAP knockout fibroblasts suppresses tail growth during random and directed migration.

STRAP regulates FAK subcellular localization

Our data suggests that loss of STRAP expression is correlated with larger centrally-located pFAK foci and defective tail retraction. Because ectopic STRAP expression in STRAP null MEFs reduces tail formation, we predicted that exogenous STRAP may induce changes in pFAK localization. To further examine the effects of STRAP expression on pFAK distribution, the stable pBABE-STRAP-HA expressing monoclones and corresponding control cultures were seeded onto fibronectin for four hours and immunostained for pFAK Y397. In accordance with our previous findings, wild type MEFs exhibit strong peripheral FAK staining, concentrated at protrusive regions while the STRAP knockout MEFs contain numerous large internal pFAK foci (Figure 26). Similar to the STRAP null MEFs, vector control cultures showed large internal pFAK puncta with small pFAK adhesions at the edges of membrane ruffles. Although the morphology of the STRAP stable clones was variable, STRAP expression appeared to increased peripheral pFAK localization and decrease central pFAK staining. These findings may suggest that STRAP affects tail growth by regulating the relative distribution of focal adhesion structures.

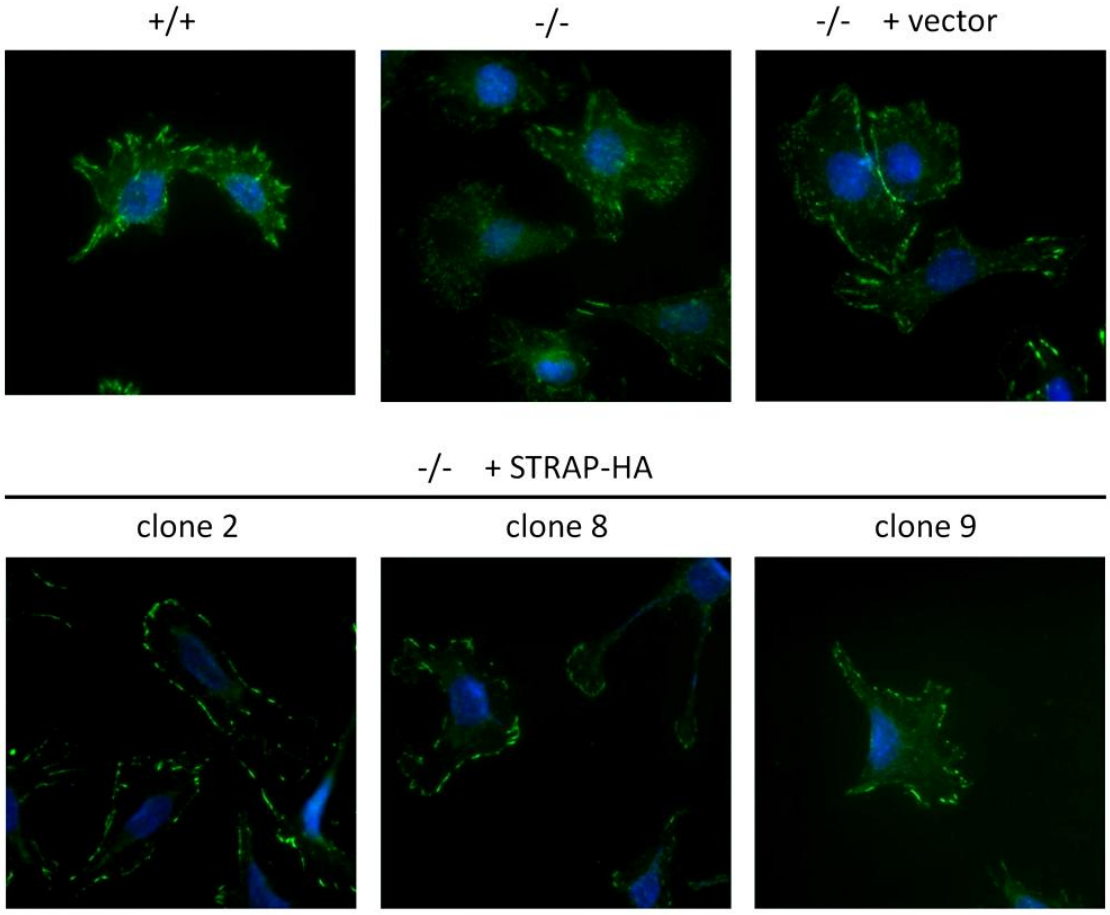


Figure 26. STRAP regulates phospho-FAK localization. Wild type, STRAP knockout, STRAP -/- pBABE vector control, and STRAP -/- pBABE-STRAP-HA expressing fibroblast cultures were seeded onto fibronectin. Four hours after seeding, the cultures were immunostained for pFAK Y397 to show the distribution of focal adhesion complexes. Ectopic overexpression of STRAP in the STRAP null MEFs was associated with a reduction in large, central adhesions and an increase in peripheral pFAK staining.

Discussion

Previous studies have shown that loss of STRAP expression prevents mouse embryonic fibroblasts from acquiring a mesenchymal morphology during development, possibly due to arrested embryonic EMT. Given that EMTs are associated with a motile phenotype, we hypothesized that STRAP expression would promote cellular motility. Initial investigation of the relative motility of wild type and STRAP null fibroblasts supported this hypothesis. Knockdown of STRAP in wild type MEFs and HT29 colon adenocarcinoma cells also decreases migration in Boyden chamber assays. However, it will be important to repeat these experiments using non-target shRNA controls and additional STRAP-specific shRNAs to address concerns regarding off-target effects of shRNA.

A defect in the basic mechanics of cell motility was initially presumed due to the reduced migration of STRAP null and STRAP knockout cell lines in Boyden chamber assays. Surprisingly, live cell imaging experiments suggest that loss of STRAP expression adversely affects persistent migration towards a gradient of serum. Reintroduction of STRAP into the STRAP knockout MEFs rescued motility in Boyden chamber assays, suggesting that STRAP is required for directed migration towards a chemoattractant. Interestingly, both wild type and STRAP knockout MEFs were able to effectively recruit Akt to the cell membrane, suggesting that upstream activation of GPCRs and PI3K occur in both cell types. It has been reported that αvβ3 integrin expression promotes persistent migration of epithelial cells whereas α5β1 integrin promotes random migration by stimulating RhoA activity at the leading edge (Danen et al., 2005). Likewise, inhibition of αvβ3 endocytic recycling has been reported to disrupt persistent migration of fibroblasts due to increased recycling of α5β1 integrin (White et

al., 2007). In light of the increased adhesion exhibited by STRAP null MEFs, it is tempting to speculate that differences in the integrin expression profile could account for the differences in migrational persistence and adhesion. However, increased α5β1 activation is associated with reduced polarity (Danen et al., 2005; White et al., 2007) and the STRAP null MEFs appeared to be more polarized than wild type fibroblasts (data not shown). It is plausible that loss of STRAP may cause random motility by activating RhoA downstream of cell polarization.

Our preliminary data also suggests that STRAP may have a role in the regulation of cell adhesion dynamics during cell spreading and migration. Loss of STRAP expression in fibroblasts increased adhesion and cell spreading on extracellular matrix proteins and inhibited tail retraction during chemotaxis. Because cell attachment to matrix is regulated by integrin receptors, it is possible that STRAP affects the expression or activation of cell surface integrin receptors. The contribution of integrins to the adhesive properties of these cells can be explored by investigating the integrin expression profile of wild type and STRAP null MEFs by flow cytometric analysis.

Despite the defect in tail release while migrating on fibronectin, STRAP knockout MEFs appeared to efficiently form adhesions at the leading edge as their rate of migration was signficantly higher than wild type fibroblasts. This may suggest that STRAP is differentially regulating adhesion structures at the front and rear of migrating cells. Immunofluorescent staining for pFAK Y397 revealed significant differences in the distribution of focal adhesion structures between wild type and STRAP null MEFs. In the absence of STRAP, fibroblasts exhibited punctate peripheral pFAK staining and larger foci distributed throughout the basal surface of the cell. The smaller peripheral foci may represent immature focal complexes while the large central foci may be focal adhesions that have undergone maturation. During directed migration, the majority of immature

focal complexes are rapidly turned over (Zaidel-Bar et al., 2003), so the presence of small punctate adhesions at dynamic membrane structures may account for the increased rate of STRAP null fibroblast motility. However, the presence of large, centrally-located foci in STRAP null MEFs may inhibit tail retraction due to increased stabilization of focal adhesions. It has recently been reported that myosin IIB stabilizes actin bundles in the cell rear and promotes the formation of large, mature cell adhesions (Vincente-Manzanares et al., 2011). Although tail retraction was not specifically examined, overexpression of myosin IIB was shown to markedly hinder focal adhesion turnover in favor of focal adhesion maturation (Vincente-Manzanares et al., 2011). Interestingly, ectopic expression of STRAP in STRAP null MEFs increased peripheral pFAK staining and decreased tail formation. STRAP was not identified as a component in purified focal adhesions (Kuo et al., 2011), so it is unlikely that STRAP regulates adhesion dynamics by directly affecting FAK localization to focal complexes.

