

Université de Montréal

**Characterization of p120-catenin, a novel RSK substrate in
the Ras/MAPK signalling pathway.**

par Beichen Gao

Département de Biologie Moléculaire
Faculté de Médecine

Mémoire présenté
en vue de l'obtention du grade de M.Sc
en Biologie Moléculaire
option Générale

Janvier, 2017

© Beichen Gao, 2017

Résumé

La voie de signalisation Ras/mitogen-activated protein kinase (Ras/MAPK) occupe un rôle central dans la régulation de différents processus biologiques tels que la croissance, la survie mais aussi la prolifération cellulaire. En réponse à des signaux extracellulaires, cette voie de signalisation mène à l'activation des protéines ERK1/2, impliquées dans l'activation de nombreux substrats cellulaires dont les protéines kinases RSK (p90 ribosomal S6 kinase). Ces protéines kinases sont, entre autres, impliquées dans l'invasion et la migration cellulaire mais les mécanismes responsables de ces phénomènes biologiques restent inconnus à ce jour.

Dans mon mémoire, je développe tout d'abord les travaux précédemment réalisés dans notre laboratoire, et identifie la protéine p120-Catenin (p120ctn), un composant majeur des jonctions adhérentes (AJ), comme un nouveau substrat de la voie Ras/MAPK. En utilisant notamment un anticorps phospho-spécifique, nous avons pu démontrer que p120ctn est phosphorylée sur la sérine 320, un nouveau site de phosphorylation, d'une manière dépendante des kinases RSK. D'autre part, nous avons trouvé que la signalisation Ras/MAPK réduit l'interaction entre les protéines p120ctn et N-cadhérine. Ainsi, nos observations suggèrent que l'activation de la voie Ras/MAPK est impliquée dans la diminution de l'adhérence entre cellules par la déstabilisation des AJ. Compte tenu du rôle primordial de la voie de signalisation Ras/MAPK dans le cancer, ce mécanisme nouvellement décrit pourrait contribuer à l'avancement des connaissances sur le développement des cancers dépendants de cette voie de signalisation.

Mots-clés : MAPK; RSK; p120ctn; jonctions adhérentes; cadhérine; phosphorylation; adhérence cellule-cellule;

Abstract

The Ras/MAPK (mitogen-activated protein kinase) signalling pathway is vital in regulating cell growth, survival and proliferation in response to extracellular signals. Positioned downstream in the pathway, the p90 ribosomal S6 kinase (RSK) family regulates cell invasion by weakening cell-cell adhesion, but the mechanisms involved remain elusive.

In this thesis, I expand upon previous work performed in our lab and identify p120ctn, a major component of adherens junctions (AJ), as a new substrate of the Ras/MAPK pathway. Using a phospho-specific antibody, we demonstrate that p120ctn is phosphorylated on a new phosphorylation site on S320 upon activation of MAPK signalling in a RSK-dependent manner. Furthermore, we show that Ras/MAPK signaling reduces p120ctn binding to N-cadherin, suggesting a new mechanism by which MAPK activity decreases cell-cell adhesion by destabilizing AJs. Finally, we designed and optimized two individual assays to be used in future experiments examining the effects of Ras/MAPK signalling on AJ function.

Taken together, our data identifies RSK as a regulator of p120ctn phosphorylation, and also implicates Ras/MAPK signalling in regulating cell-cell adhesion by destabilizing AJ through p120ctn. Given the role of Ras/MAPK signalling in cancer, this new mechanism may play a role in the development and progression of Ras-driven cancers.

Keywords : MAPK; RSK; p120ctn; adherens junction; cadherin; phosphorylation; cell-cell adhesion;

Table of Contents

Résumé	i
Abstract	ii
Table of Contents	iii
List of Tables	v
List of Figures	vi
List of Abbreviations	vii
Acknowledgements	xi
INTRODUCTION	12
Ras/MAPK Signalling and RSK Kinases	13
The Ras/MAPK signalling pathway	13
The RSK kinase family	15
RSK substrates and functions	17
The role of RSK in cancer	19
Cell-Cell Adhesion and Adherens Junctions	21
Cell-Cell Adhesion Structures	21
Adherens junctions and cadherins	23
The p120-catenin Protein	26
Structure of p120ctn.....	26
p120ctn regulation of cell-cell adhesion	28
p120ctn and cadherins.....	30
p120ctn and RhoGTPases	31
p120ctn and transcriptional regulation.....	32
Regulation of p120ctn by phosphorylation.....	33
p120ctn in cancer	36
Rationale and Objectives	38
Rationale	38
Objectives	39
MATERIALS AND METHODS	40
Cell culture and transient transfection	41
DNA constructs and recombinant proteins	42
RNA interference	43
Antibodies	44
Cell lysis and lysate preparation	45
Bradford protein assay	46
Immunoprecipitation.....	46
SDS-PAGE gel separation and immunoblotting	46
Retroviral infection of cells	47
Immunofluorescence staining	48
Calcium depletion	49
Monolayer fragmentation assay	49

Cellular impedance assay.....	50
RESULTS	52
SECTION 1: Ras/MAPK regulation of p120ctn phosphorylation.	53
S320 on p120ctn is located within a highly conserved, AGC-kinase consensus sequence.....	53
p120ctn is a substrate of the Ras/MAPK pathway.....	53
Phosphorylation of p120ctn on S320 is RSK-dependent.....	54
RSK regulates p120ctn phosphorylation on S320 in epithelial cell lines.....	55
SECTION 2: Ras/MAPK mediated effects on p120ctn.....	57
Ras/MAPK activity reduces p120ctn binding to N-cadherin	57
PMA Stimulation does not affect p120ctn localization	57
Site-specific mutation at S320 does not affect p120ctn localization	58
SECTION 3: Designing assays to examine AJ function	60
MCF-10A cells form stable, confluent monolayers in culture	61
p120ctn knockdown induces complete monolayer fragmentation.....	61
Calcium depletion increases monolayer fragmentation and triggers AJ disassembly	62
Site-specific mutation of S320 on p120ctn produce minor effects on monolayer adhesion	63
CHAPTER 3: FIGURES	65
DISCUSSION	78
General Findings.....	79
Regulation of S320 phosphorylation on p120ctn.....	80
S320 phosphorylation on p120ctn function	81
Ras/MAPK regulation of cell-cell adhesion through p120ctn.....	83
CONCLUSIONS AND PERSPECTIVES	85
Bibliography	87

List of Tables

Table 1: Effects of knockout and knockdown of p120ctn and related family members in different species.	29
Table 2: Identified phosphorylation sites on p120ctn and the effects of site-specific modifications on p120ctn function.	35
Table 3: Antibodies used in this study for immunoblots and immunofluorescence experiments.	44

List of Figures

Figure 1.	Schematic representation of the Ras/MAPK pathway and RSK substrates.....	15
Figure 2.	Schematic representation of the RSK isoforms and their structural features....	16
Figure 3.	A basic schematic of cell-cell adhesion structures, and structural elements of adherens junctions.....	23
Figure 4.	The structural features of p120ctn including main domains, subcellular localization signals, phosphorylation sites, and alternative splicing sites.....	27
Figure 5.	Our proposed model of the effects of Ras/MAPK signalling on p120ctn function and AJ stability.....	39
Figure 6.	Analysis of S320 as a putative phosphorylation site on p120ctn.....	65
Figure 7.	Activation of the Ras/MAPK pathway induces phosphorylation of p120ctn on S320.....	66
Figure 8.	Ras/MAPK phosphorylation of p120ctn on S320 is RSK-dependent.....	67
Figure 9.	Activation of the Ras/MAPK pathway induces RSK-dependent p120ctn phosphorylation on S320 in epithelial cells.....	68
Figure 10.	Ras/MAPK activity reduces p120ctn binding to N-cadherin.....	69
Figure 11.	Validation of the p120ctn antibody for use in IF.....	70
Figure 12.	p120ctn remains localized at cell-cell contacts in HEK 293 cells in response to PMA stimulation.....	71
Figure 13.	p120ctn remains localized at cell-cell contacts in A431 cells in response to PMA stimulation.....	72
Figure 14.	Myc-tagged p120ctn S320 mutants localizes to cell-cell contacts in stably expressing MDCK cells.....	73
Figure 15.	MCF-10A cells generate confluent monolayers in comparison to Caco-2, A431, and IEC6 cells.....	74
Figure 16.	p120ctn knockdown promotes monolayer fragmentation.....	75
Figure 17.	Calcium depletion affects monolayer fragmentation and cell index.....	76
Figure 18.	Stable expression of p120ctn phosphomutants in A431 cells affects monolayer adhesion.....	77

List of Abbreviations

α -cat	α -catenin
β -cat	β -catenin
AGC family	protein kinases A, G and C
AJ	adherens junction
Akt	protein kinase B
AP-1	Activator protein 1
ARM	Armadillo-domain
ARVCF	Armadillo-repeat protein deleted in velocardio-facial syndrome
BTB/POZ	Bric à brac/Pox virus and zinc finger
CAMK	Ca ²⁺ /calmodulin-dependent protein kinase
CDKs	cyclin-dependent kinases
cDNA	complementary DNA
Chk1	checkpoint kinase 1
CREB	cAMP response element-binding protein
CTKD	carboxyl-terminal kinase domain
E-cad	E-cadherin
EC	extracellular cadherin
EGF	epidermal growth factor
eIF4G1	eukaryotic translation initiation factor 4 gamma 1
EMT	epithelial-mesenchymal transition
EphA2	Ephrin type-A receptor 2
EPLIN	epithelial protein lost in neoplasm
ERK	extracellular signal-regulated kinase
EST	expressed sequence tag
FERM	four point one, Ezrin, Radixin and Moesin
Gab2	Grb2-associated binder 2
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine exchange factor
GPCR	G-protein coupled receptor

Grb2	growth factor receptor-bound protein 2
IDC	invasive ductal carcinoma
ILC	invasive lobular carcinomas
JAM	junctional adhesive molecule
JMD	juxtamembrane domain
JNK	c-Jun N-terminal kinases
KIBRA	expression enriched in kidney and brain
KIBRA	kidney and brain expressed protein
MAPK	mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
MEK1/2	MAPK/ERK kinase 1 and 2
miRNA	microRNA
Mrip	Myosin phosphatase Rho-interacting protein
mTOR	mammalian target of rapamycin
N-cad	N-cadherin
NES	nuclear export signal
NLS	nuclear localization sequence
NSCLC	non-small-cell lung cancer
NTKD	amino-terminal kinase domain
p120ctn	p120-catenin
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PKD1	3'-phosphoinositide-dependent protein kinase 1
PI3K	phosphatidylinositol-3-kinase
PKA	protein kinase A
PKC	protein kinase C
PSPL	positional scanning peptide library
PTB	phosphotyrosine-binding
PTEN	phosphatase and tensin homolog
Raf	regulator of α -fetoprotein
Raptor	regulatory associated protein of mTOR

RD	regulatory domain
Rheb	Ras homolog enriched in brain
ROCK	Rho kinase
RSK	p90 ribosomal S6 kinase
RTK	receptor tyrosine kinase
S6K	S6 kinase
SH2	Src homology 2
SH3P2	SH3 domain-containing protein 2
shRNA	short hairpin RNA
siRNA	small interfering RNA
SOS	son of sevenless
TJ	tight junction
TSC2	tuberous sclerosis complex 2
WT	wild-type
ZA	zonula adherens

Dedicated to my family, friends, and fellow dreamers

Acknowledgements

There are so many people that I would like to thank for their help and support throughout my journey. First of all, I would like to thank my supervisor Dr. Philippe Roux for his support and guidance throughout this endeavour. Philippe, thank you teaching me to always think one step further, and for showing me that I should never be afraid to ask for feedback. I'm incredibly happy for the opportunities you have given me to do cutting edge research, and I'm proud of everything that I've accomplished in the lab under your mentorship!

I would also like to thank everyone in the Roux Lab, both past and present. Thank you Dr. Geneviève Lavoie for your patience while teaching me new lab techniques and for putting up with my endless "double-checking" question requests. Thank you Antoine Méant for being my fellow dreamer/idealist/teammate in the lab, and for all the interesting discussions we've had in the lab. Thank you Neethi Nandagopal for the endless snacks and crazy late night talks. If I continue on, this section will never end! So let me just give a big thank you to Léo Aubert, Thibault Houles, Sami Nourreddine and Justine Paradis for all your nuggets of wisdom and help in the lab throughout the years.

Outside of our own lab, I would like to thank Dr. Étienne Gagnon for enthusiastically supporting all my crazy ideas throughout the years. Moreover, I have to thank his student, Jordan Quenneville, for being an amazing friend who's crazy enough to join me in all my crazy side-endeavours! I'm sure you'll be doing some extraordinary things in the next few years man!

I'm incredibly grateful for the opportunity granted to me by the IRIC institute to study, train, and grow here. It has been such an amazing experience to work in a place with such cutting-edge technology. Not only that, but I also have to thank Pascale Le Thérizien, Julie Mantovani, Patrick Lacasse and Valérie De Rop for being the best administrative and student support team ever. All of you are always so friendly and approachable, and have always provided me with great advice when I needed it!

Finally, I would like to thank my mom and dad for their endless love, support, and FaceTime calls at the most inopportune times midway through an experiment. I couldn't have done it without the two of you!

INTRODUCTION

Ras/MAPK Signalling and RSK Kinases

The Ras/MAPK signalling pathway

Mitogen-activated protein kinase (MAPK) cascades are evolutionarily conserved signal transduction pathways in eukaryotic cells, allowing them to respond to various intracellular and extracellular stimuli. Many cell-surface receptors can stimulate Ras/MAPK signalling, such as G protein-coupled receptors (GPCRs), cytokine receptors, and receptor tyrosine kinases (RTKs). Traditionally organized, and characterized, by a three-tiered set of sequentially activated and evolutionarily conserved kinases, MAPK pathways act in cells to regulate a plethora of cellular processes, including gene expression, metabolism, motility, proliferation, survival and differentiation (Reviewed in [1]). In mammals, several essential MAPKs have been characterized, including the extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun amino (N)-terminal kinases 1/2/3 (JNK1/2/3), and p38 isoforms. Of these, the ERK1/2 module is the best studied, and plays a significant role in many human cancers, as evidenced by the many tumors showing hyperactivation of these kinases [2].

The core organization of the ERK1/2 module has been extensively studied, and, like many other traditional MAPK pathways, contains three sequentially acting kinases: A/B/C-Raf, MEK1/2, and ERK1/2. While other unconventional kinases have been shown to replace Raf function in a cell-specific manner, the canonical activation and regulation of the pathway remains consistent [3].

The ERK1/2 signalling cascade is most commonly activated in response to binding of extracellular growth factors, such as platelet-derived growth factor (PDGF), epithelial growth factor (EGF) and insulin, to cell surface receptors. While it has been demonstrated that GPCRs, cytokines and osmotic stress can also initiate ERK1/2 signalling, and that cAMP can regulate ERK1/2 activity in a cell-specific manner, the most well understood method of activation usually implicates RTKs (Fig. 1) [4].

Upon ligand binding, activation and dimerization of RTKs induces autophosphorylation of Tyr residues located on the intracellular domains of the receptors. These phosphorylated residues create docking sites for Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domain-containing proteins, such as Grb2 (growth factor receptor-bound protein 2). Upon binding to RTKs, Grb2 recruits SOS (son of sevenless), a guanine exchange factor (GEF), to

the plasma membrane. SOS is best known for catalyzing the exchange of GDP for GTP bound to Ras, leading to its activation. Now active, Ras-GTP directly interacts with, and promotes the dimerization, of Raf protein kinases (A-, B- and C-Raf), which then phosphorylates its specific substrates MEK1/2 (MAPK/ERK kinase 1 and 2). The dual-specificity MEK1/2 kinases then phosphorylate the MAPKs known as ERK1/2 within a conserved Thr-Glu-Tyr (TEY) motif in their activation loop motif. Activation of ERK1/2 then leads to further propagation of the initial growth factor signal to a large variety of downstream substrates regulating, among other responses, gene transcription, cell growth, proliferation, motility and survival (Reviewed in [1]).

In the context of cancer, many tumors harbour activating mutations within the genes encoding for initiating components of the ERK1/2 module, such as Ras and Raf – pointing to the oncogenic role of this pathway in driving cancer development and progression [2]. Thus, many cancers exhibit hyperactivation of ERK1/2 and deregulation of downstream biological functions, leading to tumor growth and survival.

Academic efforts to better characterize and understand this critical signalling pathway has revealed several important downstream effectors, including the 90 kDa ribosomal S6 kinases (RSKs), a family of Ser/Thr kinases that lie immediately downstream of the ERK1/2 kinases.

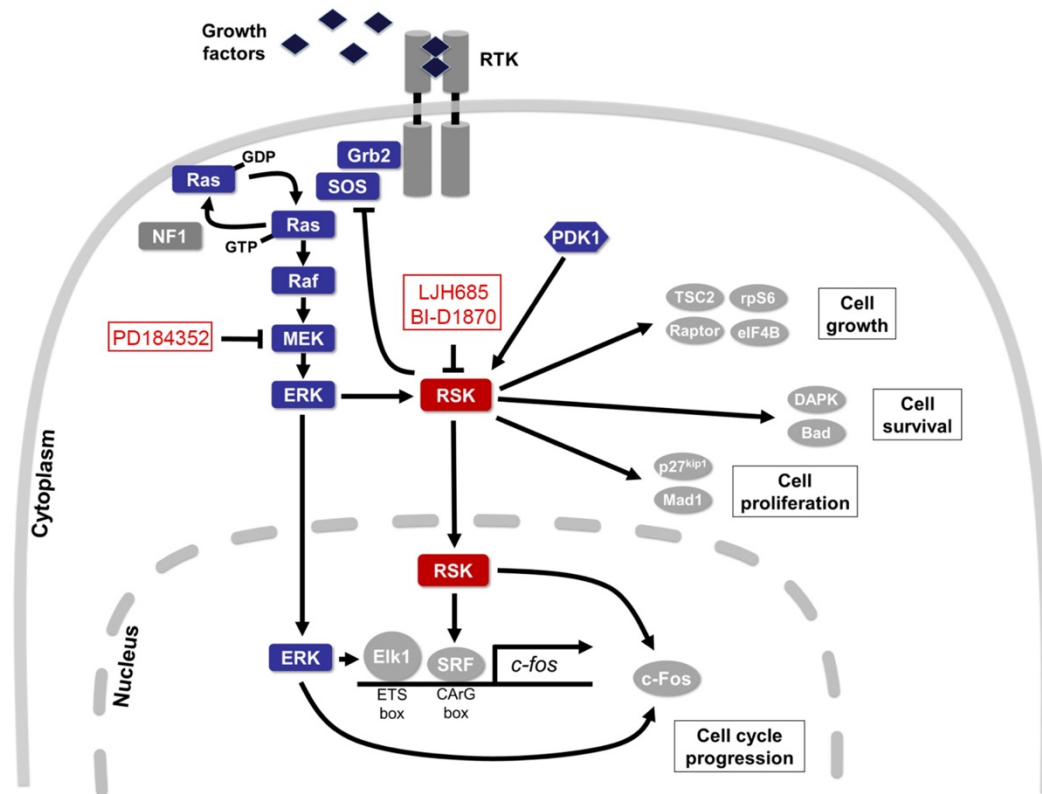


Figure 1. Schematic representation of the Ras/MAPK pathway and RSK substrates

Upon activation of cell surface RTKs, the Grb2/SOS complex is recruited to the plasma membrane and promotes GTP binding to Ras. Active Ras then initiates the signalling cascade leading to activation of the ERK1/2 MAPK signalling module, and RSK activation. Some of the known substrates of RSK are indicated here, including feedback inhibition of SOS to prevent hyperactivation of MAPK activity. Pharmacological compounds targeting MEK and RSK employed in this study are also highlighted. (Adapted from [5])

The RSK kinase family

Initially identified in 1985 as two kinases, S6KI and S6KII, that phosphorylated ribosomal protein S6 (rpS6) in *Xenopus laevis* eggs (later renamed RSK), are one of the many substrates of ERK1/2 and are effector kinases capable of regulating many cellular processes through its own substrates [6]. In human cells, there are four different isoforms (RSK1-4) that share between 73-80% sequence homology, mostly diverging in their C- and N-terminus regions [5] (Fig. 2).

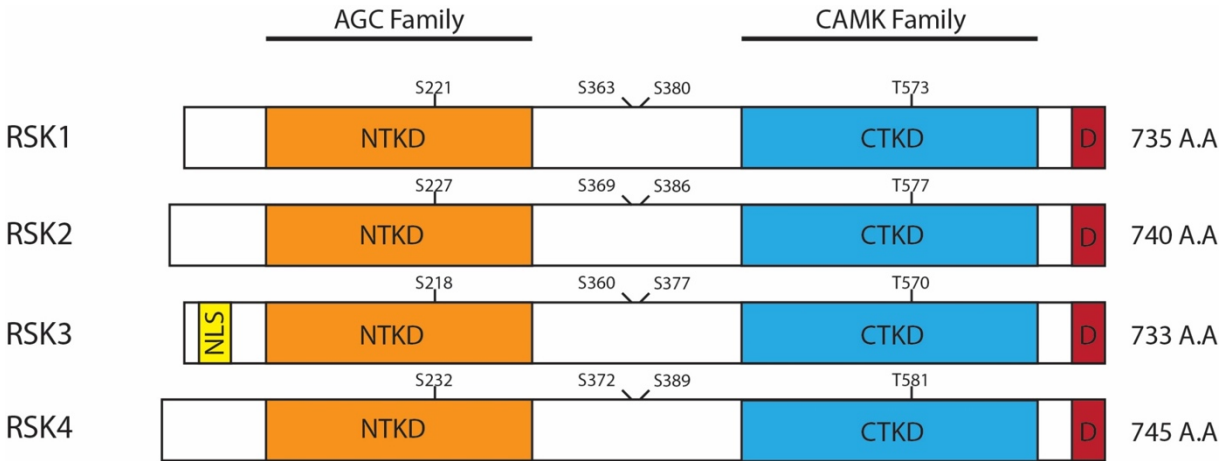


Figure 2. Schematic representation of the RSK isoforms and their structural features.

The 4 RSK isoforms all consist of the NTKD and CTKD connected by a linker region. All isoforms also contain a D-domain docking site for ERK1/2 association. Only isoform 3 contains a putative NLS domain. The phosphorylation sites required for RSK activation are indicated.

All four isoforms contain two highly conserved, yet functionally different, kinase domains connected by a linker region approximately 100-residues in length containing hydrophobic and turn motifs that are involved in RSK activation and regulation. These two distinct functional kinase domains appear to be a unique feature of RSKs, with the exception of the mitogen- and stress-activated protein kinases (MSKs), which are highly similar to the RSK [7]. The amino-terminal kinase domain (NTKD) shares high sequence homology with members of the AGC kinase family (including Akt, PKC and S6K1/2) and is the main functional domain for phosphorylation of RSK substrates and signal propagation. On the other end, the carboxyl-terminal kinase domain (CTKD) is similar to kinase domains found in the calcium/calmodulin-dependent protein kinases (CaMKs) and its only known function is to autophosphorylate RSK for the activation of NTKD in response to upstream signals from ERK1/2 [8]. Beyond the CTKD, an ERK1/2 docking site is located in the C-terminal region of RSK and is essential for the association of ERK1/2 with RSK for activation of the latter [9]. Finally, at the very end of the C-terminus is a PDZ domain-binding motif that varies slightly between the four RSK isoforms, and has been shown to play a role in regulating RSK interaction with PDZ domain-containing proteins during specific cellular processes, such as during synaptic transmission [10].

RSK activation requires a well-characterized and well-regulated sequence of phosphorylation events on four essential phosphorylation sites: Ser221, Ser363, Ser380 and Thr573 in human RSK1. Upon activation by mitogenic signals, ERK1/2 phosphorylates RSK on Thr573 in the activation loop region of the CTKD [11]. Now active, the CTKD and ERK1/2 coordinately phosphorylates Ser380 and Ser363, respectively, in the linker region of RSK [8, 12]. These phosphorylated residues generate a binding site for 3'-phosphoinositide-dependent protein kinase 1 (PDK1) – a constitutively active Ser/Thr kinase that has been shown to regulate members of the AGC kinase family [13]. Recruited PDK1 then phosphorylates Ser221 located within the activation loop of the NTKD, leading to its full activation and phosphorylation of RSK substrates [14].

Looking at their expression patterns, mRNA encoding RSK1-3 are present within all tissue types, while RSK4 appears to have low levels of expression in both embryonic and adult tissues. While RSK is ubiquitously expressed, tissue-specific differences in mRNA levels of RSK1-3 have been observed. RSK1 mRNA levels are commonly elevated in kidney, pancreas and lung tissue, while RSK2 and 3 are more predominantly expressed in the heart and skeletal muscle [15]. Northern Blot analysis identified RSK4 mRNA expression in the brain, heart, cerebellum, kidney and skeletal muscle, and is completely undetectable in the lung, liver and pancreas [16]. Specifically in the central nervous system, RSK isoforms have been shown to be expressed in different brain structures [17-19]. Studies into the expression of RSK isoforms during developmental stages also reveals distinct patterns of expression. RSK1 and 3 perhaps play a greater role in development as they are more highly expressed in fetal mouse tissue compared to RSK2 and 4 that show lower mRNA levels [20].

Taken together, these data suggest that while RSK isoforms may share high similarity, considering the differences in tissue distribution, and different expression patterns during developmental stages, the isoforms must regulate significantly different programs and serve different roles in a cell or tissue-specific manner.

RSK substrates and functions

To better understand the roles of RSKs in cells, various studies have been conducted with the aim to identify and characterize novel RSK substrates to build a greater understanding of the cellular functions controlled by RSK activity. Using synthetic peptides, the RSK1

consensus motif was initially believed to require the specific motif sequences: R/K-X-R-X-X-pS/T or R-R-X-pS/T [21]. More recently, using a positional scanning peptide library (PSPL), our lab confirmed that the RSK1 consensus motif resembles R-X-R-X-X-pS/T with a strong requirement for arginine residues at the -3 position, and a preference to phosphorylate serine over threonine [22]. Identification of the RSK consensus motif helps not only to provide confirmation of previously identified RSK substrates, but also creates the opportunity for high-throughput proteomic screening for novel RSK substrates.

Collectively, the current known substrates of RSKs indicate they play a role in regulating gene transcription, protein synthesis, cell-cycle progression, proliferation, survival, motility and migration. For the sake of brevity, I will only address a few of the identified substrates of interest here, but for further details, more in-depth reviews specifically addressing RSK substrates can be found [5, 23].

At the transcriptional level, RSK2 has been shown to phosphorylate and stabilize c-Fos in cells, a component of the AP-1 transcription factor complex and is frequently overexpressed in tumor cells. The transcription factor c-Fos upregulates cyclin D1 and promotes G1 progression in cells, driving cell proliferation [24]. Additionally, RSK2 has been shown to activate another transcription factor: cAMP response element-binding (CREB) protein through phosphorylation at Ser133 [25]. This RSK-CREB signalling pathway has been implicated in promoting tumor progression, cell survival and metastasis through upregulation of various genes [26]. Conversely, RSK3 and 4 appears to negatively regulate cell proliferation – especially in the context of cancer. RSK3 appears to function as a tumor suppressor in ovarian cancer cells, and RSK4 knockdown has been shown to promote proliferation, migration and metastasis of breast cancer cells [27, 28]

Moving on to translation, rpS6 phosphorylation by RSK at Ser235/236 promotes cap-dependent mRNA translation through assembly of the cap-binding complex [29]. Additionally, RSK promotes protein synthesis through mTOR (mammalian target of rapamycin) signalling. By phosphorylating tuberous sclerosis complex 2 (TSC2) on Ser1798, RSK inhibits its GTPase-activating activity for Rheb (Ras homolog enriched in brain), a small GTPase that is required for the activation of the mTORC1 complex [7]. By an alternate mechanism, RSK can directly phosphorylate Raptor (regulatory associated protein of mTOR), an important scaffolding protein, to promote mTORC1 assembly and activation [30].

