

The regulation of β -catenin function in vascular smooth muscle cells

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A dissertation submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Graduate Program in Biology
York University
Toronto, Ontario

November 2016

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Abstract

Vascular smooth muscle cells (VSMCs) maintain the ability to switch between a quiescent and proliferative phenotype based on external stimuli; this is an important feature for vascular repair. However, it leaves VSMCs predisposed to be affected by aberrant stimuli which can result in improper cell proliferation, contributing to atherosclerosis and restenosis following angioplasty. As such, understanding the molecular mechanisms involved in activating VSMCs is of great clinical and therapeutic importance.

β -catenin is a multifunctional protein that plays an essential role in cell adhesion at the membrane and is required for Wnt dependent target gene expression in the nucleus. Active Wnt signalling is characterized by reduced membrane and enhanced nuclear β -catenin, whose target genes are involved in epithelial-to-mesenchymal transition and cell proliferation. β -catenin has been shown to play a significant role in VSMCs during both development and wound repair, and the role of β -catenin is wholly dependent on specific protein-protein interactions in the cell, thus the aim of this project was to identify novel interacting partners and determine their effect on β -catenin function in VSMCs.

Previous work in our lab has identified the myocyte enhancer factor 2 (MEF2) family of proteins as potential β -catenin interacting partners. MEF2 proteins are transcription factors involved in vascular development during embryogenesis, but are also upregulated in the neointima following carotid artery injury in mice. MEF2A and MEF2C can also become phosphorylated and activated by the p38 mitogen activated protein kinase (MAPK) pathway.

In the first study, a direct physical interaction between MEF2 and β -catenin was documented. This interaction was mediated and enhanced by p38 MAPK phosphorylation of MEF2, which resulted in reduced membrane association and increased nuclear retention of β -catenin. Active p38 MAPK signalling in primary VSMCs was able to stimulate canonical Wnt signalling in a MEF2-dependent manner. The MEF2: β -catenin interaction also synergistically increased cell proliferation, and this interaction, as well as the activation of Wnt signalling by p38 MAPK, was observed in multiple cell types. We therefore concluded that p38 MAPK positively regulates a MEF2: β -catenin interaction to enhance canonical Wnt signalling and cell proliferation.

The purpose of the second study was to identify novel β -catenin interacting partners in VSMCs using affinity chromatography coupled to tandem LC MS/MS. The investigation revealed 131 interactions, and gene ontology (GO) enrichment analysis of these proteins emphasized translation as a highly likely biological process.

Further examination of one potential interactor, the fragile X mental retardation protein (FMRP), revealed a direct interaction with β -catenin *in vitro* and *in vivo*. FMRP has previously been shown to bind to mRNA in the pre-initiation complex and inhibit mRNA translation, and genetic loss of FMRP results in fragile X syndrome. siRNA mediated knockdown of β -catenin increased global protein synthesis, and biochemical analysis determined that β -catenin associates at the pre-initiation complex with FMRP. Wnt-dependent nuclear shuttling of β -catenin de-repressed protein synthesis, and we concluded that cytoplasmic β -catenin can inhibit translation at the cellular level. Thus, we established a completely novel role for β -catenin function in the regulation of translation by interaction with FMRP.

Acknowledgements

I would like to thank my mother (Homa), father (Darioush), and brother (Hamoon) for their unconditional love and support throughout my life; it is because of your dedication to me that I have made any accomplishments in my life, and I would be lost without you. You have raised me to become the confident and intelligent person I am today, and I hope you are proud of my achievement because it reflects on your commitment to me. I apologize to my sister in law (Sheida) for not being able to spend as much time with my beautiful nephews, Davin and Darian, as I had hoped.

I would like to thank my committee for their help and expertise these past few years, and in particular my supervisor, Prof. John McDermott. Your knowledge and guidance has been instrumental for my training, as well as your willingness to let me choose my own path during my research. I am grateful that you have been so patient with me for all these years, and I hope that you feel it has finally all paid off. I also thank my labmates throughout these years for their advice and critique during my research. In particular, I would like to thank Joe, Christina, and Mathew for their collaborations and friendship (and letting me "borrow" their reagents). Lastly, I would like to thank Tetsuaki for his tremendous support, wisdom, and friendship. I truly consider you to be my sensei, and you have made a lasting impact on my outlook in science and life.

To all my friends that have given me countless memories throughout my life so far, I thank you for the joyful times we've had together. My friends from biology (and chemistry), Mohamed, Paul, and Hossein, thanks for all the fun we had trying to stay sane doing research. My friends from "work" (aka Stuntin'), Chad, Devin, Eric, Francis, Hadi, Jim, and Kuperan, thanks for being my sanctuary outside of the lab. And to Alexander, my closest friend, thanks for being my lab partner in grade 11 chemistry. I think it really kicked off something special, and you've always helped keep things in perspective for me.

-Saviz Ehyai

Table of Contents

Abstract	ii
Acknowledgements	iv
List of abbreviations	ix

Chapter 1: Review of literature

<i>The β-catenin protein</i>	1
Figure 1: Basic structure and sequence alignment of β -catenin among 5 species ...	1
<i>Structure and protein interactions</i>	4
Figure 2: Helix structure of β -catenin armadillo repeats	5
<i>β-catenin cellular function</i>	10
Figure 3: The adherens junction	12
<i>Comparison with other armadillo repeat proteins</i>	13
Figure 4: The desmosome	15
<i>Post-translational modifications and β-catenin function</i>	15
<i>Summary of protein-protein interactions and post-translational modifications</i>	23
Figure 5: β -catenin protein interactions	24
Figure 6: β -catenin post-translational modifications	24
<i>Canonical Wnt signalling</i>	25
<i>Non-canonical Wnt signalling</i>	31
Figure 7: Summary of canonical and non-canonical Wnt signalling.....	34
<i>Cross-talk between Wnt and MAPK signalling</i>	35
<i>β-catenin function in vascular smooth muscle cells</i>	38
<i>Conclusions</i>	41
Statement of purpose	42

Chapter 2: A p38 MAPK regulated MEF2:β-catenin interaction enhances canonical Wnt signalling

<i>Rationale</i>	44
<i>Summary</i>	45
<i>Introduction</i>	46
<i>Results</i>	48
Figure 8: p38 MAPK regulates Wnt-dependent β-catenin transactivation properties and nuclear localization in primary vascular smooth muscle cells.....	54
Figure 9: MEF2 directly interacts with β-catenin in vitro and in multiple tissue types	63
Figure 10: MEF2 protein expression is required for β-catenin nuclear retention and enhanced by p38 MAPK mediated phosphorylation of MEF2	68
Figure 11: MEF2 and β-catenin cooperate to promote cell proliferation in a p38 MAPK-dependent manner, and p38 MAPK regulates Wnt target gene protein levels.....	75
<i>Discussion</i>	78
Figure 12: Schematic illustrating the cross-talk between Wnt/β-catenin and p38 MAPK/MEF2 signalling	82
<i>Materials and Methods</i>	83

Chapter 3: A Wnt-dependent β-catenin:FMRP interaction regulates translation

<i>Rationale</i>	90
<i>Summary</i>	91
<i>Introduction</i>	92
<i>Results</i>	94
Figure 13: Experimental workflow and top 50 sorted β-catenin interactors shown with GO cellular component analysis.....	97
Figure 14: GO Biological Process enrichment analysis	98
Figure 15: β-catenin co-localizes and directly interacts with FMRP	103
Figure 16: β-catenin and FMRP are co-localized in the pre-initiation complex to negatively regulate translation	108

Figure 17: Wnt dependent cytoplasmic to nuclear translocation of β -catenin de-represses translation.....	112
<i>Discussion</i>	114
<i>Materials and Methods</i>	117
 Chapter 4: Discussion and concluding statements	126
 References	130
 Appendix	
<i>A: Supplementary figures</i>	153
Figure S1: Modulation of β -catenin localization with Wnt3a stimulation and p38 MAPK inhibition (SB203580).....	153
Figure S2: Modulation of β -catenin localization with Wnt3a stimulation and p38 MAPK inhibition (SB202190)	155
Figure S3: FOPflash reporter gene activity in response to active p38 MAPK signalling.....	156
Figure S4: β -catenin localization and transactivation in response to active p38 MAPK.....	157
Figure S5: β -catenin localization and transactivation in response to active p38 MAPK.....	158
Figure S6: Determination of β -catenin as a potential substrate of p38 MAPK by in vitro kinase assay	159
Figure S7: β -catenin transactivation properties following Ser to Ala mutations of putative MAPK phospho-sites in response to p38 MAPK signalling	160
Figure S8: Ion chromatograms of tryptic β -catenin peptides containing candidate p38 MAPK phosphosites and a tryptic ATF2 peptide and phosphopeptide containing known p38 MAPK phosphosites	161
Figure S9: Purification of GST-MEF2C and 6xHis- β -catenin	163
Figure S10: In vitro binding assay	164
Figure S11: Confirmation of siRNA mediated silencing of MEF2A/C.....	164
Figure S12: TOPflash assay during Wnt stimulation using siRNA to MEF2A	165

Figure S13: c-Jun reporter assay with active p38 MAPK and β -catenin gene silencing	166
Figure S14: Effect of β -catenin gene silencing on c-Jun protein and promoter activity.....	167
Figure S15.....	168
Figures S16-S18.....	170
Figures S19-S21.....	171
<i>B: Selected materials and methods</i>	173
m ⁷ GTP-agarose pull down assay	
Puromycin incorporation for determining protein translation	
6xHis protein purification	
GST protein purification	
Cell lines and culture	
Western blot analysis	

List of abbreviations

APC	adenomatous polyposis coli
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
BBB	blood brain barrier
BCL9	B-cell CLL/lymphoma 9
BMK	big map kinase
BrdU	bromodeoxyuridine
Ca ²⁺	calcium (2+)
CamK	calmodulin dependent kinase
CBP	CREB binding protein
CIP	calf intestinal phosphatase
CK	casein kinase
coIP	co-immunoprecipitation
CRC	colorectal cancer
CRD	cysteine rich domain
CYFIP1	cytoplasmic FMRP interacting protein 1
Daam1	dishevelled associated activator of morphogenesis 1
DAPI	4',6-diamidino-2-phenylindole
DKK-1	dickkopf-1
DLL	delta like ligand
DMSO	dimethyl sulfoxide
Dsh	dishevelled
EC	extracellular
EGF	epidermal growth factor
eIF4E	eukaryotic initiation factor 4E
EMT	epithelial-to-mesenchymal
ER	endoplasmic reticulum
ERK	extracellular regulated kinase
Evi	evenness skipped
EYFP	enhanced yellow fluorescent protein
FITC	fluorescein isothiocyanate
FMRP	fragile X mental retardation protein
FXS	fragile X syndrome
Fz	Frizzled
GBP	GFP binding protein
GFP	green fluorescent protein
GO	gene ontology
GSK3	glycogen synthase kinase
GST	glutathione-S-transferase
GTP	guanosine triphosphate

HAT	histone acetyltransferase
HDAC	histone deacetylase
ICAT	inhibitor of beta-catenin and TCF4
IP	immunoprecipitation
JNK	c-Jun-N-terminal kinase
kDa	kilodalton
LC MS/MS	liquid chromatography tandem mass spectrometry
LEF	lymphoid enhancer factor
LRP	lipoprotein receptor-related protein
LUT	lookup table
MADS	MCM1, Agamous, Deficiens, SRF
MAPK	mitogen activated protein kinase
Med	mediator
MEF2	myocyte enhancer factor 2
MKK	mitogen activated protein kinase kinase
MMP	matrix metalloproteinase
mRNA	mature RNA
mTOR	mammalian target of rapamycin
NFAT	nuclear factor of activated T-cells
NLS	nuclear localization signal
PCAF	p300/CBP-associated factor
PCP	planar cell polarity
PDGF	platelet derived growth factor
pI	isoelectric point
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PP1 α	protein phosphatase 1 α
PP2A	protein phosphatase 2A
PTK7	protein tyrosine kinase 7
ROCK	RhoA associated kinase
shRNA	short hairpin RNA
siRNA	short interfering RNA
SUnSET	surface sensing of translation
TBP	TATA binding protein
TCF	T-cell factor
TRITC	tetramethylrhodamine
VSMC	vascular smooth muscle cell
Wls	wingless

Chapter 1

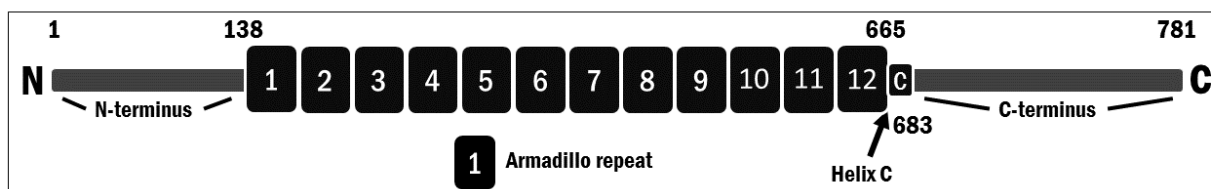
Review of literature

The β -catenin protein

β -catenin is a 781 amino acid protein, encoded by the *ctnnb1* gene in humans. β -catenin is an essential protein for eukaryotic life; it plays a key role in cell adhesion at the cell membrane and gene expression in the nucleus. β -catenin has no paralog protein that can compensate for its loss of function. Indeed, primary structure analysis of β -catenin in multiple species highlights the importance of this protein (Figure 1): there is 99% sequence conservation of β -catenin among five species, and furthermore, crystal structures of human and *Xenopus laevis* β -catenin are highly superimposable (1). Knockout studies in mice have revealed that β -catenin loss results in normal development until embryonic day 6.5 (E6.5), where gastrulation is impaired and the ectodermal layer fails to develop. By E8.5 the embryo is almost completely disorganized and most structures are unrecognizable, and near complete resorption of the failed embryo occurs by E9.5 (2). Furthermore, mutations to β -catenin can often result in atypical cell proliferation and progression of a variety of cancers, such as colorectal (3), gastric (4), and hepatocellular cancers (5). Therefore, understanding the structure and function of β -catenin is of great developmental and clinical importance.

Figure 1

A



B

<i>Homo sapiens</i>	1	A....D...E.....EE...TS
<i>Mus musculus</i>	1	A....D...E.....EE...TS
<i>Rattus norvegicus</i>	1	A....D...E.....EE...TS
<i>Danio rerio</i>	1	S...E...D.....DE...-N
<i>Xenopus laevis</i>	1	A....D...E.....DE...TN

<i>Homo sapiens</i>	61	S...T.E.....A...
<i>Mus musculus</i>	61	S...T.E.....A...
<i>Rattus norvegicus</i>	61	S...T.E.....A...
<i>Danio rerio</i>	60	N...N...E.....S...
<i>Xenopus laevis</i>	61	S...T.D.....S...

<i>Homo sapiens</i>	121	
<i>Mus musculus</i>	121	
<i>Rattus norvegicus</i>	121	
<i>Danio rerio</i>	120	
<i>Xenopus laevis</i>	121	

<i>Homo sapiens</i>	181	A.....
<i>Mus musculus</i>	181	A.....
<i>Rattus norvegicus</i>	181	A.....
<i>Danio rerio</i>	180	S.....
<i>Xenopus laevis</i>	181	A.....

<i>Homo sapiens</i>	241	..M.....
<i>Mus musculus</i>	241	..M.....
<i>Rattus norvegicus</i>	241	..M.....
<i>Danio rerio</i>	240	..V.....
<i>Xenopus laevis</i>	241	..M.....

<i>Homo sapiens</i>	301	T.....
<i>Mus musculus</i>	301	T.....
<i>Rattus norvegicus</i>	301	T.....
<i>Danio rerio</i>	300	T.....
<i>Xenopus laevis</i>	301	S.....

<i>Homo sapiens</i>	361	L...P.....
<i>Mus musculus</i>	361	L...P.....
<i>Rattus norvegicus</i>	361	P...P.....
<i>Danio rerio</i>	360	L...P.....
<i>Xenopus laevis</i>	361	L...S.....

<i>Homo sapiens</i>	421	L.....E...
<i>Mus musculus</i>	421	L.....E...
<i>Rattus norvegicus</i>	421	L.....E...
<i>Danio rerio</i>	420	P.....D...
<i>Xenopus laevis</i>	421	L.....E...