In order to determine whether STRAP regulates focal adhesion disassembly, wild type and STRAP null MEFs were treated with nocodazole to inhibit β-tubulin polymerization and focal adhesions were monitored by immunostaining for pFAK Y397 at various times after nocodazole washout. After nocodazole washout, wild type MEFs began spreading but maintained peripheral focal adhesions. Conversely, the STRAP null MEFs appeared to have a prominent ring of intense pFAK staining with weakly stained protrusions extending beyond the internal ring. This may suggest that the wild type MEFs assemble and disassemble focal adhesions at a comparable rate whereas the STRAP knockout MEFs fail to disassemble the initial cortical adhesions but continue to form immature focal contacts at distal protrusions.

In the broad context of cancer, cell migration and invasion are necessary for localized and distal tumor metastasis. Previous studies have shown that MMP secretion

by carcinoma cells increases invasive capabilities (Powell et al., 1993; Juarez et al., 1993) and may be correlated with tumor metastasis (Garbisa et al., 1992; Yoshimoto et al., 1993). However, it has also been reported that expression of MMPs in the stromal compartment promotes invasion and metastasis (Zhang et al., 2006; Gorden et al., 2007). Gaggioli et al. have previously demonstrated that stromal fibroblasts generate invasive tracks that are subsequently utilized for the collective migration of epithelial tumor cells (Gaggioli et al., 2007). As STRAP has been shown to promote MMP28 expression (Swingler et al., 2010), it is possible that STRAP overexpression in stromal fibroblasts could support tumor metastasis through increased ECM remodeling. In the event that STRAP null fibroblasts retain the ability to secrete MMPs, the apparent persistence defects may result in the generation of circuitous tracks surrounding a primary tumor . As such, loss of stromal STRAP expression could favor local metastasis whereas STRAP-expressing fibroblasts could promote colonization of distant organ systems.

Chaper IV

CONCLUSIONS AND FUTURE DIRECTIONS

Much of the available data on STRAP suggests that it confers oncogenic properties to cells by regulating diverse signaling pathways (Reiner and Datta, 2011). These experimental findings are supported by the observation that STRAP is often up-regulated in breast, colon, and lung tumors (Matsuda et al., 2000, Halder et al., 2006, Kim et al., 2007). Previous studies have suggested that (a) STRAP promotes tumor growth by regulating mitogenic ERK signaling (Halder et al., 2006) and (b) STRAP regulates a morphological switches between mesenchymal and epithelioid phenotypes (Kashikar et al., 2010). However, it had not been determined whether STRAP also regulates signaling through other MAPK pathways or whether STRAP-induced transdifferentiation was associated with changes in cell motility. Herein, I have shown that STRAP has a functional role in the regulation of c-Jun stability as well as persistent migration. In this section, the significance of these findings will be discussed in the context of cancer and future directions will be enumerated for each respective project.

Significance of STRAP-mediated c-Jun stabilization and future directions

Because deregulated c-Jun expression promotes AP-1-induced tumor development (Jin et al., 2011; Sancho et al., 2009), normal cells limit c-Jun activity by regulating c-Jun expression. I have shown that STRAP expression in fibroblasts is associated with increased c-Jun stability and increased proliferation. Because AP-1 activity promotes cell autonomous growth through transactivation of genes required for the G1/S transition, it is possible that STRAP-mediation stabilization of c-Jun could

promote cell autonomous growth of mesenchymal-derived tumors *in vivo*. If STRAP also stabilizes c-Jun in epithelial cells, overexpression of STRAP could also promote carcinoma growth. Further investigation into the effects of STRAP on c-Jun turnover in epithelial cell lines will be required to confirm this hypothesis, but the observation that STRAP regulates ERK and TGF-β signaling in fibroblasts and epithelial cell lines raises the possibility that STRAP can regulate c-Jun expression in both cell types (Datta and Moses, 2000; Halder et al., 2006).

In addition to cell autonomous growth, STRAP-mediated stabilization of c-Jun in fibroblasts could also promote paracrine tumor growth. Within normal tissues, fibroblasts maintain tissue integrity through ECM maintenance and regulate epithelial cell homeostasis through growth factor secretion. However, cancer associated fibroblasts (CAFs) have been reported to support tumorigenesis by modulating growth (Olumi et al., 1999; Cheng et al., 2005) and vascularization (Crawford et al., 2009;Zhang et al., 2011). Furthermore, studies have shown that CAFs isolated from human tumor tissues exhibit increased loss of heterozygosity at genetic hotspots (Weber et al., 2006; Weber et al., 2007), suggesting that deregulated gene expression in stroma can influence epithelial tumor progression. In the context of the tumor microenvironment, STRAP-expressing stromal fibroblasts could promote carcinoma growth through AP-1-dependent secretion of growth factors. It has previously been shown that increased AP-1 signaling in fibroblasts promotes secretion of KGF, GM-CSF, and IGF (Maas-Szabowski et al., 2001; Li et al., 2007). Importantly, these soluble factors were reported to promote keratinocyte growth and differentiation in organotypic co-cultures (Maas-Szabowski et al., 2001) as well as stimulate BPH-1 growth *in vitro* (Li et al., 2007). Although STRAP LOH has not been reported in CAFs, a 35 fold increase in KGF expression was detected in wild type MEFs compared to STRAP null MEFs (data not

shown), suggesting that stabilization of c-Jun in fibroblasts promotes AP-1 transactivation of growth factors.

Further work will be required to fully understand the mechanisms by which STRAP regulates c-Jun stability and to address the biological significance of these findings. Given that STRAP regulates cell signaling events through direct association with numerous cellular proteins, identification of STRAP binding partners involved in protein degradation may point to potential mechanisms for STRAP mediated c-Jun stabilization. To further examine the biological implications of this research, it will be necessary to confirm that STRAP promotes proliferation through increased AP-1 activity by rescuing the STRAP knockout MEF growth defect with exogenous c-Jun. It will also be important to determine whether STRAP stabilizes c-Jun in epithelial cells and whether increased c-Jun stabilization affects cell autonomous growth. It may also be worthwhile to investigate the potential effects of stromal STRAP expression on paracrine signaling. As a first step, the expression of GM-CSF, KGF, and other growth factors should be validated by RT-PCR and ELISA to confirm that STRAP expression promotes the expression and secretion of AP-1 induced soluble factors. Furthermore, the growth of normal epithelial and BPH cells should be evaluated in co-culture systems with wild type and STRAP null MEFs. If preliminary data suggest that STRAP expression affects cell autonomous or paracrine growth of epithelial cells *in vitro*, tumor growth could be evaluated *in vivo* using immune-compromised mice (Table 4).

Biological implications for STRAP-induced persistent migration and future directions

Local and systemic metastasis of tumor cells is dependent on a number of factors, including the ability of tumor cells to migrate from the primary tumor to

Animal Models: Tumor Growth

Cell Autonomous Growth			
Model	Cell type	Cell lines	Phenotype
s.c.	F	WT MEFs, STRAP KO MEFs, STRAP KO + pBABE vector, STRAP KO + pBABE-STRAP-HA, STRAP KO + pBABE-c-JUN	WT MEFs-tumorigenic in nude mice (s.c) STRAP KO-not tumorigenic
s.c. or orthotopic	E (colon)	HCT116 parental, HCT116 + off-target shRNA, HCT116 + STRAP shRNA, HCT116 + STRAP shRNA + pBABE, HCT116 + STRAP shRNA + pBABE c-JUN	moderately aggressive (orthotopic)
Paracrine Signaling			
model	Cell types	Cell lines	
orthotopic /co-tranplant	F, E (prostrate)	WT MEFs,STRAP KO MEFs, STRAP KO + pBABE, STRAP KO + pBABE-c-JUN co-transplanted with BPH-1 cells	not malignant

s.c.= subcutaneous
F=fibroblast; E=epithelial

Table 4. Mouse models for assessing the effects of STRAP on AP-1 induced cell autonomous and paracrine growth.

a new colonization site. The seed and soil hypothesis posits that tissue microenvironment exerts selective pressures on cells which serve to restrict tumor cell colonization to organ systems that provide the requisite growth conditions. Alternatively, previous studies have shown that tumor cells preferentially migrate to specific organs in response to chemoattractant signals (Orr et al., 1985; Xu et al., 2009; Gassmann et al., 2009). Herein, I have shown that STRAP regulates persistent migration, tail retraction, and focal adhesion distribution in fibroblasts. In the context of chemoattractant-driven metastasis, it is possible that STRAP expression can promote tumor metastasis through regulation of persistent migration towards tissues that secrete chemoattractants.