Finally, a newly emerging function that is of particular importance to my project is the role of RSK in promoting Ras/MAPK-dependent cell motility and invasion. Using pharmacological inhibitors, RSK1 and 2 were initially identified to play a role in Raf-dependent migration in MDCK cells by upregulating expression of ~20% of ERK regulated mRNAs, many of which being invasion/motility genes encoding proteins such as uPAR, MMP-1 and RhoC [31]. A genome-wide RNAi screen for regulators of epithelial cell migration also identified RSK as a key effector and a point of convergence of various pro-migratory signals [32]. At the post-translational level, recent studies have identified a handful of new RSK substrates, including Gab2 (Grb2-associated binder), KIBRA (expression enriched in kidney and brain), SH3P2 (SH3 domain-containing protein 2), and EphA2 (Ephrin type-A receptor 2), that are all implicated in RSK-mediated cell motility and migration [33-36]. In our own phosphoproteomic and proximity ligation screens, we have identified a new potential RSK substrate, p120-catenin (p120ctn), that plays a significant role in regulating cell motility. p120ctn is a master regulator of cell-cell adhesion and a critical component of adherens junctions (AJs) [22].

The role of RSK in cancer

As a major regulator of cell proliferation, growth and survival signals downstream of the Ras/MAPK pathway, RSK plays a significant role in cancer progression. I will provide a quick summary of RSK's contribution to cancer development here, while more detailed explorations of its role can be found in excellent reviews such as: [37, 38].

Overexpression and hyperactivation of RSK has been observed in several cancers such as breast, lung, prostate, head and neck, ovarian, melanoma, osteosarcomas and multiple myelomas (reviewed in [37]). Increased levels of RSK2 were observed in ~50% of breast and prostate cancer tissues [39, 40]. Ectopic overexpression of RSK2 also promotes proliferation and anchorage-independent cell transformation in cells [41]. Pharmacological inhibition of RSK using SL0101, a RSK specific inhibitor, in cell lines derived from breast and prostate tumours has also been shown to significantly decrease proliferation of these cells *in vitro* [5].

Beyond cell proliferation, RSK also promotes lung cancer survival via phosphorylation and inhibition of the Bcl-2 homology 3-only proapoptotic protein, Bad, in response to estrogen signalling. [42] Furthermore, research has uncovered isoform-specific effects of RSK signalling on cancer metastasis and invasion. RSK2 has been shown to be promigratory, with higher levels

of RSK2 correlating with increased metastasis of head and neck squamous cell carcinomas in patients [43]. Conversely, RSK1 appears to be a negative regulator in non-small cell lung cancer [44], while exhibiting promigratory effects in immortalized breast epithelial cells [32]. Additionally, as previously addressed, increased RSK4 expression appears to reduce the metastatic potential of breast cancer cells [45].

Overall, the RSK family kinases play a multi-functional role in cancers by various mechanisms through their downstream substrates. In particular, the contradictory effects of RSK isoforms on cancer metastasis show that our understanding of how RSK regulates changes in cell motility and invasion is still quite poor. By studying the kinase-substrate relationship between RSK and p120ctn, our goal is to advance our knowledge of the mechanisms by which RSK signalling modulates cell-cell adhesion and cell motility in the context of cancer.

Cell-Cell Adhesion and Adherens Junctions

Cell-Cell Adhesion Structures

In multicellular organisms, cell contact and adhesion to neighbouring cells are required for generating functional tissue and organs by maintaining highly organized cellular polarity and architecture. Through evolution, different types of intercellular junction structures have emerged to participate in, and to regulate cell-cell adhesion.

Strong cell-cell adhesion is most crucial in epithelial cells, one of the most ubiquitous cell types that are responsible for forming epithelial layers. The epithelial layer acts as boundary between different environments – such as the inside and outside of an organism. This barrier also controls the flux and exchange of biologically relevant molecules, nutrients, and ions into the body and organs. In vertebrates, three major types of adhesion systems have been identified to contribute to the integrity of cell-cell adhesion: adherens junctions (AJs), desmosomes (DM), and tight junctions (TJs) (Fig. 3A and B).

Years of research have identified AJs as the main, defining cell-cell adhesion structure; however, the other two structures should still be quickly addressed. TJs are claudin- and junctional adhesive molecule (JAM)-based structures are located at the most apical part of epithelial cells, and are responsible for firmly sealing the space between adjacent cells in order to establish a barrier against diffusion of molecules across the epithelial layer [46]. DMs are composed of non-classical cadherins, such as desmogleins, and are associated to intermediate filaments to form patch-like points of adhesion between cells [47].

In contrast to the two structures defined above, AJs provide strong cell-cell adhesion by forming cadherin-based adhesive structures anchored to the actin cytoskeleton that are strongly resistant to mechanical stress. In epithelial cells, AJs are linked to a circumferential “adhesive belt” of actin-filament bundles called the zonula adherens (ZA) to maintain strong adhesion. In addition to being a major cell-cell adhesion structure, the ZA forms at the apical/basal border in cells, and is important in maintaining cellular polarity by segregating the lateral membrane into apical and basal compartments [48]. AJ-dependent cell polarization also precedes the formation of TJs and desmosomes, as components of these structures need to be targeted to different compartments of the lateral membrane as defined by the ZA [49].

Despite its characteristically strong structure, numerous studies also illustrate the high plasticity of AJs. This is exemplified by the vital role of AJs in collective cell migration of various cells, such as astrocytes, neurons and neural crest cells, during development [50]. In neuronal cells, the association between AJs and the actin cytoskeleton function as mechanosensors to coordinate collective cell migration [51]. During collective cell migration, AJs are observed to undergo continuous treadmilling, a process where they are actively recycled from the rear of the cell to the front during migration. The process of AJ recycling helps not only to maintain adhesion between adjacent cells, but also maintains front-rear cell polarity and dictates the speed of migration [52, 53].

Collective cell migration is only one example showing how dynamic regulation of AJ influences cell-cell adhesion, and cell morphology. These dynamic morphogenic changes are also a hallmark of epithelial-mesenchymal transition (EMT), a well-studied physiological process that plays a crucial role in normal physiological functions as well as cancer progression.

EMT is a dramatic phenotype change of epithelial cells in response to physiological signals. Epithelial cells lose their epithelial characteristics, migrate away from the epithelial monolayer and establish new tissue at a distant site. Epithelial cells acquire many changes during EMT, including loss of apical-basal polarity, acquisition of motile behaviour, cytoskeletal re-organization, and the loss of E-cadherin-mediated cell-cell adhesion structures. Normally, EMT plays an important role in early embryogenesis during gastrulation and neural crest cell migration, and for wound healing in adult organisms, as healthy epithelial cells need to de-differentiate and migrate towards the injury site to promote new epithelial growth (Reviewed in [54]). However, in the context of human cancers, the very same process is hijacked by tumour cells for invasion and metastasis. The process of metastasis by EMT has been observed in different tissue, such as lung, prostate and breast cancers [55-57].

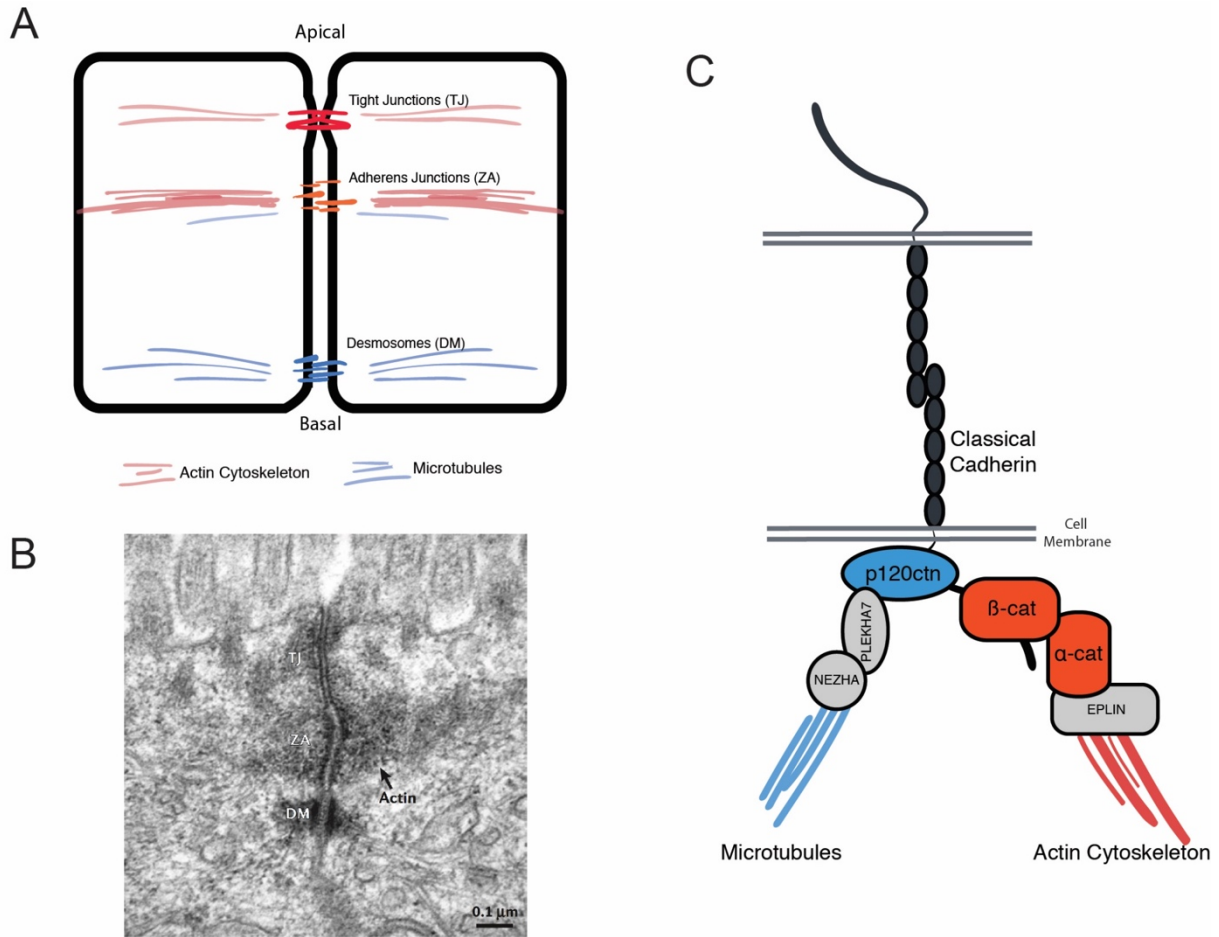


Figure 3. A basic schematic of cell-cell adhesion structures, and structural elements of adherens junctions.

(A) A schematic drawing of adjacent cells, identifying the location of three main cell-cell adhesion structures: tight junctions (TJ), adherens junctions, also known as zonula adherens (ZA), and desmosomes (DM). (B) Electron microscope image of mouse intestinal epithelium cells, indicating the three main structures at cell-cell contacts. The actin filament bundle consisting the ZA is also indicated. (C) A detailed representation of the cadherin-catenin complex including known interacting proteins that are required for the attachment of the complex to the actin cytoskeleton and microtubules.

The image in (B) was obtained from [58]

Adherens junctions and cadherins

The main AJ complex is composed of the adhesion molecule E-cadherin, which mediates strong, calcium-dependent binding between cells, and a catenin complex consisting of α -, β - and p120-catenin, that stabilizes and connects E-cadherin to the actin cytoskeleton (Fig. 3C).

The cadherins are a large family of cell-cell adhesion molecules, and play a role in tissue patterning and maintaining cell adhesion. Classical cadherins are single-pass, type I transmembrane glycoproteins that establish calcium-dependent, homophilic adhesion through binding cadherin molecules on neighbouring cells [59].

The two most well-studied types of classical cadherins are E- and N-cadherin. As its name suggests, epithelial cadherin (E-cadherin) is found in epithelial cells, and contributes to the formation of adherens junctions for tight cell adhesion in epithelial cell layers [60]. Neural (N-cadherin) is most abundant in the nervous system and forms adherens junction-like structures at synapses and other parts of the neuron [61]. There are other forms of classical cadherins that have been identified, such as cadherin-11, but there is still much to understand about the function of these other cadherins due to different patterns of expression across tissue types. A more detailed review about the greater family of cadherins can be found in [62].

Each cadherin molecule is composed of a short C-terminal cytosolic domain, and five “extracellular cadherin” (EC) domains that are essential for calcium-binding and cell-cell adhesion [59]. Upon binding of extracellular calcium ions, the EC-domains adopt a stiff, rod-like structure that extends from the cell membrane to form *trans* junctional interactions with cadherins on adjacent cells. These *trans* adhesive structures are incredibly sensitive to fluctuations in extracellular calcium levels, and have been observed to change conformation rapidly in response to removal of extracellular Ca^{2+} . When extracellular concentration of Ca^{2+} drops below 1 mM, the conformational change of cadherin EC-domains results in detachment from one another, and leads to a rapid loss of cell-cell adhesion [63].

The formation and maintenance of AJ requires the retention of cadherins at the cell membrane, as well as functional attachment of adhesive cadherins to the actin cytoskeleton for mechanical transduction. These two important aspects are regulated through a major family of cadherin interacting proteins known as the catenins, which bind to the cytoplasmic domain of classical cadherins. There is high sequence identity between the cytoplasmic domains of classical cadherins, within which are sites for binding a complex composed of α -, β -, and p120-catenins. Both β -catenin (β -cat) and p120-catenin (p120ctn) are direct interactors of cadherins, binding to different sequences in the cytoplasmic domain through their own highly conserved

armadillo (ARM) repeat domains. However, despite the sequence homology shared between β -cat and p120ctn, they serve functionally different purposes in the cadherin-catenin complex.

The linkage of cadherins to the actin cytoskeleton is mediated through β -cat and its association to α -catenin (α -cat). Initially it was believed that β -catenin binding to the distal portion of the cadherin recruits α -catenin, which serves to anchor the cadherin-catenin complex to the actin cytoskeleton either through directly binding F-actin or indirectly through other interacting proteins, such as afadin or epithelial protein lost in neoplasm (EPLIN) [64]. The specific mechanism of interaction between α -cat and F-actin is still under debate. Biochemical studies performed with AJ complexes in mouse and zebrafish indicates that α -cat binding to the AJ complex drastically decreases its affinity for F-actin. However, another study shows that a “minimal cadherin-catenin complex” composed of a E-cadherin/ α -cat fusion protein is capable of binding F-actin under force [65, 66]. Regardless, all studies agree that α -cat is a crucial component at AJ to mediate cadherin-actin cytoskeletal interaction. Meanwhile, β -cat appears to serve a supporting role at AJs by bridging cadherin to α -cat, and also harbours a collection of phosphorylation sites that converge from various signalling pathways for fine regulation of AJ. Briefly, phosphorylation of Ser/Thr in the β -cat binding domain of E-cadherin has been shown to strengthen β -cat binding, while phosphorylation of Tyr on both E-cadherin and β -cat reduces their affinity for each other, and promotes the dissociation of the cadherin-catenin complex (Reviewed in [67]).

Independent of α - and β -cat, p120ctn binds cadherins at a separate juxtamembrane domain (JMD) located in a more proximal region of the cadherin cytoplasmic domain and acts as an important regulator of both cadherin stability and its localization to the membrane. Intentional knockdown of p120ctn in a variety of mammalian cell lines results in the destruction of the cadherin-catenin complex at AJ, and a significant reduction in the protein levels of all classical cadherins, and both α - and β -cat [68, 69]. These results suggest that p120ctn plays a pivotal role in the formation and maintenance of the AJ/cadherin-catenin complex even superseding that of α - and β -cat.

The p120-catenin Protein

Structure of p120ctn

Found only in vertebrates, p120ctn is a member of a larger family of ARM-repeat containing proteins including: delta-catenin, p0071, and armadillo-repeat protein deleted in velo-cardio-facial syndrome (ARVCF). Comparing the proteomes across different vertebrate and metazoan species shows that all of these proteins evolved from a δ -catenin-like ancestor [70]. Successive evolution has generated multiple p120ctn family proteins in the vertebrate genome, while only one single δ -catenin-like protein exists in invertebrates. Interestingly, deletion of p120ctn homologues in invertebrates (p120 in *D. melanogaster* and JAC-1 in *C. elegans*) does not affect survival and only produces minor adhesion phenotypes [71, 72]. In contrast, p120ctn depletion in vertebrates causes embryonic lethality, while knockdown of other p120ctn members are better tolerated (See Table 1) [73]. This suggests that in vertebrates, p120ctn has evolved into a protein essential for life, while other members of the family appear to play less substantial roles in regulating physiological functions.

The gene coding for p120ctn, *ctnnd1*, consists of 21 exons encoding a total of 968 amino acids (Fig. 4), with alternative splicing sites occurring at exon 18 (exon A), exon 20 (exon B) which contains a nuclear export signal (NES), and exon 11 (exon C), the presence of which disrupts a nuclear-localization signal (NLS) [74]. In vertebrates, mRNA containing exon A is found to be abundantly expressed, while those with exons B and C are found at much lower copy numbers, with the only exception being in neural tissues where exon C appears to be abundantly expressed [75]. Aside from alternative splicing, the CTNND1 gene contains four unique start codons, and gives rise to four different isoforms, with isoforms 1 and 3 being the most commonly expressed in cells. There are four distinct functional regions to be found in the full-length p120ctn protein, beginning with a coiled-coil domain located near the N-terminal, a phosphorylation or regulatory domain, the ARM domain containing 9 ARM-repeats, and a C-terminal tail [74]. Due to the location of alternative start codons, the coiled-coil domain is only found in isoform 1, and the regulatory domain is present in all but isoform 4. However, the main central ARM-repeat domains critical for the interaction between p120ctn and the proximal region of cadherins remain preserved in all isoforms. However, due to variations in the other domains, there still appears to be functional differences between isoforms. For example,

evidence indicates a stronger expression of isoform 1 in mesenchymal cells expressing N-cadherin, while isoform 3 is preferentially expressed in epithelial cells and interacts more strongly with E-cadherin in these cells [76].

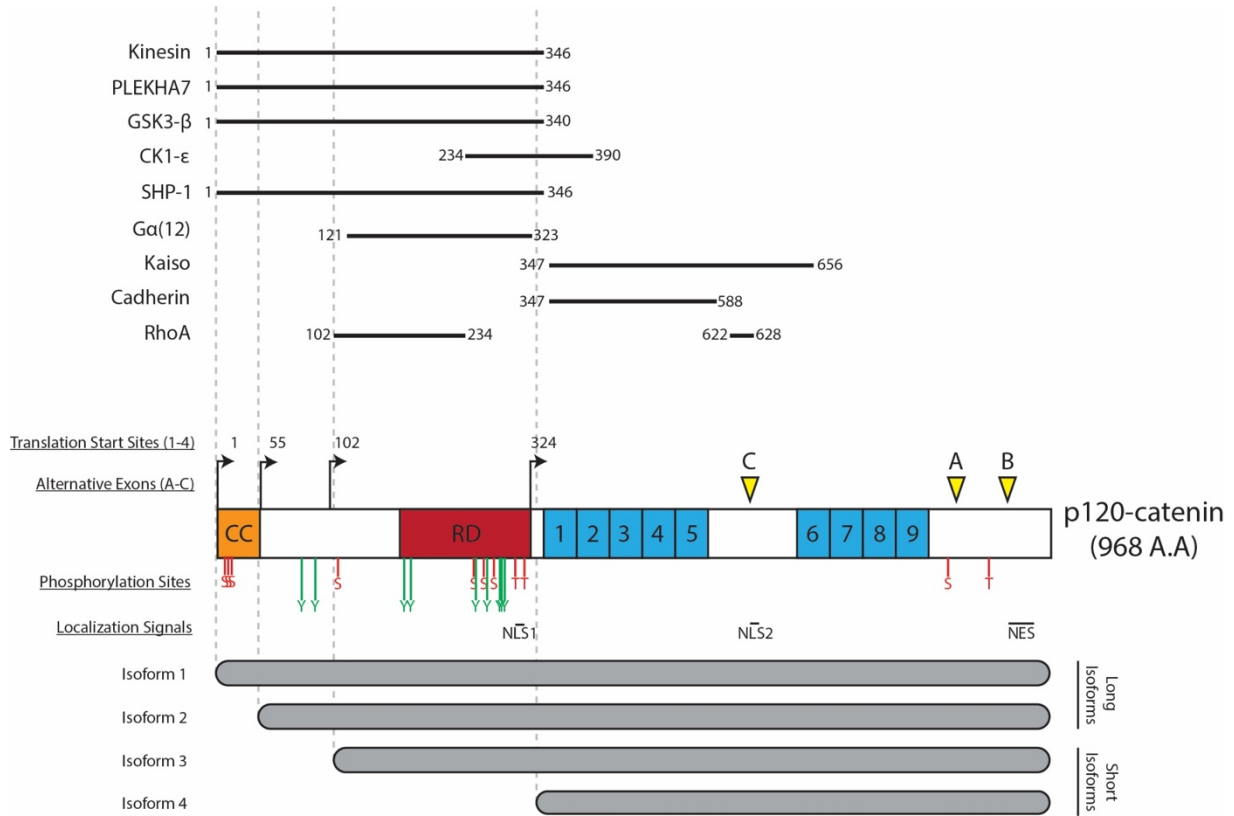


Figure 4. The structural features of p120ctn including main domains, subcellular localization signals, phosphorylation sites, and alternative splicing sites.

The CTNND1 gene for p120ctn codes for a protein up to 968 residues in length. Due to alternative splicing, four different start sites are present to give rise to four different isoforms of varying lengths. Alternatively used exons A, B and C are their locations are indicated. As a member of the armadillo-repeat (ARM) protein family, p120ctn contains 9 ARM-domains indicated from 1-9. The structure of p120ctn contains two nuclear localization signals (NLS) and one nuclear export sequence (NES). All identified Ser, Thr and Tyr phosphorylation sites are identified by S/T/Y labels. The interaction domains of major p120ctn interactors are also indicated.

CC: coiled-coil domain, RD: regulatory (phosphorylation) domain, ARM domains: 1-9.

p120ctn regulation of cell-cell adhesion

Originally discovered as a novel substrate for membrane-associated Src, p120ctn was identified as a protein that plays a role in regulating cell-cell adhesion [77]. Supporting this initial prediction, subsequent studies identified p120ctn to be an important member in the cadherin/catenin complex found at AJ [78].

Similar to β -cat, p120ctn binds to the cytoplasmic tail of classical cadherins but does not play a role in linking cadherins to the actin cytoskeleton. Instead, p120ctn maintains AJ stability by regulating cadherin turnover at the membrane through regulation of cadherin endocytosis [69]. In the absence of p120ctn binding, cadherins become rapidly internalized and are degraded by an endo-lysosomal degradation pathway [79]. Early studies have shown direct interaction between the two proteins: classical cadherin and p120ctn are required for their proper function as a complex at the cell membrane. E-cadherin re-expression in E-cadherin deficient cells causes p120ctn to be recruited to the membrane [80]. Conversely, in E-cadherin expressing cells, downregulation of p120ctn results in a decrease in the total levels of E-cadherin and a drastic decrease in cell-cell adhesion due to loss of AJs [81]. Finally, only re-expression of wild-type p120ctn, and not a mutant incapable of binding cadherin, can rescue cadherin levels in p120ctn-deficient cells [82]. This indicates that p120ctn mediated stabilization of cadherins requires direct interaction between these two proteins.

At a physiological level, the importance of p120ctn in maintaining cell-cell adhesion in vertebrates was studied using tissue-specific knockout models, as whole animal knock-out of p120ctn proved to be embryonic lethal as addressed previously. Descriptions of the effects of conditional p120ctn knockout in various mouse tissues are summarized in Table 1.

Overall, the trend suggests that conditional p120ctn knockouts produce defects in tissue morphogenesis resulting from loss in of cell-cell adhesion. Especially in epithelial tissue, the resulting defects in epithelial barrier function produces an inflammatory phenotype that further drives organ dysfunction (such as in the colon, skin, and salivary glands) and can generate a tumor promoting microenvironment [83]

Table 1: Effects of knockout and knockdown of p120ctn and related family members in different species.

Protein	Organism	Tissue	Phenotype	Reference
p120ctn	<i>Xenopus</i>	Whole animal	Disrupted gastrulation; reduced C-cadherin levels.	[84]
		Anterior neural ectoderm	Impaired optic vesicle and eye formation; changes in cranial neural crest cell migration; malformation in craniofacial cartilage.	[85]
	Mouse	Whole animal	Early embryonic lethality.	[86]
		Vascular endothelium	Embryonic lethality due to defects in embryonic vasculature; hemorrhage and defective proliferation; reduced VE- and N-cadherin levels.	[87]
		Salivary gland	Die soon after birth; neoplasia formation; abnormal epithelial polarity and changes in morphology; reduced E-cadherin levels; affected acinar differentiation.	[86]
		Small Intestine/Colon	Death within 3 weeks; reduced epithelial barrier function; intestinal bleeding and increased inflammation; reduced AJ components.	[73]
		Skin	Viable; develops skin neoplasia and hyperplasia; chronic inflammation due to NFkB activation; mitotic defects.	[88, 89]
		Liver	Viable; severely impairs in hepatic bile duct development; accelerated hepatocarcinoma development.	[90]
ARVCF	Mouse	Whole animal	Viable; no apparent abnormalities.	[91]
Delta-catenin	Mouse	Whole animal	Viable; impaired cognitive functions and abnormal synaptic plasticity; reduced N-cadherin levels.	[92]

p120ctn and cadherins

As outlined previously, p120ctn plays a significant role in maintaining cadherin expression and stability within cells by directly binding cadherins at its JMD. Crystallographic analysis of the binding interface between the E-cadherin JMD and p120ctn revealed the presence of an endocytic dileucine motif that lies in a region masked by p120ctn [93]. The exposed dileucine motif can be targeted by the adaptor protein AP-2, which promotes the clathrin-dependent endocytosis of cadherins from the membrane [94, 95]. Within the same binding interface also lies two Tyr residues (Y753/754) that, upon phosphorylation by Src, recruits the E3-ubiquitin ligase Hakai. In the absence of p120ctn, Hakai is recruited and promotes the proteasome-dependent degradation of E-cadherin [96]. However, these two mechanisms of cadherin regulation are only present in E-cadherin and are not conserved in other classical cadherins such as N- and P-cadherin.

Recently, a different endocytic DEE-motif was identified that is conserved across all classical cadherins, and re-emphasizes the role of p120ctn as an important regulator of all classical cadherins, not just E-cadherin [97]. This is reinforced by studies showing that p120ctn plays a vital role in regulating neuron growth and polarization through dynamic regulation of N-cadherin [98].

Outside of regulating cadherin dynamics through binding the JMD, a large number of interactors of p120ctn has also been identified that play a role in mediating alternative pathways of cadherin degradation. At AJs, p120ctn can recruit presenilin (PSEN1), a component of the gamma-secretase complex. The consequential assembly of the full complex results in the cleavage of E-cadherin amongst other target proteins, and promotes the disassembly of AJs [99]. Additionally, the C-terminal tail region of p120ctn can also associate with NUMB, and invokes clathrin-dependent endocytosis of the whole AJ complex – potentially for recycling back to the membrane during dynamic changes in cell-cell adhesion [100].

Finally, recent studies have also identified an interaction between the C-terminal domain of p120ctn and EPB41L5, a FERM (Four point one, Ezrin, Radixin and Moesin)-domain containing protein that has been identified to play a role in regulating cell-cell adhesion during EMT. EPB41L5 binding to p120ctn reduces its binding to E-cadherin at AJs and increases the number of E-cadherin positive vesicles detected in cells with EPB41L5 overexpression [101].

Further studies indicate this mechanism may play a significant role in driving the invasion and metastasis of breast cancer cells [102].

p120ctn and RhoGTPases

In addition to membrane localized p120ctn, cytoplasmic and nuclear pools of p120ctn have been observed across various cells, and have been shown to harbour pleiotropic functions to regulate cell adhesion, cytoskeletal remodelling and cell signalling.