<i>Homo sapiens</i>	481	
<i>Mus musculus</i>	481	
<i>Rattus norvegicus</i>	481	
<i>Danio rerio</i>	480	
<i>Xenopus laevis</i>	481	

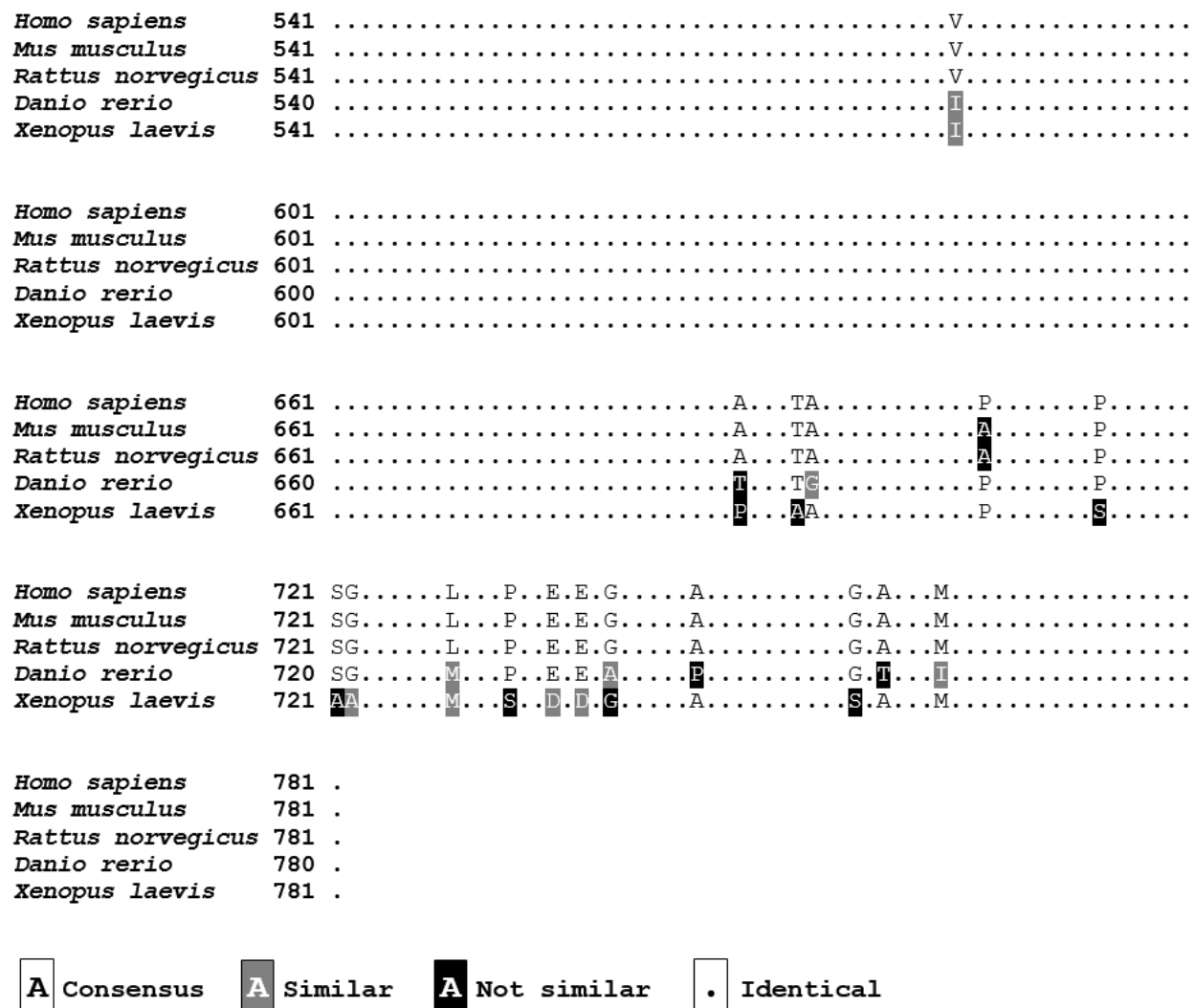


Figure 1: Basic structure and sequence alignment of β -catenin among 5 species. A. The structural domains of β -catenin are represented in the schematic with the corresponding amino acid that forms a domain boundary. **B.** The amino acid sequence of the β -catenin protein among the species shown was aligned using the T-Coffee multiple sequence alignment server (6).

Structure and protein interactions

There are four distinct domains that comprise β -catenin, which have been revealed through X-ray crystallography: an N-terminal domain spanning the first 137 amino acids, a series of 12 consecutive armadillo repeats encompassing amino acids 138-664, an α -helix immediately adjacent to the armadillo repeat region from amino acids 667-683 (known as helix C), and the C-terminal transactivation domain tail that forms the remainder of the protein (1). An important feature of β -catenin is its variable isoelectric point (pI) values across these regions: 4.4 for the N-terminus, 8.3 for the armadillo repeats, and 4.3 for the helix C/C-terminus. The significance of the higher pI in the armadillo repeats will be discussed below.

Armadillo repeats

The armadillo repeat region is entirely made up of α -helices that are connected by loops and form a superhelix structure; generally each of the 12 individual armadillo repeats is defined by three consecutive α -helices. The first helix (helix 1, H1) is a shorter sequence that makes 2 turns, the second helix (H2) makes 2-3 turns, and the final larger helix 3 (H3) makes 3-4 turns. H2 and H3 are packed tightly anti-parallel to each other and separated by a hairpin loop, while H1 is oriented perpendicular to H2 and H3, interacting with the amino-terminus of H2 and the carboxy-terminus of H3 (Figure 2). Armadillo repeats 1 and 7 do not have an H1 helix, which in the case of repeat 7, may provide extra rigidity to the overall superhelix structure. Overall, this region forms a single densely packed structural domain in which neighbouring armadillo repeats interact with one another and provide a large area for interactions with a variety of proteins. Importantly,

this large region is not symmetrically charged, and a positively charged groove spans the first 10 repeats, contributed mostly by the basic residues in each of the H3 helices, and in part by some basic residues from the H1 helices that reach into the groove (7). This feature is essential for β -catenin's ability to bind to several of its binding partners.

Figure 2

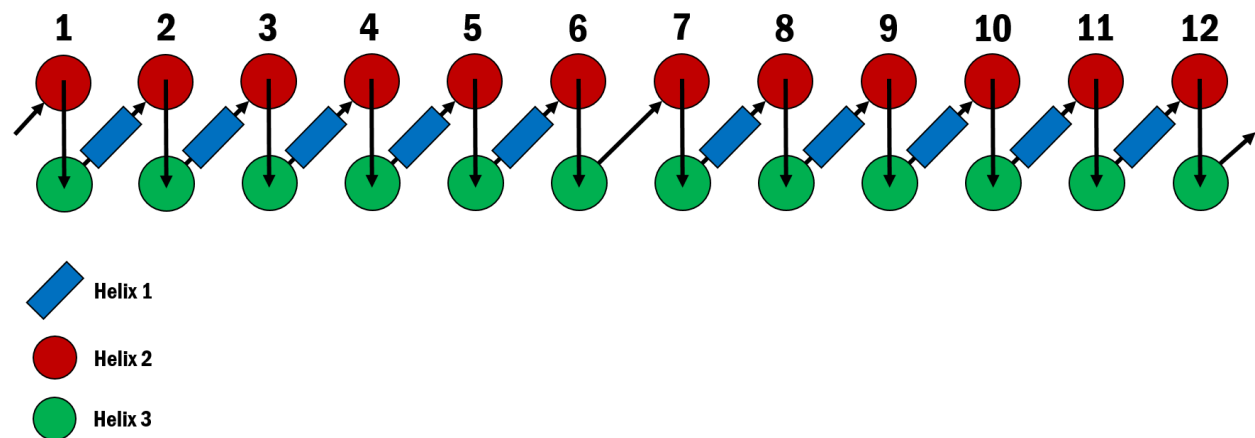


Figure 2: Helix structure of β -catenin armadillo repeats. Simplified representation of the 12 armadillo repeats in β -catenin. Helix 2 and 3 are anti-parallel to each other, while helix 1 runs perpendicular to helix 2 and 3. Note that helix is not present in armadillo repeats 1 and 7.

For example, the T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) transcription factors are well-known to be co-activated by β -catenin by direct interaction following Wnt signalling activation, resulting in target gene transcription (8). The crystal structure of the *Xenopus* TCF3: β -catenin interaction has been resolved using TCF3's β -catenin binding domain (amino acids 1-63) and the armadillo repeat region of human β -catenin (amino acids 134-664). The crystal structure revealed that TCF3 interacts along armadillo repeats 3-10 of β -catenin in an antiparallel fashion, and, importantly, aspartate 16 and glutamate 24 of TCF3 are key interacting points. Also, three aspartate residues

found between amino acids 25-29 significantly contributed to the interaction. These key interactions are owed to their negative charge which promotes a high affinity with the positive groove of the armadillo repeats (9). Similarly with TCF4, point mutations confirmed that the conserved Asp 16 and Glu 17 residues were necessary for binding to β -catenin (10).

The LEF-1 interaction with β -catenin, which is crucial for proper axis formation in *Xenopus*, occurs within the N-terminal region of LEF-1 (amino acids 1-71) and between armadillo repeats 1-12 of β -catenin (more detailed deletion analysis of β -catenin did not pinpoint specific repeats) (11). A peptide charge analysis of LEF-1 amino acids 1-71 reveals that this sequence carries a net -10 charge.

The minimal binding domain of Adenomatous polyposis coli (APC) has been characterized as an 11 amino acid fragment that is repeated three times in APC from amino acids 1020-1169. Surprisingly, even just one of these repeats fused to GST was sufficient for binding to β -catenin, and, perhaps unsurprisingly, each of these sequence repeats contained a net negative charge of at least -3 (12). APC is an important player in β -catenin regulation during canonical Wnt signalling.

Axin interacts between armadillo repeats 2 and 5 of β -catenin. There are many hydrophobic contact points that are essential for this interaction, along with a critical aspartate 474 residue on Axin that interacts with lysine 292 of β -catenin. Mutations to this aspartate residue abolishes the interaction between the two proteins (13). Axin is also important for forming a complex with β -catenin, GSK3, and CKI to regulate β -catenin in Wnt signalling (14).

The same case can be made for non-Wnt signalling partners that interact with β -catenin in the armadillo region. For example, the cytoplasmic tail of E-cadherin binds to repeats 3-12 of β -catenin (15), and this interaction stabilizes the cytoplasmic tail of E-cadherin and may be a mechanism to regulate cadherin turnover (16). Once again, interrogation of the E-cadherin cytoplasmic region shown to interact with β -catenin reveals a net -30 charge. It naturally follows that the cytoplasmic tails of several cadherins are conserved, and that they are each highly negatively charged.

N-terminus

The N-terminal tail is unstructured and does not form a stably folded structure in itself, but does interact with α -catenin and contains many important regulatory regions.

α -catenin, unlike most other exclusively armadillo region binding partners of β -catenin, interacts primarily with the N-terminus and encroaches only slightly into the first armadillo repeat of β -catenin. Extensive interaction analysis has been carried out using highly conserved *Drosophila* α - and β -catenin homolog proteins (the β -catenin homolog is named Armadillo), which determined that the interacting region spans amino acids 90-165 of Armadillo. Mutational analysis of Armadillo revealed that charged amino acids arginine, aspartate, and glutamate were essential for binding to α -catenin (17). In addition, a separate study looked at the contribution of polar amino acids threonine 120 and tyrosine 142 in this interaction and concluded that they too were essential for binding (18). Finally, and perhaps most definitively, a crystal structure of the murine α - and β -catenin interaction corroborated many of these previous observations. For example, the structure identified aliphatic helices on both α - and β -catenin to be critical binding

domains, which is comprised of the aforementioned T120 and Y142 of β -catenin and contained many of the charged amino acids used for point mutations by Pai et al. The study also narrowed the interacting region of α -catenin to be between amino acids 118-149 of β -catenin (19).

An important regulatory region lies in the N-terminus of β -catenin. Serines 33, 37, threonine 41, are each targets for phosphorylation by glycogen synthase kinase 3 (GSK3) following a “priming” phosphorylation at serine 45 by casein kinase I (CKI) (20, 21). This is a very significant event in the stability and subsequent function of β -catenin, and will be discussed in more detail in the sections “Canonical Wnt signalling” and “Post-translational modifications”.

Helix C

Prior to a 2008 study by Xing et al., in which a complete β -catenin crystal structure was analyzed, it was thought that β -catenin contained only three main domains. Essentially, helix C was never identified due to incomplete crystal structures. The apparent purpose of helix C is to cap the C-terminal end of armadillo repeat 12's H3 helix, allowing for a packed structure and hiding exposed hydrophobic residues of H3 by running parallel to it.

Aside from completing the superhelix structure of β -catenin, helix C is also responsible for some crucial protein interactions. The authors hypothesized that the inhibitor of β -catenin and TCF-4 protein (ICAT), a negative regulator of canonical Wnt signalling, interacts in part with helix C by analyzing published crystal structures of the β -

catenin:ICAT complex and superimposing those with their own crystal structure (22). Furthermore, pull-down assays using Chibby protein showed that the helix C region is essential for high affinity binding. Chibby is another inhibitor of Wnt target gene activation (23). Based on the data provided, helix C clearly plays an important role for determining β -catenin interactions and may be a key pharmacological target due to its relatively short sequence.

C-terminus

The C-terminal domain of β -catenin is chiefly responsible for recruiting the transcriptional machinery to target genes, and is more loosely structured than the armadillo repeats preceding it. To date, several transcription activating proteins have been identified as interactors with β -catenin.

The TATA binding protein (TBP) has been shown to interact with β -catenin in the armadillo 12-C-terminus region *in vitro* (24). Also, the same armadillo 12-C-terminus region interacts with the Mediator 12 (MED12) subunit of the Mediator complex, which is essential for class II gene transcription and directly recruits RNA polymerase II (25). Inhibition and silencing of MED12 resulted in a loss of Wnt-mediated β -catenin transactivation (26).

An important histone acetyltransferase (HAT) that binds to β -catenin is the CREB binding protein (CBP). This protein normally acetylates lysine residues of histones 3, 4, 2A, and 2B. These modifications relax the chromatin to allow nearby gene transcription (27). Interestingly, some studies suggested that CBP inhibits Wnt signalling, while others

have shown that it can activate Wnt signalling. The results are conflicting since one study has shown that over-expression of CBP enhances TOPflash reporter activity, and an inhibitor of CBP can abrogate this activation (28). Another reports that siRNA mediated silencing of CBP activates TOPflash (29). Ultimately, it is thought that a host of other interactions may be responsible for contributing to the particular role of CBP. Although these reports are seemingly conflicting in regards to the true nature of the CBP interaction with β -catenin, the experiments do highlight the importance of cellular context in conferring specific β -catenin function.

An important consideration is that many of these interactions do not occur exclusively in the C-terminal domain, that is, armadillo repeat 12 and helix C are also minimally necessary for a functional interaction. This was also documented experimentally through mutation analysis. Deletion of the helix C region of the Armadillo protein from the remaining C-terminus in *Drosophila* abolished Wnt mediated transactivation; restoring this region was necessary for proper transcriptional co-activity (30).

β -catenin cellular function

Membrane

β -catenin plays an integral role in cell adhesion, and provides a direct link between the cell membrane and actin cytoskeleton. β -catenin directly interacts with the cytoplasmic tail of classical cadherins.

Cadherins (named after “calcium dependent adhesion”) are single-pass transmembrane glycoproteins that form the basis of the adherens junction, and are

named according to the tissue in which they were first discovered (e.g.: N-cadherin for neurons and E-cadherin for epithelial). They have five extracellular domain repeats (EC1-5), where the most distal repeat, EC1, can homodimerize with the EC1 domain of another cadherin. The rigidity of the EC domains is dependent on Ca^{2+} , and there are conserved calcium binding domains linking each EC domain that promote the formation of a rigid rod-like shape among the ECs when bound to Ca^{2+} (31). The intracellular domain of cadherins directly bind the armadillo repeats of β -catenin; the relatively unstructured intracellular domain of cadherins allow it to wrap around all twelve armadillo repeats of β -catenin and form a very tight interaction (32).

As mentioned above, α -catenin interacts with the N-terminus/armadillo repeat 1 region of β -catenin, and so β -catenin is able to simultaneously interact with both cadherins and α -catenin. Detailed analysis has determined that α -catenin interacts directly with F-actin (33). β -catenin's unique position as the link that bridges the cell membrane with the cytoskeleton makes it a key regulator of cell adhesion. This is evident in β -catenin null mice during embryonic development, whereby cells inadvertently detach from the ectoderm and fail to form the mesoderm (2). Similarly in human stomach carcinoma cell lines, β -catenin is expressed as a mutant with an N-terminal deletion that cannot bind α -catenin, resulting in cells that distinctly grow in loose aggregates or single cells (34). Figure 3 is a schematic representation of the adherens junction.

Figure 3

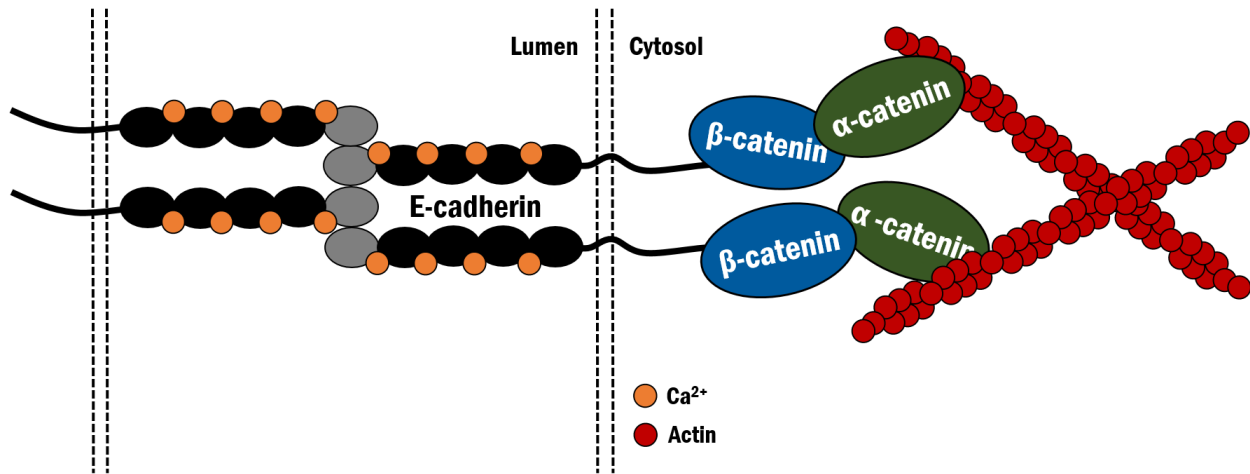


Figure 3: The adherens junction. The extracellular cadherin domain interacts with the domains of nearby cells in a calcium dependent manner. The intracellular cadherin domain interacts with β -catenin directly, which in turn interacts with actin-bound α -catenin to regulate cell adhesion.