It has previously been shown that fibroblasts generate leading tracks that are utilized by carcinoma cells for metastasis from primary tumors (Gaggioli et al., 2007). Based on this finding, STRAP expression in fibroblasts may support epithelial tumor cell metastasis by creating a path towards a metastatic site. It remains to be determined whether STRAP expression in trailing epithelial tumor cells is also required for efficient metastasis. Preliminary data using STRAP knockdown HT29 colon carcinoma cells suggests that STRAP promotes migration of epithelial cells towards chemoattractants. As such, it seems likely that STRAP expression in stromal fibroblasts and epithelial tumors would promote persistent migration and metastasis.

Future investigation into the role of STRAP in cell motility should be aimed at examining potential mechanisms regulating persistence and adhesion. To this end, it may be useful to perform affinity purification and mass spectrometry to identify STRAP-binding partners that regulate migration and focal adhesion dynamics. In addition to influencing cell signaling pathways through protein-protein interactions, STRAP may be up-regulating the expression of adhesion and motility proteins. Large scale proteomic analysis using multiplexed antibody arrays could be used to investigate differences in

the protein expression profiles between wild type and STRAP null fibroblasts. As extracellular matrix proteins have been reported to affect cell phenotype and biological response (Dekkers et al., 2006), it will be important to consider these variables when conducting future experiments. Because the Rho family of proteins are central regulators of motility, the spatial and temporal activation of RhoA, Rac1, and Cdc42 should be examined in migrating cells by FRET analysis. To further explore the role of STRAP in adhesion, it may be useful to characterize the effects of STRAP on focal adhesion assembly and disassembly in real time by using fluorescently-tagged focal adhesion proteins (Webb et al., 2004). Because the cell tails are less evident in stationery cells, it would be most informative to investigate the kinetics of adhesion formation and turnover at both the leading and trailing edges in migrating cells.

In addition to these mechanistic studies, it will be important to address the clinical relevance of these data. Loss of STRAP adversely affects embryonic development (Chen et al., 2004), possibly due to a failure in directed migration. Post-development attenuation of STRAP expression could potentially affect wound healing processes and immunological function. Conversely, overexpression of STRAP in tumors could promote tumor metastasis. Although STRAP overexpression has been reported in breast, lung, and colon tumor tissue (Matsuda et al., 2000; Halder et al., 2006), a correlation between STRAP expression level and incidence of metastasis has not been investigated. Until sufficient clinical data becomes available, animal models may serve as a useful tool to address this question. It has previously been reported that the colon adenocarcinoma cell line, HT29, spontaneously colonize the lungs of severe combined immunodeficiency (SCID) mice after subcutaneous implantation (Mitchell et al., 1997; Jojovic and Schumacher, 2000). Given that STRAP knockdown in HT29 cells decreases migration in Boyden chamber assays, it is possible that STRAP knockdown may decrease the

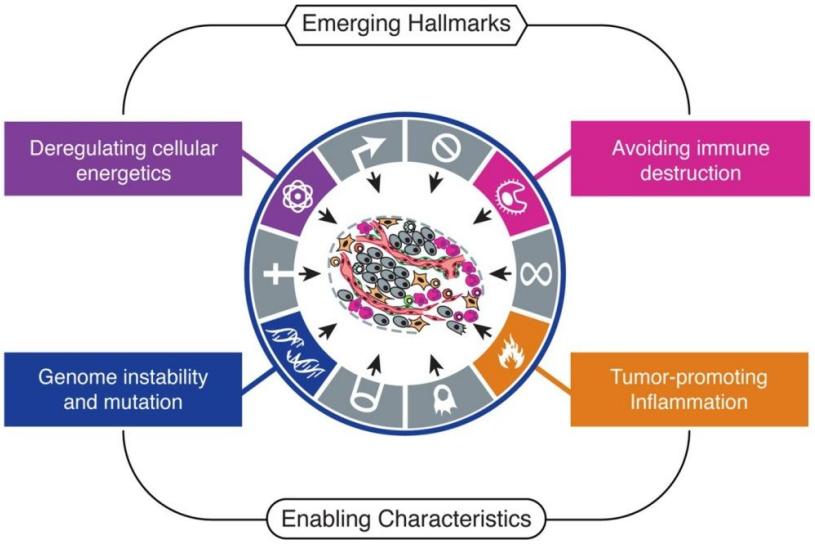
number of lung metastases relative to the appropriate controls. Swingler et al. have recently reported that STRAP functions as a co-factor for MMP28 transactivation in HeLa cells (Swingler et al., 2010). However, MMP28 is not expressed in HT29 cells (Lohi et al., 2001) and is down-regulated in inflammed colon and colon tumor epithelia (Bister et al., 2004). Provided that STRAP does not directly regulate expression of other MMPs, this model would provide a means of dissecting out the role of STRAP on tumor cell migration and metastasis independent of its effects on matrix degradation.

Concluding remarks

Collectively, the work presented herein further supports a role for STRAP in cancer development. Thus far, the literature on STRAP focuses on its cell autonomous functions. Although these studies directly examine the cell autonomous effects of STRAP on c-Jun expression and motility in fibroblasts, there is increasing evidence that stromal cells play an important role in cancer progression. It has been reported that AP-1 signaling in fibroblasts promotes growth factor secretion (Maas-Szabowski et al., 2001; Li et al., 2007) and that fibroblast-mediated matrix remodeling permits tumor cell migration (Gaggioli et al., 2007). In the context of these findings, it seems plausible that that stromal STRAP expression can contribute to tumor malignancy by stimulating paracrine growth and tumor metastasis. However, much more work will be necessary to understand the cell autonomous and microenvironmental contributions of STRAP towards tumor development.

In the framework of the hallmarks of cancer (Hanahan and Weinberg; 2011), previous studies suggest that STRAP may influence multiple aspects of cancer progression (Figure 27). Herein, I have shown that STRAP has a functional role in the

regulation of c-Jun stability and cell migration. The effects of STRAP on these biological processes have the potential to enforce sustained proliferation and promote dissemination of tumor cells from primary tumors, respectively.



Reprinted from *Cell* (2011) 144, 646-74 with permission from Elsevier

Hallmarks of Cancer	Influence of STRAP
Evading growth suppressors	Inhibits anti-proliferative TGF-β Smad signaling
Enabling replicative immortality	Not determined
Resisting cell death	Inhibits ASK1-mediated apoptosis Promotes PDK1/AKT survival pathway
Activating Invasion and Metastasis	Promotes transcription of MMP28 Promotes persistent chemotactic migration*
Sustaining proliferative signaling	Promotes ERK activation Stabilizes c-Jun*
Inducing angiogenesis	Not determined

Figure 27. Biological effects of STRAP on the hallmarks of cancer. Updated hallmarks of cancer as described by Hanahan and Weinberg. Icons corresponding to the classical hallmarks of cancer are depicted below with a description of the STRAP's contribution to these malignant properties. Asterisks (*) indicate observations from dissertation research.

REFERENCES

Angel, P., Hattori, K., Smeal, T., and Karin, M. (1988) The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. *Cell* **55**, 875-885.

Anthis, N. J., Wegener, K. L., Ye, F., Kim, C., Goult, B. T., Lowe, E. D., Vakonakis, I., Bate, N., Critchley, D. R., Ginsberg, M. H., and Campbell, I. D. (2009) The structure of an integrin/talin complex reveals the basis of inside-out signal transduction. *EMBO J* **28**, 3623–3632

Anumanthan, G. S., Halder, S. K., Friedman, D. B., and Datta, P. K. (2006) Oncogenic serine-threonine kinase receptor-associated protein modulates the function of Ewing sarcoma protein through a novel mechanism. *Cancer Res* **66**, 10824-32.

Bakin, A. V., Tomlinson, A. K., Bhowmick, N. A., Moses, H. L., Arteaga, C. L. (2000) Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* **275**, 36803-10.

Bakin, A. V., Rinehart, C., Tomlinson, A. K., Arteaga, C. L. (2002) p38 mitogen-activated protein kinase is required for TGFbeta-mediated fibroblastic transdifferentiation and cell migration. *J Cell Sci* **115**, 3193-206.