Both membrane-bound and cytoplasmic p120ctn have been shown to control small RhoGTPase activity. At the membrane, cadherin-bound p120ctn recruits p190RhoGAP through a RhoGAP binding domain located in its C-terminal tail. The recruitment of p190RhoGAP mediates localized inhibition of RhoA at the cell periphery [103]. Uncoupling of p190RhoGAP from p120ctn in human pulmonary artery endothelial cells caused a drastic reduction in endothelial barrier recovery from disruption by thrombin, and contributes to vascular barrier dysfunction [104].

In contrast, cytoplasmic p120ctn can behave like a rheostat for regulating the balance between RhoA and Rac1 signalling. In the cytoplasm, p120ctn acts as a Rho guanine nucleotide dissociation inhibitor (RhoGDI) by binding RhoA in its inactive, GDP-bound form [105]. It was observed that in an indirect manner, p120ctn can also activate Rac1 by interacting with Vav2, a Rho family guanine exchange factor (GEF). Consistent with these roles, overexpression of p120ctn in NIH-3T3 cells resulted in loss of stress fibres and focal adhesions, while inducing a more dendritic phenotype [106].

However, more recent studies have shown that p120ctn's effects on RhoGTPase signalling can vary in a tissue and cell-type specific manner. In an invasive lobular carcinoma mouse model, p120ctn may activate Rho/ROCK signalling to promote cell invasion and anchorage independent growth by inhibiting Myosin phosphatase Rho-interacting protein (Mrip) [107]. However, in MDCK cells, cytosolic p120ctn mediates Src- and Rac1-induced anchorage independent growth and anoikis resistance [108].

Collectively, the new emerging effects of p120ctn on Rho and Rac signalling builds a more complex picture of p120ctn's role as a main regulator of Rho GTPase activity. Depending on the cell type, and biochemical context, cytosolic p120ctn can have a powerful effect on the

dynamics and balance of Rho/Rac signalling, and may significantly affect actin dynamics within epithelial cells.

p120ctn and transcriptional regulation

In addition to localizing to the plasma membrane and the cytoplasm, there are NLS and NES sequences present within p120ctn that enable it to shuttle in and out of the nucleus. Similar to cytoplasmic p120ctn, nuclear p120ctn also appears to be regulated by cadherin levels in cells [109]. Additional mechanisms of p120ctn nuclear translocation appear to exist, implicating p120ctn's ability to associate with microtubules and its interaction with another transmembrane protein – MUC1 that has been shown to also facilitate the nuclear translocation of β -cat [110, 111].

Upon translocation into the nucleus, p120ctn modulates gene expression by direct interaction with Kaiso, a transcriptional repressor belonging to the Bric à brac/Pox virus and zinc finger (BTB/POZ)-zinc finger family transcription factors [112]. p120ctn binding to Kaiso has been shown to relieve Kaiso-mediated transcriptional repression of its target genes. Interestingly, the target genes currently identified in literature controlled by Kaiso includes both tumor suppressor genes such as CDH-1 (E-cadherin) and Rb, as well as known components in canonical and noncanonical Wnt signalling pathways, whose activity has been shown to promote tumor cell metastasis and invasion (Reviewed in [113]). In the context of cancer, the role of Kaiso and p120ctn also appears to be cell type dependent. Evidence of nuclear Kaiso detected in high-grade invasive ductal carcinomas (IDC) and metastatic prostate cancer supports the oncogenic role of Kaiso repression [114, 115]. Conversely, detection of Kaiso in the cytoplasm of non-small-cell lung cancers (NSCLC) and invasive lobular carcinomas (ILC) suggest that Kaiso acts as a tumor suppressor in these cells [115, 116].

From these immunohistological studies, it is clear that localization of Kaiso plays a role in human cancers. However, how p120ctn regulates Kaiso localization remains ambiguous, and requires further research in order to elucidate p120ctn's exact role in Kaiso dysregulation and cancer.

Regulation of p120ctn by phosphorylation

One of the major methods of regulating p120ctn function is through phosphorylation of residues residing in the “regulatory domain” (RD) (which are present in all isoforms of p120ctn other than isoform 4), and a few in the C-terminal tail of all isoforms. In the last decade, academic efforts have identified many residues on p120ctn located within this region that are targets of post-translational modifications. The sites, as well as the known effects of phosphorylation at these residues, are summarized in Table 2.

Phosphorylation of p120ctn occurs at the membrane, and requires membrane localization of p120ctn rather than cadherin interaction – as it has been shown that simply fusing a CAAX-sequence to p120ctn is capable of inducing p120ctn phosphorylation in cadherin-deficient cell lines [117] This suggests that many, if not all kinases that regulate p120ctn are located at the cell membrane.

The interaction between p120ctn and cadherin is not only crucial to maintain cadherin stability, but also sequesters p120ctn to the cell membrane in epithelial cells and preventing its localization to the cytoplasm and nucleus, where it can exert pleiotropic functions. Hence, it is no surprise that many of these phosphorylation sites serve to alter p120ctn interaction with AJ members, and by connection, its localization into other cellular compartments.

Looking through the data, the broad trend that emerges is that phosphorylation on Tyr residues on p120ctn is required for its supporting role at AJ – serving as docking sites for other proteins to be recruited to act on other components of AJ such as β -cat. In contrast, Ser/Thr phosphorylation modulates p120ctn localization by changing its preference for binding partners away from cadherin. This destabilizes AJ while promoting the other pleiotropic functions of p120ctn such as actin cytoskeletal remodelling and altering gene transcription to promote cell migration.

This trend is supported by data from experiments using site-specific mutants of p120ctn. In Colo205 and A431D cell lines, the expression of 6 S/T phosphorylation-deficient mutants at S252, S268, S288, T310, S312 and T910 on p120ctn exhibited stronger cell-cell adhesion. While a phosphomimetic construct that replaced 4 S/T residues (S268, S288, T310 and S312) with basic, negatively charged glutamic acid greatly reduced cell-cell adhesion [118]. In a follow-up study that was published this year, it was discovered that in conditions of strong adhesion, there was further dephosphorylation observed on p120ctn outside of the 6 S/T residues

mutated in previous studies, suggesting the presence of other phosphorylation sites that respond to establishment of strong adhesion [119]. In a similar study, replacing 8Y residues (Y96, Y112, Y228, Y257, Y280, Y291, Y296 and Y302) with unphosphorylatable phenylalanines reduces the tumorigenic potential of p120ctn in breast cancer cells [120].

An interesting new insight into the mechanism of tumorigenic tyrosine phosphorylation on p120ctn is the discovery of p120ctn forming two distinct junctional complexes surrounding the ZA belt in MDCK and Caco-2 cells. A complex located just apical to the ZA contains unphosphorylated p120ctn that recruits PLEKHA7 and a microprocessor complex DROSHA and DGCR8 to regulate miR-30b expression in cells suppressing cell transformation markers. On the basolateral side, a population of more Tyr-phosphorylated p120ctn is co-localized with active Src and other proteins that promote cell-cycle progression, endocytosis, and is associated with cell transformation [121]. It is possible that the role of regulating Tyr-phosphorylation on p120ctn impacts the homeostasis between these two complexes, and the imbalance between the two leads to changes in cell phenotype.

Understanding how various cell signalling pathways converge on p120ctn have been an ongoing effort and area of focus in academia, and have been the subject of many reviews in the past decade [122, 123]. Not only is there a large collection of phosphorylation sites present, our understanding of the temporal relationships between these modifications, and how they translate to cellular responses to extracellular signals remain vague. As a major integrator of signals, it has been suggested that not only do individual modification sites matter in regulating p120ctn, but the collective pattern of phosphorylation on p120ctn affects its resulting function.

Table 2: Identified phosphorylation sites on p120ctn and the effects of site-specific modifications on p120ctn function.

Phosphorylated Residue	Kinase	Functional Effect	Reference
S8/S11/S15	CK1 α & GSK3 β	Phosphorylation at these residues promote p120ctn degradation.	[124]
S252	Unknown	Phosphorylated in response to VEGF stimulation. Phosphomutants fails to localize to the membrane.	[125]
S268/S269	PKC ϵ	Regulated by Wnt activity. Phosphorylation decreases binding affinity to E-cadherin and increases p120ctn binding to Kaiso - facilitating Kaiso nuclear export.	[126]
S288	Pak4/5	Alters p120ctn localization into cytoplasm and nucleus.	[127]
T310	GSK3	Required for the dissociation of p120ctn from N-cadherin during N-cadherin recycling in astrocyte migration.	[52]
S312	Unknown	Phosphorylation seems to occur in concert with phosphorylation at T310.	[128]
S879	PKC α	Promotes dissociation of VE-cadherins from AJ.	[129]
T916	Unknown	Unknown	[128]
Y112	Fyn	Phosphorylation lowers p120 association to RhoA, Rac and Vav2 and promotes E-cadherin binding.	[130]
Y217	Fyn/Src	Releases p120ctn from E-cadherin. But a phosphomimetic mutant (Y217E) also shows decreased binding of Rac1 and Vav2.	[131]
Y228	Src	Promotes p120ctn binding to RhoA and increases its inhibition. Constitutively phosphorylated in many carcinoma cell lines.	[130, 132]
Y96	Src Regulated	Re-expression of a p120ctn construct with mutations at all 8 Tyr residues (p120ctn-8F) in breast cancer cells produced less colonies in soft agar assay.	[120, 133]
Y257			
Y280			
Y296			
Y302			

p120ctn in cancer

In normal cells, formation of adhesion triggers signalling events that suppress cell growth and migration, but in tumor cells, this regulation is impaired as cells lose adhesive structures, and increases growth and motility. Based on its ability to regulate E-cadherin function, p120ctn was initially identified as a tumor suppressor by maintaining cell-cell adhesion. However, it is also implicated in regulating Rho-GTPase signalling to regulate cell migration and plays a role in Src-mediated cell transformation. Therefore, p120ctn appears to have both tumor suppressing and tumorigenic roles in the cell.

In accordance with its role stabilizing E-cadherin, evidence of p120ctn as a tumor-suppressor is found in studies into p120ctn deletion *in vivo* using mouse models (See Table 1). In the salivary gland, p120ctn deletion results in severe loss of adhesion due to E-cadherin destabilization, and created neoplastic lesions [86]. Additional studies performed in the oral cavity, esophagus, and forestomach also generated invasive squamous neoplastic lesions, as well as a tumorigenic inflammatory microenvironment, in response to tissue-specific p120ctn deletions [83]. The same deletion and knockdown of p120ctn performed in lung tumor cell lines resulted in loss of E-cadherin, and further promoted cell invasion and metastasis [134]. Immunohistochemical studies have shown that p120ctn is downregulated in certain tumors such as breast, prostate and lung cancers [135-137]. Together, these results suggest that the loss of p120ctn in cells is a significant event in some cancers and leads to increased tumor aggressiveness.

Over the course of cancer development, the loss and downregulation of E-cadherin is observed in many tumors. In response to decreased E-cadherin levels in cells, p120ctn translocation to the cytoplasm is strongly linked to transforming pre-invasive lesions into metastatic tumors in lobular breast carcinoma [138]. This suggests that p120ctn mislocalization to the cytoplasm causes dysfunctional Rho-GTPase signal regulation, and thus driving the development of a metastatic phenotype in tumors. In colon cancer cells undergoing EMT, the loss of E-cadherin coincides with a decrease of RhoA activity and an increase in cytoplasmic p120ctn, which is correlated with increased tumor aggressiveness. Knockdown of p120ctn by RNAi in these cells could restore stress fiber formation, and significantly reduce motility – indicating that cytoplasmic p120ctn is a major driver of cell invasion upon loss of E-cadherin [139]. As addressed in a previous section, cytoplasmic p120ctn is also implicated in driving

invasive lobular breast carcinoma by inhibiting Mrip, and promoting ROCK1-mediated anoikis resistance [107]. Cytoplasmic p120ctn is also observed and correlated with poor prognosis in other cancers, such as pancreatic cancer, and lung squamous cell carcinoma. [137, 140].

In addition to mislocalizing upon E-cadherin loss, p120ctn can drive EMT by playing an essential role in mediating a cadherin switch. Since it is possible for different cadherins to compete for binding with a limited pool of p120ctn in the cell, overexpression of mesenchymal cadherins, such as R- and N-cadherins, can induce E-cadherin destabilization [141]. An isoform switch between isoforms 1 and 3 of p120ctn can also promote the cadherin switch process by selectively stabilizing N- or E-cadherins, respectively. In lung cancer cells, expression of p120ctn-1A promoted EMT and increased N-cadherin expression, while p120ctn-3A inhibited cell invasion and promoted E-cadherin expression [142]. Another isoform-specific tumorigenic function was observed when comparing the expression of isoforms 1 and 4 in renal cancer, where only cytoplasmic isoform 1 was able to effectively inhibit RhoA activity and activate Rac1 due to RhoA binding to the N-terminal domain that is lacking in isoform 4 [143]. Similarly, analysis of p120ctn isoforms in breast cancer cell lines revealed preferential expression of larger isoforms 1 and 2 in more invasive cells [144].

Finally, in some cancers, p120ctn can exert tumorigenic properties even in the presence of E-cadherin. In inflammatory breast cancer, overexpression of the RNA-binding protein eIF4G1 induces an upregulation of p120ctn that further stabilizes E-cadherin. In this specific type of cancer, it appears the stabilization of E-cadherin paradoxically promotes its progression through an unknown mechanism [145]. In squamous cell carcinomas, p120ctn mediated stabilization of E-cadherin is essential for collective cell migration and invasion [146].

Collectively, the data from decades of studying p120ctn in cancer reveals a nebulous number of potential mechanisms by which p120ctn can influence cancer progression. Given the large number of functions and regulatory roles that p120ctn plays within the cell, the mechanism by which it drives cancer development appears to be highly context dependent, and cell-type specific. However, in many of these cases, it appears that regulation affecting p120ctn binding to E-cadherin and localization can significantly alter the tumorigenic roles that p120ctn plays in a context-dependent manner. Studies into the phosphorylation status of p120ctn in cancers may come to reveal new connections by which signals impinging on p120ctn can drive cancer development and metastasis.

Rationale and Objectives

Rationale

The Ras/MAPK signalling pathway is responsible for regulating a variety of cellular processes and responses, including regulating cell motility. As a kinase located downstream of the Ras/MAPK pathway, RSK has been shown to play a role in Ras/MAPK-mediated cell spreading and cell motility. However, currently the specific mechanisms by which RSK causes cell-spreading and loss of cell-cell adhesion in cells remain unknown. Our own proteomics studies have identified a new phosphorylation site on p120ctn that is regulated in response to RSK activity.

p120ctn is a crucial component of AJs and its dysregulation and mislocalization away from the plasma membrane is often linked to cancer progression, and more adverse outcomes. As a major stabilizer of classical cadherins in many cell types, its interaction with partner cadherins are crucial to maintaining proper cell function. Previous studies into phosphorylation sites present within the regulatory domain of p120ctn suggests these post-translational modifications (PTM) regulate p120ctn function by affecting its interaction with cadherins. In previous studies conducted by our lab, we have identified a potential novel phosphorylation site – S320 – on p120ctn that is also located within this crucial regulatory region. We hypothesized that phosphorylation of p120ctn at this site by Ras/MAPK activity promotes its dissociation from cadherins, and leads to AJ disassembly and overall loss in cell-cell adhesion (Fig. 1.5).

The main goal of my M.Sc. project is to validate p120ctn as a bona-fide substrate of the Ras/MAPK signalling pathway, and understand how p120ctn is regulated at the functional level by Ras/MAPK activity. Finally, if possible, I wanted to strive to understand how Ras/MAPK mediated regulation of p120ctn affects AJ function and how this translates to impacts on cellular function in the context of cell-cell adhesion and cell migration.

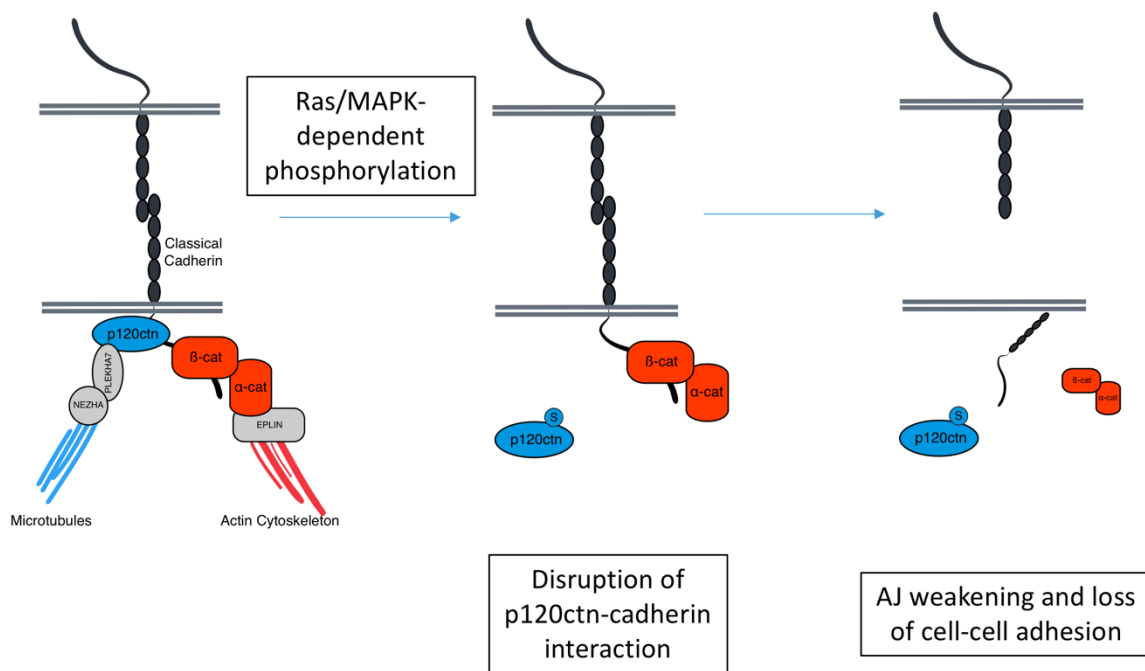


Figure 5. Our proposed model of the effects of Ras/MAPK signalling on p120ctn function and AJ stability.

The location of S320 within the regulatory domain of p120ctn suggests that phosphorylation at this site most likely will affect its binding to cadherins, and induce AJ destabilization. We present this model whereby increased phosphorylation of S320 in response to Ras/MAPK activity induces AJ disassembly by inhibiting p120ctn binding to cadherins at the surface.

Objectives

There are three main objectives to my thesis:

1. Given that we have identified through two separate proteomic screens that p120ctn may be a substrate of RSK, we intend to confirm and demonstrate that the Ras/MAPK pathway, through RSK, regulates phosphorylation at a specific site: S320 on p120ctn
2. As the proposed phosphorylation site, S320, is located within the regulatory domain of p120ctn, we intend to investigate how Ras/MAPK activity may affect p120ctn function, and how S320 phosphorylation may contribute to the observed effects.
3. Since p120ctn is a major regulatory protein at AJs, we intend to investigate how Ras/MAPK activity, and how S320 phosphorylation of p120ctn can affect AJ function by designing specific assays for AJ turnover and integrity.

MATERIALS AND METHODS

Cell culture and transient transfection

HEK293, HEK293T, HEK293 Phoenix, HeLa, MCF7, MDCK, IEC6, A431, A375 and Colo829 cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 4.5g/L glucose, supplemented with 5% [v/v] fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotic mix.

MCF-10A cells obtained from Dr. Guillaume Charras (UCL, London, UK) were cultured in DMEM-F12 medium supplemented with 5% horse serum [v/v; Invitrogen], 20 ng/mL human recombinant epidermal growth factor [EGF; R&D Systems, Minneapolis, MN], 10g/mL bovine insulin [Sigma], 0.5g/mL hydrocortisone [Sigma], and 1% penicillin/streptomycin antibiotic mix [Invitrogen]. To maintain optimal growth, the cells were refed with fresh, complete media every 72 hours.

Caco2 cells were graciously provided by Dr. Samantha Gruenheid (McGill University, Montréal, Canada) and were maintained in Minimum Essential Medium – alpha modification, with nucleosides (AMEM) supplemented with 10% [v/v] FBS and 1% penicillin/streptomycin antibiotic mix. To maintain optimal cell growth, the cells were refed every 48-72 hours with fresh complete media as needed.

To maintain epithelial phenotype and prevent unwanted spontaneous transformation in epithelial cell lines, MCF-7, MCF-10A, A431, Caco-2 and IEC6 cells were grown to 100% confluency before being used in experiments, or passed into carry plates. When passing them into new plates, the cells were passed at a density of no less than one-third of a confluent plate, to maintain a constant, high-level of confluency of cells in culture.

For transient expression of p120ctn and RSK constructs in our characterization experiments, 2.5×10^6 HEK293 cells were seeded onto 10cm plates and allowed to grow for 24 hours before transfecting with the desired plasmids by calcium phosphate co-precipitation. 24 hours post-transfection, the media was changed to either full growth media or starvation media as required by experiment. In each experiment, all transfections were performed on the same day and time for direct comparison between conditions. Additionally, when necessary, mock transfections were also performed as a control using calcium phosphate transfection reagents only.

DNA constructs and recombinant proteins

The original plasmid encoding full-length, untagged p120ctn was kindly provided by Albert Reynolds (Vanderbilt University, Nashville, USA) in a pENTR-gateway plasmid backbone (pENTR-p120ctn-1AB) [104]. The HA-tagged avRSK1 and mRSK2 (both wildtype and kinase-dead) constructs were used from our own lab and have been described previously [33].

First, to validate the sequence encoded within the pENTR vector provided, we amplified the p120ctn region by PCR using the following primers:

Forward	5' – GGG CCC CAA ATA ATG ATT – 3'
Reverse	5' – GTA ACA TCA GAG ATT TTG AGA CAC – 3'

The Sanger Sequencing results were aligned to the CTNND1 gene provided from NCBI databases to confirm sequence identity with the cDNA sequence for human p120ctn.

To generate the 6Myc-tagged p120ctn constructs used in the project, we inserted p120ctn into the pcDNA3.1-6Myc backbone by PCR cloning. To perform the ligation, both the p120ctn insert and pcDNA3.1-6Myc insert was digested by EcoRI and XbaI. The p120ctn encoded within the pENTR gateway vector was amplified by PCR using the following primers:

Forward	5' – GCG CGA ATT CGG ACG ACT CAG AGG TGG AGT – 3'
Reverse	5' – CCC GTC TAG ACT AAA TCT TCT GCA TGG AGG – 3'

To subclone 6Myc-p120ctn into a pLPC-puro backbone to produce retroviral particles used in generating stable cell lines, we again employed PCR cloning. This time, the 6myc-p120ctn insert and pLPC-puro target vector were subject to single-digestion by NheI. The primers used to amplify 6myc-p120ctn are:

Forward	5' – CCT GGC TAG CAT TTA AAG CTA TGG AGC AAA AGC – 3'
Reverse	5' – CGG CGC TAG CCT AAA TCT TCT G – 3'

During sub-cloning, we validated successful amplification, digestion and ligation by running the DNA products on agarose gels. To confirm the resulting plasmids, DNA was extracted from at least 3 positive bacterial colonies transformed with the ligation product and sent to sequencing to validate the final plasmid sequence. The primers used for sequencing were

common primers for pcDNA3.1 and pLPC-puro vectors obtained from Addgene. (T7 and CMV primers respectively)

To produce site-specific point mutants at S320, the QuikChange methodology was used. To generate point-mutants in both pLPC and pcDNA3.1 constructs, the following primers were used:

S/A Forward	5' – CCT CGT CGG CGC CTC AGG GCC TAT GAA GAC ATG ATT GGT G – 3'
S/A Reverse	5' – CAC CAA TCA TGT CTT CAT AGG CCC TGA GGC GCC GAC GAG G – 3'
S/D Forward	5' – CCT CGT CGG CGC CTC AGG GAC TAT GAA GAC ATG ATT GGT G – 3'
S/D Reverse	5' – CAC CAA TCA TGT CTT CAT AGT CCC TGA GGC GCC GAC GAG G – 3'

To validate the resulting mutation located at S320, we designed forward and reverse primers to perform Sanger sequencing for a specific section of the final p120ctn construct containing the mutation site of interest. The primers used were:

Forward	5' – GGG AGC AGC GTG GAT CTG – 3'
Reverse	5' – AGG CCC TCC TTT GCG CAG – 3'

RNA interference

Small interfering RNA (siRNA)-mediated knockdown of RSK1 and RSK2 were performed using previously validated, 21-nucleotide cRNAs with symmetrical 2-nucleotide overhangs obtained from Qiagen. [33] The siRNA specific for p120ctn were also obtained from Qiagen, and validated through experimental knockdowns performed in HEK293 cells using a validated antibody.

To perform the knockdowns, HEK293 cells were transfected with 50 nM of siRNA per 10cm dish by calcium phosphate co-precipitation. The cells were then harvested 48 hours post-transfection. HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions, and the cells were harvested 48 hours post-transfection.

To generate cells with stable knockdown of p120ctn, five small hairpin RNA (shRNA) constructs were purchased from MISSION shRNA library (Sigma). To generate lentiviral particles containing the appropriate shRNA for infection of HEK293, HeLa, MCF-7 and MCF-10A cells, HEK293T cells were co-transfected with both the shRNA and lentiviral packaging constructs by calcium phosphosphate. 12h-hours post-transfection, the media was removed and replaced with fresh media and the HEK293T cells were left to produce lentiviral particles containing shRNA for 24 hours, after which the media was taken and applied to 10cm plates of target cells.

72 hours after lentiviral infection, the cells were selected with fresh media containing appropriate concentrations of puromycin: HEK293: 1.5 µg/mL, HeLa: 2.0 µg/mL, MCF-7: 1.5 µg/mL, MCF-10A: 1.0 µg/mL. After a week of selection, the cells were maintained in passage for future experiments, or were plated at desired densities to use for experiments.

Antibodies

Table 3: Antibodies used in this study for immunoblots and immunofluorescence experiments.

Target Protein	Source	Immunoblot Dilution	Immunofluorescence Dilution
p120ctn	Santa Cruz	1:3000, 5% milk	1:500
Phospho-p120ctn (S320)	Cell Signalling	1:1000, 5% BSA	N/A
E-cadherin	BD Bioscience	1:1000, 5% BSA	N/A
E-cadherin	Sigma	1:1000, 5% BSA	1:500
N-cadherin	BD Bioscience	1:1000, 5% BSA	1:500
Anti-Myc	9E10 monoclonal	1:3000, 5% milk	1:500
Anti-HA	12CA5 monoclonal	1:1000, 5% milk	N/A
Anti-Tubulin	Sigma	1:2000, 5% milk	N/A
Akt	Cell Signalling	1:1000, 5% BSA	N/A
Phospho-Akt (S473)	Cell Signalling	1:1000, 5% BSA	N/A

ERK1/2	Cell Signalling	1:2000, 5% milk	N/A
Phospho-ERK1/2 (T202/Y204)	Cell Signalling	1:1000, 5% BSA	N/A
S6K	Cell Signalling	1:1000, 5% BSA	N/A
Phospho-S6K (T389)	Cell Signalling	1:1000, 5% BSA	N/A
rpS6	Cell Signalling	1:1000, 5% BSA	N/A
Phospho-rpS6 (S235/236)	Cell Signalling	1:1000, 5% BSA	N/A

Cell lysis and lysate preparation

To prepare lysates for immunoprecipitation or immunoblot analysis, cells were lysed using BLB lysis buffer. First, media was removed from plated cells by aspiration, and then cells were rinsed with ice cold, Phosphate Buffered Saline (PBS) pH 7.4 to remove any remaining media. Afterwards, cells were lysed using an appropriate amount of working BLB lysis buffer (10 mM K_3PO_4 , 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β -glycerophosphate, 0.5% Nonidet P-40, 0.1% Brij 35, 0.1% deoxycholic acid, 1 mM sodium orthovanadate [Na_3VO_4], 1mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail tablet [Roche]). Processed cells were collected using a cell scraper, and the resulting total cell lysate were centrifuged at 14800 RPM for 10 minutes at 4°C. After centrifugation, the supernatant was collected and total protein levels in the cell lysate were determined via Bradford protein assay. Finally, the supernatant was used for further immunoprecipitation experiments, or diluted with 2x Laemmli's reducing sample buffer (5x buffer consists of 60 mM Tris-HCL, pH6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) and boiled at 95°C for 5 min. After boiling, the final samples were centrifuged at 14800 RPM for 1 min before loading on to SDS-PAGE gels for immunoblot analysis.