Nucleus

Outside of the cadherin complex, β -catenin is an effector of Wnt signalling, many of whose target genes are involved in cell proliferation and epithelial-to-mesenchymal transition (EMT). Briefly, in the absence of Wnt signal, β -catenin is highly associated with a “destruction complex” that results in its N-terminal phosphorylation by GSK3, subsequent ubiquitination, and proteasomal degradation. Upon Wnt binding to the Frizzled (Fz) and lipoprotein receptor-related protein (LRP) receptors, β -catenin is freed from the complex and is no longer phosphorylated by GSK3. β -catenin then accumulates in the cytoplasm and translocates into the nucleus.

Once inside the nucleus, β -catenin binds the TCF/LEF family of transcription factors and co-activates TCF/LEF target genes. Many of these Wnt target genes are highly

expressed in various cancers, including genes that encode c-Myc and matrix metalloproteinase (MMP) 2 and 9 (35-37). The precise mechanism in which β -catenin moves into the nucleus is still unknown, although it is an importin and nuclear localization signal (NLS) independent mechanism. Some studies speculate that it is the specific set of proteins in which β -catenin interacts with that determine where it is retained (38-40).

Cytoplasm

A study has suggested a role for β -catenin in the cytoplasmic shuttling of newly synthesized E-cadherin from the endoplasmic reticulum (ER) to the plasma membrane. This may act as an important regulatory step for E-cadherin function that may be modulated through Wnt signalling (41).

There is no other known function of β -catenin in the cytoplasm outside of its regulation by canonical Wnt signalling, which will be discussed in its own section. It is believed that once β -catenin protein is made in the cytoplasm, subsequent signalling events and post-translational modifications promote β -catenin to the adherens junction or nucleus, where protein-protein interactions determine its function at those sites.

Comparison with other armadillo repeat proteins

Plakoglobin (γ -catenin)

Plakoglobin also contains 12 armadillo repeats and shares significant sequence homology with β -catenin in this region (83%) and 65% homology overall, but itself lacks the strong

transactivation domain that β -catenin possesses. Some studies have shown that plakoglobin can weakly activate canonical Wnt signalling by interaction with TCF/LEF (42, 43), but it cannot compensate for loss of β -catenin. Similar to β -catenin, plakoglobin interacts directly with classical cadherins and α -catenin to regulate cell adhesion (44). In addition to this, plakoglobin plays a central role in desmosomes, which are junctions required for strong adhesion between cells that can resist shearing forces. Plakoglobin interacts with the desmosomal cadherins desmocollin and desmoglein and links them to intermediate filaments (such as keratin) by also interacting with desmoplakin, thus providing a strong connection between the desmosome and intermediate filaments (45, 46). Much like how plakoglobin cannot compensate for β -catenin function in the nucleus, β -catenin is unable to interact with these factors and compensate for plakoglobin at the desmosome.

p120-catenin (δ -catenin)

The p120-catenin family of proteins contain 9 armadillo repeats and are sub-divided into two groups. One group, known as the classical p120-catenin subfamily, are responsible for binding to cadherins and mediating their phosphorylation and nearby β -catenin through Fer and Fyn kinase (47). The second is known as the plakophilin subfamily and works in concert with plakoglobin to maintain the desmosome complex by interacting with desmoplakin, but not with the desmosomal cadherins (48, 49) (Figure 4).

Studies have also suggested a nuclear role for p120-catenins, such as the ability to interact with the transcriptional regulator Kaiso and prevent it from repressing target

gene expression, some of which are Wnt targets. It is thought that p120-catenin accomplishes this by physical interaction and sequestration into the cytoplasm (50).

Figure 4

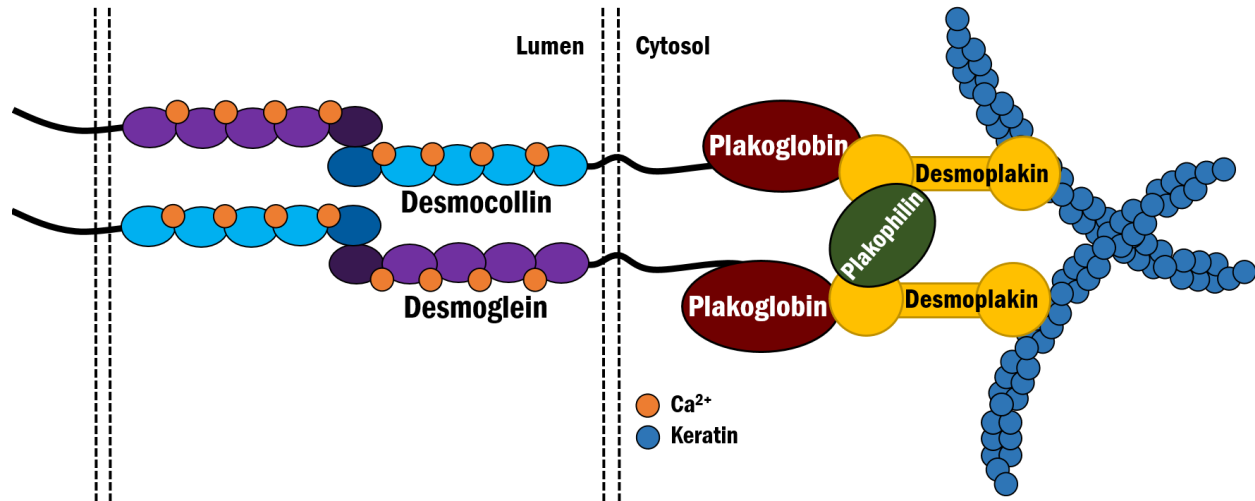


Figure 4: The desmosome. The extracellular domains of desmosomal cadherins (desmocollin and desmoglein) interact with those of nearby cells. Their intracellular domains interact directly with plakoglobin, which in turn interacts with intermediate filament (e.g. keratin)-bound desmoplakin. Plakophilin stabilizes this junction by interacting with desmoplakin.

Post-translational modifications and β -catenin function

Phosphorylation and membrane function

Cadherin phosphorylation

At the membrane, a number of studies have shown that various phosphorylation of the cytoplasmic tail of E-cadherin can enhance binding to β -catenin. For example, one study concluded that E-cadherin is phosphorylated at serine 684 by casein kinase II (CKII) and serines 686 and 692 by GSK3, *in vitro*. Mutational analysis in which these residues were substituted with alanine revealed a significant reduction in binding with purified β -

catenin protein (32). A separate study investigated the cytoplasmic E-cadherin region spanning amino acids 833-862. They also determined through mutation analysis that CKII phosphorylates E-cadherin at serines 840, 863, and 855, while GSK3 phosphorylates it at serine 849. Further investigation demonstrated that β -catenin binding affinity was enhanced with E-cadherin phosphorylated, and greatly abolished in alanine mutants. Lastly, stable expression of wild-type or S853A/S55A E-cadherin mutants in Wnt-1 cultured cells resulted in a significant reduction in cell adhesion in the mutant cell line (51).

Phosphorylation of VE-cadherin (vascular endothelial) at tyrosines 658 and 731 by Src kinase activation has been reported to prevent binding with β -catenin. Phosphomimetic mutations to these sites resulted in an increased mesenchymal state in cells, similar to cell lacking VE-cadherin (52).

β -catenin phosphorylation

Threonine 120 and tyrosine 142 phosphorylation of β -catenin is an important regulatory feature for adherens junction complex stability, and has been extensively studied. As mentioned earlier, α -catenin binds to β -catenin amino acids 118-149. Analysis of the sequence within that region revealed two potential phosphorylation sites: threonine 120 and tyrosine 142. The mutation of these sites to alanine resulted in a loss of interaction with α -catenin *in vitro*. The authors summarized that these two sites may be significant for proper α -helix formation in β -catenin and changes to these sites could lead to a progression of cancers (18).

Further investigation into the tyrosine 142 site revealed that it is directly phosphorylated by the Fer and Fyn tyrosine kinases; Fyn kinase is a member of the Src

family of tyrosine kinase oncogenes and is involved in critical signalling pathways, such as T-cell receptor signalling (53). Moreover, ectopic expression of these kinases in cell culture reduced the interaction between β - and α -catenin by co-immunoprecipitation. The study also concluded that Fer and Fyn kinases are constitutively docked with p120-catenin, and Ras mediated phosphorylation of p120-catenin enhances its association with E-cadherin, thus allowing Fer and Fyn to phosphorylate nearby β -catenin to disrupt the cadherin complex (54).

Another important regulatory region governing the cadherin complex is β -catenin phosphorylation at tyrosine 654 by Src. This modification has been hypothesized to negatively regulate the interaction between β -catenin and E-cadherin. *In vitro* kinase analysis determined direct phosphorylation at both tyrosine 86 and 654, however, only phospho-tyrosine 654 reduced the β -catenin/E-cadherin interaction. This was confirmed by creating point mutations of tyrosine 86 or tyrosine 654 to phenylalanine on β -catenin. Furthermore, *in vivo* phosphatase inhibition proved that tyrosine 654 phosphorylation was essential for E-cadherin binding in cells. The authors observed that tyrosine 654 is within armadillo repeat 12 and part of the E-cadherin binding domain on β -catenin. They surmised that since the positive charge of the armadillo repeats is what allows β -catenin to bind many of its interacting partners, subsequent phosphorylation and introduction of negative charges disrupts the binding. Indeed, they had used tyrosine 654 to aspartate β -catenin mutants to show that E-cadherin interacts significantly less than with tyrosine to phenylalanine mutants (55).

The balance between phospho and non-phospho tyrosine 654 is regulated by protein tyrosine phosphatase 1B (PTP1B) and upstream by Fer. A study has shown that

Fer can directly phosphorylate PTP1B to enhance its interaction with cadherin and allow it to continuously de-phosphorylate β -catenin at tyrosine 654. This constant de-phosphorylation maintains β -catenin interaction at the adherens junction (56). Therefore, Fer seems to play significant yet opposing roles on regulating β -catenin association at the cell membrane.

Phosphorylation at tyrosine 654 is also important for regulating β -catenin localization in dendrites. When hippocampal neurons were depolarized using potassium chloride, there was a redistribution of β -catenin from dendritic shafts to spines. This event is in concert with an increased association of β -catenin to α -catenin at the adherens junction. Further investigation revealed that use of a tyrosine kinase inhibitor provided similar effects of β -catenin localization as depolarization. This prompted the authors to hypothesize that phosphorylation at tyrosine 654 is the cause for β -catenin distribution. Use of point mutated β -catenin confirmed this, as tyrosine to aspartate mutants failed to distribute following depolarization, while tyrosine to phenylalanine mutants could. The phenotypic result of this redistribution was increased post-synaptic density following depolarization, an important feature of organizing neurotransmitter synapses on the synaptic cleft (57). Thus, tyrosine 654 is an essential regulatory site for β -catenin and cadherin binding in the cell adherens junction.

Tyrosine 489 has also been shown to be important in regulating the N-cadherin (neuronal): β -catenin interaction through a mechanism involving a complex comprised of the transmembrane receptor Roundabout, its ligand Slit, and the N-cadherin binding Abelson kinase. This signalling pathway is thought to be important for growth cone

regulation in axons, which in part involves Abelson phosphorylation of β -catenin to abolish interaction with N-cadherin (58).

Phosphorylation and nuclear function

We have seen evidence that demonstrates tyrosine 654 phosphorylation of β -catenin preventing adherens junction formation through reduced interaction with cadherin. A secondary effect of phosphorylation at tyrosine 654 is enhanced binding to TBP. Furthermore, a tyrosine 654 to glutamate mutant β -catenin enhanced TOPflash reporter activity more than wild-type β -catenin (59).

Much like how tyrosine 654 reduces the cadherin interaction and increases nuclear β -catenin activity, the same is true for tyrosine 142 phosphorylation. The B-cell CLL/lymphoma 9 protein-2 (BCL9-2) is an important β -catenin interacting partner in the nucleus and co-activator of Wnt target gene expression (60). Further investigation has shown that tyrosine 142 to alanine mutants have reduced interaction with BCL9-2, while a tyrosine to glutamate mutant interacts more highly than wild-type β -catenin. The alanine mutant can also be sequestered away from BCL9-2 by α -catenin, resulting in reduced TOPflash activity. Lastly, the phospho- β -catenin/BCL9-2 interaction was determined to play a crucial role in mesoderm patterning during zebrafish embryogenesis (61). Clearly, based on the results above, the equilibrium between phosphorylated and non-phosphorylated β -catenin at these sites greatly determines cellular function and the identity of its interacting partners, therefore conferring a contextual role for β -catenin.

Protein kinase B, also known as Akt, is an important regulator of mTOR signalling and plays a role in cell survival and metabolism (62). Akt has been shown to directly

phosphorylate β -catenin at serine 552 in response to epidermal growth factor (EGF) stimulation in multiple cell lines. Through immunofluorescent imaging analysis, the authors demonstrated that serine 552 to alanine β -catenin mutants were almost exclusively localized in the cell membrane, while phosphomimetic serine to aspartate mutants were highly localized in both the cytosol and nucleus. Finally, TOPflash reporter activity and tumour cell invasion was increased in S552D mutants compared to S552A or wild-type β -catenin (63). Although it was not mentioned in the article, it is very likely that this mutation results in a loss of cadherin binding, which then promotes β -catenin's nuclear activity.

Protein kinase A (PKA) also phosphorylates β -catenin, in this case at serine 675. One study examined the stability of β -catenin following phosphorylation by PKA, and determined that this event results in enhanced β -catenin stability by inhibiting its ubiquitination. A side effect of this stability is enhanced co-operativity with TCF4 on Wnt target gene expression (64). A separate study suggested that PKA also phosphorylates β -catenin at serine 552 in addition to serine 675, however, other studies have refuted the ability of PKA to robustly phosphorylate serine 552 *in vivo*. Nevertheless, the paper asserted that these phosphorylation events lead to increased β -catenin transcriptional activity and, specifically with the serine 675 phosphorylation, enhanced interaction with CBP (65).

In 2008, new research suggested that Rac1 signalling could significantly and directly influence β -catenin during limb outgrowth. In addition to genetic interactions with β -catenin and dickkopf-1, the study revealed that Rac1 activation by Wnt7a could result in

the dual phosphorylation and activation of c-Jun-N-terminal kinase 2 (JNK2), a member of the mitogen activated protein kinase (MAPK) family. These kinases are proline directed kinases: they preferentially recognize and phosphorylate serines or threonines immediately C-terminal to proline residues (so-called SP/TP sites). Of the three SP sites on β -catenin (serines 191, 246, and 605), JNK2 was shown to directly phosphorylate β -catenin at serines 191 and 605. This event causes nuclear localization of β -catenin and is critical for Wnt target gene activation (66).

Perhaps the most well-known phosphorylation event for β -catenin is the dual kinase phosphorylation at serines 33, 37, threonine 41 and serine 45 by GSK3 and CKI, respectively. Under baseline conditions, cytoplasmic β -catenin is actively associated with a destruction complex that is comprised in part of GSK3 and CKI, which constitutively phosphorylate β -catenin and target it for degradation. This prevents cytoplasmic accumulation of β -catenin, and in turn maintains low expression of Wnt target genes, many of which are generally involved in EMT. Furthermore, over-expression of a scaffolding protein in the destruction complex, Axin, resulted in higher phosphorylation of β -catenin at these sites. The study verified that priming occurs by CKI and is facilitated by the Axin scaffold, allowing both CKI and GSK3 to be in close proximity to β -catenin. Loss of CKI in *Drosophila* produced a phenotype similar to loss of Axin and GSK3 (20).

Protein phosphatase 2A (PP2A) can dephosphorylate β -catenin at multiple sites, including S33, 37, T41, and S45. PP2A is also found in the β -catenin destruction complex and is an important regulatory factor for regulating β -catenin protein stability during canonical Wnt signalling (67).

Ubiquitination

Following β -catenin phosphorylation at S33, 37, T41, and S45, it becomes targeted for degradation by the β -transducin repeat containing E3 ubiquitin protein ligase (β -TrCP). This E3 ubiquitin ligase is also found in the destruction complex, however, it does not recognize β -catenin in its unphosphorylated form. Following phosphorylation, β -TrCP binds to β -catenin and ubiquitinates the protein at lysines 19 and 49. Double mutants of these lysines results in stabilized protein, enhanced TOPflash activity, and significantly reduced total ubiquitination (68). In the absence of β -TrCP, Wnt signalling is greatly induced in *Xenopus* embryos, and they perform axis duplication during embryogenesis. This corresponds with increased stability of β -catenin protein in 9 day embryos (69).