Bohren, K. M., Nadkarni, V., Song, J. H., Gabbay, K. H., and Owerbach, D. (2004) A M55V polymorphism in a novel SUMO gene (SUMO-4) differentially activates heat shock transcription factors and is associated with susceptibility to type I diabetes mellitus. *J Biol Chem* **279**, 27233-8.

Boyer, B., and Thiery, J. P. (1995) Epithelium-mesenchyme interconversion as example of epithelial plasticity. *APMIS* **101**, 257-68.

Brock, C., Schaefer, M., Reusch, H. P., Czupalla, C., Michalke, M., Spicher, K., Schultz, G., and Nürnberg, B. (2003) Roles of Gβγ in membrane recruitment and activation of p110/p101 phosphoinositide 3-kinase. *J Cell Biol* **160**, 89-99.

Buess, M., Terracciano, L., Reuter, J., Ballabeni, P., Boulay, J. L., Laffer, U., Metzger, U., Herrmann, R., and Rochlitz, C. (2004) STRAP is a strong predictive marker of adjuvant chemotherapy benefit in colorectal cancer. *Neoplasia* **6**, 813-20.

Carissimi, C., Baccon, J., Straccia, M., Chiarella, P., Maiolica, A., Sawyer, A., Rappsilber, J., and Pellizzoni, L. (2005) Unrip is a component of SMN complexes active in snRNP assembly. *FEBS Lett* **579**, 2348-54.

Carragher, N. O., Levkau, B., Ross, R., Raines, E. W. (1999) Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. J Cell Biol **147**, 619-30.

Carrillo, R. J., Dragan, A. I., and Privalov, P. L. (2010) Stability and DNA-Binding Ability of the bZIP Dimers Formed by the ATF-2 and c-Jun Transcription Factors. *J Mol Biol* **396**, 431-40.

Carter, R., Cosenza, S. C., Pena, A., Lipson, K., Soprano, D.R., and Soprano, K. J. (1991) A potential role for c-Jun in cell cycle progression through late G1 and S. *Oncogene* **6**, 229-35.

Chang, F., Lemmon, C. A., Park, D., and Romer, L. H. (2007) FAK potentiates Rac1 activation and localization to matrix adhesion sites: a role for betaPIX. *Mol Biol Cell* **18**, 253-64.

Chen, H-C, Appeddu, P. A., Parsons, J. T., Hildebrand, J. D., Schaller, M. D., and Guan, J-L (1995) Interaction of the focal adhesion kinase with cytoskeletal protein talin. *J Biol Chem* **270**, 16995-16999.

Chen, W. V., Delrow, J., Corrin, P. D., Frazier, J. P., and Soriano, P. (2004) Identification and validation of PDGF transcriptional targets by microarray-coupled gene-trap mutagenesis. *Nat Genet* **36**, 304-12.

Cheng, N., Bhowmick, N. A., Chytil, A., Gorksa, A. E., Brown, K. A., Muraoka, R., Arteaga, C. L., Neilson, E. G., Hayward, S. W., Moses, H. L. (2005). Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks. *Oncogene* **24**, 5053-68.

Cheresh, D. A., Leng, J., and Klemke, R. L. (1999) Regulation of cell contraction and membrane ruffling by distinct signals in migratory cells. *J Cell Biol* **146**, 1107-1116.

Choi, J. H., Yang, Y. R., Lee, S. K., Kim, S. H., Kim, H., Cha, J. Y., Oh, S. W., Ha, J. R., Ryu, S. H., and Suh, P. G. (2008) Potential inhibition of PDK1/Akt signaling by phenothiazines suppresses cancer cell proliferation and survival. *Ann NY Acad Sci* **1138**, 393-403.

Cobben, J. M., van der Steege, G., Grootscholten, P., de Visser, M,, Scheffer, H., and Buysm, C. H. (1995) Deletions of the survival of motor neuron gene in unaffected siblings of patients with spinal muscular atrophy. *Am J Hum Genet* **57**, 805-8

Cordenosi, M., Dupont, S., Maretto, S., Insinga, A., Imbriano, C., and Piccolo, S. (2003) Links between tumor suppressors: p53 is required for TGF-beta gene responses by cooperating with Smads. *Cell* **113**, 301-14.

Crawford, Y., Kasman, I., Yu, L., Zhong, C., Wu, X., Modrusan, Z., Kaminker, J., Ferrara, N. (2009) PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. *Cancer Cell* **15**, 21-34.

Danen, E. H. J., van Rhenen, J., Franken, W., Huveneers, S., Sonneveld, P., Jalink, K., and Sonnenberg, A. (2005) Integrins control motile strategy through a Rho–cofilin pathway. *J Cell Biol* **169**, 515–526.

Datta, P. K., Chytil, A., Gorska, A. E., and Moses, H. L. (1998) Identification of STRAP, a novel WD domain protein in transforming growth factor-beta signaling. *J Biol Chem* **273**, 34671-4.

Datta, P. K., and Moses, H. L. (2000) STRAP and Smad 7 synergize in the inhibition of transforming growth factor beta signaling. *Mol Cell Biol* **20**, 3157-67.

Davies, C. C., Chakraborty, A., Cipriani, F., Haigh, K., Haigh, J. J., and Behrens, A. (2010) Identificatio of a co-activator that links growth factor signaling to c-Jun/AP-1 activation. *Nat Cell Biol* **12**, 963-74.

Dekkers, B. G. J., Schaafsma, D., Nelemans, S. A., Zaagsma, J., and Meurs, H. (2006) Extracellular matrix proteins differentially regulate airway smooth muscle phenotype and function. *Am J Physiol Lung Cell Mol Physiol* **292**, L1405–L1413.

Derijard, B., Hibi, M., Wu, I.H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**, 1025-1037.

Derynck, R., and Zhang, Y. E. (2003) Smad-dependent and Smad-independent pathways in TGF-β family signaling. *Nature* **425**, 577-84.

Duband, J. L., Monier, F., Delannet, M., and Newgreen, D. (1995) Epithelium-mesenchyme transition during neural crest development. *Acta Anat* **154**, 63-78.

Elliott, R. L., and Blobe, G. C. (2005) Role of transforming growth factor beta in human cancer. *J Clin Oncol* **23**, 2078-93.

Engel, M. E., Datta, P. K., and Moses, H. L. (1998) Signal transduction by transforming growth factor-beta: a cooperative paradigm with extensive negative regulation. *J Cell Biochem Suppl* **30-31**, 111-22.

Erdogan, M., Pozzi, A., Bhowmick, N., Moses, H. L., and Zent, R. (2008). Transforming growth factor-beta (TGF-beta) and TGF-beta-associated kinase 1 are required for R-Ras-mediated transformation of mammary epithelial cells. *Cancer Res* **68**, 6224-31.

Evans, J. R., Mitchell, S. A., Spriggs, K. A., Ostrowski, J., Bomsztyk, K., Ostarek, D., and Willis, A. E. (2003) Members of the poly (rC) binding protein family stimulate the activity of the c-myc internal ribosome entry segment *in vitro* and *in vivo*. *Oncogene* **22,** 8012-20.

Fang, D., Kerppola, T.K. (2004) Ubiquitin-mediated fluorescence complementation reveals that Jun ubiquitinated by Itch/AIP4 is localized to lysosomes. *Proc Natl Acad Sci* **101**, 14782-14787.

Fleming, Y. M., Ferguson, G. J., Spender, L. C., Larsson, J., Karlsson, S., Ozanne, B. W., Grosse, R., and Inman, G. J. (2009) TGF-beta-mediated activation of RhoA signaling is required for efficient (V12)HaRas and (V600E)BRAF transformation. *Oncogene* **28**, 983-93.

Fuchs, S.Y., Dolan, L., Davis, R.J., and Ronai, Z. (1996) Phosphorylation-dependent targeting of c-Jun ubiquitination by Jun N-kinase. *Oncogene* **13**, 1531-1535.

Funamoto, S., Milan, K., Meili, R., and Firtel, R. A. (2001) Role of phosphatidylinositol 3′ kinase and a downstream pleckstrin homology domain containing protein in controlling chemotaxis in *Dictyostelium*. *J Cell Bio.* **153**, 795–810.

Funamoto, S., Meili, R., Lee, S., Parry, L. and Firtel, R. A. (2002) Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell* **109**, 611–623.

Garbisa, S., Scagliotti, G., Masiero, L., Di Francesco, C., Caenazzo, C., Onisto, M., Micela, M., Stetler-Stevenson, W. G., and Liotta, L. A. (1992) Correlation of serum metalloproteinase levels with lung cancer metastasis and response to therapy. *Cancer Res* **52**, 4548-9

Gaggioli, C., Hooper, S., Hidalgo-Carcedo, C., Grosse, R., Marshall, J. F., Harrington, K., and Sahai, E. (2007) Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nat Cell Biol* **9**, 1392-400.