Bradford protein assay

To quantify protein concentration in cell lysate samples, Bradford reagent (Bio-Rad Laboratories Inc.) was diluted at a 1:5 ratio in Milli-Q water, and aliquoted in 1000 μ L aliquots in individual Eppendorf Tubes.

First, to obtain a concentration curve for the Bradford assay, known concentrations of BSA, ranging from a final concentration of 1 μ g to 20 μ g, were added to the tubes containing diluted Bradford reagent. The relative absorbance of each sample was determined using light at 595 nm, and the resulting absorbance measurements were used to create a protein concentration curve corresponding to absorbance.

Then, 2 μ L of cell lysate samples in lysate buffer was added to the tubes containing diluted Bradford reagent and mixed. Afterwards, the relative absorbance of each sample was assessed, and using the previously generated curve, the protein concentrations in each sample was determined. If protein levels between samples needed to be equalized, the adjustments needed were calculated using the protein concentrations determined through the Bradford assay.

Immunoprecipitation

For immunoprecipitations, 700 μ L of cell lysates obtained from BLB lysis of cells from 10cm plates were incubated with the indicated antibodies for 2 hours at 4°C using a rotating platform. Afterwards, 45 μ L of protein A-Sepharose CL-4B beads (GE Healthcare) were added and incubated for another 1 hour at 4°C with constant rotation.

To obtain the final immunoprecipitated sample, the solution with beads were centrifuged at 4000 RPM for 2 minutes to precipitate the beads, and the supernatant were removed then washed three times with working BLB lysis buffer (described previously). After washing, the beads were resuspended in 50uL of 2x Laemmli's reducing sample buffer and boiled at 95°C for 5 min. The beads were then centrifuged at 14800 RPM for 1 min before loading on to SDS-PAGE gels for immunoblot analysis.

SDS-PAGE gel separation and immunoblotting

To identify and quantify proteins of interest in cell lysate or immunoprecipitation samples, the samples were loaded and electrophoresed on 10% polyacrylamide gels at 55-85 volts in 1X SDS-Page running buffer (25 mM Tris Base, 200 mM Glycine, and 0.1% SDS)

overnight to achieve proper separation of protein targets. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes by wet electrotransfer at 0.75A for 2 hours.

After transferring, the membranes were then blocked in 1X TBST (20 mM Tris pH 7, 150 mM NaCl and 0.1% Tween) with 5% skim milk powder by rocking for 1 hour at room temperature. Then, membranes were incubated overnight at 4°C with constant rocking in primary antibody solution in 1X TBST with either 5% skim milk powder, or 5% BSA at effective working dilutions as per “antibodies” section above.

Following overnight incubation with primary antibody, the membranes were washed 3 times for 5 min with 1X TBST, and then incubated with secondary HRP-conjugated antibodies of specific species against the desired primary antibody. The secondary antibody is diluted at 1:2000 in 1X TBST with 5% milk, and membranes were left with constant rocking for 1 hour at room temperature.

To reveal the immunoblots, the membranes were washed 3 times with 1X TBST for 5 minutes before incubating them for a few seconds in enhanced chemiluminescence (ECL) solutions. The membranes were then developed on films at varying exposure times to visualize the protein bands.

Retroviral infection of cells

To generate cell lines stably expressing exogenous Myc-tagged p120ctn constructs, A431, MDCK and MCF-10A cells were infected using pLPC-puro vectors packaged in retroviral particles – to varying levels of success.

To generate pLPC-puro containing retroviral particles, 4.0×10^6 HEK293 Phoenix cells were seeded in 10mL of complete DMEM media in 10cm cell culture plates and grown for 24 hours prior to transfection. Then, each plate of cells was transfected with 20 ug of pLPC-puro vectors containing the 6Myc-p120ctn wildtype and mutants or an empty vector as a control, using calcium phosphate co-precipitation. Cells were then left to incubate for 24 hours post transfection, and the media was replaced with 10mL of new complete media specific to the cells of interest. At the same time, cells from the target cell lines were seeded in 10cm cell culture plates at appropriate densities that would result in 40-50% confluency the next day in 10 mL of the appropriate growth medium before infection.

To perform the retroviral infections, 16 hours after plating, the media was removed from the target cells, and the now retrovirus containing media was collected from the 293 Phoenix cells, and passed through a 0.45 micron filter before being added directly to the target cells. Additionally, a final concentration of 4 $\mu\text{g}/\text{mL}$ of Polybrene was added to the infection media to assist with the infection. 10mL of fresh media was put back in the 293 Phoenix plates to continue viral production. 6 hours later, the first infection media was removed from the target cell plates and a second round of retroviral infection was performed by adding media from the 293 Phoenix cells to the target cells as before. Finally, after 24 hours of incubation with the new infection medium, the target cells were selected by adding media with an appropriate concentration of puromycin as determined by a kill curve experiment. The concentration of puromycin used for selection of each cell line are as follows: MDCK: 1.5 $\mu\text{g}/\text{mL}$, MCF-10A: 1.0 $\mu\text{g}/\text{mL}$, A431: 1.0 $\mu\text{g}/\text{mL}$.

After 72 hours of selection, the plates with surviving cells were washed 2 times with PBS, and replaced with fresh media containing the same concentration of puromycin to maintain expression of the pLPC-puro constructs in surviving cells. If confluent, the cells were trypsinized and divided into new plates and selection was continued. Approximately one week after initial selection, the cells were divided into larger 15cm plates for making cell stock, or replated into appropriate plates for use in experiments.

Immunofluorescence staining

To visualize cellular localization of proteins of interest, cells were seeded and cultured in 12-well plates, on glass coverslips coated with poly-D-lysine to improve cell adhesion to the coverslip during the washing and staining process. After at least 48 hours of incubation (in cells that were not experimentally treated), the cells were washed three times with ice-cold PBS pH 7.4, and then fixed with 3.7% paraformaldehyde in PBS solution for 15 minutes at room temperature (RT). Afterwards, the cells were again washed three times with PBS to remove the paraformaldehyde and then permeabilized by incubating in 0.3% Triton-X100/PBS solution for 5 minutes at RT.

Post-permeabilization, cells were washed three times with 0.05% PBS-Tween (PBS-T) washing solution, followed by a 1 hour long incubation in Blocking Buffer (1% Bovine serum albumin (BSA)/PBS). The cells were then washed again three times with PBS-T, then incubated

with desired primary antibodies at an appropriate concentration diluted in 1% BSA/PBS blocking buffer for 1 hour at RT. After three more washes with PBS-T, the cells were then incubated with the secondary antibody at an appropriate dilution in 1% BSA/PBS for 30 minutes at RT. The secondary antibodies conjugated to Alexa-Fluor dyes (488 or 555nm; Life Technologies) to identify target molecules were used at a 1:500 dilution. F-actin was labelled with Texas Red-X phalloidin (Life Technologies), used at 1:400 dilution. Finally, the coverslips were washed three more times in PBS-T before being mounted onto glass microscopy slides with Vectashield (Vector Laboratories) mounting medium containing DAPI.

Images of antibody-stained cells were taken using the DeltaVision (Applied Precision) microscope equipped with a CoolSNAP HQ2 camera (Photometrics) using 40x and 60x Aplanachromat objectives. Images were captured and analysed using SoftWoRx software (Applied Precision) before processing using Adobe Photoshop.

Calcium depletion

EGTA is a divalent ion chelator with high specificity for Ca^{2+} ions and we utilized this mechanism to reduce Ca^{2+} concentration in the growth media to specific levels to induce AJ disassembly and cell-cell adhesion loss in cells.

Briefly, calculated amounts of 0.2mM EGTA pH 7.4 working solution were added to the full growth media of confluent cell monolayers to achieve desired final concentrations of EGTA in the media.

Monolayer fragmentation assay

To perform the monolayer fragmentation assays, cells were seeded in 60mm cell culture plates to reach >90% confluency in 48 hours, as determined through a cell titration experiment. For MCF10A cells, 3.0×10^6 cells were seeded per plate, and p120ctn expressing A431 stable cell lines were seeded at a density of 1.5×10^6 cells per plate. Cells were plated in quadruplicate for each condition: three for fragmentation counts, and one for a total cell number count.

To isolate the monolayers from the plates, cells were washed twice with warm PBS (pH 8.0) and then incubated for 20-40 minutes with 2 mL of media containing dispase (2.4U/mL, Roche) until the monolayer visibly began to detach. After detachment, 5 mL of warm PBS (pH 8.0) was added slowly to fully release the monolayer, and the total 7 mL were transferred into

15 mL Falcon conical tubes (Fisher Scientific). The solutions were then centrifuged at 1000 RPM for 5 min to pellet the monolayer, and the remaining supernatant was carefully removed by aspiration.

Before monolayers were subject to dispersion, 1 mL of complete media was added back to each tube to re-suspend the monolayer. For calcium depletion experiments, EGTA was added to the media at the desired concentration, and for the desired time before monolayers were disrupted. Each monolayer was then subject to disruption by pipetting up and down 15 times with a P1000 pipetman. Immediately after disruption, 10 μ L of the final mixture was taken and single cells were counted using a haemocytometer.

Percentage fragmentation of each triplicate within a condition was then calculated against the cell count obtained from a fourth 60mm plate that is trypsinized to obtain a total cell count, rather than trypsinizing dispase treated cells.

Cellular impedance assay

Changes in impedance of an epithelial monolayer was measured using the xCELLigence RTCA SP System (ACEA Biosciences). Briefly, the xCELLigence system functions by assessing changes in the electron flow between microelectrodes embedded at the bottom of wells in a 96-well specialized E-plate (ACEA Biosciences). In the plates, cells will impede the electron flow between electrodes resulting in an increase in detected electrode impedance that is translated into a variable known as “Cell Index” (CI) by the xCELLigence system. Commonly, cell growth, adhesion and changes in morphology can be assessed using this system, and are reflected in changes in CI.

To use the system to monitor changes in cell behaviour, a measurement is first made for the impedance of each well in the presence of only growth media before cell seeding to determine a background CI, this is then subtracted from future CI measurements during the experiment after cell attachment.

In cell titration experiments, different densities of A431, MCF-10A, IEC-6 and Caco-2 cells were seeded in quadruplicate wells and left to attach for 30 minutes at RT before being placed in the incubator at 37°C. CI measurements were taken by the xCELLigence system every 15 minutes for 96 hours. Cell confluency was indicated by a long-term plateau in CI, and time

to confluency was assessed through analysis of recorded CI curves to select optimal cell density to seed for future experiments.

In calcium depletion experiments using EGTA, MCF-10A cells were seeded in quadruplicate per condition at the optimal density and allowed to grow for 40 hours to reach confluency. During the growth period, CI measurements were taken every 15 minutes to monitor growth and establishment of confluency. Following EGTA addition, CI measurements were taken every 15 seconds for 8 hours by the xCELLigence system to closely monitor changes in cell-cell adhesion. Normalized cell index values were obtained by dividing by the cell index at the time of treatment and baseline-corrected by subtracting the cell index obtained from vehicle-treated conditions.

To program experiments using the xCELLigence system, and to analyze the data collected, the RTCA Software Version 2.0 (ACEA Biosciences) was used. Post-processed data points (measured in CI) were then manually exported into Microsoft Excel 2016 for analysis.

RESULTS

SECTION 1: Ras/MAPK regulation of p120ctn phosphorylation.

S320 on p120ctn is located within a highly conserved, AGC-kinase consensus sequence

Since there are many kinases responsible for regulating phosphorylation of proteins in cells, we started by identifying potential kinases which can regulate phosphorylation of this site on p120ctn. First, we asked if S320 was located within a consensus recognition sites for any known kinases, or kinase families. By using Scansite [147] to perform an analysis for minimal consensus recognition sites on p120ctn at high-stringency, we found that S320 is a putative phosphorylation site nestled in a basophilic AGC-kinase motif (reported as an Akt Kinase Motif) (Fig 6A).

Next, we asked if S320 was an evolutionarily conserved site by performing an *in silico* alignment of p120ctn proteins encoded by the CTNND1 gene across 5 different vertebrate species using BioWorkBench [148]. No invertebrates were included as p120ctn is only present in vertebrate species. The alignment results show that S320 is conserved in the regulatory domain of p120ctn from all five species (Fig 6B and C). Interestingly, we also see conservation of the residues surrounding S320, together making up the R-X-R-X-X-pS/T consensus motif shared by the AGC family of kinases, further supporting the ScanSite analysis.

p120ctn is a substrate of the Ras/MAPK pathway

Following the *in silico* analysis of S320 as a potential phosphorylation site on p120ctn, we set out to confirm that this site is phosphorylated *in vivo*. To monitor phosphorylation at S320, we used a phospho-specific antibody raised against phosphorylated S320 on p120ctn.

First, HEK293 and HeLa cells were starved overnight then stimulated with different agonists to activate Ras/MAPK and PI3K/mTOR signalling (Fig 7A). Activation of each pathway is indicated by the levels of phospho-ERK1/2 (T202/Y204) and phospho-Akt (S473), respectively. We found that stimulation of cells with the phorbol ester PMA and epidermal growth factor (EGF) induced significant phosphorylation of S320 on endogenous p120ctn. Comparatively, treating cells with 10% fetal bovine serum (FBS) only produced detectable levels of S320 phosphorylation in HeLa cells, while insulin stimulation appears to be unable to induce phosphorylation at this site. In both cell types, phosphorylation of p120ctn at S320 correlated closely with ERK1/2 activation. To further examine this correlation, we performed a

time-course analysis of p120ctn phosphorylation after PMA and EGF stimulation (Fig 7B). From this, we found that S320 phosphorylation correlated more closely with levels of phospho-RSK (S380) than phospho-ERK1/2 (T202/Y204). Since phospho-RSK (S380) is a marker of RSK activity, our results implicate not only Ras/MAPK signalling in regulating S320 phosphorylation, but also suggests that RSK is the AGC-kinase that regulates this site.

Since Akt and S6K are also relevant AGC kinases that can be activated by PMA and EGF stimulation, we tested if Akt could phosphorylate p120ctn at S320 by strongly activating PI3K/mTOR signalling in HEK293 cells with increasing doses of Insulin (Fig 7C). We found that Akt activation by this method was still unable to promote phosphorylation of S320, indicating that it is not the AGC kinase responsible for regulating this site. Additionally, the lack of increase in phospho-Akt (S473) in response to increasing Insulin indicates we have induced maximum activation of the pathway, albeit no effect on p120ctn phosphorylation.

However, since we still observe active S6K (T389) in response to PMA stimulation in the previous experiment (Fig 7C) we investigated its involvement in regulating S320 phosphorylation by using pharmacological inhibitors targeting the Ras/MAPK and PI3K/mTOR pathways (Fig 7D). From the results, we see that treatment with a PI3K/mTOR dual specificity inhibitor (PI-103) or mTOR specific inhibitors (Ku-0063794 and Rapamycin) inhibits Akt and S6K activity without inhibiting S320 phosphorylation. In contrast, treatment with a MEK1/2-specific inhibitor (PD184352) almost completely eliminates PMA- and EGF-induced p120ctn phosphorylation on S320.

Together, these experiments not only imply that growth factor induced phosphorylation of p120ctn on S320 is Ras/MAPK dependent, but also suggests that RSK is the likely AGC kinase involved in phosphorylating this site.

Phosphorylation of p120ctn on S320 is RSK-dependent

To confirm the involvement of RSK in regulating p120ctn phosphorylation on S320, we exposed starved HEK293 cells with two RSK inhibitors (BI-D1870 and LJM685) before treating them with PMA and EGF (Fig 8A). We found that successful RSK inhibition, indicated by reduced phosphorylated rpS6 (S235/236), significantly reduced phospho-p120ctn (S320) levels.

As addressed in the introduction, there are 4 different isoforms of RSK present within mammalian cells, so we asked if phosphorylation on this site was isoform specific. From

previous studies in our lab, we know that RSK1 and 2 are the isoforms most highly expressed in HEK293 cells. Therefore, we utilized previously validated siRNA targeting RSK1 and 2 to perform either individual or additive knockdowns in HEK293 cells (Fig 8B). We found that, while knockdown of either isoform produced a marked decrease in p120ctn phosphorylation induced by PMA or EGF stimulation, a greater effect was seen when both isoforms were knocked down concurrently. This suggests not only that RSK is required for Ras/MAPK mediated phosphorylation of p120ctn on S320, but that both isoforms of RSK contribute to regulation of this site.

Conversely, we also transiently overexpressed wild-type (WT) or kinase-deficient (KD) RSK1 and 2 in HEK293 to test if RSK activity was sufficient to induce S320 phosphorylation (Fig 8C and D). Successful transfection of the RSK constructs are indicated by the anti-HA immunoblot. An increase in total RSK activity in cells transfected with WT RSK is indicated by the increase in phosphorylated rpS6 (S235/236) and decreased phosphorylated ERK1/2 (T202/Y204) due to known feedback mechanisms. We found that WT RSK could not only increase S320 phosphorylation in response to PMA or EGF stimulation, but also induced phosphorylation at this site under starved conditions. However, the KD RSK fails to produce the same augmentation of p120ctn phosphorylation, indicating that active RSK is sufficient to promote phosphorylation of this site. Additionally, these results also confirm that RSK-mediated phosphorylation of p120ctn on S320 is not isoform specific.

RSK regulates p120ctn phosphorylation on S320 in epithelial cell lines

Since p120ctn plays an important role in regulating AJ function, which are crucial in epithelial cells for maintaining cell function and polarity, it was important for us to choose a suitable cell line, which we can use for future functional experiments. While HeLa cells are of an epithelial origin, and HEK 293 cells can adopt an epithelial morphology, p120ctn function and AJ formation have been extensively characterized and studied using more epithelial cell lines such as A431 and MCF-7 cells. A431 cells are an epidermoid carcinoma cell line, and MCF-7 cells are human breast adenocarcinoma cells that retain many characteristics of the mammary epithelium. Both cell lines have been used to study p120ctn function [146, 149], and form clearly defined cell-cell adhesion structures.

To verify that p120ctn is phosphorylated by the same mechanism in these cells, we decided to recapitulate the agonist and inhibitor experiments conducted previously in A431 and MCF-7 cells. We pre-treated both cell lines with a MEK specific inhibitor (PD184352) and a RSK specific inhibitor (BI-D1870) before stimulating the cells with PMA or EGF (Fig 9A and B). We see that both PMA and EGF can stimulate p120ctn phosphorylation on S320 in these cells lines, and that inhibition of both MEK and RSK also effectively prevented this modification. Interestingly, unlike in HEK293 and HeLa cells, in these two cell lines EGF stimulation produced greater levels of p120ctn phosphorylation on S320 compared to PMA. Additionally, in both cell lines, we only detected one band of p120ctn, compared to two that we had seen in HEK293 and HeLa cells. This is most likely because epithelial cells are known to only express the shorter isoform (isoform 3) of p120ctn, while more mesenchymal cells express both isoforms 1 and 3 [75].

SECTION 2: Ras/MAPK mediated effects on p120ctn

Ras/MAPK activity reduces p120ctn binding to N-cadherin

Since cadherins are one of the most important binding partners of p120ctn in the cell, and are crucial to p120ctn function, we chose to evaluate how Ras/MAPK activity may affect this interaction. To address whether Ras/MAPK phosphorylation of p120ctn affects its binding with cadherin, we immunoprecipitated p120ctn from HEK293 cells that were stimulated with PMA for up to two hours, and analyzed the amount of N-cadherin that was bound to the immunoprecipitates (Fig 10A). We found that PMA stimulation quickly reduced the amount of N-cadherin bound to immunoprecipitated p120ctn. Moreover, we also found that PMA stimulation beyond an hour reduced N-cadherin levels in the total cell lysate, suggesting that N-cadherin may be degraded under long-term PMA stimulation. This effect may be attributed to the reduction in p120ctn, resulting in destabilization of unbound N-cadherin. To avoid the possibility of N-cadherin degradation affecting our results, we chose to shorten PMA stimulation in the following experiments.

Next, we asked if Ras/MAPK inhibition could prevent the reduction in p120ctn binding to N-cadherin. This time we pre-treated starved HEK 293 cells with the MEK inhibitor (PD184352) for 30 minutes before stimulating with PMA for up to another 30 minutes (Fig. 10B). Within this short-term of PMA stimulation, we still found a significant loss in p120ctn binding to N-cadherin. Inhibition of MEK partially restored N-cadherin binding to p120ctn, but the PMA-induced loss was not completely prevented. This shows that Ras/MAPK signalling, in part, contributes to PMA-induced loss of binding between p120ctn and N-cadherin. However, our results also suggest that PMA stimulation induces other pathways that converge on p120ctn to regulate its binding to N-cadherin.

PMA Stimulation does not affect p120ctn localization

Another way by which p120ctn function can be altered and regulated by phosphorylation is by altering its localization in different cellular compartments. As addressed in the introduction, p120ctn has been shown to have different functions and different roles depending on its localization within the cell. This led us to question if Ras/MAPK signalling influenced p120ctn localization. We assessed this by staining for endogenous p120ctn in two different cell lines: HEK293 (N-cadherin positive) and A431 (E-cadherin positive) using a p120ctn specific

antibody.

First, to validate the p120ctn antibody, we immunostained HeLa and MCF-7 cell lines that were infected by 3 different shRNA to specifically knockdown p120ctn (Fig 11). While not all shRNA were effective at knocking down p120ctn, as indicated by the western blot results for total cell lysate, in cells with a drastic reduction of p120ctn, we also saw a loss of p120ctn staining by IF. From this, we can conclude that the antibody we are using is indeed specific for endogenous p120ctn.

With a validated antibody on hand, we proceeded to stimulate HEK293 and A431 cells with PMA for up to 8 hours after a 16-hour starvation period, and stained for p120ctn to investigate if there was a change in its localization away from cell membrane. To visualize the cell membrane and areas of cell-cell contact, we also stained for F-actin in cells. From the resulting IF images, we see that even with prolonged PMA stimulation, we are unable to visualize a significant change in localization of p120ctn. In HEK293 cells, much of the p120ctn remain localized at the cell membrane, co-localizing with F-actin (Fig 12). In A431 cells, which are highly epithelial, endogenous p120ctn localizes to sides of the cell in contact with adjacent cells, and not the protruding external edges of cells (Fig 13). Interestingly, in A431 cells, some p120ctn localize specifically to the tips of filopodia-like protrusions that form points of contact with adjacent cells (Indicated by white arrows). These structures have been identified as “punctate AJs” and are a cadherin-based cell-cell adhesion structure in epithelial cells that precedes for the formation of continuous regions of cell-cell contact [150]. This suggests that in epithelial cells, p120ctn remains localized at cadherin-rich areas in response to PMA stimulation.

Site-specific mutation at S320 does not affect p120ctn localization

To investigate if site-specific phosphorylation at S320 affected p120ctn localization, independently from Ras/MAPK activity, we generated stable cell lines in MDCK cells expressing wild-type or phospho-mutants of Myc-tagged p120ctn (Fig 14A). We then visualized the localization of the exogenous p120ctn by staining the cells with a Myc-specific antibody.

First, we validated the specificity of the Myc antibody for our p120ctn constructs by comparing Myc-staining in MDCK cells infected with an empty vector and those stably expressing wild-type Myc-p120ctn (Fig 14B). In the latter cell line, we observed a significant

concentration of the immunofluorescence signal at the cell membrane, where we expect our p120ctn construct to localize, indicating the Myc antibody is efficient and specific for the detection of our p120ctn constructs.

Having the validated Myc antibody on hand, we proceeded to stain for Myc-tagged p120ctn in our three cell lines with similar levels of expression of each p120ctn construct (Fig 14C). In each cell line, we observed an enrichment of Myc-tagged p120ctn at the cell membrane. This suggests that under standard growing conditions, phosphorylation at S320 does not regulate p120ctn localization in epithelial cells.

SECTION 3: Designing assays to examine AJ function

To examine effects of p120^{ctn} phosphorylation on AJ function in more dynamic cell contexts, such as during cell migration or AJ disassembly, we designed two assays to evaluate two major properties of AJ function: the strength of AJ adhesion, and AJ turnover.

To assess the strength of adhesion at actin-dependent cell-cell contacts, previous studies employed a dispase-based monolayer fragmentation assay [151]. Since AJs are a major structure in cell-cell adhesion, and provides epithelial monolayers with resistance to shear force, this assay uses dispase to isolate confluent monolayers, and then evaluates an epithelial monolayer's resistance to tearing and shear force. The resulting % fragmentation of the monolayer correlates to the strength AJ-mediated adhesion. More recently, investigators successfully employed this method to quantify and compare the relative adhesion strength of different E-cadherin based cell-cell adhesion structures [152]. Given the specificity of this assay for E-cadherin based cell-cell contacts, we chose to design a fragmentation assay in this project.

Next, to study epithelial monolayer integrity, the transepithelial resistance (TER) assay is commonly employed. TER evaluates changes in epithelial integrity space by measuring the electron flow through the paracellular space between adjacent cells. As illustrated in the introduction section, cell-cell adhesion structures contribute to the integrity of epithelial sheets by closing the paracellular space between adjacent cells. Studies into regulation of cell-cell adhesion have used TER to measure the rate of assembly and disassembly of TJ and AJ, correlating with changes in epithelial barrier function [153]. More recently, a new impedance-based method using the xCELLigence RTCA system was shown to generate the same quantitative results as a TER assay when performed in concurrent experiments studying cell-cell adhesion structure [154]. With the same system available to us, we set out to design an impedance-based TER assay using the xCELLigence RTCA system.

Having selected the two assays, our final aim is to apply them to study how Ras/MAPK signalling and S320 phosphorylation on p120^{ctn} specifically affects AJ function in relevant epithelial cell lines.

MCF-10A cells form stable, confluent monolayers in culture

A crucial requirement before beginning to validate and optimize the assays is the need to obtain stable, epithelial monolayers to work with. This begins with selecting an epithelial cell line that exhibits contact inhibition, and can be sustained for a long period.

Since the xCELLigence RTCA system can generate real time measurements of cell growth, we leveraged its capabilities to monitor the growth pattern of different cell lines in culture. We seeded three different densities of MCF-10A, Caco-2, A431 and IEC-6 cells in quadruplicate into wells in a 96-well E-plate for the xCELLigence system. Then, generated growth curves by taking Cell Index (CI) measurements every 15 minutes for 72 hours as the cells grew towards confluency. At the same time, the same proportion of cells were seeded into clear bottomed, 96-well plates to confirm cell confluency by microscopy.

From the resulting growth curves generated for each cell line, we see that the MCF-10A curve forms a stable plateau after 40 hours, indicating confluent growth (Fig 15A). In comparison, the data from A431 cells show an unexpected drop in CI, perhaps indicating cell death, or detachment, then resuming growth (Fig 15B). Similarly, Caco-2 cells appear to reach confluency, but then begins to rapidly decrease in impedance, possibly also due to cell detachment (Fig 15C). Finally, the IEC-6 cells appear to grow slowly, and after 72 hours is still growing towards confluency (Fig 15D).

Through microscopy, we were able to visualize the quality of cell growth in the wells, paralleling that of the 96-well E-plates (Fig 15E-H). IEC-6, A431, and Caco-2 cells all form incomplete monolayers after 72 hours of growth, with visible spaces between cells (indicated by arrows). However, in 72 hours, MCF-10A cells have grown to a confluent stable monolayer, further supporting the results of the xCELLigence measurements.