The ubiquitin ligase UBR5 can also ubiquitinate β -catenin at lysines 29 and 11 in the nucleus, while also causing the nuclear localization of GSK3. These two acts are thought to promote β -catenin stability and nuclear activity (70).

Acetylation

As mentioned above, CBP is a known transcriptional partner of β -catenin and directly interacts with its C-terminus. The acetylation of β -catenin at lysine 49 by CBP regulates *c-myc* expression levels, but not other Wnt targets. Mutant β -catenin which cannot be acetylated by CBP enhance *c-myc* expression in cells. This observation was deduced due to the prevalence of lysine 49 mutations of β -catenin in anaplastic thyroid carcinomas. Thus, the ability to upregulate c-Myc and hence cell proliferation is seen as a partial mechanism for the propagation of this carcinoma (71).

Finally, the p300/CBP-associated factor (PCAF) has also been shown to acetylate β -catenin, at lysine 49 (similar to CBP) and at lysine 19. While CBP acetylation at lysine 49 could only activate *c-myc* expression and not other Wnt targets, dual acetylation by PCAF could enhance TOPflash activity. The interaction between β -catenin and PCAF was enhanced following stimulation of Wnt signalling, which increased β -catenin protein stability. The acetylation events are thought to prevent β -catenin ubiquitination and proteasomal degradation, and PCAF gene silencing abrogates cancer cell migration and tumour growth (72).

Summary of protein-protein interactions and post-translational modifications

The schematic representation of β -catenin below highlights the regions that are responsible for binding to the interacting partners mentioned above (Figure 5). We can see from the schematic the competition of different interacting partners in the armadillo region that can confer entirely different functions on β -catenin. Post-translational modifications play a role in determining which specific interactions β -catenin will partake, and what its subsequent role will be. Figure 6 summarizes the many post-translational modifications discussed thus far.

Figure 5

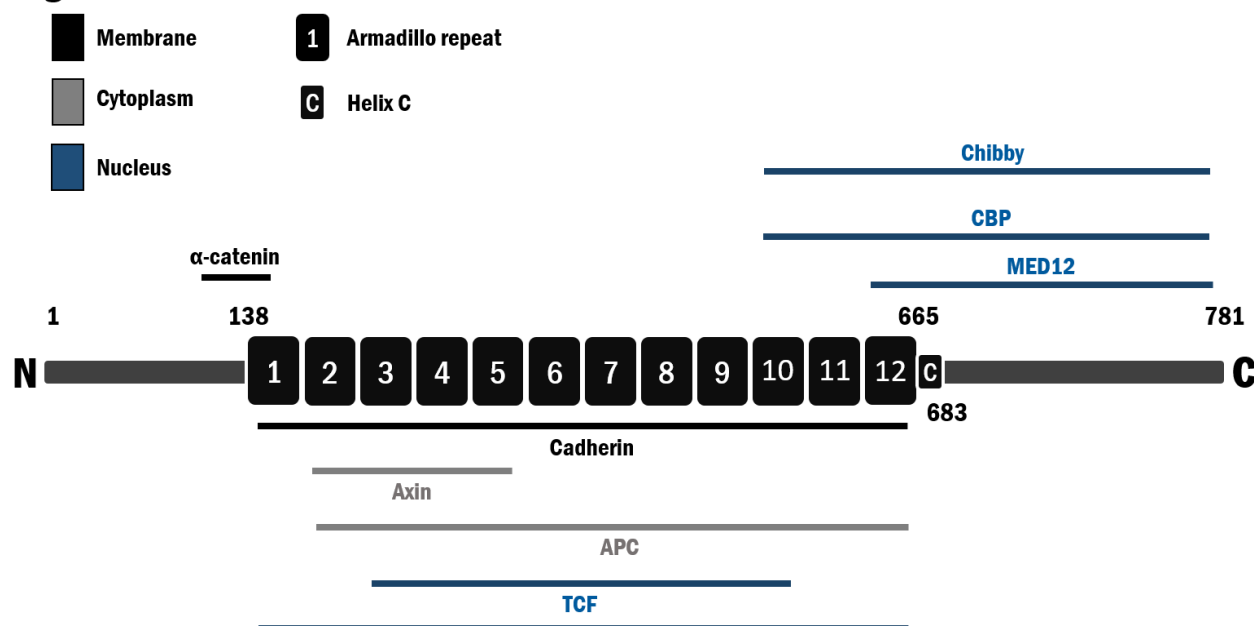


Figure 5: β -catenin protein interactions. β -catenin interacts with a variety of proteins at the cell membrane, cytoplasm, and nucleus. Some key interactions are depicted, mapped to the region in which they interact with β -catenin.

Figure 6

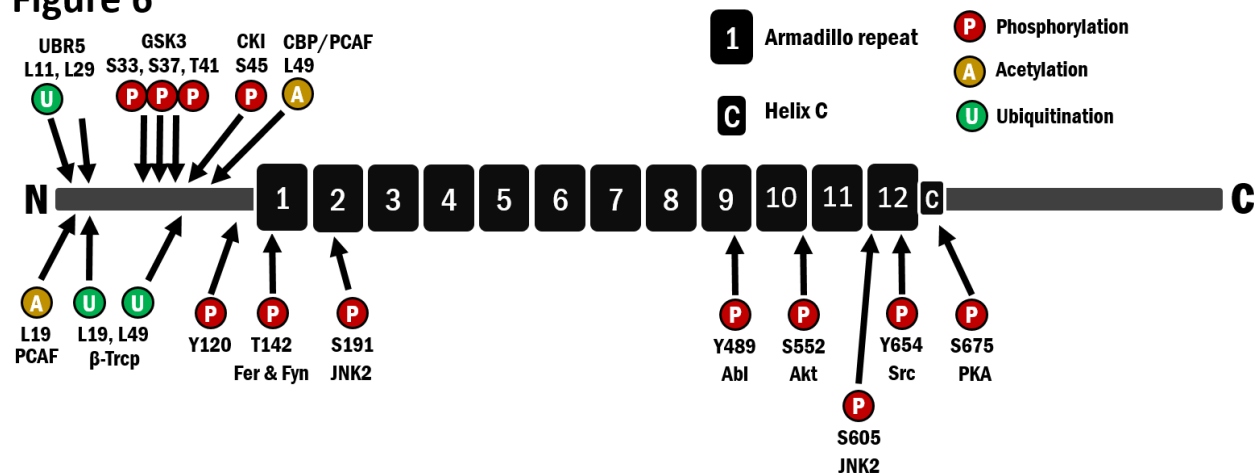


Figure 6: β -catenin post-translational modifications. β -catenin function, stability, and protein interactions are regulated by various post-translational modifications. Key modifications are depicted above and are mapped to specific sites on β -catenin.

Canonical Wnt signalling

Canonical Wnt signalling plays an essential role during embryonic development, including proper progression of gastrulation, paraxial mesoderm development, and limb bud development (36). Post-development, Wnt signalling is responsible for the regulation of target genes involved in EMT (73), cell cycle progression (74, 75), and reduction of cell-cell contact (76). Aberrations to the signalling pathway are found in numerous cancers, most notably breast, colorectal, lung, and prostate cancer (77).

Wnt ligands

Wnts make up a family of 19 glycoproteins that share a similar distribution of 23-24 cysteine residues which are essential to proper folding and function. These glycoproteins are defined by their amino acid sequences and not by function, are all 350-400 amino acids in length (78), and have a conserved cysteine residue which is subsequently palmitoylated to increase hydrophobicity and thus solubility (79).

Once these Wnts are produced and modified, they are ready to be secreted by signalling cells. Until recently, it was unknown how these modified proteins were released into the extracellular milieu by signalling cells, until the discovery of the evenness interrupted (Evi)/wntless (Wls) transmembrane protein in *Drosophila* by two separate research groups in 2006 (80, 81). Wls encodes a seven-pass transmembrane protein that is a requirement for Wnt secretion, but is not needed in Wnt receiving cells. Mutant Wls cells have a marked increase of Wnt ligand without an increase in Wnt gene expression, and the distribution of Wnt protein outside of the source cells was decreased (80).

Wnt receptors Frizzled and LRP

Upon reaching the membrane of a target cell, Wnts bind preferentially to the Frizzled (Fz) family of membrane receptors, of which there are ten members. Fzs are seven-pass transmembrane proteins containing an N-terminal extension known as the cysteine-rich domain (CRD), to which Wnts bind to directly (82). Although required for high affinity binding of Wnts, it has been shown that mutation of this CRD can still induce the signalling pathway, and it has been hypothesized that the main function of the CRD is to concentrate Wnts to the vicinity of other nearby binding sites of the receptor complex that are critical for signal transduction (83).

Another integral membrane receptor is the single membrane pass lipoprotein receptor-related protein (LRP) 5 or 6 (84, 85), which physically interacts with Fz to initiate Wnt signalling. Studies have shown that forced association of both Fz and LRP results in activation of Wnt signalling (86, 87), and that these two membrane proteins interact to form a Wnt receptor complex (88). Mutational analysis of LRP6 revealed that truncating the extracellular domain while leaving in the transmembrane and intracellular components intact results in constitutively active Wnt signalling, and Wnt binding to the membrane receptor complex results in a conformational change in the LRP intracellular domain that removes allosteric inhibition (89).

The cytoplasmic tail of LRP is dependent upon phosphorylation of five PPPSPxS motifs by both CKI and GSK3 (90). This phosphorylation event results in a much higher affinity between GSK3 and LRP's cytoplasmic tail, thus preventing phosphorylation of β -catenin (91). Removing only the intracellular component of LRP produces an inactive, or

dominant-negative, form (88). Similarly, mutation of even two of the five PPPSPxS motifs abolishes signalling, while mutation of only one motif severely abrogates activity. It has been hypothesized that all five motifs are necessary to induce phosphorylation of one another and interact with Axin in a synergistic manner (92).

Interestingly, there are thought to be two distinct pools of GSK3, plasma membrane and cytosolic associated, in which there are opposing roles in regards to Wnt regulation. The plasma membrane associated pool is thought to activate signalling by phosphorylation of CKI and LRP, while the destruction complex pool phosphorylates CKI and β -catenin to inhibit signalling. In both cases, CKI is constitutively phosphorylated, however its role varies depending on location and isoform. CKI γ is a membrane bound isoform, and is responsible for directly phosphorylating the LRP6 motifs upon Wnt stimulation, thus promoting binding of Axin to the cytoplasmic tail of LRP6 (93). CKI α , δ , and ϵ are exclusively cytosolic and are responsible for phosphorylating and priming β -catenin at serine 45, which provides a docking site for GSK3 phosphorylation of β -catenin at serine 33 and 37, threonine 41, and subsequent ubiquitination by β -TrCP and proteasomal degradation (90, 94).

The phosphorylation of LRP is a significant event in canonical Wnt signalling, as it results in the deactivation of the β -catenin destruction complex by drawing Axin to itself at the membrane. Tamai et al. showed that PPPSPxS phosphorylation of LRP6 results in co-immunoprecipitation with Axin, not seen in un-phosphorylated forms, suggesting that these motifs act as docking sites for Axin binding when phosphorylated (95). Additionally, studies have shown that Fz can directly interact with Disheveled (Dsh), another member

of the destruction complex that is involved in helping to promote Axin-GSK3 recruitment to the Fz/LRP membrane complex and to promote LRP6 phosphorylation and signal transduction (96).

The destruction complex

The β -catenin destruction complex is composed of tumour suppressor proteins Axin and APC, Ser/Thr kinases GSK3 and CKI, PP2A, Dsh, and β -TrCP. The pool of GSK3 involved in Wnt signalling acts independently from the pool that is responsible for the other known functions of GSK3, namely glycogen synthesis, cell cycle control, and inflammation. In these non-Wnt pathways, GSK3 is regulated by phosphorylation at Serine 21 and 9 (GSK3 α and GSK3 β , respectively) by Akt, resulting in its inhibition (97).

Axin acts as a scaffold protein and contains binding sites to CKI, GSK3, and β -catenin. The binding of these three proteins to Axin results in an increased local concentration that promotes efficient phosphorylation and subsequent inhibition of β -catenin (14, 98, 99). The efficiency of β -catenin phosphorylation by CKI and GSK3 has been shown experimentally to be significantly increased in the presence of Axin (14, 100, 101). Importantly, Axin binds to regions in GSK3 and CKI that are far from their catalytic subdomains, therefore Axin acts purely as a scaffolding protein and does not alter the kinase activity of these proteins (100, 102). Axin also contains a so-called DIX domain that is necessary for binding to Dsh (103), and an RGS domain near its amino terminus which binds to APC (104). The interaction of Axin with APC is required for phosphorylation of APC (103), an important feature of Wnt signalling.

APC is a large protein, weighing in at 310 kDa, and contains a central 1000 amino acid region that binds to β -catenin and Axin (105). Phosphorylation of APC at specific 20-mer repeats by both CK1 and GSK3 enhances its affinity for β -catenin by up to 1500 fold (106). There are Axin binding domains within APC's sequence termed SAMP repeats. These repeats, in combination with its β -catenin interacting domains, are essential for the destruction of β -catenin, and mutations to these domains results in oncogenesis (104, 107). It is thought that these SAMP repeats prevent PP2A from de-phosphorylating β -catenin and that APC therefore spatially prevents PP2A from accessing the N-terminal region of β -catenin (108). The ability for APC to interact with β -catenin and Axin, in conjunction with the ability of Axin to bind to CKI and GSK3, provides a mechanism in which β -catenin can be readily found by these kinases and phosphorylated, resulting in its subsequent degradation. The 20-mer repeats on APC are necessary for β -catenin's ubiquitination and subsequent degradation, although these repeats do not directly bind to β -catenin itself. Studies have shown that mutations or truncations to the repeats results in a failure of β -TrCP to co-immunoprecipitate with β -catenin, which suggests that they are essential for promoting the interaction of β -TrCP to β -catenin (109).

Mutational analysis of APC and Axin suggests that they are both involved in retaining β -catenin in the cytoplasm (110, 111). Although both Axin and APC contain CRM1-dependent nuclear export signal sequences, and despite the initial hypothesis that these sequences implicate both Axin and APC in β -catenin shuttling, experiments using fluorescence recovery after photobleaching have shown that APC and Axin enrich cytoplasmic β -catenin in a CRM1 independent manner and that neither protein

enhanced nucleocytoplasmic shuttling of β -catenin. Therefore, it is suggested that Axin and APC are involved in cytoplasmic retention of β -catenin (40, 111).

Target genes

The stability of β -catenin by Wnt signalling results in the activation of Wnt target genes through interactions with TCF/LEF transcription factors; however, β -catenin cannot bind DNA directly and serves as a co-activator (112). In the absence of Wnt signalling, TCF/LEF activity is repressed by Groucho binding, which is mediated by histone deacetylases (HDACs) (113). Upon Wnt stimulation, accumulated cytoplasmic β -catenin moves into the nucleus and is thought to displace Groucho from TCF/LEF as well as recruit several coactivators such as the histone acetylase CBP by direct interaction (29).

Countless Wnt target genes have been discovered that contain TCF/LEF regulatory sites, most of which are involved in cell proliferation. Examples include *c-myc* (114), *cyclin d1* (74), *matrix metalloproteinase 7* (115), *gastrin* (116), *vegf* (117), *folliculin* (118), and *e-cadherin* (down-regulated) (119). Hyperactive Wnt signalling therefore contributes to uncontrollable cell proliferation, which can lead to various cancers.

Mutations to β -catenin in cancer and disease

Aberrant Wnt signalling is known to be a main cause of colorectal cancer (CRC), mainly due to mutations in *apc* and *ctnnb1* (35). Mutations in the *ctnnb1* gene are present in almost 10% of CRC tumours and mutations in *apc* contribute to 85% of CRC (120). Most mutations of β -catenin occur at S37 and T41, affecting the phosphorylation sites of GSK3 β and thus preventing β -catenin degradation (121).

In fact, β -catenin mutations are found in a whole host of cancers and diseases. Mutations that affect β -catenin's ability to be phosphorylated by GSK3 are particularly prominent. For example, several human melanoma cell lines contain mutations at S37 of β -catenin that prevent phosphorylation and degradation (122). Up to 50% of ovarian carcinomas also have S37 and T41 mutations (123). S33, S37, and T41 mutations have been found in human pilomatricomas, a type of benign skin tumour (124). The same is true for thyroid carcinomas, as up to 46% of tumours were seen to have these mutation in β -catenin coinciding with significant reduction in membrane bound β -catenin (125).

Activating mutations of the Wnt pathway are seen in other cancers as well. For example, upper gastrointestinal tumours have a high number of β -catenin mutations, up to 20% of gastric carcinomas have activating mutations in *ctnnb1* (126). Additionally, human melanoma samples have mutations in *apc* and *ctnnb1* (122).

In summary, mutations in β -catenin's GSK3 regulatory sites result in stabilization of the protein and an active Wnt signalling phenotype.

Non-canonical Wnt signalling

There exist two signalling pathways that are considered “non-canonical” in that they do not involve LRPs as co-receptors and β -catenin as a downstream effector. Specific Wnts bind to the Fz receptor to initiate a separate signalling cascade, which will be discussed briefly below. They are the planar cell polarity (PCP) and the Wnt/calcium pathways.