Gao, B., Lee, S-M., and Fang, D. (2006) The tyrosine kinase c-Able protects c-Jun from ubiquitination-mediated degradation in T cells. *J Biol Chem* **281**, 29711-29718.

Gassmann, P., Haier, J., Schlüter, K., Domikowsky, B., Wendel, C., Wiesner, U., Kubitza, R., Engers, R., Schneider, S. W., Homey, B., and Müller, A. (2009) CXCR4 regulates the early extravasation of metastatic tumor cells in vivo. *Neoplasia* **11**, 51-61.

Gorden, D. L., Fingleton, B., Crawford, H. C., Jansen, D. E., Lepage, M., and Matrisian, L. M. (2007) Resident stromal cell-derived MMP-9 promotes the growth of colorectal metastases in the liver microenvironment. *Int J Cance* **121**, 495-500.

Grimmler, M., Otter, S., Peter, C., Muller, F., Chari, A., and Fischer, U. (2005) Unrip, a factor implicated in cap-independent translation, associates with the cytosolic SMN complex and influences its intracellular localization. *Hum Mol Genet* **14**, 3099-111.

Guo, D., Han, J., Adam, B. L., Colburn, N. H., Wang, M. H., Dong, Z., Eizirik, D. L., She, J. X., and Wang, C. Y. (2005) Proteomic analysis of SUMO4 substrates in HEK293 cells under serum starvation-induced stress. *Biochem Biophys Res Commun* **337**, 1308-18.

Halder, S. K., Beauchamp, R. D., and Datta, P. K. (2005) Smad7 induces tumorigenicity by blocking TGF-beta-induced growth inhibition and apoptosis. *Exp Cell Res* **307**, 231-46.

Halder, S. K., Anumanthan, G., Maddula, J., Mann, J., Chytil, A., Gonzalez, A. L., Washington, M. K., Moses, H. L., Beauchamp, R. D., and Datta, P. K. (2006) Oncogenic function of a novel WD-domain protein, STRAP, in human carcinogenesis. *Cancer Res* **66**, 6156-66.

Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of cancer: the next generation. *Cell* **144**, 646-674.

Hamadi, A., Bouali, M., Dontenwill, M., Stoeckel, H., Takeda, K., and Rondé, P. (2005) Regulation of focal adhesion dynamics and disassembly by phosphorylation of FAK at tyrosine 397. *J Cell Sci* **118**, 4415-25.

Han, W., Jung, E. M., Cho, J. Lee, J. W., Hwang, K. T., Yang, S. J., Kang, J. J., Bae, J. Y., Jeon, Y. K., Park, A., Nicolau, M., Jeffrey, S. S., and Noh, D. Y. (2008) DNA copy number alterations and expression of relevant genes in triple-negative breast cancer. *Genes Chromosomes Cancer* **47**, 490-9.

Hayashi, I., Vuori, K., and Liddington, R. C. (2002) The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. *Nat Struct Biol* **9**, 101-106.

Henegariu, O., VAnce, G. H., Heier, D., Pera, M., and Heerema, N. A. (1998) Triple-color FISH analysis of 12p amplification in testicular germ-cell tumors using 12p band-specific painting probes. *J Mol Med* **76**, 648-55.

Hennessy, C., Henry, J. A., May, F. E., Westley, B. R., Angus, B., and Lennard, T. W. (1991) Expression of the anti-metastatic gene nm23 in human breast cancer: an association with good prognosis. *J Natl Cancer Inst* **83**, 281-5.

Hildebrand, J. D., Schaller, M. D., and Parsons, J. T. (1995) Paxillin, a Tyrosine Phosphorylated Focal Adhesionassociated Protein Binds to the Carboxyl Terminal Domain of Focal Adhesion Kinase. *Mol Biol Cell* **6**, 637-647.

Hunt, S. L., Hsuan, J. J., Totty, N., and Jackson, R. J. (1999) unr, a cellular cytoplasmic RNA-binding protein with five cold-shock domains, is required for internal initiation of translation of human rhinovirus RNA. *Genes Dev* **13**, 437-48.

Hsu, S., Huang, F., Wang, L., Banerjee, S., Winawer, S., and Friedman, E. (1994) The role of nm23 in transforming growth factor beta 1-mediated adherence and growth arrest. *Cell Growth Differ* **5**, 909-17.

Huang, C-C., Wang, J-M., Kikkawa, U., Mukai, H., Shen, M-R., Morita, I., Chen, B-K., and Chang, W-C. (2008) Calcineurin-mediated dephosphorylation of c-Jun Ser-243 is required for c-Jun protein stability and cell transformation. *Oncogene* 27, 2422-2429.

Hudson, L. .G., Newkirk, K. M., Chandler, H. L., Choi, C., Fossey, S. L., Parent, A. E., Kusewitt, D. F. (2009) Cutaneous wound reepithelialization is compromised in mice lacking functional Slug (Snai2). *J Dermat Sci* **56**,19-26.

Humphries, J. D., Wang, P., Streuli, C., Geiger, B., Humphries, M. J., and Ballestrem, C. (2007) Vinculin controls focal adhesion formation by direct interactions with talin and actin. *J Cell Biol* **179**, 1043-1057.

Jin, J.Y., Ke, H., Hall, R.P., and Zhang, J.Y. (2011) c-Jun Promotes whereas JunB Inhibits Epidermal Neoplasia. *J Invest Dermatol*.

Johnson, R. S., van Lingen, B., Papaioannou, V. E., Spiegelman, B. M. (1993) A null mutation at the c-jun locus causes embryonic lethality and retarded cell growth in culture. *Genes Dev* **7**, 1309-1317.

Juarez, J., Clayman, G., Nakajima, M., Tanabe, K. K., Saya, H., Nicolson, G. L., and Boyd, D. (1993) Role and regulation of expression of 92-kDa type-IV collagenase (MMP-9) in 2 invasive squamous-cell-carcinoma cell lines of the oral cavity. *Int J Cancer* **55**, 10-8.

Jung, H., Seong, H. A., and Ha, H. (2007) NM23-H1 tumor suppressor and its interacting partner STRAP activate p53 function. *J Biol Chem* **282**, 35293-307.

Jung, H., Seong, H. A., Manoharan, R., and Ha, H. (2010) Serine-threonine kinase receptor-associated protein (STRAP) inhibiits apoptosis signal-regulating kinase 1 function through direct interaction. *J Biol Chem* **285**, 54-70.

Lamph, W. W., Wamsley, P., Sassone-Corsi, P., Verma, I. M. (1988) Induction of proto-oncogene JUN/AP-1 by serum and TPA. *Nature* **334**, 629-31.

Lauffenberger, D. A. and Horowitz, A. F. (1996) Cell migration: a physically integrated molecular process. *Cell* **84**, 359-369.

Li, W., Wu, C. L., Febbo, P. G., Olumi, A. F. (2007) Stromally expressed c-Jun regulates proliferation of protstrate epithelial cells. *Am J Pathol* **171**, 1189-98.

Lipinski, C. A., and Loftus, J. C. (2010) The PYK2 FERM domain: A novel therapeutic target. *Expert Opin Ther Targets* **14**, 95-108.

Llic, D. Furuta, Y., Kanazawa, S., Tekeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., and Aizawa, S. (2002) Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* **377**, 539 - 544.

Lopez-Bergami, P., Huang, C., Goydos, J. S., Yip, D., Bar-Eli, M., Herylyn, M., Smalley, K. S. M., Mahale, A., Eroshkin, A., Aaronson, S., and Ronai, Z. (2007) Rewired ERK-JNK signaling pathways in melanoma. *Cancer Cell* **11**, 447-60.

Kalluri, R., and Zeisberg, M. (2006) Fibroblasts in cancer. *Nat Rev Cancer* **6**, 392-401.

Katiyar, S., Casimiro, M. C., Dettin, L., Ju, X., Wagner, E.F., Tanaka, H., and Pestell, R. G. (2010) C-jun inhibits mammary apoptosis in vivo. *Mol Biol Cell* **21**, 4264-4274.

Kattla, J. J., Carew, R. M., Heljic, M., Godson, C., and Brazil, D. P. (2008) Protein kinase B/Akt activity is involved in renal TGF-beta1-driven epithelial -mesenchymal transition *in vitro* and *in vivo*. *Am J Physio Renal Physiol* **295**, F2515-25.

Kashikar, N. D., Reiner, J., Datta, A., and Datta, P. K. (2010) Serine threonine receptor-associated protein (STRAP) plays a role in the maintenance of mesenchymal morphology. *Cell Signal* **22**, 138-49.