From these results, we can conclude that MCF-10A cells, when seeded at the selected density can produce a confluent monolayer after 40-50 hours of growth. Additionally, the CI curves indicate that confluency can be maintained in these cells for approximately 24 hours or more without any significant changes in cell adhesion or cell death.

p120ctn knockdown induces complete monolayer fragmentation

To assess if the monolayer fragmentation assay is sensitive to the presence of AJs, we stably knocked down p120ctn in MCF-10A cells by shRNA to induce an overall loss of AJs in

the epithelial monolayer. By microscopy, the loss of AJs is reflected in the sh984 and sh988 cell lines taking on a more mesenchymal appearance, and the sh987 cells appear more “rounded” in shape indicative of loss of adhesion with adjacent cells (Fig 16A). To verify that the changes in cell morphology are not due to off-target effects, the knockdown of p120ctn was confirmed by western blot, with sh987 and sh988 cells showing undetectable levels of p120ctn. (Fig 16B).

Each respective cell line was seeded onto 60mm plates and allowed to grow to >90% confluency before being treated with dispase and then subjected to disruption (Fig 16C). In the cell lines where p120ctn was efficiently knocked down (at almost undetectable levels), we observed almost complete fragmentation of the monolayer in response to disruption, compared to the non-target control cells that retained high levels of cell-cell adhesion. This suggests that AJs contributes almost entirely to monolayer stability, and that changes in AJ stability will be reflected in fragmentation of epithelial sheets.

Calcium depletion increases monolayer fragmentation and triggers AJ disassembly

AJ are calcium-dependent adhesive structures, and require extracellular calcium to maintain adhesion and prevent AJ turnover at the membrane. As another way of validating the both assays, we evaluated if they could detect AJ disassembly and adhesion loss over time in response to calcium depletion.

To test the monolayer fragmentation assay, we first released the monolayers by dispase, since this enzyme requires Ca^{2+} for its activity, and would be inhibited by EGTA addition. Afterwards, the monolayers were incubated in complete media, and 4mM of EGTA was added at 30-, 15- and 5-minute time points to deplete all Ca^{2+} from the media. Following incubation, the monolayers were disrupted using a pipette per the monolayer fragmentation protocol, and the final % fragmentation was calculated against a total cell count obtained by counting trypsinized cells (Fig 17A). We obtained a gradual increase in monolayer fragmentation over time after EGTA addition, indicating that calcium depletion decreases monolayer integrity.

It is well known that E-cadherin degradation and recycling occurs because of AJ destabilization and internalization, so to verify that the increase in fragmentation that we observe is specifically due to AJ disruption and not cadherin degradation, we analyzed the levels of E-cadherin and p120ctn in MCF-10A cells treated with 4mM of EGTA (Fig 17C). After 30

minutes of EGTA treatment, we see that E-cadherin levels remain stable in MCF-10A cells, while some degradation appears to occur after one hour.

Therefore, taken together, we conclude that the observed increase in fragmentation of monolayers within 30 minutes of EGTA treatment is specific to disassembly and loss of the AJ adhesion structure, and not due to E-cadherin degradation.

To confirm the specificity of the xCELLigence RTCA system to monitor changes in AJs, we set out to determine if induction of AJ disassembly generates a distinctive decrease in Cell Index (CI) detected by the xCELLigence system. Since the CI is correlated with monolayer impedance, we anticipate that AJ disassembly will rapidly increase the paracellular space between cells, decreasing impedance, and will be reflected as a rapid decrease in CI.

As previously stated in the introduction, AJ adhesion and stability require sufficient concentrations of extracellular Ca^{2+} , more specifically greater than 1mM of $[\text{Ca}^{2+}]$. To validate the xCELLigence system, we added different concentrations of EGTA to decrease $[\text{Ca}^{2+}]$ to trigger AJ disassembly. Since EGTA chelates $[\text{Ca}^{2+}]$ at a 1:1 stoichiometric ratio, we could calculate the amount of EGTA required to reduce $[\text{Ca}^{2+}]$ to below the critical concentration of 1.0 mM. Knowing that our growth medium contains 1.8 mM of Ca^{2+} , we induced AJ disassembly by adding EGTA to a final concentration of 1 mM and 2 mM. As a control, we added EGTA to a “sub-optimal” concentration of 0.5 mM (Fig 17B). As anticipated, we observed a significant and rapid reduction in the normalized CI of MCF-10A monolayers treated with 1 mM or 2 mM of EGTA. In contrast, addition of 0.5 mM of EGTA did not produce a significant difference in CI from untreated cells. These results suggest that changes in the CI of confluent monolayers are specific to changes in cell-cell adhesion, and can reflect AJ disassembly. Interestingly, both 1 mM and 2 mM of EGTA produced the same decrease in CI, indicating that AJs disassembly occurs as an “all-or-none” response when $[\text{Ca}^{2+}]$ drops below the critical level of 1.0 mM, rather than occurring in a dose-dependent manner.

Site-specific mutation of S320 on p120ctn produce minor effects on monolayer adhesion

Once the assays for cell-cell adhesion were validated, I was able to perform a preliminary experiment to investigate if site-specific phosphorylation at S320 on p120ctn affected cell-cell adhesion. First, we generated stable cell lines in A431 cells expressing wild-type or phospho-

mutants of p120ctn. Since p120ctn levels have been shown to impact cell-cell adhesion, we first verified that the cells expressed equal levels of exogenous p120ctn constructs by immunoblotting using a Myc-specific antibody (Fig 18A).

After confirming that the cells express equal levels of exogenous p120ctn, we proceeded to seed each cell line in 60mm plates to obtain confluent monolayers. In hopes to perceive larger differences between the S320A and S320D isoforms, we starved the cells for 16-hours before releasing the monolayers by dispase and performing a fragmentation assay. From the resulting data, we see that overexpression of all p120ctn constructs appear to promote cell-cell adhesion (Fig 18B). The S/D mutant produced significantly lower percentage fragmentation compared to empty vector cells and wild-type cells. In contrast, the S/A mutant failed to produce significant stabilization of cell-cell adhesion compared to empty vector cells. While we did not observe major changes in the degree of fragmentation, nonetheless these preliminary results indicate that phosphorylation solely at this site perhaps produces minor effects in cell-cell adhesion.

CHAPTER 3: FIGURES

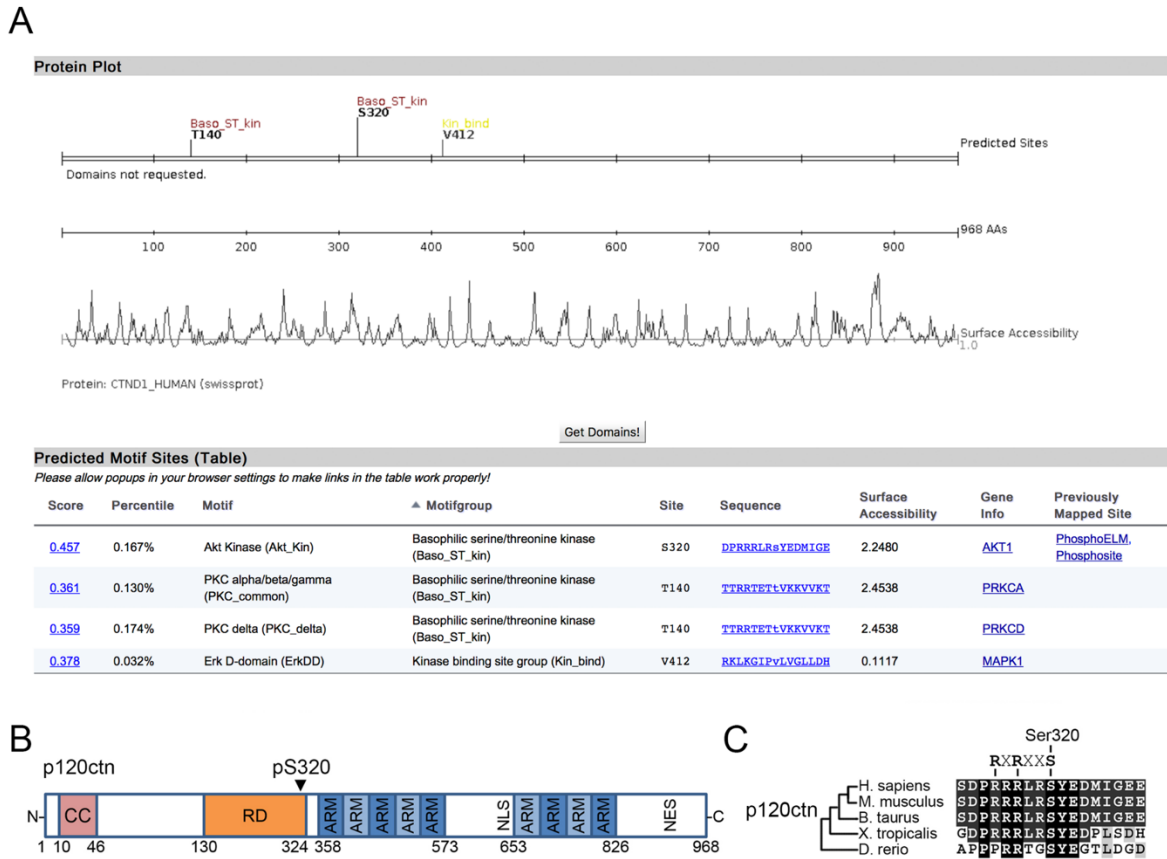


Figure 6. Analysis of S320 as a putative phosphorylation site on p120ctn.

Computational analysis of the protein sequence of p120ctn. (A) Using *Scansite3* to search for consensus motifs present in p120ctn with high stringency parameters identifies S320 as a putative phosphorylation site on p120ctn. (B) Schematic of p120ctn indicating the location of S320. Known domains of p120ctn are also illustrated. (C) Alignment of p120ctn amino acid sequences from different vertebrate species reveals evolutionary conservation of S320 and the AGC-kinase consensus motif.

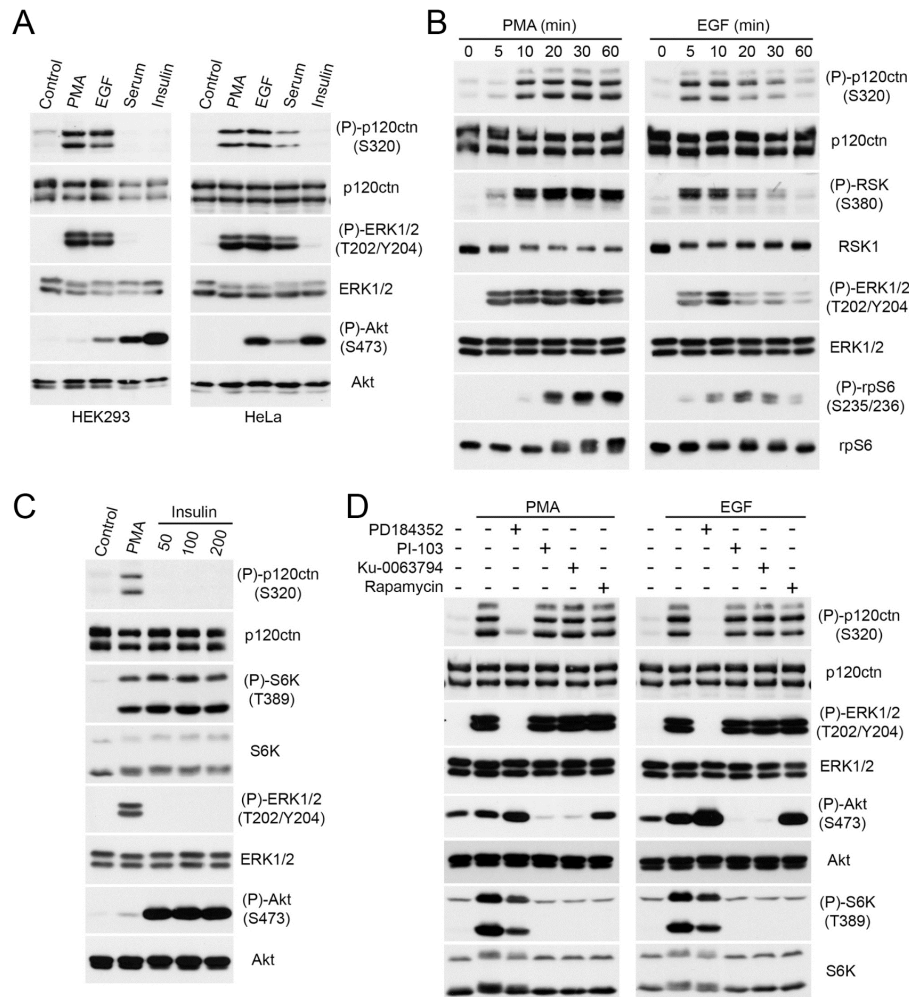


Figure 7. Activation of the Ras/MAPK pathway induces phosphorylation of p120ctn on S320.

(A) HEK293 and HeLa cells were serum starved overnight before being stimulated for 30 min with PMA (100ng/mL), FBS (10%), Insulin (50ng/mL) or 10 min with EGF (25ng/mL). p120ctn phosphorylation on S320 was assessed by immunoblotting total cell lysates using a phospho-specific antibody. (B) HEK293 cells were serum starved then stimulated with PMA and EGF for up to 60 minutes. Activation of Ras/MAPK signaling and p120ctn phosphorylation was assessed at each time point using the appropriate phospho-specific antibodies. (C) As in panel A, but cells were stimulated with increasing concentrations of Insulin (50, 100, 200ng/mL) for 30 minutes. (D) HEK293 cells were serum starved overnight. Then, cells were pre-treated with PD184352 (10 μ M), PI-103 (10 μ M), Ku-0063794 (5 μ M) or Rapamycin (25 μ M) for 30 min before stimulation with PMA (100ng/mL) for 30 min or EGF (25ng/mL) for 10 min. p120ctn phosphorylation on S320 was assessed by immunoblotting total cell lysates using a phospho-specific antibody.

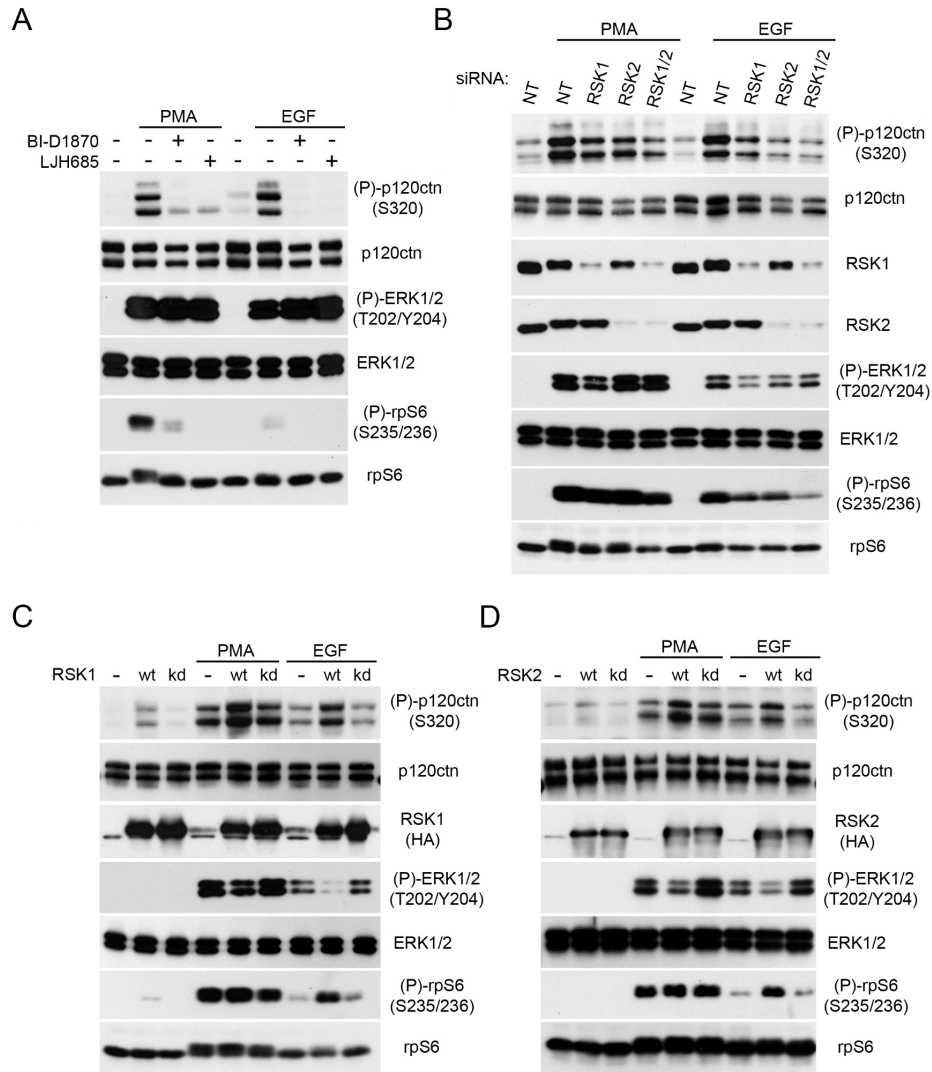


Figure 8. Ras/MAPK phosphorylation of p120ctn on S320 is RSK-dependent.

(A) HEK293 cells were serum starved overnight. Then, cells were pre-treated with BI-D1870 (10 μ M) or LJM685 (10 μ M) for 30 min before stimulation with PMA (100ng/mL) for 30 min or EGF (25ng/mL) for 10 min. p120ctn phosphorylation on S320 was assessed by immunoblotting total cell lysates using a phospho-specific antibody. RSK inhibition is assessed by immunoblotting for phosphorylation of rpS6 (S235/236). (B) HEK293 cells were transfected with either a scrambled siRNA or siRNAs targeting RSK1 and/or RSK2. Cells were serum starved overnight, then stimulated with PMA (100ng/mL) for 30 min or EGF (25ng/mL) for 10 min. p120ctn phosphorylation was assessed as in panel A. RSK knockdown efficiency was assessed by immunoblotting using antibodies specific for each isoform. (C and D) HEK293 cells were transfected with wild-type or kinase-deficient RSK1 (in C) or RSK2 (in D). Cells were then starved overnight before stimulating with PMA (100ng/mL) or EGF (25ng/mL). Successful RSK transfection was confirmed by immunoblotting with a HA-antibody. p120ctn phosphorylation was assessed as in panel A.

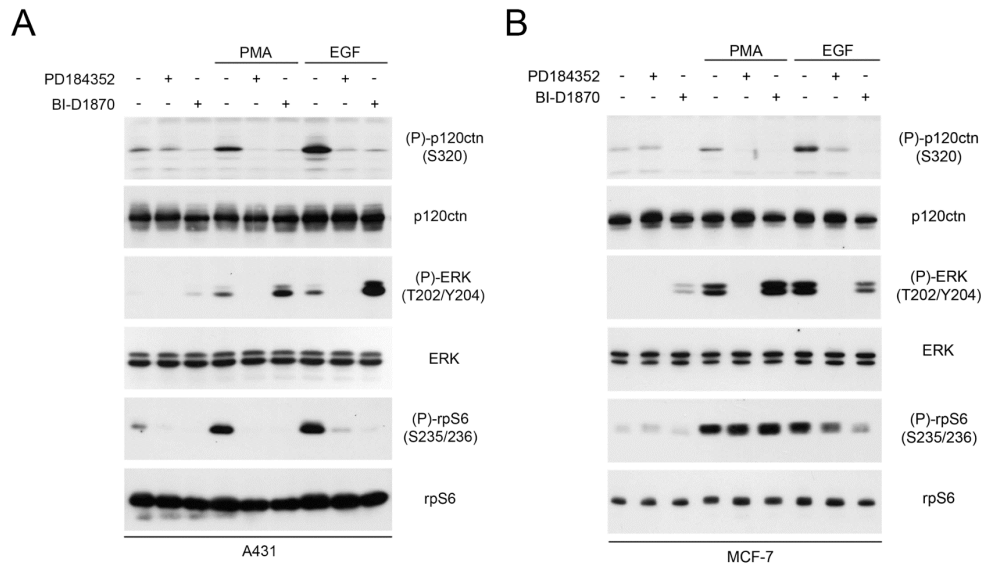


Figure 9. Activation of the Ras/MAPK pathway induces RSK-dependent p120ctn phosphorylation on S320 in epithelial cells.

(A) A431 cells and (B) MCF-7 cells were serum starved overnight. Then, the cells were pre-treated with PD184352 (10 μ M) or BI-D1870 (10 μ M) for 30 min before stimulation with PMA (100ng/mL) for 30 min or EGF (25ng/mL) for 10 min. p120ctn phosphorylation on S320 was assessed by immunoblotting total cell lysates using a phospho-specific antibody. Effective MEK and RSK inhibition is evaluated by immunoblotting for phosphorylated ERK and rpS6 using appropriate antibodies.

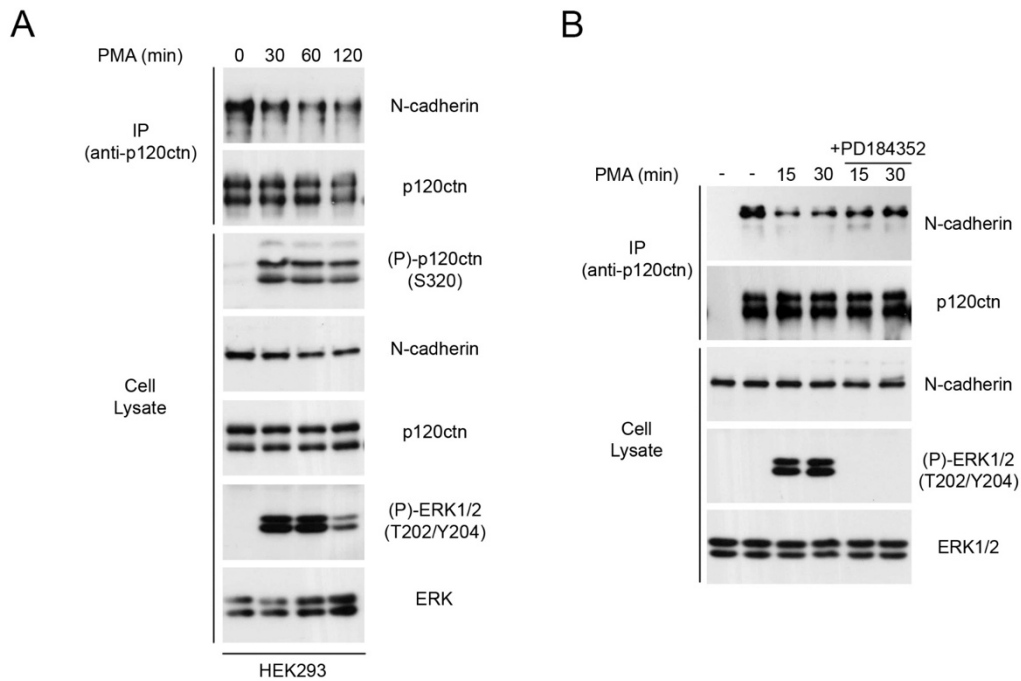


Figure 10. Ras/MAPK activity reduces p120ctn binding to N-cadherin.

(A) HEK293 cells were serum starved overnight before being stimulated with PMA (100ng/mL) over a time course of up to two hours. N-cadherin associated with endogenous p120ctn immunoprecipitates were assayed by immunoblotting. Total cellular levels of p120ctn and N-cadherin were also assessed by immunoblotting the cell lysate. (B) HEK293 cells were serum starved overnight then pre-treated with PD184352 (10 μ M) for 30 min before stimulation by PMA (100ng/mL) for 15 or 30 min. N-cadherin and p120ctn levels were assessed as in panel A.

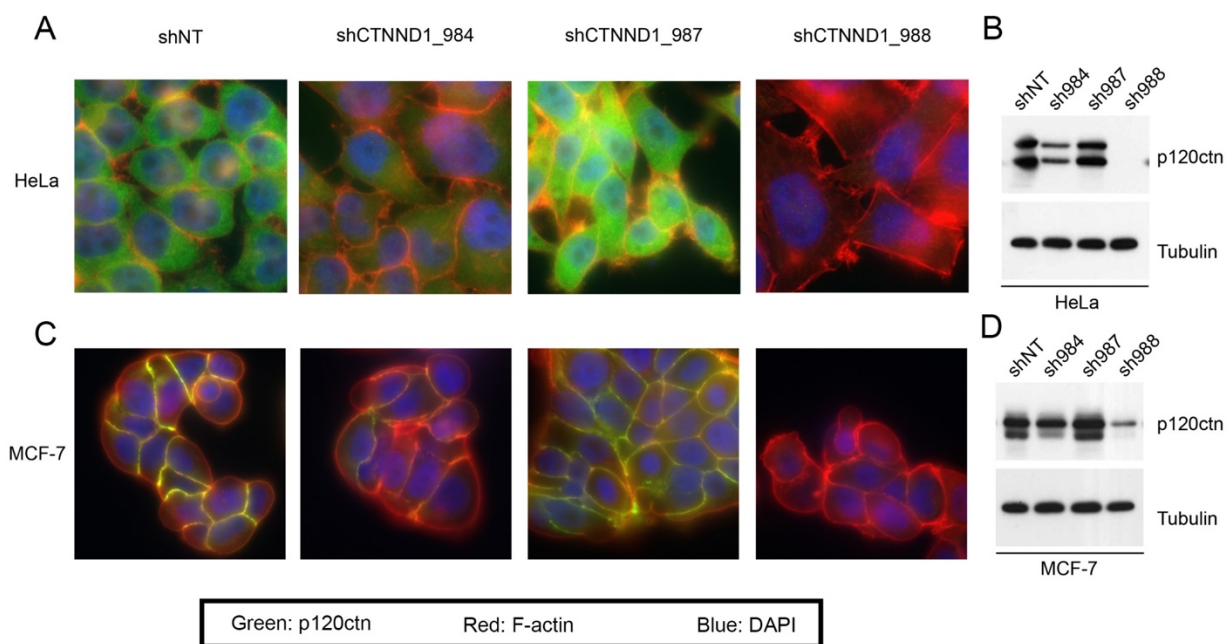


Figure 11. Validation of the p120ctn antibody for use in IF.

HeLa and MCF-7 cells were infected with non-target (NT) shRNA or shRNA specific to p120ctn (sh984, sh987 and sh988) to generate stable knockdown of endogenous p120ctn. (A) HeLa and (C) MCF-7 cells were grown on glass coverslips before being fixed and stained using phalloidin to visualize the actin cytoskeleton, anti-p120ctn to visualize endogenous p120ctn, and DAPI to visualize nuclei. Only merge images are shown. Successful knockdown of p120ctn in (B) HeLa and (D) MCF-7 cells were assessed by immunoblotting using anti-p120ctn.