Planar cell polarity pathway

Planar cell polarity defects are characterized in *Drosophila* as the mis-orientation of epithelial cells such as primordia of wings, legs, and eyes. For example in the wing, cells are oriented proximally-distally and grow hair that is pointed distally. In defective PCP, these hairs no longer grow in the same direction and are randomly oriented (127). The PCP pathway is initiated with Wnt4, Wnt5, or Wnt11 ligand binding to the Fz receptor followed by co-receptor dimerization with various candidates. These include the tyrosine-protein kinase transmembrane receptor ROR2 (128), protein tyrosine kinase 7 (PTK7) (129), and tyrosine protein kinase Ryk (130). This results in the recruitment of Dsh to the membrane by interaction of its PDZ (PSD-95, DLG, ZO1) and DEP (Dishevelled, EGL-10, Pleckstrin) domains and activates two GTPase pathways in parallel, the Rho and Rac pathways. The Rho pathway is activated through the Dsh associated activator of morphogenesis 1 (Daam1) protein, which interacts with both Dsh and Rho. This leads to activation of the RhoA-associated kinase (ROCK) and downstream cytoskeletal organization (131). The Rac pathway is activated independently of Daam1 and stimulates downstream JNK, which is required for proper cell polarity during gastrulation (132, 133).

Wnt/calcium pathway

These non-canonical Wnts also have the ability to stimulate Ca^{2+} release from the cell and initiate phosphatidylinositol signalling through trimeric G-proteins (134). This in turn activates downstream protein kinase C (PKC) and calcium-dependent kinase II (CamKII) signalling. Some studies also suggest that the PDZ and DEP domains of Dsh contribute to the activation of these pathways (135). PKC is hypothesized to promote proper tissue

separation during *Xenopus* gastrulation (136), while activation of the CamKII pathway results in the positive regulation of transcription factors such as nuclear factor of activated T-cells (NFAT) which promotes ventral cell fate in developing *Xenopus* embryos (137).

These two non-canonical Wnt pathways highlight the diverse nature of Wnts themselves, and the multifunctional characteristics of the Fz receptor. It is evident that specific co-receptors of Fz can help determine the downstream effects of Wnt signalling in both a β -catenin dependent and independent manner. The canonical and non-canonical Wnt pathways are summarized in Figure 7.

Figure 7

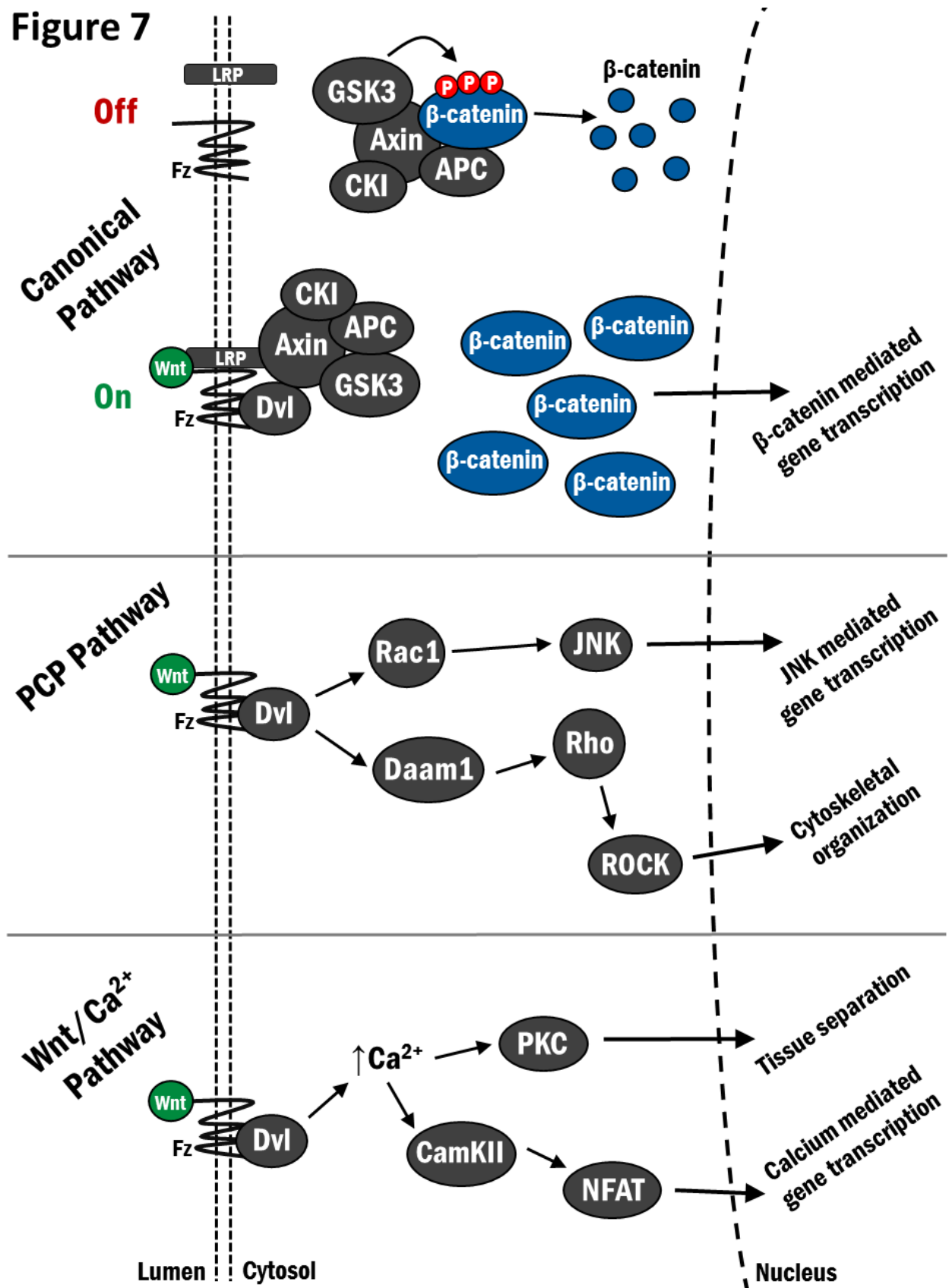


Figure 7: Summary of canonical and non-canonical Wnt signalling. The canonical Wnt pathway involves the LRP receptor and β -catenin as the effector in gene transcription. When this pathway is off, cytoplasmic β -catenin associated with a destruction complex is quickly phosphorylated and degraded. Upon Wnt binding to the Fz receptor, the destruction complex is pulled towards the membrane, thus allowing β -catenin to accumulate and translocate into the nucleus, where it co-activates specific target genes. In the PCP pathway, Wnt binding to Fz results in Dsh activation and downstream JNK and Rho/ROCK activation, leading to proper cell polarity. In the Wnt/calcium pathway, Wnt binding to the Fz receptor leads to Dsh dependent calcium signalling. This activates the PKC and CamKII pathways, which leads to tissue separation and ventral cell fate.

Cross-talk between Wnt and MAPK signalling

Mitogen activated protein kinases (MAPKs) have been identified as a family of four distinct groups of kinases that regulate the cellular response to a wide variety of stimuli, including growth factors, osmotic stress, ultraviolet radiation, chemical agents, and nutrients (138). The MAPK families are comprised of p38, extracellular signal-regulated kinase 1/2 (ERK 1/2), ERK5, and JNK. While they are themselves controlled by upstream signalling distinct from the Wnt signalling pathway, there are a variety of studies that have suggested an intersection between these groups of MAPKs and Wnt signalling.

One paper has suggested that three MAPK families, ERK1/2, p38, and JNK are capable of directly phosphorylating the PPPSPxS motif of the cytoplasmic tail of LRP6. This results in propagation of the Wnt signal by promoting the recruitment of Axin to the cell membrane, and enhances β -catenin dependent Wnt target gene activation. Mutational analysis and pharmacological inhibition of these MAPKs greatly reduced Wnt signal output. Therefore, this study suggests that these MAPKs are crucial for the transmission of Wnt signalling at the receptor level (139).

p38 MAPK

The p38 mitogen activated protein kinases (p38 MAPKs) are 38 kDa proteins (p38 α , β , δ , and γ) that are activated by dual phosphorylation of a threonine-glycine-tyrosine (TGY) motif by the upstream MAPK kinases, MKK3 and MKK6. Once activated, p38 MAPK proceeds to phosphorylate a host of downstream targets in both the cytoplasm and the nucleus. Many of its targets are transcription factors, and thus p38 MAPK serves to regulate gene expression through these transcription factors in response to external stimuli. p38 MAPK can also target other kinases, which in turn modulate key cellular processes such as cell cycle progression, chromatin remodeling, mRNA translation, and cytoskeletal organization (140).

A number of studies have determined that p38 MAPK may be playing a direct role on regulating Wnt signalling at various levels. One such study investigated the role of p38 MAPK on the effects of canonical Wnt signalling. Use of Wnt3a ligand resulted in activation of p38 MAPK activity measured by its ability to phosphorylate a known substrate. Further investigation revealed that this effect was in a G α_q and G α_s dependent manner. They then used the pharmacological p38 α/β inhibitor SB2036580, a pyridinyl imidazole inhibitor that binds to the p38 ATP binding pocket to abolish catalytic activity (141). Use of this inhibitor was able to prevent Wnt3a dependent TOPflash activity which coincided with a reduction in β -catenin protein levels. siRNA mediated silencing of p38 MAPK also reduced TOPflash activity as well as β -catenin protein. Interestingly, in their last series of experiments, they showed that GSK3 β activity was reduced in response to activated p38 MAPK, and they hypothesized that p38 MAPK's mechanism of action was somehow through inhibiting GSK3 β (142).

The importance of this hypothesis was presented in a paper published around the same time in which the authors discovered that p38 MAPK was capable of directly phosphorylating GSK3 β at serine 389. The effects of this phosphorylation was significant, as it was shown to greatly enhance β -catenin stability, and increase c-Myc and LEF protein levels (143).

Finally, a study in osteotropic prostate cancer cells asserted that p38 MAPK has the opposite effect on Wnt signalling in this system. Pharmacological inhibition of p38 MAPK resulted in reduced expression of the Wnt inhibitor dickkopf-1 (DKK-1). DKK-1 is a secreted protein that acts to repress Wnt signalling by interacting with LRP6 to prevent its interaction with Fz (144). Conversely, p38 MAPK activation could enhance DKK-1 expression, thus inhibiting the Wnt pathway. Tumours of prostate cancer patients were analyzed and shown to have elevated levels of both DKK-1 and p38 MAPK mRNA (145). Interestingly, while the data supporting their hypothesis was compelling, no experiments were performed that explored the effects of differential DKK-1 expression on downstream Wnt activity. Nevertheless, the assertion that p38 MAPK influences the Wnt signalling pathway is persuasive.

ERK 1/2 and JNK

As mentioned in the post-translational modifications section above, JNK2 has been shown to directly phosphorylate and activate β -catenin in response to Rac1 signalling. Similar to p38 MAPK, a separate study claims that JNK can repress canonical Wnt signalling by promoting nuclear export of β -catenin, thereby blocking axis duplication in developing *Xenopus* embryos; knockdown of JNK activates Wnt target gene expression (146).

A mechanism in which ERK 1/2 regulates Wnt signalling was elucidated, whereby ERK 1/2 directly interacts with and phosphorylates GSK3 β at threonine 43 to prime it for inactivation by p90RSK phosphorylation at serine 9. This increases β -catenin protein levels and enhances TOPflash reporter activity as well as Wnt target protein levels (147).

β -catenin function in vascular smooth muscle cells

Role of β -catenin during vascular development

A β -catenin target gene during embryogenesis is *dll4*, which encodes the delta like ligand 4 (DLL4) protein. This is one of the main ligands in Notch signalling, an important pathway during development that contributes to cell fate determination (148), embryo polarity (149), and somitogenesis (150). β -catenin gain-of-function mutations were genetically and conditionally introduced in mice endothelial cells and studied. By E9.5, significant vascular defects were present that resulted in lethality, such as improper vessel branching, altered lumen size, and improper arterial-venous specification. Stabilized β -catenin mice were investigated further and subjected to qPCR analysis. In addition to increased *axin2* expression, which was expected, several Notch signalling factors were also upregulated, including *dll4*, *hey1*, and *hes*. The experiments concluded that β -catenin directly activates *dll4* expression, which in turn targets the downstream Notch factors (151). Therefore, balance between β -catenin/Wnt and Notch signalling is essential during embryogenesis and in particular vascular development.

During development of the blood brain barrier (BBB), β -catenin plays an essential role in directly regulating *cldn3* expression, which encodes the Claudin 3 protein that is a key

member of the tight junctions that maintain the BBB. It was specifically the transcriptional properties of β -catenin, not its function at the cell membrane, which was responsible for this, and was mediated by Wnt3a stimulation (152).

β -catenin also plays an essential role at the adherens junction during vascular development. Loss of β -catenin in mouse embryos result in a changed adherens junction structure in which a severe loss of α -catenin and replacement with desmoplakin was observed. This change greatly diminished cell-cell adhesion strength and altered the blood vessel lumen. There were also a large amount of hemorrhagic blood vessels. It is speculated that loss of β -catenin forces increased plakoglobin to adherens junctions, and correspondingly desmoplakin, which leads to a loss of actin-based cell adhesion (153).

Role of β -catenin in wound repair

There are reports that β -catenin plays a role in adult VSMC proliferation following vascular damage, atherosclerosis, or restenosis following angioplasty. For example, analysis of balloon-injured carotid arteries in mice revealed a significant reduction in *r-cadherin* expression and a corresponding increase in nuclear β -catenin and *cyclin d1*. Studies in other systems have shown cadherin expression to be targeted by canonical Wnt signalling (119, 154). The result was an increase in VSMC proliferation measured by BrdU staining (155).

Another study reported that inhibition of β -catenin using ICAT or dominant negative TCF4 could reduce VSMC proliferation by up to 80%. This was thought to be through *cyclin d1* upregulation and *p21* downregulation (156). Balloon injury also

upregulates β -catenin mRNA levels within seven days, and this is suggested as a main driver of TCF4 dependent *cyclin d1* expression (157).

A study has suggested that the large protocadherin Fat1 is important in regulating VSMC migration and proliferation in response to injury. Fat1 has been shown to promote migration while limiting proliferation, and may accomplish this by interacting with and inhibiting nuclear β -catenin function. Fat1 does not interact with the same affinity as other cadherins however, and therefore this may be a regulatory response to vascular injury that prevents over-activity of β -catenin (158).

β -catenin also plays a role in regulating N-cadherin based cell-cell contact following injury. N-cadherin is highly expressed in VSMCs, but unlike other cadherins, it is upregulated in proliferating VSMCs. It is thought to regulate cell migration by creating transient cell-cell contacts in proliferating cells (159). This “cadherin switch” is a hallmark of EMT, and is associated with increased invasion and migration properties, in part due to N-cadherin’s ability to activate extracellular signal-regulated kinase (ERK) signalling and matrix metalloproteinase (MMP) expression in cells and its ability to provide a leading edge in migrating cells (160, 161).

Growth factor activation of β -catenin

The platelet-derived growth factor (PDGF) has been shown to promote VSMC proliferation through regulation of β -catenin localization and activity. In cultured human saphenous vein cells, treatment with PDGF could dismantle the cadherin cell adhesion complex and cause a membrane-to-nuclear shift in β -catenin. This corresponded with increased β -catenin interaction with LEF1 protein in the nucleus, and, presumably, an activation of Wnt target genes (162).

Conclusions

Based on the background knowledge presented here on β -catenin structure and function, it is evident that this protein plays an essential role in all cell types and is absolutely required for proper development. The multifunctional aspect of this protein, being able to directly mediate cell-cell contact while also regulating target gene expression, makes it a unique “jack-of-all-trades” protein. It is extensively post-translationally modified, and the effects of these modifications range from protein stability to transcriptional activity. Many β -catenin interacting partners have been identified in the past decades, and each one has a seemingly important role for modulating β -catenin activity. Since β -catenin itself contains no catalytic domain, there is an emphasized importance on the particular interactions β -catenin undergoes at any given time; these interactions are directly responsible for conferring a specific function on β -catenin.

Statement of purpose

β -catenin has been presented as a multifunctional protein that is directly involved in a wide range of cellular events. It is an integral component of the cadherin cell adhesion complex at the membrane to regulate cell-cell contact and cell motility. In the nucleus, its potent transactivation domain alters target gene expression in response to upstream signals. It is the main effector of the Wnt signalling pathway, but has also been shown to be regulated by other signalling pathways such as the Akt and JNK pathways. Mutations to β -catenin have profound effects on the cell, and many different cancers develop as a result of mutations that prevent β -catenin proteasomal degradation, and hence constitutive activity in the nucleus. The significance of β -catenin activity is not limited to a single tissue, cell type, or disease, and therefore it remains a hotly researched protein by researchers in many fields of study. These important roles of β -catenin extend to vascular smooth muscle cells as it plays an essential role in Type II epithelial to mesenchymal transition during vascular repair following injury, and understanding the various mechanisms in which β -catenin can be regulated is a significant field of research among vascular biologists.

Since β -catenin contains a potent transactivation domain yet is not a transcription factor itself, protein-protein interactions are a major contributor to its function with regards to gene expression in the nucleus. The importance of protein-protein interactions is also evident at the membrane, where structure associated proteins interact with β -catenin to maintain or alter cell shape and motility. The cellular localization of β -catenin largely determines its subsequent function, and yet, there is no clear mechanism to describe this movement. The cytoplasmic to nuclear shift of β -catenin in response to Wnt

signalling activation is independent of a nuclear localization signal or importin, but is rather mediated through various protein-protein interactions.