Kim, C. J., Choi, B. J, Song, J. H., Park, Y. K., Cho, Y. G., Nam, S. W., Yoo, N. J., Lee, J. Y., and Par,, W. S. (2007) Overexpression of serine-threonine receptor kinase-associated protein in colorectal cancers. *Pathol Int*, **57**, 178-82.

Khokhar, A., Chen, N., Yuan, J. P., Li, Y., Landis, G. N., Beaulieu, G., Kaur, H., and Tower, J. (2008) Conditional switches for extracellular matrix patterning in Drosophila melanogaster. *Genetics* **178**, 1283-93.

Klingbeil, C. K., Hauck, C. R., Hsia, D. A., Jones, K. C., Reider, S. R., and Schlaepfer, D. D. (2001) Targeting Pyk2 to beta 1-integrin-containing focal contacts rescues fibronectin-stimulated signaling and haptotactic motility defects of focal adhesion kinase-null cells. *J Cell Biol* **152**, 97-110.

Kuo, J-C., Han, X., Hsaio, C-T, Yates III, J. R., and Waterman, C. M. (2011) Analysis of the myosin-II responsive focal adhesion proteome reveals a role for β-PIX in negative regulation of focal adhesion maturation. *Nat Cell Biol* **13**, 383-393.

Kuznetsov, G., Xu, Q., Rudolph-Owen, L., Tendyke, K., Lui, J., Towle, M., Zhao, N., Marsh, J., Agoulnik, S., Twine, N., Parent, L., Chen, Z., Shie, L., Jiang, Y., Zhang, H., Du, H., Boivin, R., Wang, Y., Romo, D., and Littlefield, B. A. (2009) Potent *in vitro* and *in vivo* anticancer activities of des-methyl, des-amino pateamine A, a Synthetic analogue of marine natural product pateamine A. *Mol Cancer Ther* **5**, 1250-60.

Leone, A., Flatow, U., King, C. R., Sandeen, M. A., Margulies, I. M., Liotta, L. A., and Stteg, P. S. (1991) Reduced tumor incidence, metastatic potential, and cytokine responsiveness of nm23-transfected melanoma cells. *Cell* **65**, 25-35.

Leone, A., Flatow, U., VanHoutte, K., and Steeg, P. S. (1993) Transfection of nm23-H1 into the human MDA-MD-435 breast carcinoma cell line: effects on tumor metastatic potential, colonization and enzymatic activity. *Oncogene* **8**, 2325-33.

Li, Z., Dong, X., Wang, Z., Liu, W., Deng, N., Ding, Y., Tang, L., Hla, T., Zeng, R., Li, L., and Wu, D. (2005) Regulation of PTEN by Rho small GTPases. *Nat Cell Bio.* **7**, 399–404.

Liu, Y., Wang, J., Wu, M., Wan, W., Sun, R., Yang, D., Sun, X., Ma, D., Ying, G., and Zhang, N. (2009) Down-regulation of 3-phosphoinositide-dependent protein kinase-1 levels inhibits migration and experimental metastasis of human breast cancer cells. *Mol Cancer Res* **7,** 944-54.

Lombardi, D., Palescandolo, E., Giordano, A., and Paggi, M. G. (2001) Interplay between the antimetastatic nm23 and the retinoblastoma-related Rb2/p130 genes in promoting neuronal differentiation of PC12 cells. *Cell Death Differ* **8**, 470-6.

Low, W. K., Dang, Y., Schneider-Poetsch, T., Shi, Z., Choi, N. S., Merrick, W. C., Romo, D., and Liu, J. O. (2005) Inhibition of eukaryotic translation initiation by the marine natural product pateamine A. *Mol Cell* **20**, 709-22.

Low, W. K., Dang, Y., Schneider-Poetsch, T., Shi, Z., Choi, N. S., Rzasa R. M., Shea, H. A., Li, S., Park, K., Ma, G., Romo, D., and Lui, J. O. (2007) Isolation and identification of eukaryotic initiation factor 4A as a molecular target for the marine natural product Pateamine A. *Methods Enzymol* **431**, 303-24.

Lucas, B., Grigo, K., Erdmann, S., Lausen, J., Klein-Hitpass, L., and Ryffel, G. U. (2005) HNF4 alpha reduces proliferation of kidney cells and affects genes deregulated in renal cell carcinoma. *Oncogene* **24**, 6418-31.

Maas-Szabowski, N., Szabowski, A., Stark, H-J., Andrecht, S., and Kolbus, A., Schorpp-Kistner, M., Angel, P., and Fusenig, N. E. (2001) Organotypic cocultures with genetically modified mouse fibroblasts as a tool to dissect molecular mechanisms regulating keratinocyte growth and differentiation. *J Invest Dermatol* **116**, 816-820.

Matsuda, S., Katsumata, R., Okuda, T., Yamamoto, T., Miyazaki, K., Senga, T., Machida, K., Thankt, A. A., Nakatsugawa, S., and Hamaguchi, M. (2000) Molecular cloning and characterization of human MAWD, a novel protein containing WD-40 repeats frequently overexpressed in breast cancer. *Cancer Res* **62**, 13-7.

Minden, A., Lin, A., Smeal, T., Derijard, B., Cobb, M., Davis, R., and Karin, M. (1994) c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases. *Mol Cell Biol* **14**, 6683-6688.

Mishra, A., Bharti, A. C., Saluja, D., and Das, B. C. (2010) Transactivation and expression patterns of c-Jun and Fos/AP-1 super family protein in human oral cancer. *Int J Cancer* **126**, 819-29.

Mitra,S. K., Hanson, D. A., and Schlaepfer, D. D (2005) Focal Adhesion Kinase in command and control of cell motility. *Nat Mol Cell Biol* **6**, 56-68.

Nakao, A., Roijer, E., Imamura, T., Souchelnytskyi, S., Stenman, G., Heldin, C. H., and ten Dijke, P. (1997) Identification of Smad2, a human Mad-related protein in the transforming growth factor beta signaling pathway. *J Biol Chem* **272**, 2896-900.

Natrajan, R., Lambros, M. B., Rodrigues-Pinilla, S. M., Moreno-Bueno, G., Tan, D. S., Marchio, C., Vatcheva, R., RAyter, S., Mahler-Araujo, B., Fulford, L. G., Hungermann, D., Mackay, A., Grigoriadis, A., Fendwick, K., Tamber, N., Hardisson, D., Tutt, A., Palacios, J., Lord, C. J., Buerger, H., Ashworth, A., and Feis-Filho, J. S. (2009) Tiling path genomic profiling of grade 3 invasive ductal breast cancers. *Clin Cancer Res* **15**, 2711-22.

Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tisty, T. D., and Cunha, G. R. (1999) Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* **59**, 5002–5011.

Orr, F. W., Adamson, I. Y., and Young, L. (1985) Pulmonary inflammation generates chemotactic activity for tumor cells and promotes lung metastasis. *Am Rev Respir Dis* **131**, 607-11.

O'Neil, J., Grim, J., Strack, P., Rao, S., Tibbitts, D., Winter, C., Hardwick, J., Welcker, M., Meijerink, J. P., Pieters, R., Draetta, G., Sears, R., Clurman, B. E., and Look, A. T. (2007) FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. *J Exp Med* **204**, 1813-24.

Papassava, P., Gorgoulis, V.G., Papaevangeliou, D., Vlahopoulos, S., van Dam, H., and Zoumpourlis, V. (2004) Overexpression of activating transcription factor-2 is required for tumor growth and progression in mouse skin tumors. *Cancer Res* **64**, 8573-8584.

Peifer, C., and Alessi, D. R. (2009) New anti-cancer roel for PDK1 inhibitors: preventing resistance to tamoxifen. *Biochem J* **417**, e5-7.

Pellizzoni, L., Kataoka, N., Charroux, B., and Dreyfuss, G. (1998) A novel function for SMN, the spinal muscular atrophy gene product, in pre-mRNA splicing. *Cell* **95**, 615-24.

Pierini, L. M., Lawson, M. A., Eddy, R. J., Hendey, B., and Maxfield, F. R. (2000) Oriented endocytic recycling of α5β1 in motile neutrophils. *Blood* **95**, 2471-2480.

Poulos, C., Chang, C. L., Zhang, S., Gersell, D. J., and Ulbright, T. M. (2006) Analysis of ovarian teratomas for isochromosome 12p: evidence supporting a dual histogenetic pathway for teratomatous elements. *Mod Pathol* **19**, 766-71.

Powell, W. C., Knox, J. D., Navre, M., Grogan, T. M., Kittelson, J., Nagle, R. B., Bowden, G. T. (1993) Expression of the metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. *Cancer Res* **53**, 417-22.