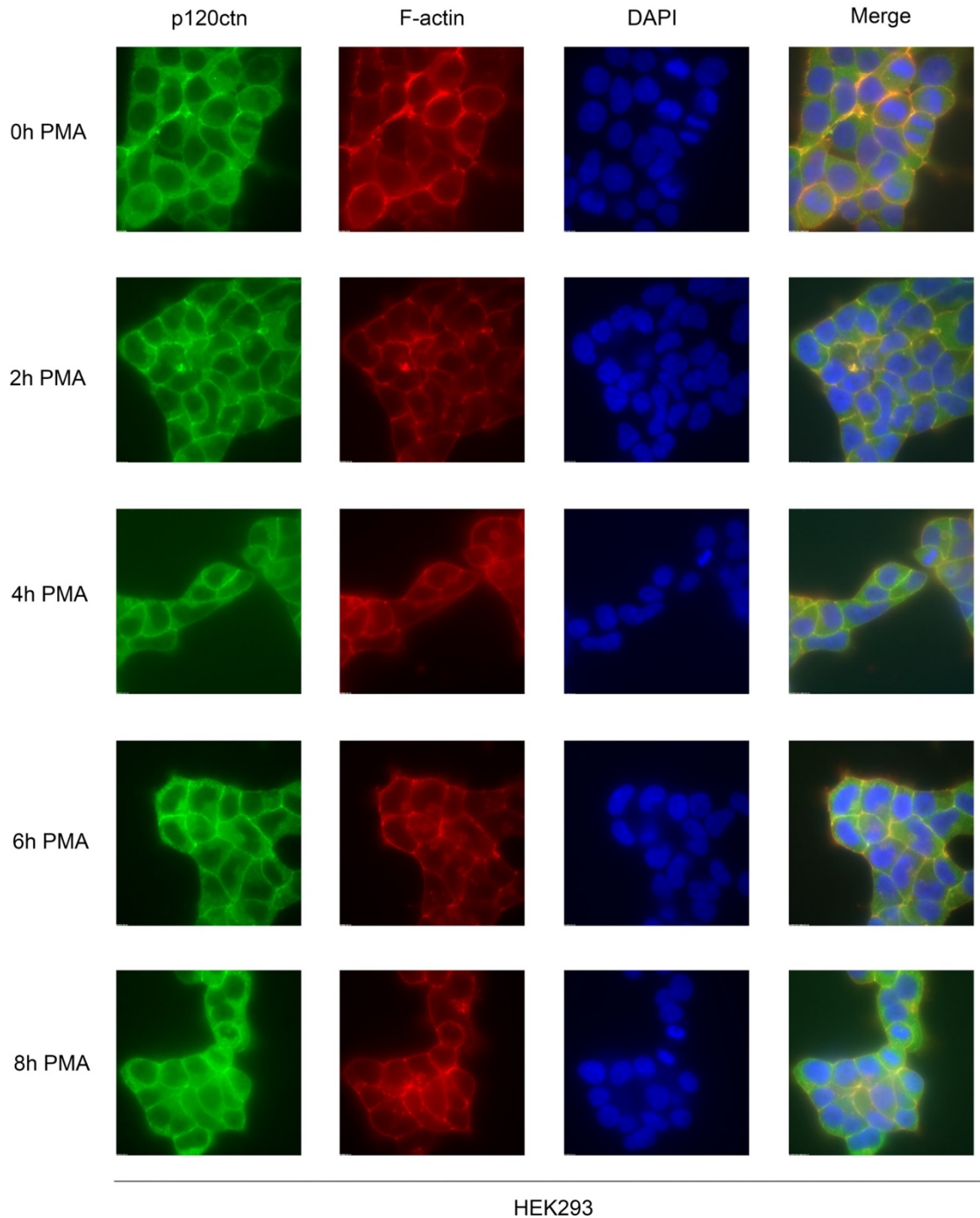
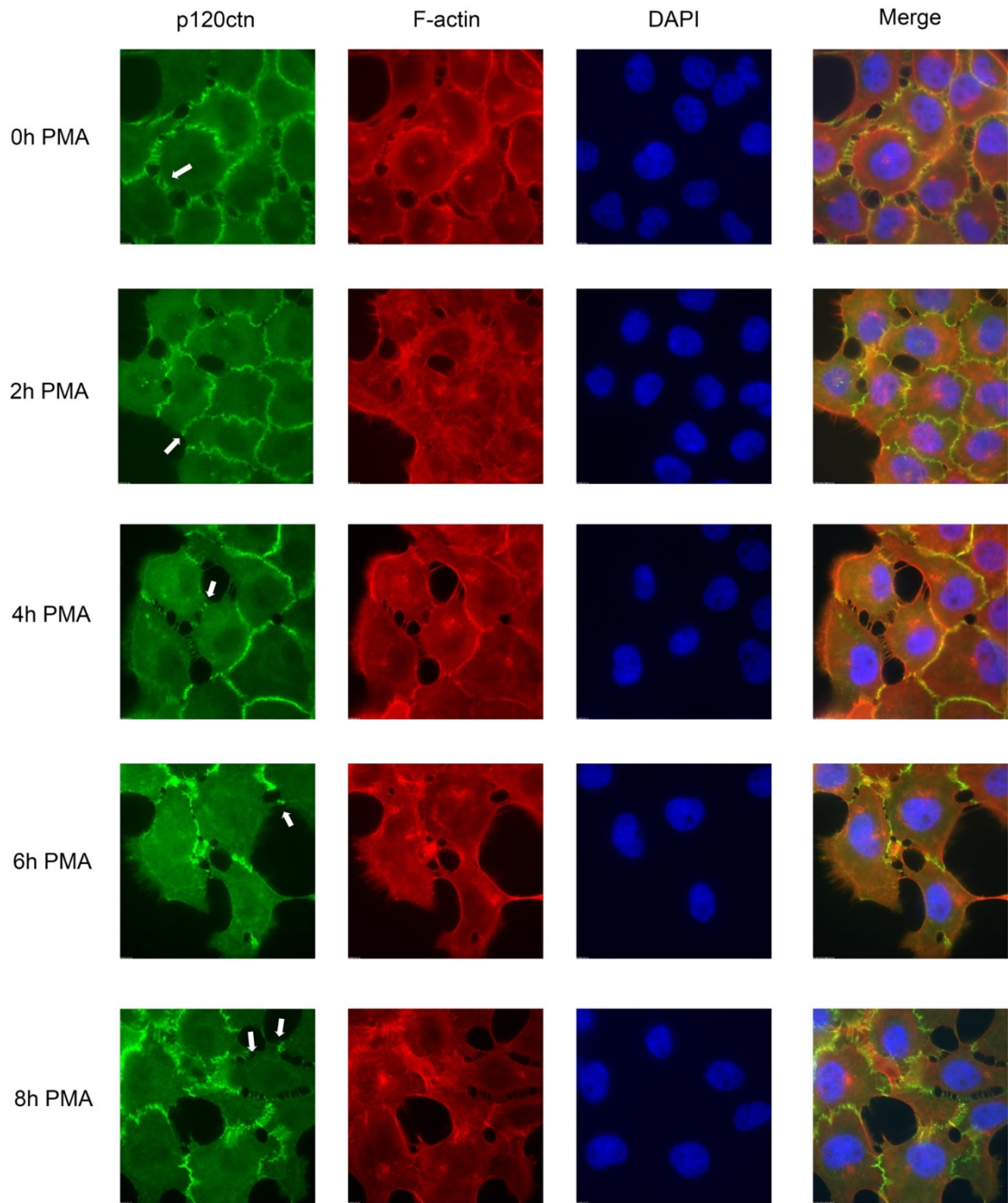


Figure 12. p120ctn remains localized at cell-cell contacts in HEK 293 cells in response to PMA stimulation

HEK293 cells seeded onto glass coverslips were serum starved overnight before stimulating with PMA (100ng/mL) over an 8 hour time-course. Cells were then fixed and stained with anti-p120ctn to monitor endogenous p120ctn localization, phalloidin to visualize the actin cytoskeleton, and DAPI to visualize nuclei.



A431

Figure 13. p120ctn remains localized at cell-cell contacts in A431 cells in response to PMA stimulation

A431 cells were seeded onto glass coverslips and serum starved overnight before stimulating with PMA (100ng/mL) over an 8 hour time-course. Cells were then fixed and stained with anti-p120ctn to monitor endogenous p120ctn localization, phalloidin to visualize the actin cytoskeleton, and DAPI to visualize nuclei. Arrows are added to indicate and emphasize p120ctn localization at “punctate AJs”.

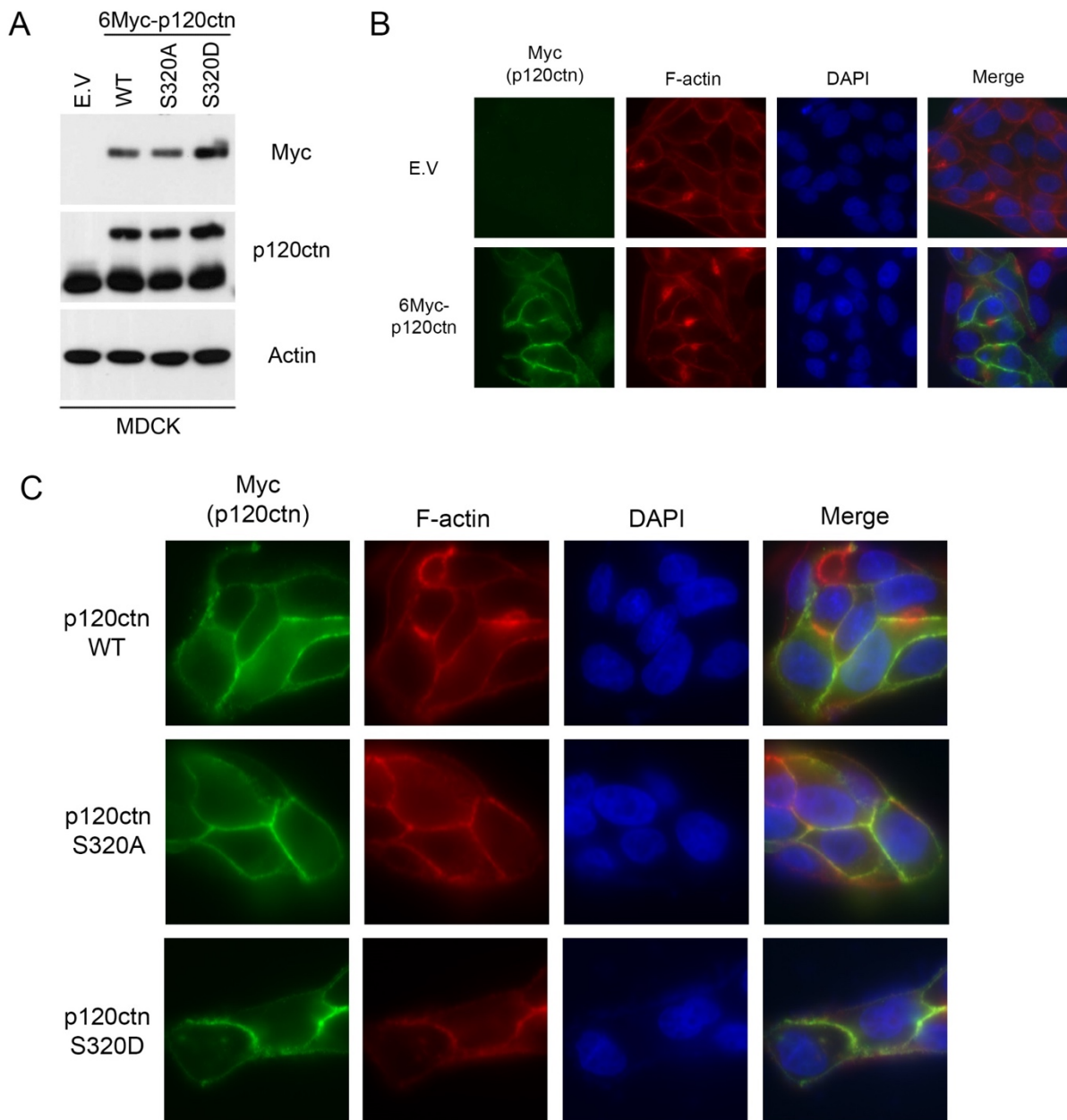


Figure 14. Myc-tagged p120ctn S320 mutants localizes to cell-cell contacts in stably expressing MDCK cells.

(A) We generated MDCK cells stably expressing an empty vector, p120ctn-wt, p120ctn-S320A or p120ctn-S320D mutants. Expression levels of exogenous p120ctn is assessed using both anti-Myc and anti-p120ctn antibodies. (B) Confocal images of fixed MDCK cells expressing an empty vector, or myc-tagged p120ctn-wt. Cells were stained with anti-Myc to visualize p120ctn, phalloidin to visualize the actin cytoskeleton, and DAPI to visualize nuclei. (C) Confocal images of MDCK cells expressing wt, S320A or S320D mutants of p120ctn. Cells were stained same as in panel B.

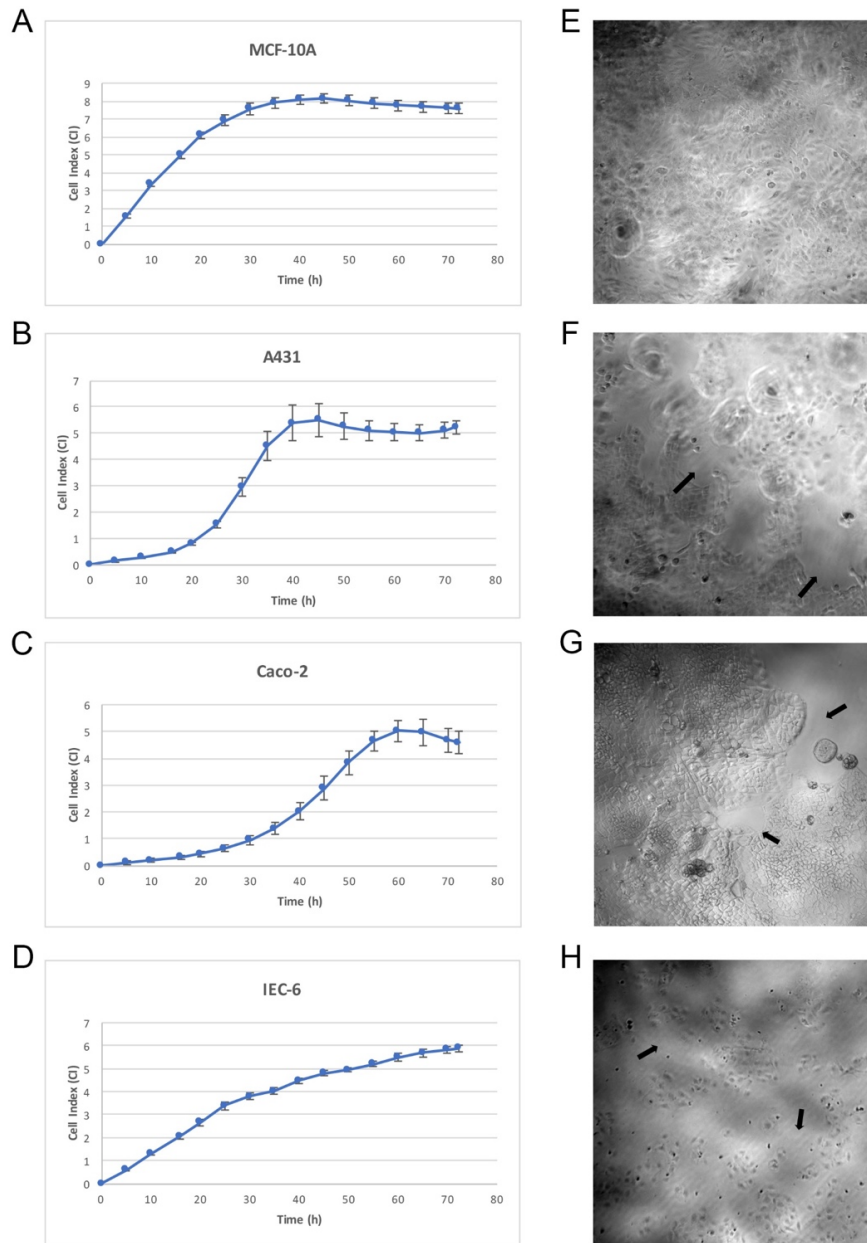


Figure 15. MCF-10A cells generate confluent monolayers in comparison to Caco-2, A431, and IEC6 cells.

(A) MCF-10A, (B) A431, (C) Caco-2 and (D) IEC-6 cells were seeded in quadruplicates into 96-well E-plates and left to grow for 72 hours at 37°C, 5% CO₂, in the xCELLigence RTCA system. (ACEA Biosciences) CI measurements were taken every 15 min for 72 hours to monitor cell growth. At the same time, (E) MCF-10A, (F) A431, (G) Caco-2 and (H) IEC-6 cells were seeded into clear-bottom 96-well plates and incubated in the same incubator as the xCELLigence cells for 72 hours. Brightfield photos were taken after 72 hours of growth to evaluate cell growth and confluency in each plate. Arrows show spaces left between monolayers and indicate lack of confluency.

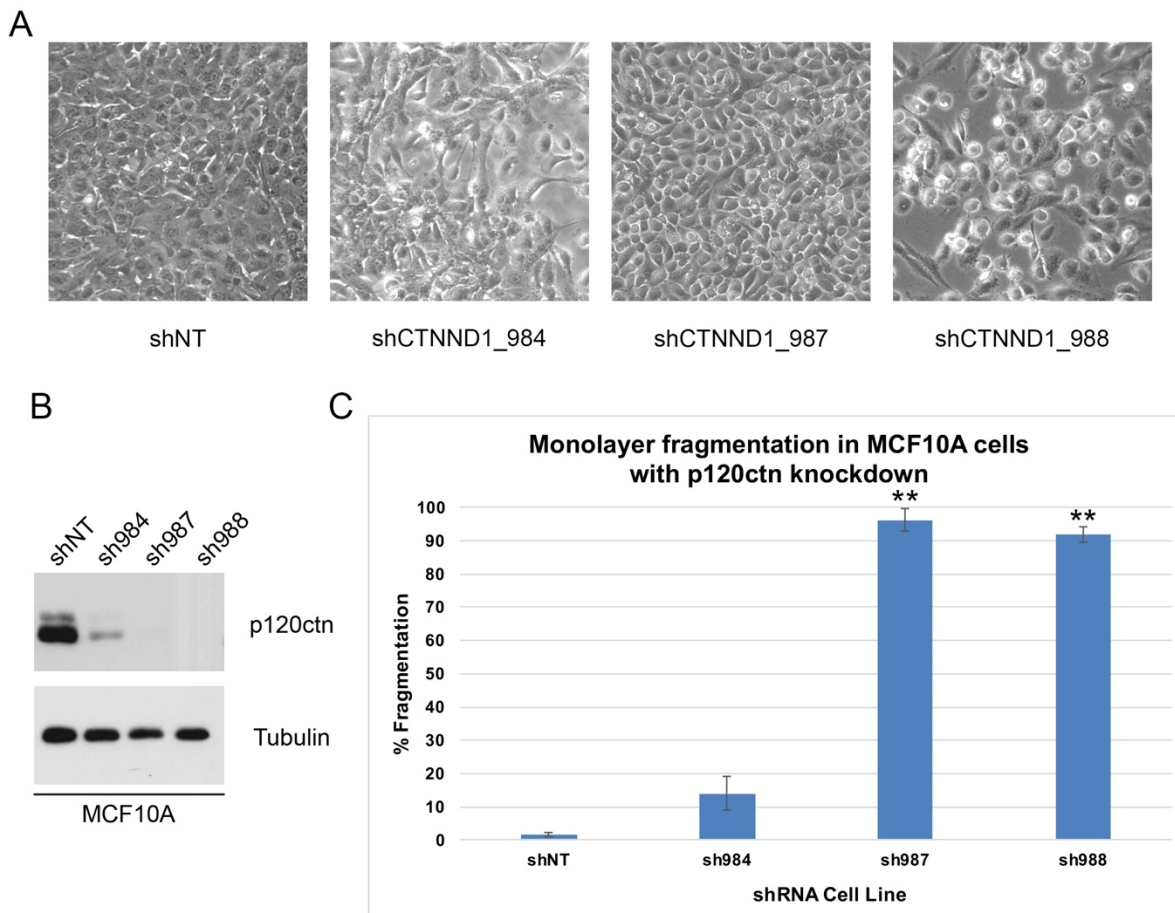


Figure 16. p120ctn knockdown promotes monolayer fragmentation.

MCF-10A cells were infected with either non-target (NT) shRNA or three different shRNAs targeting p120ctn (sh984, sh987, sh988) to generate cells with a stable knockdown of endogenous p120ctn. Successful p120ctn knockdown is evaluated by (A) Brightfield microscopy illustrating changes in cell morphology and (B) immunoblot using anti-p120ctn. (C) Each MCF-10A cell line was seeded into 60mm plates in quadruplicate and grown to confluency. At confluency, the monolayers from three plates were released by incubation with dispase, subject to disruption by Pipetman, and single cells were counted. Resulting percent fragmentation was calculated against a total cell count obtained by trypsinizing one untreated plate from each condition. Values are displayed as mean + SEM. * $p < 0.05$, ** $p = 0.001-0.05$ compared to control (shNT); two-tailed Student's t-test.

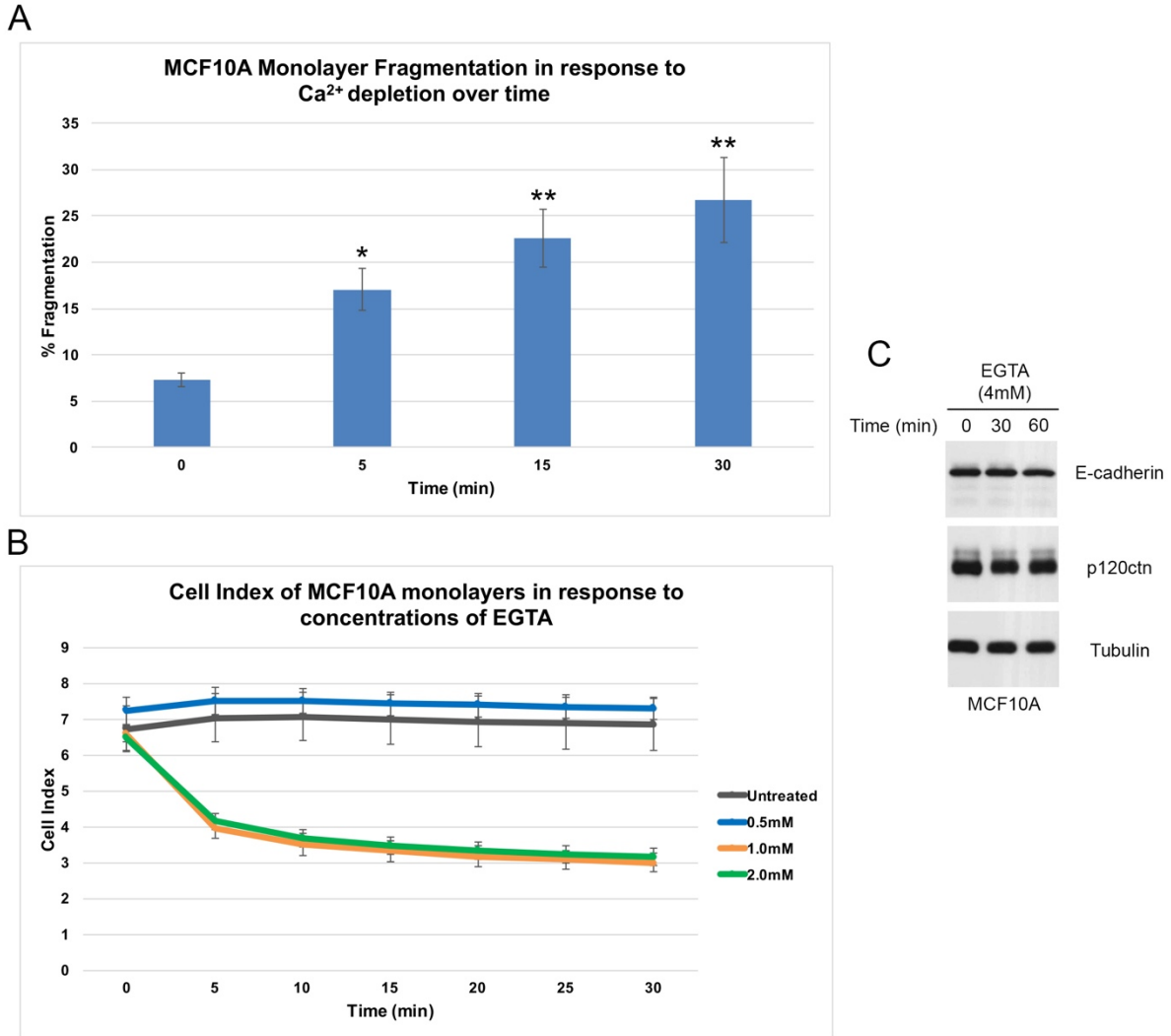


Figure 17. Calcium depletion affects monolayer fragmentation and cell index.

(A) MCF-10A cells were grown to confluency in 60mm plates then monolayers were released by incubation in 2mL of dispase (2.4U/mL). Monolayers were then incubated in media treated with EGTA (4mM) for 5, 15 or 30 min before being subject to disruption with a Pipetman. After disruption, single cells were counted and percentage fragmentation was calculated against total cell number obtained from a confluent plate treated with trypsin. Values are displayed as mean + SEM. * $p < 0.05$, ** $p = 0.001-0.05$ versus control (T=0min); two-tailed Student's t-test. (B) MCF-10A cells were seeded in quadruplicate into 96-well E-plates and allowed to grow for 48 hours to confluency. After 48 hours, EGTA was added at 0.5, 1 or 2mM concentration and CI was recorded every 15 sec for 30 min by the xCELLigence RTCA system. (C) MCF-10A cells were grown to confluency in 60mm plates, then treated with EGTA (4mM) for 30 or 60 min. Cell lysates were immunoblotted for E-cadherin and p120ctn using specific antibodies.

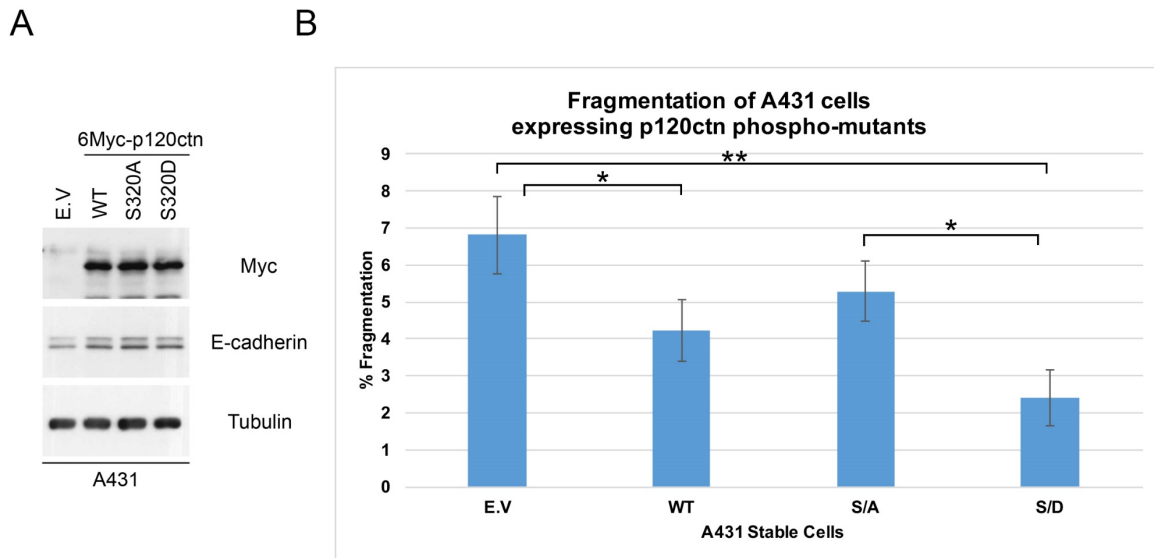


Figure 18. Stable expression of p120ctn phosphomutants in A431 cells affects monolayer adhesion.

(A) Expression levels of p120ctn constructs and E-cadherin in A431 cells were evaluated by immunoblotting using the antibodies indicated. (B) A431 cells stably expressing empty vector (E.V), p120ctn wild-type (WT) and phosphomutants (S/A and S/D) were grown to confluency in 60mm plates then monolayers were released by incubation in 2mL of dispase (2.4U/mL) before being subject to disruption with a Pipetman. After disruption, single cells were counted and percentage fragmentation was calculated against total cell number obtained from a confluent plate treated with trypsin. Values are displayed as mean + SEM. * $p < 0.05$, ** $p = 0.001-0.05$; two-tailed Student's t-test.