Previous work in our lab has identified myocyte enhancer factor 2 (MEF2) as an important regulator of VSMC gene expression through protein kinase A (PKA) and calcium/RhoA signalling (163, 164). We had previously performed a Yeast two-hybrid analysis using the highly conserved N-terminal domain of MEF2D as bait and discovered β -catenin as an interacting partner. My project therefore entailed verifying and characterizing this interaction. Furthermore, since p38 MAPK has been shown in the past to potently activate MEF2, and since there is cross-talk between p38 MAPK and Wnt signalling, its potential role in regulating this interaction was investigated. This research is summarized in Chapter 2.

I was next interested in identifying other novel β -catenin interacting partners in VSMCs using tandem LC MS/MS. The goal was to investigate potential interactions that could reveal previously unknown functions of β -catenin in the cell. We identified a possible interaction with the fragile X mental retardation protein, and the role of this interaction in regards to β -catenin function is presented in Chapter 3.

Chapter 2

A p38 MAPK regulated MEF2:β-catenin interaction enhances canonical Wnt signalling*

by

**Saviz Ehyai¹, Mathew G. Dionyssiou², Joseph W. Gordon³,
Declan Williams⁴, K. W. Michael Siu, and John C. McDermott⁵**

Rationale:

As mentioned in the statement of purpose, we recognized the significance of protein-protein interactions in the regulation of β-catenin activity. Since we had discovered that the N-terminal domain of MEF2D interacts with β-catenin by a Yeast two-hybrid screen, we performed a series of experiments to ascertain the significance of this interaction that culminated in the following manuscript. We concluded that p38 MAPK signalling promotes the nuclear retention of β-catenin by phosphorylating MEF2, which in turn directly interacts with β-catenin to promote target gene expression and cell proliferation. This manuscript was accepted for publication in *Molecular and Cellular Biology* in November 2015 and published in January 2016 as a spotlight article.

*Published in *Molecular and Cellular Biology*, (2016) 36(2): 330-346.

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¹Experimental design, manuscript draft, figures 8-12, and supplementary S1-S5, S7, S9-S14.

²Experimental design, manuscript draft, and supplementary S6.

³Experimental design.

⁴Supplementary S8.

⁵Experimental design and manuscript draft.

Summary

Canonical Wnt/ β -catenin signalling plays a major role in various biological contexts such as embryonic development, cell proliferation, and cancer progression. Previously, a connection between p38 MAPK signalling and Wnt-mediated activation of β -catenin has been implied but poorly understood. In this study, we investigate potential cross talk between p38 MAPK and Wnt/ β -catenin signalling. Here, we show that loss of p38 MAPK α/β function reduces β -catenin nuclear accumulation in Wnt3a stimulated primary vascular smooth muscle cells (VSMCs). Conversely, active p38 MAPK signalling increases β -catenin nuclear localization and target gene activity in multiple cell types. Furthermore, the effect of p38 MAPK α/β on β -catenin activity is mediated through phosphorylation of a key p38 MAPK target, myocyte enhancer factor 2 (MEF2). Here, we report a p38 MAPK mediated phosphorylation-dependent interaction between MEF2 and β -catenin in multiple cell types and primary VSMCs, resulting in: (a) increased β -catenin nuclear retention, which is reversed by siRNA mediated MEF2 gene silencing; (b) increased activation of MEF2 and Wnt/ β -catenin target genes, and; (c) increased Wnt stimulated cell proliferation. These observations provide mechanistic insight into a fundamental level of cross talk between p38 MAPK/MEF2 signalling and canonical Wnt signalling.

Introduction

Characterization of the canonical Wnt signalling pathway over the last two decades has revealed a fundamental role in many physiological and pathophysiological processes. Molecular defects in Wnt genes or their associated downstream effectors, most notably β -catenin, often have profound consequences being linked with a myriad of developmental disorders and human diseases, including hippocampal development, epithelial tube formation, and cancer (124, 165-168).

The canonical Wnt pathway involves a family of 19 Wnt ligands which are cysteine rich glycoproteins that bind to the Frizzled receptor proteins, of which there are 10 family members. The ligand:receptor interaction comprises part of a larger signalling complex containing other receptor related proteins such as the low-density lipoprotein receptor-related protein (LRP) 5 or 6 single pass transmembrane proteins. β -catenin, a bi-functional protein that, in combination with E-cadherin and α -catenin serves as a component of the cell adhesion machinery, also performs an essential nodal function in the canonical Wnt pathway downstream of the receptor complex. In brief, without active Wnt signalling, β -catenin is phosphorylated by glycogen synthase kinase 3 (GSK3) and casein kinase I (CKI) in an adenomatous polyposis coli (APC)/Axin “destruction complex”, which facilitates interaction with β -transducin repeat containing E3 ubiquitin protein ligase (β -TrCP) and subsequent ubiquitin-mediated proteasomal degradation (36, 169, 170). Conversely, pathway activation by the Wnt:Frizzled interaction dismantles the destruction complex, leading to enhanced levels of cellular β -catenin and subsequent accumulation in both the cytoplasm and particularly the nuclear compartment. In

combination with transcription factors such as lymphoid enhancer-binding factor (LEF)/T-cell factor (TCF), and several other nuclear protein interactions, β -catenin acts as a powerful regulator of Wnt target genes, such as *cyclin d1* (75), *c-myc* (114), *axin2* (171), and *c-jun* (172), in a wide range of tissues (22, 23, 173).

Nuclear accumulation of β -catenin is a central tenet of the canonical Wnt pathway, however, the nuclear β -catenin level has largely been assumed to result from destruction complex disassembly and cytoplasmic accumulation. Consideration of β -catenin nuclear localization as a potential regulatory step in canonical Wnt signalling, and also how β -catenin is retained in the nucleus, are unclear (38, 174). For a pathway that fulfills such a prominent role in many cellular processes, it seems unlikely that the facile cytoplasmic accumulation of β -catenin due to suppressed degradation is sufficient for precise regulation of the nuclear levels, especially in view of the fact that this step is heavily regulated for many transcriptional regulators (175, 176). Indeed, some studies have suggested additional control of β -catenin localization occurs in a nuclear localization signal (NLS) and importin-independent manner and by association with various proteins, however, the precise mechanism is still unknown (38-40).

Here, we report a nexus of control of β -catenin nuclear localization by cross-talk with the p38 mitogen activated protein kinase (MAPK)/myocyte enhancer factor 2 (MEF2) signalling pathway that is dependent on a direct protein-protein interaction with MEF2 and intact p38 MAPK activity in primary vascular smooth muscle and several cell lineages. These observations define a novel mechanism of β -catenin regulation with important implications for canonical Wnt signalling pathway modulation.

Results

p38 MAPK modulates Wnt-dependent β -catenin activity and nuclear localization

In order to determine whether p38 MAPK does play a role in regulating β -catenin, we utilized a well characterized synthetic reporter gene named TOPflash, which responds to Wnt dependent β -catenin activation and contains 7 TCF/LEF consensus binding sites driving luciferase expression (177). Initially, the A10 vascular smooth muscle cell line was transfected with TOPflash and then cultured under low serum conditions followed by treatment with a p38 MAPK inhibitor (SB203580, 10 μ M) or its inactive analog (SB202474, 10 μ M). Under these baseline conditions, canonical Wnt signalling was activated by treatment with purified Wnt3a protein (200 ng/mL). Wnt3a stimulation with inactive inhibitor increased TOPflash activity 11-fold ($p \leq 0.0001$), which was significantly reduced in cells treated with Wnt3a and the active p38 MAPK inhibitor ($p \leq 0.0001$; Fig. 8A, left panel). Primary vascular smooth muscle cells (VSMCs) isolated from mouse aortae were similarly treated with p38 MAPK inhibitor following serum withdrawal and stimulated with Wnt3a protein. Cell lysates were subjected to Western blot analysis to show p38 MAPK activity by analyzing a known substrate, activating transcription factor 2 (ATF2), which is phosphorylated by p38 MAPK at Thr69/71 (178)(Fig. 8A, right panel). Wnt3a stimulation has indeed been previously shown to lead to p38 MAPK activation, and thus promote ATF2 phosphorylation (142). Moreover, we reported de-repression of p38 MAPK by pharmacological GSK3 inhibition (179). Thus there seem to be a number of possible mechanisms by which the activity of p38 MAPK may be modulated by Wnt signalling.

Since nuclear accumulation is fundamental for β -catenin activity, we subsequently tested whether the effect of p38 MAPK inhibition on β -catenin activity was due to a change in its cellular localization. Exploratory experiments in A10 cells indicated that modulation of p38 MAPK activity affected β -catenin localization (Fig. S1), and this was confirmed in primary VSMCs by confocal immunofluorescence microscopy using conditions as described above. Initially, β -catenin is localized to the nucleus due to Wnt3a stimulation when compared to control cells that were maintained in serum depleted media to establish baseline levels of signalling (Fig. 8B, top panel). Strikingly, inhibition of p38 MAPK resulted in a large reduction in β -catenin nuclear accumulation. This dramatic change in β -catenin localization was illustrated by a colour scheme dependent on fluorescent intensity (LUT, blue to red; Fig. 8B, bottom left panel), and by quantification of relative nuclear/cytosolic fluorescence in all of the images taken, which indicated a near 4-fold reduction in nuclear β -catenin levels in Wnt3a stimulated cells that were treated with p38 MAPK inhibitor ($p \leq 0.0001$; Fig. 8B, bottom right panel). These results prompted us to hypothesise that p38 MAPK activity is required for Wnt3a mediated β -catenin nuclear localization.

We next decided to analyze whether there was a biological effect of p38 MAPK inhibition on Wnt3a stimulated cells. To this end, we performed a cell survival assay in which A10 cells were equally seeded at 75,000 cells and grown in serum free media overnight prior to being either stimulated with purified Wnt3a and treated with p38 MAPK inhibitor or its inactive analog every two days, over a period of six days; cells were counted at these intervals and the results are displayed in Fig 8C (left panel). Treatment with Wnt3a and inactive p38 MAPK inhibitor resulted in a significantly higher cell count than control cells that were serum withdrawn over the course of the experiment.

Interestingly, cells that were Wnt3a stimulated and treated with the active p38 MAPK inhibitor had a much lower cell count, nearly as low as cells that were serum withdrawn. A parallel experiment was performed in which β -catenin localization was determined using immunofluorescence. The number of cells that were nuclear β -catenin positive were counted for each condition throughout the course of six days, and the results show that approximately 80% of cells that are Wnt3a treated with inactive p38 MAPK inhibitor are nuclear β -catenin positive, while only approximately 30% of Wnt3a stimulated cells in the presence of active p38 MAPK inhibitor are positive (Fig. 8C, right panel). These results demonstrate that the loss of nuclear β -catenin localization observed in p38 MAPK inhibited cells has a profound effect on cell proliferation, and therefore has important biological implications.

To further validate these data we used a different p38 MAPK inhibitor (SB202190, 10 μ M) with a different mechanism of inhibition (prevents p38 MAPK phosphorylation by upstream MKKs) in order to determine if these data would be replicated. Primary smooth muscle cells were transfected with the TOPflash reporter gene and cultured in low serum conditions prior to being treated with different combinations of Wnt3a and p38 MAPK inhibitor, similar to the experiment in Fig. 8A. Wnt3a stimulation results in a large increase in reporter gene activity, and, similar to SB203580, inhibition of p38 MAPK by SB202190 results in a significant reduction in reporter gene activity ($p \leq 0.0001$; Fig. 8D, left panel). Western blot analysis of the cell lysates confirm activation of p38 MAPK by Wnt3a stimulation, and its inhibition using SB202190 by analyzing the phosphorylation status of ATF2 (Fig. 8D, right panel). We further demonstrated that this effect is correlated with β -catenin localization, and we performed an immunofluorescence assay using this p38 MAPK inhibitor. Again, inhibition of p38 MAPK by SB202190 results in a

loss of nuclear β -catenin localization in Wnt3a stimulated cells, with a greater than 3-fold decrease in the nuclear/cytosolic ratio of β -catenin with p38 MAPK inhibition ($p \leq 0.0001$; Figs. S2 and 8E).

To pursue this idea differently, we asked if platelet derived growth factor (PDGF), which is a known activator of p38 MAPK signalling (180) and has been shown to promote nuclear β -catenin localization (162), might also be modulated by p38 MAPK inhibition. In this experiment, A10 cells were treated with purified PDGF (20 ng/mL). When compared to control cells, PDGF stimulation enhanced nuclear accumulation of β -catenin over 6-fold, and this effect was also counteracted by p38 MAPK inhibition ($p \leq 0.0001$, Fig. 8F). To determine whether this similar pattern in localization had an effect on β -catenin *transactivation*, we expressed the TOPflash reporter gene and stimulated cells with Wnt3a and p38 MAPK inhibitor (SB203580). PDGF treatment results in a significant increase in reporter activity, although less than what is observed with Wnt3a treatment ($p \leq 0.0001$; Fig. 8G). Importantly, p38 MAPK inhibition results in a loss of reporter gene activity, similar to the effects seen with Wnt3a. Collectively, based on the data collected thus far, we conclude that a loss of p38 MAPK function has an effect on both β -catenin cellular localization and *transactivation* properties, and that pharmacological inhibition of p38 MAPK can abrogate canonical Wnt mediated β -catenin activity.

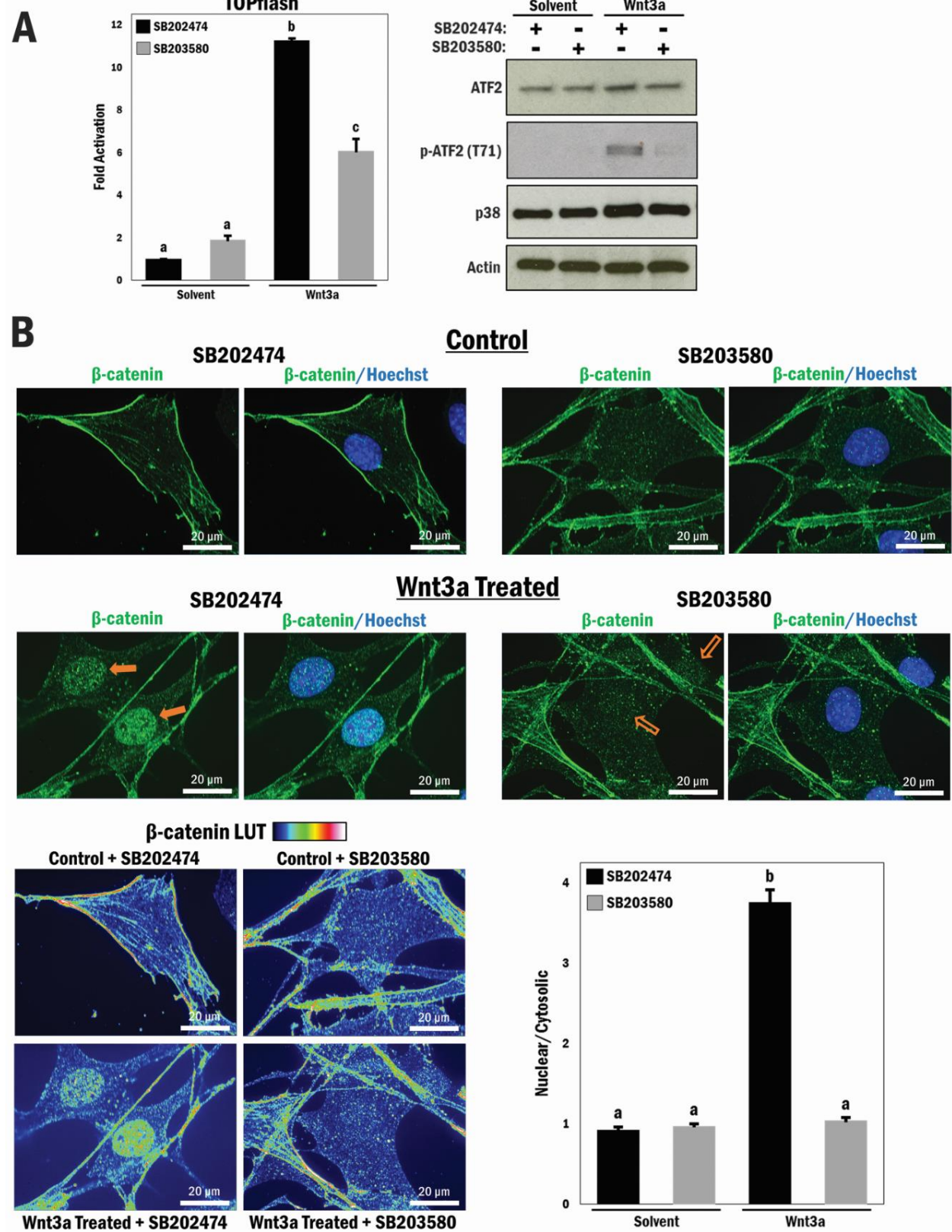
Activation of p38 MAPK results in increased β -catenin nuclear localization and Wnt responsive gene activity