Ren, X. D., Kiosses, W. B., Sieg, D. J., Otey, C. A., Schlaepfer, D. D., and Schwartz, M. A. (2000) Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J Cell Sci* **113**, 673-8.

Reiner, J., and Datta, P. (2011) TGF-beta-dependent and -independent roles of STRAP in cancer. *Front Biosci* **16**, 105-15.

Rodrigues, N. R., Owen, N., Talbot, K., Ignatius, J., Dubowitz, V., and Davies, K. E. (1995) Deletions in the survival of motor neuron gene on 5q13 in autosomal recesive spinal muscular atrophy. *Hum Mol Genet* **4**, 631-4.

Rosengard, A. M., Krutzsch, H. C., Shearn, A., Biggs, J. R., Barker, E., Margulies, I. M. King, C. R., Liotta, L. A., and Steeg, P. S. (1989) Reduced Nm23/Awd protein in tumour metastasis and aberrant Drosophila development. *Nature* **342**, 177-80.

Ryseck, R. P., Hirai, S. I., Yaniv, M., and Bravo, R. (1988) Transcriptional activation of c-Jun during the G0/G1 transition in mouse fibroblasts. *Nature* **334**, 535-7.

Ryseck, R. P., and Bravo, R. (1991). c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins. *Oncogene* **6**, 533-42.

Sabapathy, K., Hochedlinger, K., Nam, S.Y., Bauer, A., Karin, M., and Wagner, E. F. (2004) Distinct roles for JNK1 and JNK2 in regulating JNK activity and c-Jun-dependent cell proliferation. *Mol Cell* **15**, 713-725.

Sai, J., Raman, D., Liu, Y., Wikswo, J., and Richmond, A. (2008) Parallel Phosphatidylinositol 3-Kinase (PI3K)-dependent and Src-dependent Pathways Lead to CXCL8-mediated Rac2 Activation and Chemotaxis. *J Biol Chem* **283**, 26538–26547.

Sala, A. (2005) B-MYB, a transcription factor implicated in regulating cell cycle, apoptosis, and cancer. *Eur J Cancer* **16**, 2479-84.

Sancho, R., Nateri, A.S., de Vinuesa, A.G., Aguilera, C., Nye, E., Spencer-Dene, B., and Behrens, A. (2009) JNK signalling modulates intestinal homeostasis and tumourigenesis in mice. *EMBO J* 28, 1843-1854.

Savagner, P., Kusewitt, D. F., Carver, E. A., Magnino, F., Choi, C., Gridley, T., and Hudson, L. G. (2005) Developmental transcription factor slug is required for effective re-epithelialization by adult keratinocytes. *J Cell Physiol* **202**, 858-66.

Schaller, M. D. (2001) Paxillin: a focal adhesion-associated adaptor protein. *Oncogene* 20, 6459-6472.

Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R. and Parsons, J. T. *(*1994*).* Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* **14***,* 1680-*1688.*

Sieg, D. J., Hauck, C. R., and Schlaepfer, D. D. (1999) Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J Cell Sci* **112,** 2677-91.

Sieg, D. J., Ilić, D., Jones, K. C. , Damsky, C. H., Hunter, T., and Schlaepfer, D. D. (1998) Pyk2 and Src-family protein-tyrosine kinases compensate for the loss of FAK in fibronectin-stimulated signaling events but Pyk2 does not fully function to enhance FAK-cell migration. *EMBO J* 17, 5933-47.

Seong, H. A., Jung, H., Choi, H. S., Kim, K. T., and Ha, H. (2005) Regulation of transforming growth factor-beta signaling and PDK1 kinase activity by physical interaction between PDK1 and serine-threonine kinase receptor-associated protein. *J Biol Chem* **280**, 42897-908.

Seong, H. A., Jung, H., Kim, K. T., and Ha, H., 3-Phospoinositide-dependent PDK1 negatively regulates transforming growth factor-beta-induced signaling in a kinase-

dependent manner through physical interaction with Smad proteins. *J Biol Chem* **282**, 12272-89.

Seong, H. A., Jung, H., and Ha, H. (2007) NM23-H1 tumor suppressor physically interacts with serine-threonine kinase receptor-associated protein, a transforming growth factor-beta (TGF-beta) receptor-interacting protein, and negatively regulates TGF-beta signaling. *J Biol Chem* **282**, 12075-96.

Seong, H. A., Jung, H., Ichijo, H., and Ha, H. (2010) Reciprocal negative regulation of PDK1 and ASK1 signaling by direct interaction and phosphorylation. *J Biol Chem* **285**, 2397-414.

Seong, H. A., Manoharan, R., and Ha, H. (2010) B-MYB positively regulates serine-threonine kinase receptor-associated protein (STRAP) activity through direct interaction. *J Biol Chem* **286**, 7439-56.

Serrels, B., Serrels, A., Brunton, V. G., Holt, M., McLean, G. W., Gray, C. H., Jones, G. E., and Frame, M. C. (2007) Focal adhesion kinase controls actin assembly via a FERM-mediated interaction with the Arp2/3 complex. *Nat Cell Biol* **9,** 1046-56.

Servant, G., Weiner, O. D., Herzmark, P., Balla, T., Sedat, J. W., and Bourne, H. R. (2000) Polarization of Chemoattractant Receptor Signaling During Neutrophil Chemotaxis. *Science* **287**, 1037-1040.

Shaulian, E., and Karin, M. (2002) AP-1 as a regulator of cell life and death. *Nat Cell Biol* **4**, E131-E136.

Sheetz, M. D., Felsenfeld, D., Galbraith, C. G., and Choquet, D (1999) Cell migration as a five step cycle. *Biochem Soc Symp* **65**, 233-243

Slisz, M., Rothenberger, E., and Hutter, D. (2008) Attenuation of p38 MAPK activity upon contact inhibition in fibroblasts. *Mol Cell Biochem* **308**, 65-73.

Smit, M. A., Peeper, D. S. (2011). Zeb1 is required for TrkB-induced epithelial-mesenchymal transition, anoikis resistance and metastasis. *Oncogene* [epub ahead of print]

Smith, M. J., and Prochownick, E. V. (1992) Inhibition of c-Jun causes reversible proliferative arrest and withdrawal from the cell cycle. *Blood* **79,** 2107-15.

Snyder, E. L., Sandstrom, D. J., Law, K., Fiore, C., Sicinska, E., Brito, J., Bailey, D., Fletcher, J. A., Loda, M., Rodig, S. J., Dal Cin, P., and Fletcher, C. D. (2009) c-Jun amplification and overexpression are oncogenic in liposarcoma but not always sufficient to inhibit the adipocytic differentiation programme. *J Pathol* **218**, 292-300.

Song, J. H., Schnittke, N., Zaat, A., Walsh, C. S., and Miller, C. W. (2008) FBXW7 mutation in adult T-cell and B-cell acute lymphocytic leukemias. *Leuk Res* **32**, 1751-5.

Spath, G. F., and Weiss, M. C. (1998) Hepatocyte nuclear factor 4 provokes expression of epithelial marker genes, acting as a morphogen in dedifferentiated hepatoma cells. *J Cell Biol* **140**, 935-46.

Sprague, E. R., Redd, M. J., Johnson, A. D., and Wolberger, C. (2000) Structure of the C-terminal domain of Tup1, a corepressor of transcription in yeast. *Embo J* **19**, 3016-27.

Stirnimann, C. U., Petsalaki, E., Russell, R. B., Müller, C. W. (2010) WD proteins propel cellular networks. *Trends Biochem Sci* **35**, 565-574.

Swingler, T. E., Kevorkian, L., Culley, K. L., Illman, S. A., Young, D. A., Parker, A. E., Lohi, J., and Clark, I. M. (2010) MMP28 gene expression is regulated by Sp1 transcription factor acetylation. *Biochem J* **427**, 391-400.

Tachibana, K., Sato, T., D'Avirro, N. and Morimoto, C. (1995) Direct associaiton of pp125FAK with paxillin, the focal adhesion targeting mechanism of pp125FAK. *J Exp Med* **182**, 1089-1100.

Takeda, K., Sasaki, A., T., Ha, H., Seung, H-A, and Firtel, R. A. (2007) Role of Phosphatidylinositol 3-Kinases in Chemotaxis in *Dictyostelium. J Biol Chem* **282**, 1874–11884

Tavares, A. L., Mercado-Pimentel, M. E., Runyan, R. B., and Kitten, G. T. (2006) TGF beta-mediated RhoA expression is necessary for epithelial-mesenchymal transition in the embryonic chick heart. *Dev Dyn* **235**, 1589-98.