DISCUSSION

General Findings

Stimulation of Ras/MAPK signalling has been shown to induce pro-motile and pro-invasive properties in epithelial cells. Located downstream in the Ras/MAPK pathway, RSK is identified as an important effector in mediating Ras-induced cell motility by regulating the transcription of several potent pro-invasive genes [31]. More recently, it was also shown that RSK promotes cell migration by specifically weakening the strength of cell-cell contacts and promoting cell scattering through a yet unknown mechanism [155]. Our identification of a putative phosphorylation site regulated by RSK activity on p120ctn, a major component of AJs, in a phosphoproteomic screen suggests a novel, post-translational mechanism by which RSK can regulate cell-cell adhesion in epithelial cells [22]. We hypothesized that RSK-dependent phosphorylation at S320 on p120ctn regulates its function and impacts AJ stability and overall cell-cell adhesion.

By performing an alignment across vertebrate species, we show that S320 on p120ctn is an evolutionarily conserved residue, and located in a AGC-kinase consensus sequence. While phosphorylation of p120ctn at this residue has been observed in many high-throughput proteomic studies listed on the PhosphoSitePlus database, no studies have yet confirmed the phosphorylation of this site using low-throughput methods [156]. In our study, by using a phospho-specific antibody raised against S320 on p120ctn, we verified S320 as a bona fide, novel phosphorylation site on p120ctn regulated by Ras/MAPK signalling. Furthermore, by using RSK-specific pharmacological inhibitors, our experiments show phosphorylation of S320 is highly dependent on RSK activity, and suggests with a high degree of confidence that RSK is the AGC-kinase that specifically regulates phosphorylation of this site.

Functionally, we provide evidence that Ras/MAPK activation rapidly decreases p120ctn binding to N-cadherin, without altering its localization away from the membrane. This demonstrates a new mechanism by which Ras/MAPK signalling regulates cell-cell adhesion by affecting the stability of cadherins at AJs. Additionally, it suggests that Ras/MAPK signalling can change p120ctn binding partners at the membrane, perhaps influencing local RhoGTPase activity at the cell membrane to promote cell spreading in coordination with loss in cell-cell adhesion.

Finally, we designed and validated two assays to assess the effects of Ras/MAPK activity and S320 phosphorylated p120ctn on AJ turnover and cell-cell adhesion. By employing

these assays, our preliminary results suggest that S320 phosphorylation produces minor effects in cell-cell adhesion in A431 stable cell lines.

Regulation of S320 phosphorylation on p120ctn

Our discovery of S320 as a bona fide novel phosphorylation site on p120ctn introduces a new element into the already complex landscape of p120ctn phosphorylation. In the last decade, studies into p120ctn phosphorylation have mainly focused on understanding and elucidating the regulation and function of the original 8Y and 8S/T sites identified by the Reynolds lab [133]. Since then, the only new phosphorylation sites identified are located at the very tip of the N-terminal domain of p120ctn (S8, S11, S15) and are only found in the longest isoform [124]. In contrast, we find that S320 is phosphorylated on both major long and short isoforms in human cells, indicating this site plays a more general regulatory role on all p120ctn isoforms.

Our results challenge the previous conclusion that all S/T sites are constitutively phosphorylated in unstimulated cells, and are downregulated in response to growth factor signalling [69]. Instead, we show that S320 is rapidly and highly phosphorylated in a RSK-dependent manner upon activation of the Ras/MAPK pathway.

Compared to HEK293 and HeLa cells, stimulation of A431 and MCF-7 cells with EGF elicits higher levels of S320 phosphorylation compared to PMA treatment. Particularly in A431 cells, we observe greater phosphorylation of S320 by EGF even though our immunoblots show equal Ras/MAPK activation in the PMA treated cells. (Fig. 9) This suggests the presence of other signals converging on p120ctn that influences Ras/MAPK mediated phosphorylation of S320.

One potential explanation of this disparity in S320 phosphorylation is the inhibitory role of PKC signalling in epithelial cells. Many studies have implicated PKC as a major negative regulator of phosphorylation on S/T residues in p120ctn. Stimulation of vascular endothelial cells with VEGF promotes overall dephosphorylation of p120ctn on S/T residues by a PKC-dependent mechanism [129, 157]. More specific to our experiments, treating cells with phorbol esters such as PMA to stimulate of PKC activity induced dephosphorylation of p120ctn on residues S268, S288 and T310 [117]. The mechanism by which PKC activity dephosphorylates these residues remain unknown, but it suggests that it signals to an unidentified phosphatase

targeted to this region. Since S320 is also closely located within this region, it is highly likely that this residue is subject to the same regulation. Our results do not show levels of PKC activity in A431 cells, but our use of PMA would undoubtedly activate the same mechanism. Therefore, our results suggest the possibility that S320 is RSK phosphorylation site that is subject to negative regulation by a PKC-dependent mechanism. However, further experiments using inhibitors of PKC-activity would allow us to confirm the role that PKC signalling has on regulation of S320 phosphorylation on p120ctn. Additionally, performing these experiments in a wide variety of cell lines would allow us to explore whether this antagonistic mechanism is cell-type specific.

S320 phosphorylation on p120ctn function

Given its location in the “regulatory domain” of p120ctn, we expected phosphorylation on S320 of p120ctn to impact cell-cell adhesion by affecting its binding to its main interactor: cadherin. When we stably expressed p120ctn molecules with S320A and S320D mutations in A431 cells, our preliminary results show that overexpression of the phosphomimetic form of p120ctn promoted cell-cell adhesion, while the phospho-deficient mutant reduced cell-cell adhesion compared to wildtype p120ctn.

This was surprising because, as addressed in the introduction, phosphorylation of other sites in the regulatory domain near S320 has been shown to promote p120ctn dissociation from cadherins. For example, phosphorylation on T310 by GSK3, just ten residues upstream of S320 significantly decreases p120ctn binding to N-cadherin and promotes AJ disassembly during collective migration of mouse astrocytes and fibroblasts [52]. Further upstream, phosphorylation on S288 of p120ctn is detected in the nucleus, suggesting this PTM plays a role in p120ctn nuclear translocation [158]. However, in these examples, a distinct change in p120ctn localization is observed in response to phosphorylation of S288 or T310. In comparison, our IF results show both phosphomutants, and wt p120ctn remain localized to the membrane in MDCK cells. Results from a recent study suggested that p120ctn phosphorylation could induce conformational changes of the extracellular cadherin domains by an “inside-out” regulatory mechanism [118]. Since our phosphomutants appear to remain localized at the membrane, perhaps S320 regulates cell-cell adhesion in a similar manner rather than disrupting AJ stability. By performing a calcium-switch experiment to assess changes in monolayer impedance, we

could evaluate the rate of AJ turnover in these cells to confirm the prediction that S320 phosphorylation does not disrupt AJ stability.

Furthermore, studies evaluating the effects of individual site mutants of p120ctn on cell-cell adhesion showed that modulation of each site does not produce large changes in the morphology of p120ctn-deficient A431 cells [117]. In contrast, mass mutations on several sites in the regulatory domain produced striking effects on cell-cell adhesion in A431 cells expressing phospho-deficient (6S/T>A: S252A, S268A, S288A, T310A, S312A, and T916A) and phospho-mimetic (4S/T>E: S268E, S288E, T310E, and S312E) p120ctn constructs [118]. These results illustrate the impact the collective phosphorylation status of p120ctn can have on the final cellular response. In the scope of our experiments, the phosphorylation status of other sites on p120ctn are undetermined. Therefore, it is possible that the effects of p120ctn phosphorylation on S320 are masked by modifications on other sites. To resolve this, future experiments using a p120ctn construct with 6S/T>A mutations on the other known phosphorylation sites would help us isolate the effects of S320 phosphorylation on cell-cell adhesion.

Also, unlike the studies performed by Petrova *et al.*, our own cell-cell adhesion experiments used A431 cells stably expressing p120ctn phosphomutants over high levels of endogenous p120ctn [118]. Due to the low stoichiometric ratio of exogenous to endogenous p120ctn, it is possible that endogenous p120ctn masks the effects of our phosphomutant p120ctn constructs. Thus, future experiments should be performed in p120ctn deficient cells, such as SW-48 – a naturally p120ctn-deficient cell line, to better isolate the effects of the phosphomutant constructs [82].

Recent studies have indicated that p120ctn can localize in distinct E-cadherin based complexes that are localized differently into apical and basolateral fractions [121]. In light of this discovery, our localization experiments appear to have been too narrow in scope to evaluate the apical-basal localization of p120ctn. While we show that p120ctn is localized at the membrane of stable cell lines, we failed to assess the apical/basolateral position of the mutants. Since p120ctn localization appears to be affected by its phosphorylation status, an interesting application of IF in future experiments will be to examine the apical-basal location of p120ctn phosphomutants in stable cell lines.

Ras/MAPK regulation of cell-cell adhesion through p120ctn

Since Ras/MAPK signalling is implicated in promoting cell migration, we expect that a MAPK-RSK-p120ctn mechanism would play a role in mediating this change in cellular function. Our biochemical studies confirm p120ctn as a substrate of the Ras/MAPK pathway, and we show that its phosphorylation occurs rapidly in response to RSK activation. Through co-immunoprecipitation experiments, we show that activation of this MAPK-RSK-p120ctn signalling axis in response to PMA stimulation produces rapid loss in N-cadherin binding to p120ctn in HEK293 cells. This binding between p120ctn and cadherin is crucial for AJ stabilization in all mammalian cells [68]. Therefore, our data illustrates a new mechanism by which Ras/MAPK activity can rapidly affect cell-cell adhesion by promoting AJ disassembly. Future experiments to assess p120ctn binding to cadherins, and AJ stability under conditions of RSK-specific inhibition or in RSK knockdown cells will allow us to confirm if this destabilizing mechanism is RSK-dependent.

In contrast, other known RSK substrates which regulate cell-cell adhesion operate by indirect mechanisms that are much slower acting. For example, EphA2 is a RTK that promotes cell migration through its own distinct set of ligands and signalling pathways, including reciprocal signalling with Akt [159, 160]. SH3P2 phosphorylation by RSK induces cytoskeletal rearrangement and lamellipodia formation by promoting myosin-1E activity at the plasma membrane [35].

The regulatory role of Ras/MAPK signalling at AJs is not a new discovery however, and has been implied in many studies. In cells with constitutive Ras/MAPK activity, rescue of cell-cell adhesion requires not only E-cadherin overexpression but also inhibition of ERK [161]. A more recent study also showed that ERK-inhibition prompts the translocation of E-cadherin bound β -cat to the membrane and areas of cell-cell contact [162]. Together, these studies suggest that ERK-inhibition promotes cadherin stability and AJ formation, possibly by restoring p120ctn binding to E-cadherin.

Conversely, ERK activity has been shown to destabilize the interaction between α - and β -cat indirectly through CK2 in A431 cells, promoting Wnt pathway activation by increasing β -cat/TCF [163]. In this study, significant dissociation between the two catenins were observed after 6 hours of EGF stimulation. In comparison, we show significant loss in N-cadherin binding

to p120ctn in under 15 minutes of PMA stimulation. This suggests perhaps that Ras/MAPK signalling to p120ctn acts to destabilize and primes AJs to be further disrupted by these other mechanisms.

Since it is likely that Ras/MAPK signalling to the p120ctn regulatory domain mediates this mechanism, future experiments using isoform 4 of p120ctn lacking the N-terminal regulatory domain would help us confirm this hypothesis. Perhaps isoform 4 will not be susceptible to MEK-induced AJ destabilization, and potentially abrogates MEK-induced β -cat/TCF activity by sequestering active β -cat to the cell membrane.

Usually, uncoupling of p120ctn from cadherin is associated with promoting its pleiotropic functions through other interactors. Our IF results in HEK293 cells show that even in response to PMA stimulation, p120ctn remains localized at the cell membrane. Many studies highlight the way interactors such as mucin 1, RhoA, p190RhoGAP, and PLEKHA7 compete with cadherin to bind p120ctn [103, 121, 164]. Given that some of these interactors, such as p190RhoGAP and RhoA are regulators of RhoGTPase dynamics, this “switch” would allow Ras/MAPK signalling to coordinate disassembly of cell-cell adhesion complexes with promoting localized cytoskeletal remodelling at the cell membrane to initiate cell spreading.

This is not impossible as MEK/ERK signalling has been shown to regulate Rac1 activity at the cell periphery [165]. MEK inhibition significantly reduced EGF-induced migration of cells, and inhibited extensive cortical actin reassembly at the leading edge of cells. Since RhoA competes with cadherin to bind p120ctn, it is possible that loss of cadherin binding allows p120ctn to inhibit RhoA instead in response to Ras/MAPK signalling. Alternatively, promoting p120ctn binding to p190RhoGAP would also inhibit RhoA activity at the plasma membrane.

In order to better understand changes in p120ctn binding partners, future GST-pulldown or IP-M/S experiments comparing proteins bound to p120ctn under different stimulation conditions could be performed. Additionally, by using site specific phosphomutants (ex. At S320), we could evaluate the effects of modifications at individual sites on p120ctn function.

CONCLUSIONS AND PERSPECTIVES

In this thesis, we present the discovery of a new phosphorylation site in the regulatory domain of p120ctn on S320 that is regulated by Ras/MAPK activity. Furthermore, even though we did not perform an *in vitro* kinase assay, we present compelling evidence that phosphorylation of p120ctn on S320 is specifically regulated by RSK kinases.

Through our functional experiments, we show that MAPK activity destabilizes AJ by rapidly disrupting the binding between p120ctn and N-cadherins. However, in preliminary results using site-specific mutants of S320 on p120ctn, we find phosphorylation at this site promotes cell-cell adhesion. Moving forward, it will be important to reproduce the functional experiments by expressing phosphomutants in p120ctn deficient cell lines to better isolate the effects of the site-specific mutation. Additionally, experiments using isoform 4 of p120ctn will allow us to better understand the function of the p120ctn regulatory domain in mediating Ras/MAPK-dependent AJ disassembly.

Collectively, the findings I have presented illustrate a new MAPK-RSK-p120ctn mechanism by which Ras/MAPK signalling can influence cell-cell adhesion and drive cell transformation by destabilizing cell junctions. Additionally, since dysregulation of p120ctn is greatly implicated in promoting cancer development and metastasis, this regulatory mechanism illustrates a new way by which Ras/MAPK signalling contributes to cancer progression.

Bibliography

1. Cargnello M, Roux PP: **Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases.** *Microbiol Mol Biol Rev* 2011, **75**(1):50-83.
2. Hoshino R, Kohno M: **Constitutive activation of the ERK-MAP kinase pathway in human tumors.** *Seikagaku* 2000, **72**(6):460-465.
3. Raman M, Chen W, Cobb MH: **Differential regulation and properties of MAPKs.** *Oncogene* 2007, **26**(22):3100-3112.
4. T Y, S N, DP W, FA B, BD C, JC P: **Cyclic AMP activates B-Raf and ERK in cyst epithelial cells from autosomal-dominant polycystic kidneys.** *Kidney Int* 2003, **63**:1983-1994.
5. Romeo Y, Zhang X, Roux PP: **Regulation and function of the RSK family of protein kinases.** *Biochem J* 2012, **441**(2):553-569.
6. Erikson E, Maller JL: **A protein kinase from Xenopus eggs specific for ribosomal protein S6.** *Proc Natl Acad Sci U S A* 1985, **82**(3):742-746.
7. Roux PP, Ballif BA, Anjum R, Gygi SP, Blenis J: **Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase.** *Proc Natl Acad Sci U S A* 2004, **101**(37):13489-13494.
8. Vik TA, Ryder JW: **Identification of serine 380 as the major site of autophosphorylation of Xenopus pp90rsk.** *Biochem Biophys Res Commun* 1997, **235**(2):398-402.
9. Gavin AC, Nebreda AR: **A MAP kinase docking site is required for phosphorylation and activation of p90(rsk)/MAPKAP kinase-1.** *Curr Biol* 1999, **9**(5):281-284.
10. Thomas GM, Rumbaugh GR, Harrar DB, Haganir RL: **Ribosomal S6 kinase 2 interacts with and phosphorylates PDZ domain-containing proteins and regulates AMPA receptor transmission.** *Proc Natl Acad Sci U S A* 2005.
11. Sutherland C, Alterio J, Campbell DG, Le Bourdelles B, Mallet J, Haavik J, Cohen P: **Phosphorylation and activation of human tyrosine hydroxylase in vitro by mitogen-activated protein (MAP) kinase and MAP-kinase-activated kinases 1 and 2.** *Eur J Biochem* 1993, **217**(2):715-722.
12. Dalby KN, Morrice N, Caudwell FB, Avruch J, Cohen P: **Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK.** *J Biol Chem* 1998, **273**(3):1496-1505.
13. Frodin M, Jensen CJ, Merienne K, Gammeltoft S: **A phosphoserine-regulated docking site in the protein kinase RSK2 that recruits and activates PDK1.** *Embo J* 2000, **19**(12):2924-2934.
14. Jensen CJ, Buch MB, Krag TO, Hemmings BA, Gammeltoft S, Frodin M: **90-kDa ribosomal S6 kinase is phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1.** *J Biol Chem* 1999, **274**(38):27168-27176.
15. Moller DE, Xia CH, Tang W, Zhu AX, Jakubowski M: **Human rsk isoforms: cloning and characterization of tissue-specific expression.** *American Journal of Physiology* 1994, **266**(2 Pt 1):351-359.

16. Dummler BA, Hauge C, Silber J, Yntema HG, Kruse LS, Kofoed B, Hemmings BA, Alessi DR, Frodin M: **Functional characterization of human RSK4, a new 90-kDa ribosomal S6 kinase, reveals constitutive activation in most cell types.** *J Biol Chem* 2005, **280**(14):13304-13314.
17. Heffron D, Mandell JW: **Differential localization of MAPK-activated protein kinases RSK1 and MSK1 in mouse brain.** *Brain research* 2005, **136**(1-2):134-141.
18. Zeniou M, Ding T, Trivier E, Hanauer A: **Expression analysis of RSK gene family members: the RSK2 gene, mutated in Coffin-Lowry syndrome, is prominently expressed in brain structures essential for cognitive function and learning.** *Hum Mol Genet* 2002, **11**(23):2929-2940.
19. Dugani CB, Paquin A, Kaplan DR, Miller FD: **Coffin-Lowry syndrome: a role for RSK2 in mammalian neurogenesis.** *Dev Biol* 2010, **347**(2):348-359.
20. Kohn M, Hameister H, Vogel M, Kehrer-Sawatzki H: **Expression pattern of the Rsk2, Rsk4 and Pdk1 genes during murine embryogenesis.** *Gene Expr Patterns* 2003, **3**(2):173-177.
21. Leighton IA, Dalby KN, Caudwell FB, Cohen PT, Cohen P: **Comparison of the specificities of p70 S6 kinase and MAPKAP kinase-1 identifies a relatively specific substrate for p70 S6 kinase: the N-terminal kinase domain of MAPKAP kinase-1 is essential for peptide phosphorylation.** *FEBS Lett* 1995, **375**(3):289-293.
22. Galan JA, Geraghty KM, Lavoie G, Kanshin E, Tcherkezian J, Calabrese V, Jeschke GR, Turk BE, Ballif BA, Blenis J *et al*: **Phosphoproteomic analysis identifies the tumor suppressor PDCD4 as a RSK substrate negatively regulated by 14-3-3.** *Proc Natl Acad Sci U S A* 2014, **111**(29):E2918-2927.
23. Carriere A, Ray H, Blenis J, Roux PP: **The RSK factors of activating the Ras/MAPK signaling cascade.** *Front Biosci* 2008, **13**:4258-4275.
24. Chen RH, Juo PC, Curran T, Blenis J: **Phosphorylation of c-Fos at the C-terminus enhances its transforming activity.** *Oncogene* 1996, **12**(7):1493-1502.
25. Xing J, Ginty DD, Greenberg ME: **Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase.** *Science* 1996, **273**:959-963.
26. Li D, Jin L, Alesi GN, Kim YM, Fan J, Seo JH, Wang D, Tucker M, Gu TL, Lee BH *et al*: **The prometastatic ribosomal S6 kinase 2-cAMP response element-binding protein (RSK2-CREB) signaling pathway up-regulates the actin-binding protein fascin-1 to promote tumor metastasis.** *J Biol Chem* 2013, **288**(45):32528-32538.
27. Bignone PA, Lee KY, Liu Y, Emilion G, Finch J, Soosay AE, Charnock FM, Beck S, Dunham I, Mungall AJ *et al*: **RPS6KA2, a putative tumour suppressor gene at 6q27 in sporadic epithelial ovarian cancer.** *Oncogene* 2007, **26**(5):683-700.
28. Zhu J, Li QY, Liu JL, Wei W, Yang HW, Tang W: **RSK4 knockdown promotes proliferation, migration and metastasis of human breast adenocarcinoma cells.** *Oncol Rep* 2015, **34**(6):3156-3162.
29. Roux PP, Shahbazian D, Vu H, Holz MK, Cohen MS, Taunton J, Sonenberg N, Blenis J: **RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation.** *J Biol Chem* 2007, **282**(19):14056-14064.

30. Carriere A, Cargnello M, Julien LA, Gao H, Bonneil E, Thibault P, Roux PP: **Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation.** *Curr Biol* 2008, **18**(17):1269-1277.
31. Doehn U, Hauge C, Frank SR, Jensen CJ, Duda K, Nielsen JV, Cohen MS, Johansen JV, Winther BR, Lund LR *et al*: **RSK is a principal effector of the RAS-ERK pathway for eliciting a coordinate promotile/invasive gene program and phenotype in epithelial cells.** *Mol Cell* 2009, **35**(4):511-522.
32. Smolen GA, Zhang J, Zubrowski MJ, Edelman EJ, Luo B, Yu M, Ng LW, Scherber CM, Schott BJ, Ramaswamy S *et al*: **A genome-wide RNAi screen identifies multiple RSK-dependent regulators of cell migration.** *Genes Dev* 2010, **24**(23):2654-2665.
33. Zhang X, Lavoie G, Fort L, Huttlin EL, Tcherkezian J, Galan JA, Gu H, Gygi SP, Carreno S, Roux PP: **Gab2 phosphorylation by RSK inhibits Shp2 recruitment and cell motility.** *Mol Cell Biol* 2013, **33**(8):1657-1670.
34. S Y, M J, L Z, Y C, DO W, J K, J D: **Phosphorylation of KIBRA by the extracellular signal-regulated kinase (ERK) - ribosomal S6 kinase (RSK) cascade modulates cell proliferation and migration.** *Cell Signalling* 2014, **26**(2):343-351.
35. Tanimura S, Hashizume J, Arichika N, Watanabe K, Ohyama K, Takeda K, Kohno M: **ERK signaling promotes cell motility by inducing the localization of myosin 1E to lamellipodial tips.** *J Cell Biol* 2016, **214**(4):475-489.
36. Y Z, N Y, T T, T H, S Y, Y H, S Y, J F, K K, I S *et al*: **Crucial roles of RSK in cell motility by catalysing serine phosphorylation of EphA2.** *Nature Communications* 2015, **6**:7679.
37. Sulzmaier FJ, Ramos JW: **RSK isoforms in cancer cell invasion and metastasis.** *Cancer Res* 2013, **73**(20):6099-6105.
38. Eisinger-Mathason TS, Andrade J, Lannigan DA: **RSK in tumorigenesis: connections to steroid signaling.** *Steroids* 2010, **75**(3):191-202.
39. Smith JA, Poteet-Smith CE, Xu Y, Errington TM, Hecht SM, Lannigan DA: **Identification of the first specific inhibitor of p90 ribosomal S6 kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation.** *Cancer Res* 2005, **65**(3):1027-1034.
40. Clark DE, Errington TM, Smith JA, Frierson HF, Jr., Weber MJ, Lannigan DA: **The Serine/Threonine Protein Kinase, p90 Ribosomal S6 Kinase, Is an Important Regulator of Prostate Cancer Cell Proliferation.** *Cancer Res* 2005, **65**(8):3108-3116.
41. Cho YY, Yao K, Kim HG, Kang BS, Zheng D, Bode AM, Dong Z: **Ribosomal s6 kinase 2 is a key regulator in tumor promoter induced cell transformation.** *Cancer Res* 2007, **67**(17):8104-8112.
42. Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME: **Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms.** *Science* 1999, **286**(5443):1358-1362.
43. Kang S, Elf S, Lythgoe K, Hitosugi T, Taunton J, Zhou W, Xiong L, Wang D, Muller S, Fan S *et al*: **p90 ribosomal S6 kinase 2 promotes invasion and metastasis of human head and neck squamous cell carcinoma cells.** *J Clin Invest* 2010, **120**(4):1165-1177.

44. Lara R, Mauri FA, Taylor H, Derua R, Shia A, Gray C, Nicols A, Shiner RJ, Schofield E, Bates PA *et al*: **An siRNA screen identifies RSK1 as a key modulator of lung cancer metastasis.** *Oncogene* 2011, **30**(32):3513-3521.
45. Thakur A, Sun Y, Bollig A, Wu J, Biliran H, Banerjee S, Sarkar FH, Liao DJ: **Anti-invasive and antimetastatic activities of ribosomal protein S6 kinase 4 in breast cancer cells.** *Clin Cancer Res* 2008, **14**(14):4427-4436.
46. H S, T N, K T, Y Y, A T, R I, N D, S T, O N, Y F: **Crystal structure of a claudin provides insight into the architecture of tight junctions.** *Science* 2014, **344**(6181):304-307.
47. E D, DK T, AP K: **The desmosome.** *Cold Spring Harbour Perspectives Biology* 2009, **1**(2):a002543.
48. Adams CL, Chen YT, Smith SJ, Nelson WJ: **Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein.** *J Cell Biol* 1998, **142**(4):1105-1119.
49. Yonemura S, Itoh M, Nagafuchi A, Tsukita S: **Cell-to-cell adherens junction formation and actin filament organization: similarities and differences between non-polarized fibroblasts and polarized epith.** *J Cell Sci* 1995, **108**:127-142.
50. Kawauchi T, Hoshino M: **Molecular pathways regulating cytoskeletal organization and morphological changes in migrating neurons.** *Dev Neurosci* 2008, **30**(1-3):36-46.
51. le Duc Q, Shi Q, Blonk I, Sonnenberg A, Wang N, Leckband D, de Rooij J: **Vinculin potentiates E-cadherin mechanosensing and is recruited to actin-anchored sites within adherens junctions in a myosin II-dependent manner.** *J Cell Biol* 2010, **189**(7):1107-1115.
52. Peglion F, Llense F, Etienne-Manneville S: **Adherens junction treadmilling during collective migration.** *Nat Cell Biol* 2014, **16**(7):639-651.
53. Camand E, Peglion F, Osmani N, Sanson M, Etienne-Manneville S: **N-cadherin expression level modulates integrin-mediated polarity and strongly impacts on the speed and directionality of glial cell migration.** *J Cell Sci* 2012, **125**(Pt 4):844-857.
54. Hay ED: **The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it.** *Developmental dynamics : an official publication of the American Association of Anatomists* 2005, **233**(3):706-720.
55. Beck TN, Chikwem AJ, Solanki NR, Golemis EA: **Bioinformatic approaches to augment study of epithelial-to-mesenchymal transition in lung cancer.** *Physiol Genomics* 2014, **46**(19):699-724.
56. Bitting RL, Schaeffer D, Somarelli JA, Garcia-Blanco MA, Armstrong AJ: **The role of epithelial plasticity in prostate cancer dissemination and treatment resistance.** *Cancer Metastasis Rev* 2014, **33**(2-3):441-468.
57. Taube JH, Herschkowitz JI, Komurov K, Zhou AY, Gupta S, Yang J, Hartwell K, Onder TT, Gupta PB, Evans KW *et al*: **Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metaplastic breast cancer subtypes.** *Proc Natl Acad Sci U S A* 2010, **107**(35):15449-15454.
58. Takeichi M: **Dynamic contacts: rearranging adherens junctions to drive epithelial remodelling.** *Nat Rev Mol Cell Biol* 2014, **15**(6):397-410.