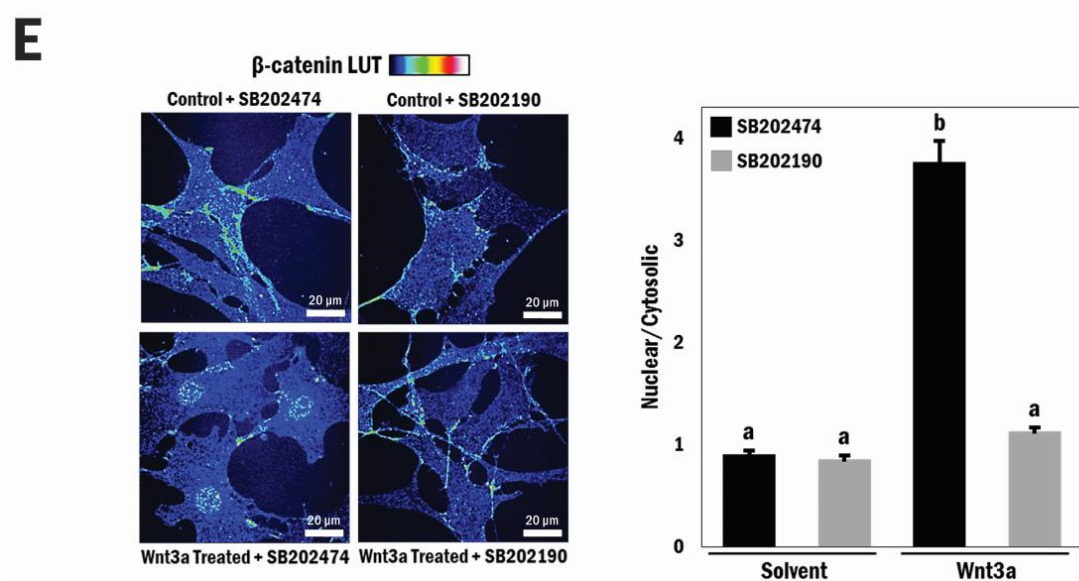
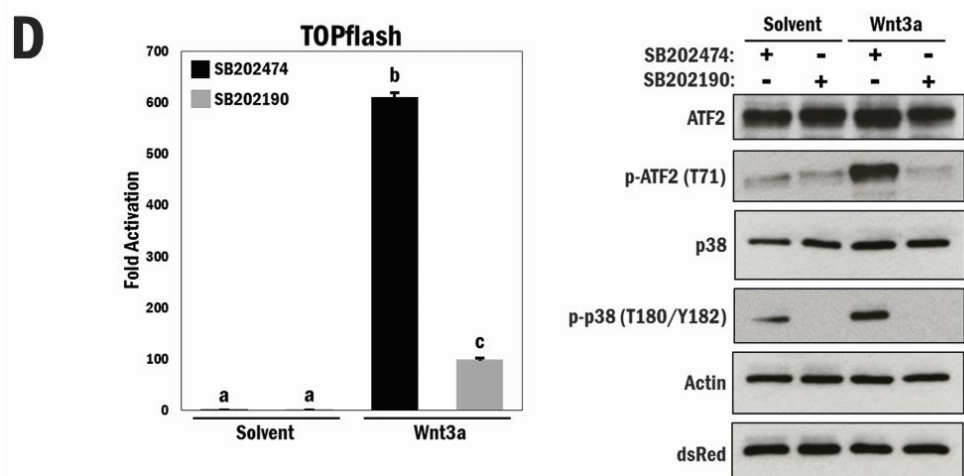
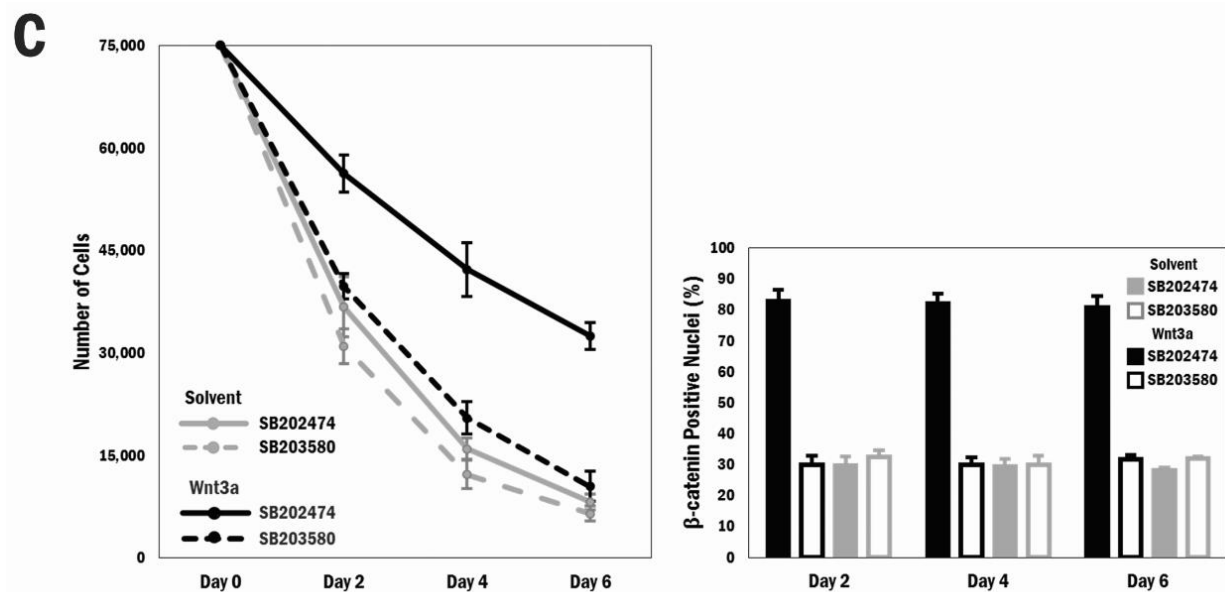
In view of the loss of function data described above, we carried out p38 MAPK gain of function experiments to test if nuclear β -catenin levels and *transactivation* properties are enhanced. First, we ectopically expressed p38 MAPK and a constitutively active form of its upstream kinase, dual specificity mitogen activated protein kinase kinase 6 (MKK6, EE), in primary VSMCs and looked at TOPflash reporter gene activity. Over-expression of MKK6 alone increased reporter gene activity ($p \leq 0.01$) while co-expression of MKK6 and p38 MAPK increased reporter gene activity significantly more than MKK6 alone ($p \leq 0.01$; Fig. 8H, top left panel), despite the fact that these cells were cultured in high serum conditions and would have high baseline p38 MAPK activity. Activation of p38 MAPK was confirmed by Western blotting (Fig. 8H, bottom left panel). To test whether the increase in reporter gene activity is specific to p38 MAPK and not a different target of MKK6, these cells were again transfected with MKK6, but with an inactive form of p38 MAPK in which its MKK6 phospho-acceptor sites are mutated (T180A/Y182F, p38 MAPK AGF). Over-expression of the p38 MAPK AGF mutant does not have an effect on TOPflash reporter activity, leading us to conclude that the effects seen with ectopic MKK6 expression are through p38 MAPK ($p \leq 0.01$; Fig 8H, top right panel). Expression patterns were confirmed by Western blot analysis, and levels of phospho-p38 are unchanged when compared to a positive A10 lysate control, indicating that the p38 MAPK mutant is unaffected by MKK6 expression (Fig. 8H, bottom right panel).

We next assessed A10 cells that were transfected with MKK6 and p38 MAPK, and marked by co-transfection with dsRed, and stained for endogenous β -catenin [fluorescein isothiocyanate (FITC), green] and nuclei [4'-6-diaminidino-2-phenylindole (DAPI), blue]. MKK6/p38 MAPK positive cells showed higher nuclear β -catenin localization compared to untransfected cells in the same field (Fig. 8I, left panel). In addition, cells co-transfected with both MKK6 and p38 MAPK with TOPflash showed a synergistic increase in reporter gene activity ($p \leq 0.0001$; Fig. 8I, top right panel). This indicates that activation of p38 MAPK potently enhances β -catenin *transactivation* properties, an effect that was not observed when using the FOPflash control reporter gene, which contains 6 mutated TCF/LEF sites (Fig. S3). p38 MAPK activation was confirmed by Western blotting (Fig. 8I, bottom right panel).

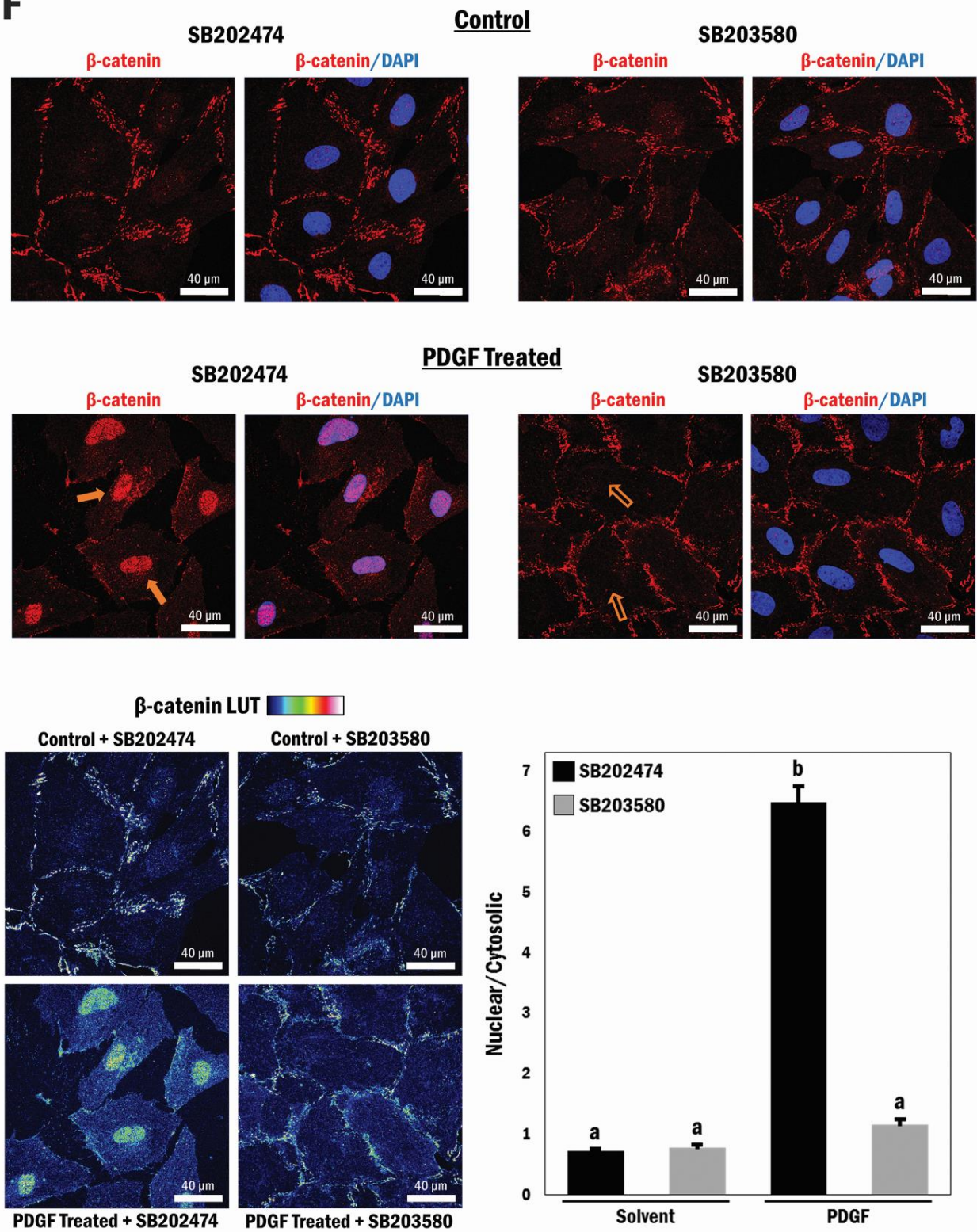
To determine if the effect of p38 MAPK signalling on β -catenin localization and activity is limited to smooth muscle cells, we repeated the gain of function experiments in both 10T1/2 (embryonic fibroblast, mouse) and human embryonic kidney (HEK) 293T (embryonic kidney, human) cells. Our observations confirmed by multiple techniques that the effect on β -catenin localization and activity by p38 MAPK hyperactivation was common to these other cell types as well ($p \leq 0.05$ and $p \leq 0.01$; Figs. S4 and S5, respectively). Taken together, these data support the conclusion that p38 MAPK plays a potent role in modulating Wnt-dependent β -catenin localization and *transactivation* properties in primary and several other cell lineages.

Figure 8

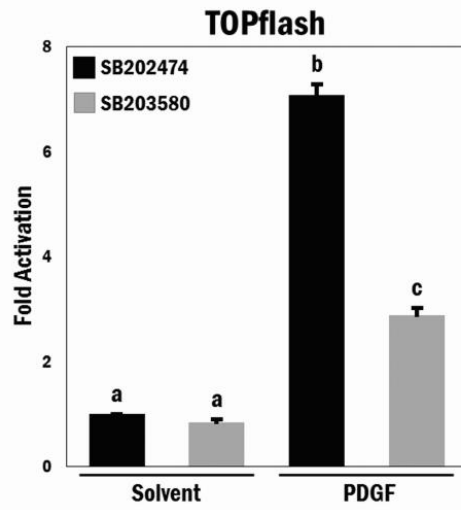




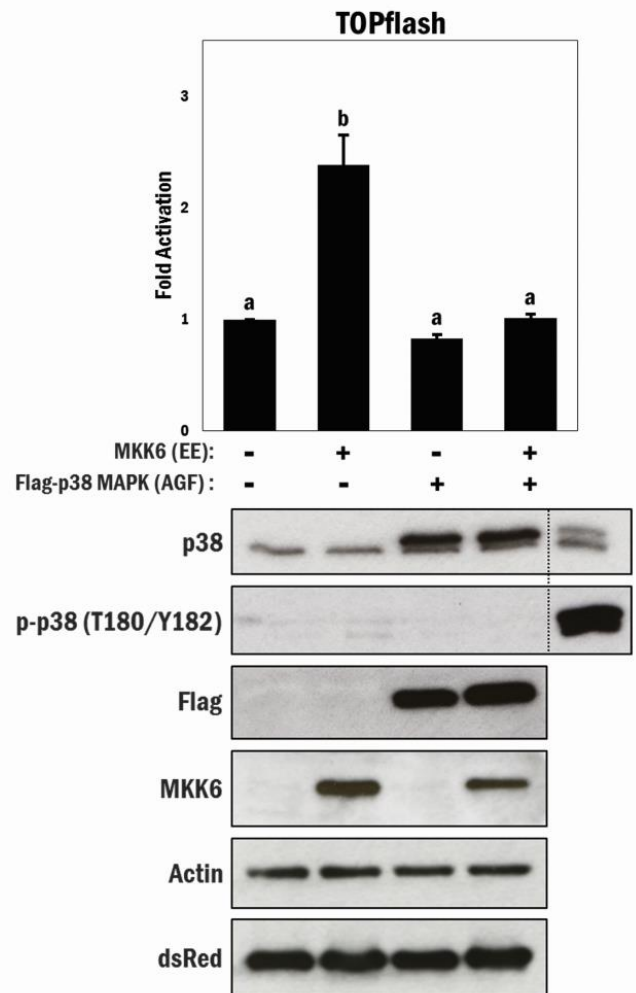
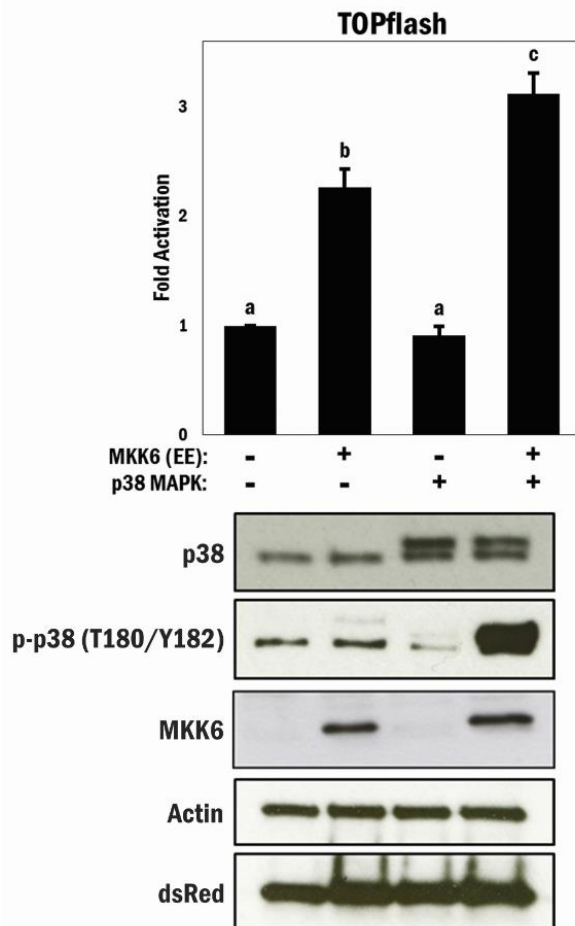
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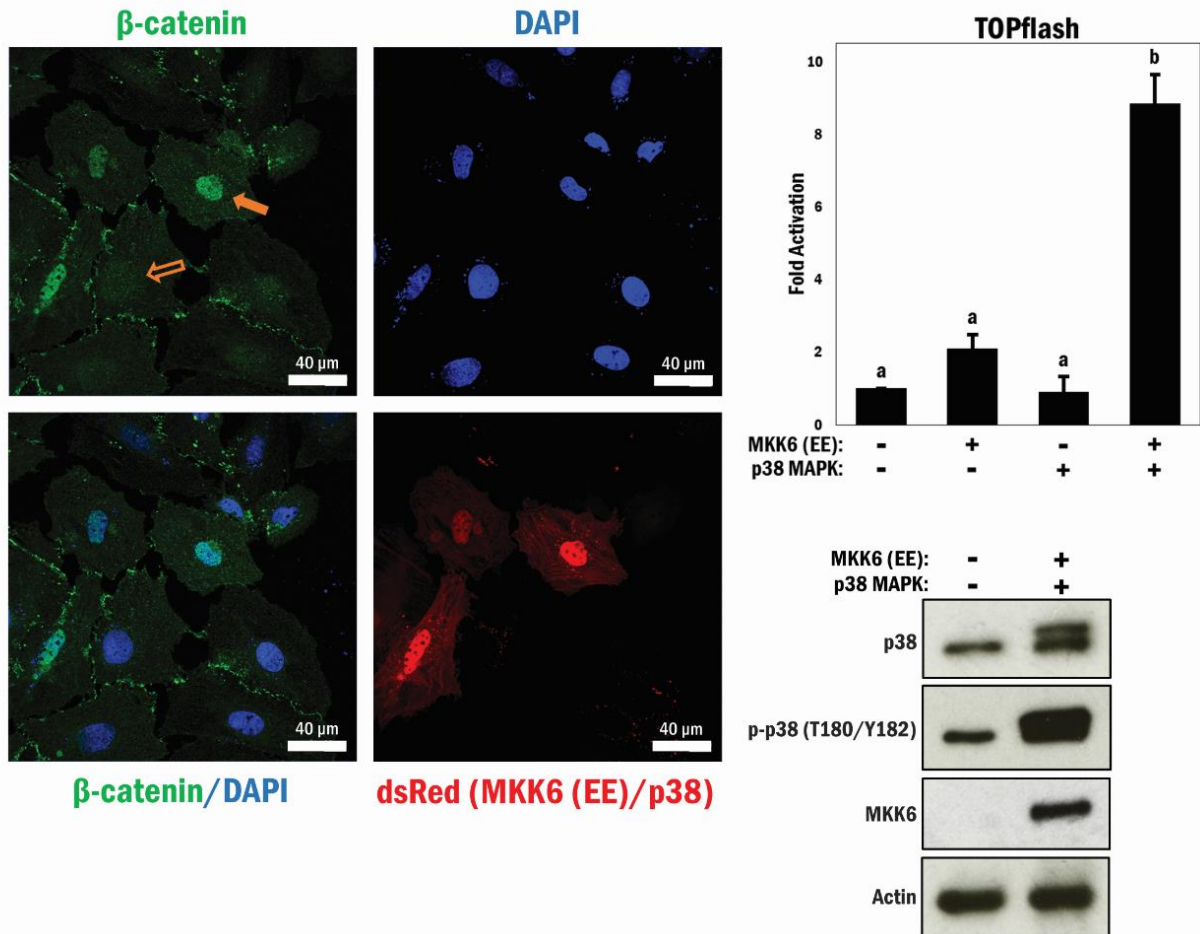