Teixeira, A., Chaverot, N., Strosberg, A.D., and Cazaubon, S. (2000) Differential regulation of cyclin D1 and D3 expression in the control of astrocyte proliferation induced by endothelin-1. *J Neurochem* **74**, 1034-1040.

Tomar, A., Lim, S. T., Lim, Y., and Schlaepfer, D. D. (2009) A FAK-p120RasGAP-p190RhoGAP complex regulates polarity in migrating cells. *J Cell Sci* **122**, 1852-62.

Vincente-Manzanares, M., Newell-Litwa, K., Bachir, A. I., Whitmore, L. A., and Horwitz, A. R. (2011) Myosin IIA/IIB restrict adhesive and protrusive signaling to generate front-back polarity in migrating cells. *J Cell Biol* **193**, 381-96.

Wang, S. E., Xiang, E. B., Guix, M., Olivares, M. G., Parker, J., Chung, C. H., Pandiella, A., and Arteaga, C. L. (2008) Transforming growth factor beta engages TACE and ErbB3 to activate phosphatidylinositol-3 kinase/Akt in ErbB2-overexpressing breast cancer and desensitizes cells to trastuzumab. *Mol Cell Biol* **28**, 5605-20.

Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C.E., Parsons, J. T., and Horwitz, A. F. (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol* **6**, 154-61.

Weber, F., Shen, .L, Fukino, K., Patocs, A., Mutter, G. L., Caldes, T., and Eng, C. (2006) Total-genome analysis of BRCA1/2-related invasive carcinomas of the breast identifies

tumor stroma as potential landscaper for neoplastic initiation. *Am J Hum Genet* **78**, 961-72.

Weber, F., Xu, Y., Zhang, L., Patocs, A., Shen, L., Platzer, P., and Eng, C. (2007) Microenvironmental genomic alterations and clinicopathological behavior in head and neck squamous cell carcinoma. *JAMA* **297**, 187-95.

Wei, W., Jin, J., Schlisio, S., Harper, J.W., Kaelin Jr, W.G. (2005) The v-Jun point mutation allows c-Jun to escape GSK3-dependent recognition and destruction by the Fbw7 ubiquitin ligase. *Cancer Cell* **8**, 25-33.

Wendt, M. K., Taylor, M. A., Schiemann, B. J., and Schiemann, W. P. (2011 Downregulation of epithelial cadherin is required to initiate metastatic outgrowth of breast cancer. Mol Cell Bio [epub ahead of print]

Wertz, I.E., O'Rourke, K.M., Zhang, Z., Dornan, D., Arnott, D., Deshajies, R. J., and Dixit, V. M. (2004) Human De-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. *Science* **303**, 1371-1374.

Westhoff, M. A., Serrels, B., Fincham, V. J., Frame, M. C., and Carragher, N. O. (2004) SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. *Mol Cell Biol* **24**, 8113-33.

Westmoreland, J. J., Wang, Q., Bouaffour, M., Baker, S. J., and Sosa-Pineda, B. (2009) Pdk1 activity controls proliferation, surival, and growth of developing pancreatic cells. *Dev Biol* **334**, 285-98.

White, D. P., Caswell, P. T. and Norman, J. C. (2007) αvβ3 and α5β1 integrin recycling pathways dictate downstream Rho kinase signaling to regulate persistent cell migration. *J Cell Biol* **177**, 515-525.

Wood, C. K., Turner, C. E., Jackson, P., and Critchley, D. R. (1994) Characterisation of the paxillin-binding site and the C-terminal focal adhesion targeting sequence in vinculin. *J Cell Sci* **107**, 709-717.

Wu, X., Suetsugu, S., Cooper, L. A., Takenawa, T., and Guan, J. L. (2004) Focal adhesion kinase regulation of N-WASP subcellular localization and function. J Biol Chem 279, 9565-76.

Xia, Y., Wang, J., Xu, S., Johnson, G.L., Hunter, T., and Lu, Z. (2007) MEKK1 mediates the ubiquitination and degradation of c-Jun in response to osmotic stress. *Mol Cell Biol* **27**, 510-517.

Xie, L., Law, B. K., Chytil, A. M., Brown, K. A., Aakre, M. E., and Moses, H. L. (2004) Activation of the Erk pathway is required for TGF-beta-1-induced EMT *in vitro*. *Neoplasia* 6, 603-10.

Xu, Z., Shen, M. X., Ma, D. Z., Wang, L. Y., and Zha, X. L. (2003) TGF-beta1-promoted epithelial-to-mesenchymal transformation and cell adhesion contribute to TGF-beta1-enhanced cell migration in SMMC-7721 cells. *Cell Res* **13**, 343-50.

Xu, L., Zhou, Y., Liu, Q., Luo, J. M., Qing, M., Tang, X. Y., Yao, X. S., Wang, C. H., and Wen Z. K. (2009) CXCR4/SDF-1 pathway is crucial for TLR9 agonist enhanced metastasis of human lung cancer cell. *Biochem Biophys Res Commun* **382**, 71-6.

Yan, Z., Kim, G. Y., Deng, X., and Friedman, E. (2002) Transforming growth factor beta 1 induces proliferation in colon carcinoma cells by Ras-dependent, smad-independent down-regulation of p21cip1. *J Biol Chem* **277**, 9870-9.

Yang, Y., Lu, S., Li, M. F., and Wang, Z. H. (2009) Effecs of tumor metastasis suppressor gene nm23-H1 on invasion and proliferation of cervical cancer cell lines. *Ai Zheng* **28**, 702-7.

Yoo, S., K., Deng, Q., Cavnar, P. J., Wu, Y. L., Hahn, K. M., Huttenlocher, A. (2010) Differential Regulation of Protrusion and Polarity by PI(3)K during Neutrophil Motility in Live Zebrafish. *Dev Cell* **18**, 226-236.

Yoshimoto, M., Itoh, F., Yamamoto, H., Hinoda, Y., Imai, K., and Yachi, A. (1993) Expression of MMP-7(PUMP-1) mRNA in human colorectal cancers. *Int J Cancer* **54**, 614-8.

Zaidel-Bar, R., Ballestrem, C., Kam, Z., and Geiger B (2003) Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *J Cell Sci* **116,** 4605-4613.

Zamir, E., and Geiger, B (2001) Molecular complexity and dynamics of cell-matrix Adhesions. *J Cell Sci* **114**, 3583-90.

Zhang, W., Matrisian, L. M., Holmbeck, K., Vick, C. C., and Rosenthal, E. L. (2006) Fibroblast-derived MT1-MMP promotes tumor progression in vitro and in vivo. *BMC Cancer* **6**, 52.

Zhang, Y., Feng, X., We, R., and Derynck, R. (1996) Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. *Nature* **383**, 168-72.

Zhang, W., Lee, J.C., Kumar, S., Gowen, M. (1999) ERK pathway mediates the activation of Cdk2 in IGF-1-induced proliferation of human osteosarcoma MG-63 cells. *J Bone Miner Res* **14**, 528-535.

Zhang, Y., Pu, X., Shi, M., Chen, L., Song, Y., Qian, L., Yuan, G., Zhang, H., Yu, M., Hu, M., Shen, B., and Guo, N. (2007) Critical role of c-Jun overexpression in liver metastasis of human breast cancer xenograft model. *BMC Cancer* **7**, 145.

Zhang, Y., Tang, H., Cai, J., Zhang, T., Guo, J., Feng, D., and Wang, Z. (2011) Ovarian cancer-associated fibroblasts contribute to epithelial ovarian carcinoma metastasis by promoting angiogenesis, lymphangiogenesis and tumor cell invasion. *Cancer Lett* **303**, 47-55.

Zohn, I. E., Li, Y., Skolnik, E. Y., Anderson, K. V., Han, J., and Niswander, L. (2006) p38 and a p38-interacting protein are critical for downregulation of E-cadherin during mouse gastrulation. *Cell* **125**, 957-69.

Zhu, F., Choi. B. Y., Ma, W-Y., Zhao, Z., Zhang, Y., Cho, Y. Y., Choi, H. S., Imamoto, A., Bode, A. M., and Dong, Z. (2006) COOH-Terminal Src kinase-mediated c-Jun phosphorylation promotes c-Jun degradation and inhibits cell transformation. *Cancer Res* **66**, 5729-36.

Zwerts, F., Lupu, F., De Vriese, A., Pollefeyt, S., Moons, L., Altura, R. A., Jiang, Y., Maxwell, P. H., Hill, P., Oh, H., Rieker, C., Collen, D., Conway, S. J., and Conway, E. M. (2007) Lack of endothelial cell survivin causes embryonic defects in angiogenesis, cardiogenesis, and neural tube closure. *Blood* **109**, 4742-52.