59. Shapiro L, Fannon AM, Kwong PD, Thompson A, Lehmann MS, Grubel G, Legrand JF, Als-Nielsen J, Colman DR, Hendrickson WA: **Structural basis of cell-cell adhesion by cadherins.** *Nature* 1995, **374**(6520):327-337.
60. Gumbiner B, Stevenson B, Grimaldi A: **The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex.** *J Cell Biol* 1988, **107**(4):1575-1587.
61. Uchida N, Honjo Y, Johnson KR, Wheelock MJ, Takeichi M: **The catenin/cadherin adhesion system is localized in synaptic junctions bordering transmitter release zones.** *J Cell Biol* 1996, **135**(3):767-779.
62. van Roy F: **Beyond E-cadherin: roles of other cadherin superfamily members in cancer.** *Nat Rev Cancer* 2014, **14**(2):121-134.
63. Kim SA, Tai CY, Mok LP, Mosser EA, Schuman EM: **Calcium-dependent dynamics of cadherin interactions at cell-cell junctions.** *Proc Natl Acad Sci U S A* 2011, **108**:9857-9862.
64. Abe K, Takeichi M: **EPLIN mediates linkage of the cadherin catenin complex to F-actin and stabilizes the circumferential actin belt.** *Proc Natl Acad Sci U S A* 2008, **105**(1):13-19.
65. Miller PW, Pokutta S, Ghosh A, Almo SC, Weis WI, Nelson WJ, Kwiatkowski AV: **Danio rerio α E-catenin is a monomeric F-actin binding protein with distinct properties from Mus musculus α E-catenin.** *J Biol Chem* 2013, **288**(31):22324-22332.
66. Buckley CD, Tan J, Anderson KL, Hanein D, Volkmann N, Weis WI, Nelson WJ, Dunn AR: **Cell adhesion. The minimal cadherin-catenin complex binds to actin filaments under force.** *Science* 2014, **346**(6209):1254211.
67. Coopman P, Djiane A: **Adherens Junction and E-Cadherin complex regulation by epithelial polarity.** *Cell Mol Life Sci* 2016, **73**(18):3535-3553.
68. Davis MA, Ireton RC, Reynolds AB: **A core function for p120-catenin in cadherin turnover.** *J Cell Biol* 2003, **163**(3):525-534.
69. Xiao K, Allison DF, Buckley KM, Kottke MD, Vincent PA, Faundez V, Kowalczyk AP: **Cellular levels of p120 catenin function as a set point for cadherin expression levels in microvascular endothelial cells.** *J Cell Biol* 2003, **163**(3):535-545.
70. Carnahan RH, Rokas A, Gaucher EA, Reynolds AB: **The molecular evolution of the p120-catenin subfamily and its functional associations.** *PLoS One* 2010, **5**(12):e15747.
71. Myster SH, Cavallo R, Anderson CT, Fox DT, Peifer M: **Drosophila p120catenin plays a supporting role in cell adhesion but is not an essential adherens junction component.** *J Cell Biol* 2003, **160**(3):433-449.
72. Pettitt J, Cox EA, Broadbent ID, Flett A, Hardin J: **The Caenorhabditis elegans p120 catenin homologue, JAC-1, modulates cadherin-catenin function during epidermal morphogenesis.** *J Cell Biol* 2003, **162**(1):15-22.
73. Smalley-Freed WG, Efimov A, Burnett PE, Short SP, Davis MA, Gumucio DL, Washington MK, Coffey RJ, Reynolds AB: **p120-catenin is essential for maintenance of barrier function and intestinal homeostasis in mice.** *J Clin Invest* 2010, **120**(6):1824-1835.

74. Keirsebilck A, Bonne S, Staes K, van Hengel J, Nollet F, Reynolds A, van Roy F: **Molecular cloning of the human p120ctn catenin gene (CTNND1): expression of multiple alternatively spliced isoforms.** *Genomics* 1998, **50**(2):129-146.
75. Montonen O, Aho M, Uitto J, Aho S: **Tissue distribution and cell type-specific expression of p120ctn isoforms.** *J Histochem Cytochem* 2001, **49**(12):1487-1496.
76. Aho S, Levansuo L, Montonen O, Kari C, Rodeck U, Uitto J: **Specific sequences in p120ctn determine subcellular distribution of its multiple isoforms involved in cellular adhesion of normal and malignant epithelial cells.** *J Cell Sci* 2002, **115**(Pt 7):1391-1402.
77. Reynolds AB, Herbert L, Cleveland JL, Berg ST, Gaut JR: **p120, a novel substrate of protein tyrosine kinase receptors and of p60v-src, is related to cadherin-binding factors beta-catenin, plakoglobin and armadillo.** *Oncogene* 1992, **7**(12):2439-2445.
78. Reynolds AB, Daniel J, McCrea PD, Wheelock MJ, Wu J, Zhang Z: **Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes.** *Mol Cell Biol* 1994, **14**(12):8333-8342.
79. Xiao K, Garner J, Buckley KM, Vincent PA, Chiasson CM, Dejana E, Faundez V, Kowalczyk AP: **p120-Catenin regulates clathrin-dependent endocytosis of VE-cadherin.** *Mol Biol Cell* 2005, **16**(11):5141-5151.
80. Thoreson MA, Anastasiadis PZ, Daniel JM, Ireton RC, Wheelock MJ, Johnson KR, Hummingbird DK, Reynolds AB: **Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion.** *J Cell Biol* 2000, **148**:189-202.
81. Reynolds AB, Carnahan RH: **Regulation of cadherin stability and turnover by p120ctn: implications in disease and cancer.** *Semin Cell Dev Biol* 2004, **15**(6):657-663.
82. Ireton RC, Davis MA, van Hengel J, Mariner DJ, Barnes K, Thoreson MA, Anastasiadis PZ, Matrisian L, Bundy LM, Sealy L *et al*: **A novel role for p120 catenin in E-cadherin function.** *J Cell Biol* 2002, **159**(3):465-476.
83. Stairs DB, Bayne LJ, Rhoades B, Vega ME, Waldron TJ, Kalabis J, Klein-Szanto A, Lee JS, Katz JP, Diehl JA *et al*: **Deletion of p120-catenin results in a tumor microenvironment with inflammation and cancer that establishes it as a tumor suppressor gene.** *Cancer Cell* 2011, **19**(4):470-483.
84. Fang X, Ji H, Kim SW, Park JI, Vaught TG, Anastasiadis PZ, Ciesiolka M, McCrea PD: **Vertebrate development requires ARVCF and p120 catenins and their interplay with RhoA and Rac.** *J Cell Biol* 2004, **165**(1):87-98.
85. Ciesiolka M, Delvaeye M, Van Imschoot G, Verschuere V, McCrea P, van Roy F, Vleminckx K: **p120 catenin is required for morphogenetic movements involved in the formation of the eyes and the craniofacial skeleton in Xenopus.** *J Cell Sci* 2004, **117**(Pt 18):4325-4339.
86. Davis MA, Reynolds AB: **Blocked acinar development, E-cadherin reduction, and intraepithelial neoplasia upon ablation of p120-catenin in the mouse salivary gland.** *Developmental cell* 2006, **10**(1):21-31.
87. Oas RG, Xiao K, Summers S, Wittich KB, Chiasson CM, Martin WD, Grossniklaus HE, Vincent PA, Reynolds AB, Kowalczyk AP: **p120-Catenin is required for mouse vascular development.** *Circ Res* 2010, **106**(5):941-951.
88. Perez-Moreno M, Davis MA, Wong E, Pasolli HA, Reynolds AB, Fuchs E: **p120-catenin mediates inflammatory responses in the skin.** *Cell* 2006, **124**(3):631-644.

89. Perez-Moreno M, Song W, Pasolli HA, Williams SE, Fuchs E: **Loss of p120 catenin and links to mitotic alterations, inflammation, and skin cancer.** *Proc Natl Acad Sci U S A* 2008, **105**(40):15399-15404.
90. van Hengel J, Van den Broeke C, Pieters T, Libbrecht L, Hofmann I, van Roy F: **Inactivation of p120 catenin in mice disturbs intrahepatic bile duct development and aggravates liver carcinogenesis.** *European journal of cell biology* 2016.
91. Pieters T, van Hengel J, van Roy F: **Functions of p120ctn in development and disease.** *Front Biosci (Landmark Ed)* 2012, **17**:760-783.
92. Israely I, Costa RM, Xie CW, Silva AJ, Kosik KS, Liu X: **Deletion of the neuron-specific protein delta-catenin leads to severe cognitive and synaptic dysfunction.** *Curr Biol* 2004, **14**(18):1657-1663.
93. Miyashita Y, Ozawa M: **Increased internalization of p120-uncoupled E-cadherin and a requirement for a dileucine motif in the cytoplasmic domain for endocytosis of the protein.** *J Biol Chem* 2007, **282**(15):11540-11548.
94. Kelly BT, McCoy AJ, Spate K, Miller SE, Evans PR, Honing S, Owen DJ: **A structural explanation for the binding of endocytic dileucine motifs by the AP2 complex.** *Nature* 2008, **456**(7224):976-979.
95. Chiasson CM, Wittich KB, Vincent PA, Faundez V, Kowalczyk AP: **p120-catenin inhibits VE-cadherin internalization through a Rho-independent mechanism.** *Mol Biol Cell* 2009, **20**(7):1970-1980.
96. Fujita Y, Krause G, Scheffner M, Zechner D, Leddy HE, Behrens J, Sommer T, Birchmeier W: **Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex.** *Nat Cell Biol* 2002, **4**(3):222-231.
97. Nanes BA, Chiasson-MacKenzie C, Lowery AM, Ishiyama N, Faundez V, Ikura M, Vincent PA, Kowalczyk AP: **p120-catenin binding masks an endocytic signal conserved in classical cadherins.** *J Cell Biol* 2012, **199**(2):365-380.
98. Elia LP, Yamamoto M, Zang K, Reichardt LF: **p120 catenin regulates dendritic spine and synapse development through Rho-family GTPases and cadherins.** *Neuron* 2006, **51**(1):43-56.
99. Kiss A, Troyanovsky RB, Troyanovsky SM: **p120-catenin is a key component of the cadherin-gamma-secretase supercomplex.** *Mol Biol Cell* 2008, **19**(10):4042-4050.
100. Sato K, Watanabe T, Wang S, Kakeno M, Matsuzawa K, Matsui T, Yokoi K, Murase K, Sugiyama I, Ozawa M *et al*: **Numb controls E-cadherin endocytosis through p120 catenin with aPKC.** *Mol Biol Cell* 2011, **22**(17):3103-3119.
101. Hirano M, Hashimoto S, Yonemura S, Sabe H, Aizawa S: **EPB41L5 functions to post-transcriptionally regulate cadherin and integrin during epithelial-mesenchymal transition.** *J Cell Biol* 2008, **182**(6):1217-1230.
102. Hashimoto A, Hashimoto S, Sugino H, Yoshikawa A, Onodera Y, Handa H, Oikawa T, Sabe H: **ZEB1 induces EPB41L5 in the cancer mesenchymal program that drives ARF6-based invasion, metastasis and drug resistance.** *Oncogenesis* 2016, **5**(9):e259.
103. Wildenberg GA, Dohn MR, Carnahan RH, Davis MA, Lobdell NA, Settleman J, Reynolds AB: **p120-catenin and p190RhoGAP regulate cell-cell adhesion by coordinating antagonism between Rac and Rho.** *Cell* 2006, **127**(5):1027-1039.
104. Zebda N, Tian Y, Tian X, Gawlak G, Higginbotham K, Reynolds AB, Birukova AA, Birukov KG: **Interaction of p190RhoGAP with C-terminal domain of p120-catenin**

- modulates endothelial cytoskeleton and permeability. *J Biol Chem* 2013, **288**(25):18290-18299.**
105. Anastasiadis PZ, Moon SY, Thoreson MA, Mariner DJ, Crawford HC, Zheng Y, Reynolds AB: **Inhibition of RhoA by p120 catenin.** *Nat Cell Biol* 2000, **2**(9):637-644.
106. Noren NK, Liu BP, Burridge K, Kreft B: **p120 catenin regulates the actin cytoskeleton via Rho family GTPases.** *J Cell Biol* 2000, **150**(3):567-580.
107. Schackmann RC, van Amersfoort M, Haarhuis JH, Vlug EJ, Halim VA, Roodhart JM, Vermaat JS, Voest EE, van der Groep P, van Diest PJ *et al*: **Cytosolic p120-catenin regulates growth of metastatic lobular carcinoma through Rock1-mediated anoikis resistance.** *J Clin Invest* 2011, **121**(8):3176-3188.
108. Dohn MR, Brown MV, Reynolds AB: **An essential role for p120-catenin in Src- and Rac1-mediated anchorage-independent cell growth.** *J Cell Biol* 2009, **184**(3):437-450.
109. van Hengel J, Vanhoenacker P, Staes K, van Roy F: **Nuclear localization of the p120(ctn) Armadillo-like catenin is counteracted by a nuclear export signal and by E-cadherin expression.** *Proc Natl Acad Sci U S A* 1999, **96**(14):7980-7985.
110. Franz CM, Ridley AJ: **p120 catenin associates with microtubules: inverse relationship between microtubule binding and Rho GTPase regulation.** *J Biol Chem* 2004, **279**(8):6588-6594.
111. Li Y, Kuwahara H, Ren J, Wen G, Kufe D: **The c-Src tyrosine kinase regulates signaling of the human DF3/MUC1 carcinoma-associated antigen with GSK3 beta and beta-catenin.** *J Biol Chem* 2001, **276**(9):6061-6064.
112. Daniel JM, Reynolds AB: **The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor.** *19* 1999, **5**(3614-3623).
113. Schackmann RC, Tenhagen M, van de Ven RA, Derksen PW: **p120-catenin in cancer - mechanisms, models and opportunities for intervention.** *J Cell Sci* 2013, **126**(Pt 16):3515-3525.
114. Wang H, Liu W, Black S, Turner O, Daniel JM, Dean-Colomb W, He QP, Davis M, Yates C: **Kaiso, a transcriptional repressor, promotes cell migration and invasion of prostate cancer cells through regulation of miR-31 expression.** *Oncotarget* 2016, **7**(5):5677-5689.
115. Vermeulen JF, van de Ven RA, Ercan C, van der Groep P, van der Wall E, Bult P, Christgen M, Lehmann U, Daniel J, van Diest PJ *et al*: **Nuclear Kaiso expression is associated with high grade and triple-negative invasive breast cancer.** *PLoS One* 2012, **7**:e37864.
116. Dai SD, Wang Y, Miao Y, Zhao Y, Zhang Y, Jiang GY, Zhang PX, Yang ZQ, Wang EH: **Cytoplasmic Kaiso is associated with poor prognosis in non-small cell lung cancer.** *BMC Cancer* 2009, **9**:178.
117. Xia X, Carnahan RH, Vaughan MH, Wildenberg GA, Reynolds AB: **p120 serine and threonine phosphorylation is controlled by multiple ligand-receptor pathways but not cadherin ligation.** *Exp Cell Res* 2006, **312**(17):3336-3348.
118. Petrova YI, Spano MM, Gumbiner BM: **Conformational epitopes at cadherin calcium-binding sites and p120-catenin phosphorylation regulate cell adhesion.** *Mol Biol Cell* 2012, **23**(11):2092-2108.

119. Maiden SL, Petrova YI, Gumbiner BM: **Microtubules Inhibit E-Cadherin Adhesive Activity by Maintaining Phosphorylated p120-Catenin in a Colon Carcinoma Cell Model.** *PLoS One* 2016, **11**(2):e0148574.
120. Kourtidis A, Yanagisawa M, Huvelde D, Copland JA, Anastasiadis PZ: **Pro-Tumorigenic Phosphorylation of p120 Catenin in Renal and Breast Cancer.** *PLoS One* 2015, **10**(6):e0129964.
121. Kourtidis A, Ngok SP, Pulimeno P, Feathers RW, Carpio LR, Baker TR, Carr JM, Yan IK, Borges S, Perez EA *et al*: **Distinct E-cadherin-based complexes regulate cell behaviour through miRNA processing or Src and p120 catenin activity.** *Nat Cell Biol* 2015, **17**(9):1145-1157.
122. Hong JY, Oh IH, McCrea PD: **Phosphorylation and isoform use in p120-catenin during development and tumorigenesis.** *Biochim Biophys Acta* 2016, **1863**(1):102-114.
123. Alema S, Salvatore AM: **p120 catenin and phosphorylation: Mechanisms and traits of an unresolved issue.** *Biochim Biophys Acta* 2007, **1773**(1):47-58.
124. Hong JY, Park JI, Cho K, Gu D, Ji H, Artandi SE, McCrea PD: **Shared molecular mechanisms regulate multiple catenin proteins: canonical Wnt signals and components modulate p120-catenin isoform-1 and additional p120 subfamily members.** *J Cell Sci* 2010, **123**(Pt 24):4351-4365.
125. Chidiac R, Zhang Y, Tessier S, Faubert D, Delisle C, Gratton JP: **Comparative Phosphoproteomics Analysis of VEGF and Angiopoietin-1 Signaling Reveals ZO-1 as a Critical Regulator of Endothelial Cell Proliferation.** *Mol Cell Proteomics* 2016, **15**(5):1511-1525.
126. Del Valle-Perez B, Casagolda D, Lugilde E, Valls G, Codina M, Dave N, de Herreros AG, Dunach M: **Wnt controls the transcriptional activity of Kaiso through CK1epsilon-dependent phosphorylation of p120-catenin.** *J Cell Sci* 2011, **124**(Pt 13):2298-2309.
127. Wong LE, Reynolds AB, Dissanayaka NT, Minden A: **p120-catenin is a binding partner and substrate for Group B Pak kinases.** *J Cell Biochem* 2010, **110**(5):1244-1254.
128. Xia X, Mariner DJ, Reynolds AB: **Adhesion-associated and PKC-modulated changes in serine/threonine phosphorylation of p120-catenin.** *Biochemistry* 2003, **42**(30):9195-9204.
129. Vandembroucke St Amant E, Tauseef M, Vogel SM, Gao XP, Mehta D, Komarova YA, Malik AB: **PKCalpha activation of p120-catenin serine 879 phospho-switch disassembles VE-cadherin junctions and disrupts vascular integrity.** *Circ Res* 2012, **111**(6):739-749.
130. Castano J, Solanas G, Casagolda D, Raurell I, Villagrasa P, Bustelo XR, Garcia de Herreros A, Dunach M: **Specific phosphorylation of p120-catenin regulatory domain differently modulates its binding to RhoA.** *Mol Cell Biol* 2007, **27**(5):1745-1757.
131. Valls G, Codina M, Miller RK, Del Valle-Perez B, Vinyoles M, Caelles C, McCrea PD, Garcia de Herreros A, Dunach M: **Upon Wnt stimulation, Rac1 activation requires Rac1 and Vav2 binding to p120-catenin.** *J Cell Sci* 2012, **125**(Pt 22):5288-5301.

132. Mariner DJ, Davis MA, Reynolds AB: **EGFR signaling to p120-catenin through phosphorylation at Y228.** *J Cell Sci* 2004, **117**(Pt 8):1339-1350.
133. Mariner DJ, Anastasiadis P, Keilhack H, Bohmer FD, Wang J, Reynolds AB: **Identification of Src phosphorylation sites in the catenin p120ctn.** *J Biol Chem* 2001, **276**(30):28006-28013.
134. Liu Y, Li QC, Miao Y, Xu HT, Dai SD, Wei Q, Dong QZ, Dong XJ, Zhao Y, Zhao C *et al*: **Ablation of p120-catenin enhances invasion and metastasis of human lung cancer cells.** *Cancer Sci* 2009, **100**(3):441-448.
135. Dillon DA, D'Aquila T, Reynolds AB, Fearon ER, Rimm DL: **The expression of p120ctn protein in breast cancer is independent of alpha- and beta-catenin and E-cadherin.** *Am J Pathol* 1998, **152**(1):75-82.
136. Kallakury BV, Sheehan CE, Ross JS: **Co-downregulation of cell adhesion proteins alpha- and beta-catenins, p120CTN, E-cadherin, and CD44 in prostatic adenocarcinomas.** *Hum Pathol* 2001, **32**(8):849-855.
137. Wang EH, Liu Y, Xu HT, Dai SD, Liu N, Xie CY, Yuan XM: **Abnormal expression and clinicopathologic significance of p120-catenin in lung cancer.** *Histol Histopathol* 2006, **21**(8):841-847.
138. Sarrio D, Perez-Mies B, Hardisson D, Moreno-Bueno G, Suarez A, Cano A, Martin-Perez J, Gamallo C, Palacios J: **Cytoplasmic localization of p120ctn and E-cadherin loss characterize lobular breast carcinoma from preinvasive to metastatic lesions.** *Oncogene* 2004, **23**(19):3272-3283.
139. Bellovin DI, Bates RC, Muzikansky A, Rimm DL, Mercurio AM: **Altered localization of p120 catenin during epithelial to mesenchymal transition of colon carcinoma is prognostic for aggressive disease.** *Cancer Res* 2005, **65**(23):10938-10945.
140. Mayerle J, Friess H, Buchler MW, Schnekenburger J, Weiss FU, Zimmer KP, Domschke W, Lerch MM: **Up-regulation, nuclear import, and tumor growth stimulation of the adhesion protein p120 in pancreatic cancer.** *Gastroenterology* 2003, **124**(4):949-960.
141. Maeda M, Johnson E, Mandal SH, Lawson KR, Keim SA, Svoboda RA, Caplan S, Wahl JK, 3rd, Wheelock MJ, Johnson KR: **Expression of inappropriate cadherins by epithelial tumor cells promotes endocytosis and degradation of E-cadherin via competition for p120(ctn).** *Oncogene* 2006, **25**(33):4595-4604.
142. Zhang Y, Zhao Y, Jiang G, Zhang X, Zhao H, Wu J, Xu K, Wang E: **Impact of p120-catenin isoforms 1A and 3A on epithelial mesenchymal transition of lung cancer cells expressing E-cadherin in different subcellular locations.** *PLoS One* 2014, **9**(2):e88064.
143. Yanagisawa M, Huvelde D, Kreinest P, Lohse CM, Cheville JC, Parker AS, Copland JA, Anastasiadis PZ: **A p120 catenin isoform switch affects Rho activity, induces tumor cell invasion, and predicts metastatic disease.** *J Biol Chem* 2008, **283**(26):18344-18354.
144. Paredes J, Correia AL, Ribeiro AS, Schmitt F: **Expression of p120-catenin isoforms correlates with genomic and transcriptional phenotype of breast cancer cell lines.** *Cell Oncol* 2007, **29**(6):467-476.

145. Silvera D, Arju R, Darvishian F, Levine PH, Zolfaghari L, Goldberg J, Hochman T, Formenti SC, Schneider RJ: **Essential role for eIF4GI overexpression in the pathogenesis of inflammatory breast cancer.** *Nat Cell Biol* 2009, **11**(7):903-908.
146. Macpherson IR, Hooper S, Serrels A, McGarry L, Ozanne BW, Harrington K, Frame MC, Sahai E, Brunton VG: **p120-catenin is required for the collective invasion of squamous cell carcinoma cells via a phosphorylation-independent mechanism.** *Oncogene* 2007, **26**(36):5214-5228.
147. Obenaus JC, Cantley LC, Yaffe MB: **Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs.** *Nucleic Acids Res* 2003, **31**(13):3635-3641.
148. Subramaniam S: **The Biology Workbench--a seamless database and analysis environment for the biologist.** *Proteins* 1998, **32**(1):1-2.
149. Boguslavsky S, Grosheva I, Landau E, Shtutman M, Cohen M, Arnold K, Feinstein E, Geiger B, Bershadsky A: **p120 catenin regulates lamellipodial dynamics and cell adhesion in cooperation with cortactin.** *Proc Natl Acad Sci U S A* 2007, **104**(26):10882-10887.
150. Taguchi K, Ishiuchi T, Takeichi M: **Mechanosensitive EPLIN-dependent remodeling of adherens junctions regulates epithelial reshaping.** *J Cell Biol* 2011, **194**(4):643-656.
151. Huen AC, Park JK, Godsel LM, Chen X, Bannon LJ, Amargo EV, Hudson TY, Mongiu AK, Leigh IM, Kelsell DP *et al*: **Intermediate filament-membrane attachments function synergistically with actin-dependent contacts to regulate intercellular adhesive strength.** *J Cell Biol* 2002, **159**(6):1005-1017.
152. Erami Z, Timpson P, Yao W, Zaidel-Bar R, Anderson KI: **There are four dynamically and functionally distinct populations of E-cadherin in cell junctions.** *Biol Open* 2015, **4**(11):1481-1489.
153. Zheng B, Cantley LC: **Regulation of epithelial tight junction assembly and disassembly by AMP-activated protein kinase.** *Proc Natl Acad Sci U S A* 2007, **104**(3):819-822.
154. Sun M, Fu H, Cheng H, Cao Q, Zhao Y, Mou X, Zhang X, Liu X, Ke Y: **A dynamic real-time method for monitoring epithelial barrier function in vitro.** *Anal Biochem* 2012, **425**(2):96-103.
155. Caslavsky J, Klimova Z, Vomastek T: **ERK and RSK regulate distinct steps of a cellular program that induces transition from multicellular epithelium to single cell phenotype.** *Cell Signal* 2013, **25**(12):2743-2751.
156. Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, Latham V, Sullivan M: **PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse.** *Nucleic Acids Res* 2012, **40**(Database issue):D261-270.
157. Wong EY, Morgan L, Smales C, Lang P, Gubby SE, Staddon JM: **Vascular endothelial growth factor stimulates dephosphorylation of the catenins p120 and p100 in endothelial cells.** *Biochem J* 2000, **346 Pt 1**:209-216.
158. Zhang PX, Wang Y, Liu Y, Jiang GY, Li QC, Wang EH: **p120-catenin isoform 3 regulates subcellular localization of Kaiso and promotes invasion in lung cancer**

- cells via a phosphorylation-dependent mechanism. *Int J Oncol* 2011, **38**(6):1625-1635.
159. Miao H, Li DQ, Mukherjee A, Guo H, Petty A, Cutter J, Basilion JP, Sedor J, Wu J, Danielpour D *et al*: **EphA2 mediates ligand-dependent inhibition and ligand-independent promotion of cell migration and invasion via a reciprocal regulatory loop with Akt.** *Cancer Cell* 2009, **16**(1):9-20.
160. Miao H, Gale NW, Guo H, Qian J, Petty A, Kaspar J, Murphy AJ, Valenzuela DM, Yancopoulos G, Hambardzumyan D *et al*: **EphA2 promotes infiltrative invasion of glioma stem cells in vivo through cross-talk with Akt and regulates stem cell properties.** *Oncogene* 2015, **34**(5):558-567.
161. Li Q, Mattingly RR: **Restoration of E-cadherin cell-cell junctions requires both expression of E-cadherin and suppression of ERK MAP kinase activation in Ras-transformed breast epithelial cells.** *Neoplasia* 2008, **10**(12):1444-1458.
162. Lemieux E, Cagnol S, Beaudry K, Carrier J, Rivard N: **Oncogenic KRAS signalling promotes the Wnt/beta-catenin pathway through LRP6 in colorectal cancer.** *Oncogene* 2015, **34**(38):4914-4927.
163. Ji H, Wang J, Nika H, Hawke D, Keezer S, Ge Q, Fang B, Fang X, Fang D, Litchfield DW *et al*: **EGF-induced ERK activation promotes CK2-mediated disassociation of alpha-Catenin from beta-Catenin and transactivation of beta-Catenin.** *Mol Cell* 2009, **36**(4):547-559.
164. Liu X, Yi C, Wen Y, Radhakrishnan P, Tremayne JR, Dao T, Johnson KR, Hollingsworth MA: **Interactions between MUC1 and p120 catenin regulate dynamic features of cell adhesion, motility, and metastasis.** *Cancer Res* 2014, **74**(5):1609-1620.
165. Ray RM, Vaidya RJ, Johnson LR: **MEK/ERK regulates adherens junctions and migration through Rac1.** *Cell Motil Cytoskeleton* 2007, **64**(3):143-156.