Figure 8: p38 MAPK regulates Wnt-dependent β -catenin transactivation properties and nuclear localization in primary vascular smooth muscle cells.

A. (Left panel) A10 cells were transfected with the TOPflash reporter gene for 3 hr, followed by treatment with different combinations of Wnt3a (200 ng/mL), solvent (PBS, control), SB203580 (p38 MAPK inhibitor, 10 μ M) or SB202474 (control, 10 μ M) for 16 hr in serum-free media, as indicated; b,c=p<0.0001. (Right panel) Primary VSMCs were serum withdrawn (12 hr) and pre-treated with SB203580 or SB202474 (10 μ M, 45 min) then stimulated with Wnt3a (200 ng/mL) or solvent (PBS) for 4 hr. Cell lysates were subjected to Western blot analysis, as indicated. **B.** (Top panel) Primary VSMCs were serum withdrawn for 12 hr and pre-treated with SB203580 or SB202474 (10 μ M, 45 min) then stimulated with Wnt3a (200 ng/mL) or solvent (PBS) for 4 hr. Cells were fixed and stained with Hoechst 33342 (blue, nucleus) and FITC (green, β -catenin). Filled arrows indicate presence of nuclear β -catenin, empty arrows indicate lack thereof. (Bottom left panel) A rainbow (blue to red) lookup table (LUT) was used to indicate relative β -catenin

intensity. (Bottom right panel) Relative nuclear to cytosolic fluorescence levels were measured and quantified (n=33, 32, 31, 32 from left to right); b=p≤0.0001. **C.** (Left panel) A10 cells were seeded at 75,000 cells, serum withdrawn (12 hr) then pre-treated with SB203580 or SB202474 (10 μM, 45 min) and stimulated with Wnt3a (200 ng/mL) or solvent (PBS). This was repeated every 2 days for a total of 6 days, and cells were counted at each interval. (Right panel) A10 cells were cultured and treated as in the left panel, fixed and stained at each interval for nucleus (Hoechst 33342, blue), β-catenin (FITC, green), and counted for number of β-catenin nuclear positive cells for each condition and time interval (n=158, 210, 145, 146, 159, 201, 149, 160, 175, 167, 123, 145 from left to right). **D and E.** Primary VSMCs were prepared as in A and B, respectively, but were treated with an alternative p38 MAPK inhibitor (SB202190, 10 μM) or its control (SB202474, 10 μM) (n=26, 39, 34, 35 from left to right); b=p≤0.0001. **F.** A10 cells were prepared as in B, but were stimulated with either PDGF (20 ng/mL) or solvent (0.1% BSA in 4 mM HCl) for 4 hr prior to fixing and staining with DAPI and TRITC (n=35, 28, 36, 24 from left to right); b=p≤0.0001. **G.** A10 cells were transfected with the TOPflash reporter gene for 3 hr, followed by treatment with different combinations of PDGF (20 ng/mL), solvent (0.1% BSA in 4 mM HCl), SB203580 (10 μM), or SB202474 (10 μM) for 16 hr in serum-free media, as indicated; b,c=p≤0.0001. **H.** (Top left panel) TOPflash reporter gene activity was measured in primary VSMCs that were transfected with different combinations of active MKK6 (EE) and p38 MAPK as indicated; b,c=p≤0.01, with corresponding Western blots to show relative over-expression (bottom left panel). (Top right panel) TOPflash reporter gene activity was measured in primary VSMCs that were transfected with different combinations of active MKK6 (EE) and mutant Flag-p38 MAPK (AGF) as indicated; b=p≤0.01, with corresponding Western blots to show relative over-expression; A10 lysates were used as controls for expression of p38 and p-p38 (bottom right panel). **I.** (Left panel) A10 cells were co-transfected with MKK6 (EE) and p38 MAPK and marked for ectopic expression (dsRed, red), DAPI was used for nuclear staining and FITC was used for β-catenin staining (green); (Top right panel) TOPflash reporter gene activity was measured in A10 cells that were transfected with different combinations of p38 MAPK and active MKK6 (EE) as indicated; b=p≤0.0001. (Bottom right panel) Western blot analysis to show activation of p38 MAPK.

β -catenin is not a direct p38 MAPK substrate

We initially supposed that p38 MAPK might directly phosphorylate β -catenin, and that this phosphorylation was required for β -catenin accumulation in the nucleus. To assess this idea, we performed an *in vitro* kinase assay using purified β -catenin, p38 MAPK, and [γ - 32 P]ATP. When compared to a known p38 MAPK substrate as a positive control (MEF2C), weak β -catenin phosphorylation was apparent, but we suspected this to be background (Fig. S6). To further explore this, an *in silico* analysis of the β -catenin primary amino acid sequence revealed three possible p38 MAPK phosphorylation sites at S191, S246, and S605. These residues were individually mutated to alanine and the resulting mutants used in TOPflash reporter assays with ectopically expressed MKK6 (EE) and p38 MAPK. There was no change in the p38 MAPK responsiveness of β -catenin with the engineered mutations (Fig. S7), indicated that p38 MAPK may not directly phosphorylate β -catenin. Lastly, our group has extensive experience of detecting phosphorylation events by mass spectrometry (181-183) and, despite robust detection of ATF2 phosphorylation in our parallel assays, there was no evidence of corresponding p38 MAPK mediated β -catenin phosphopeptides by highly sensitive LC-MS/MS (Fig. S8). Therefore, we conclude that β -catenin is not a direct substrate of p38 MAPK.

MEF2 is a direct β -catenin interacting partner

Previous observations from our group led us to perform the next set of experiments. The first was that MEF2 was reported by us and others to be potently regulated by p38 MAPK (184-187). The second was that we observed a high confidence interaction between the highly conserved N-terminus of MEF2 and β -catenin in a yeast two hybrid screen, in

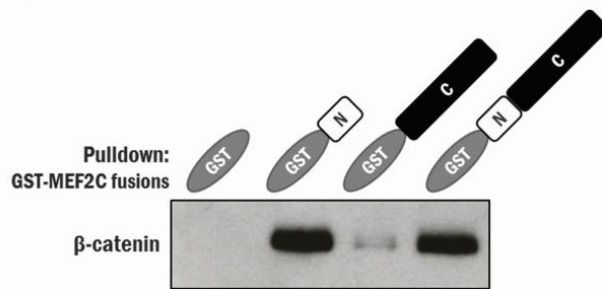
which we previously reported a novel interaction between MEF2 and big mitogen activated protein kinase 1 (BMK1) (188), histone deacetylase 4 (HDAC4) (183), and protein phosphatase 1 α (PP1 α) (189). Also, another group independently documented an interaction between MEF2 and β -catenin in a hepatocellular carcinoma cell line (190). We next sought to further validate the interaction between β -catenin and MEF2 biochemically. To determine if the β -catenin:MEF2 interaction was direct, bacterially produced glutathione-S-transferase (GST)-MEF2C and 6xHistidine (6xHis)- β -catenin fusion proteins were used in pull down assays (Fig. S9). This analysis indicated that β -catenin directly interacts with GST-full length MEF2C and also a GST-N-terminus-MEF2C fusion (aa 1-86) with high affinity. A weak, probably insignificant, interaction with the GST-C-terminus-MEF2C (aa 87-465) was observed and no interaction with a GST only control (Fig. 9A, repeated in Fig. S10). To determine if the interaction occurs in a cellular context, GSK-3 degradation resistant, “activated” β -catenin (β -catenin act) and MEF2C were expressed in CV-1 Origin SV40-7 (COS7) cells and their interaction was confirmed by co-immunoprecipitation assays (Fig. 9B). Subsequently, we tested for an endogenous interaction between MEF2 and β -catenin in whole cell lysates of the A10 cell line in which we previously detected expression of both proteins. Western analysis of the MEF2C immunoprecipitate for β -catenin, and the reverse, in which β -catenin was immunoprecipitated and probed for MEF2C, resulted in detection of the respective interacting partners, indicating a robust interaction between MEF2C and β -catenin (Fig. 9C).

We were further interested in determining if this interaction occurs with other MEF2 family proteins, since the region shown to interact with β -catenin is the highly conserved N-terminal MADS-MEF2 domain that is common to all MEF2 proteins, and if

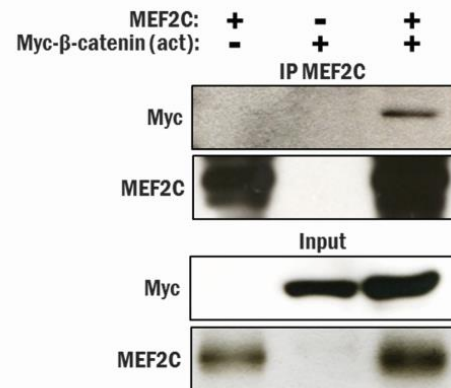
this interaction occurs in other cell types. Readily detectable endogenous interactions between β -catenin and MEF2A, as well as β -catenin and MEF2D, were observed in primary vascular smooth muscle whole cell lysates (Fig. 9D, top panel). This result was repeated in A10, HEK 293T, and 10T1/2 cell lines (Fig. 9D, second, third, and fourth panels, respectively). Overall, these data establish that β -catenin interacts with MEF2A, C, and D, most likely through their highly conserved N-terminal region, that the interaction occurs in several cell lines of different lineages, and in primary vascular smooth muscle cells. We therefore hypothesized that MEF2 potentially enhances β -catenin accumulation in the nucleus by direct physical interaction.

Figure 9

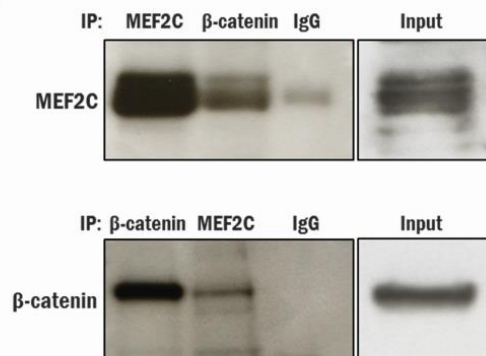
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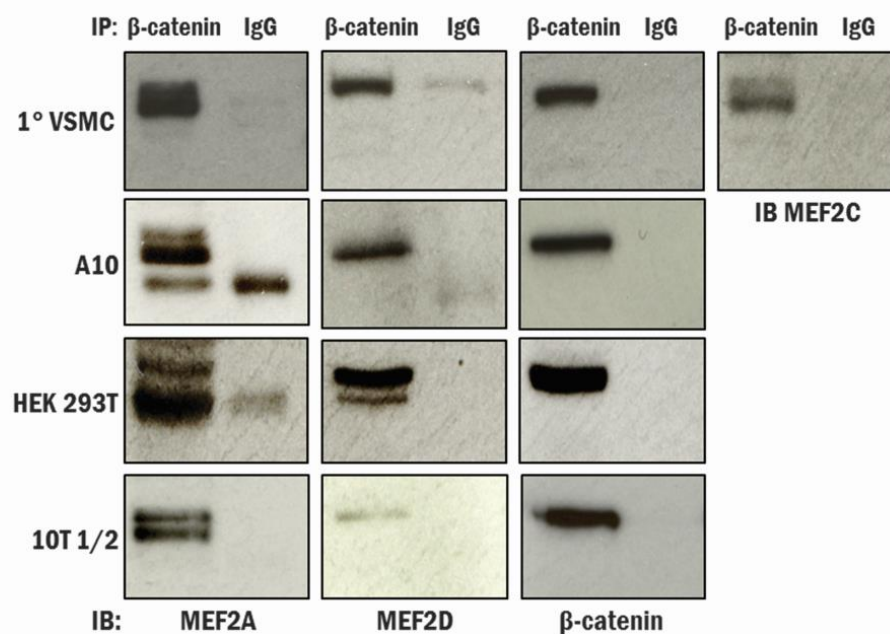


Figure 9: MEF2 directly interacts with β -catenin *in vitro* and in multiple tissue types. **A.** An *in vitro* GST pull down assay was performed using purified 6x-His- β -catenin and either GST (control), the N-terminal (aa 1-86), C-terminal (aa 87-465), or full length (aa 1-465) GST-MEF2C fusion proteins. **B.** A co-immunoprecipitation assay was performed in COS7 cells using MEF2C antibody (immunoprecipitation, IP) to precipitate ectopically expressed MEF2C and determine whether an interaction with ectopically expressed, degradation resistant, Myc- β -catenin (act) occurs. **C.** A co-immunoprecipitation assay was performed in A10 cells using both MEF2C and β -catenin antibodies for immunoprecipitation to test for an interaction between the endogenous proteins. **D.** Co-immunoprecipitation assays were performed using β -catenin antibody to test an interaction between endogenous β -catenin and either MEF2A/C/D in primary VSMCs (top panel), A10 (second panel), HEK 293T (third panel), and 10T1/2 (bottom panel) cells.

MEF2:β-catenin interaction enhances β-catenin accumulation in the nucleus

As alluded to above, previous studies have shown that phosphorylation of MEF2 by p38 MAPK results in potent activation of MEF2, and we have now shown that activation of p38 MAPK results in enhanced nuclear localization of β-catenin, even though it is not a direct substrate of p38 MAPK. Therefore, given the observation that β-catenin interacts with MEF2, we postulated that there might be a p38 MAPK mediated phospho-dependent interaction between β-catenin and MEF2 that enhances the nuclear accumulation of β-catenin. To investigate this idea, A10 cells were grown in high serum conditions and transfected with short interfering RNAs (siRNAs) directed against MEF2A and MEF2C, which reduced the levels of these proteins (Fig. S11), followed by immunofluorescence microscopy to detect β-catenin localization. Cells transfected with the siRNAs were marked (dsRed), and endogenous β-catenin localization was analyzed by antibody detection (FITC, green). Combined reduction of MEF2A and MEF2C expression resulted in a substantial and significant loss of nuclear β-catenin localization when compared to cells transfected with the scrambled siRNA control ($p \leq 0.0001$, Fig. 10A).

Next, we tested if loss of MEF2A and MEF2C might modulate Wnt-dependent β-catenin accumulation in the nucleus. To investigate this, siMEF2A and siMEF2C transfected cells were stimulated with Wnt3a protein or solvent (control) prior to analysis of β-catenin subcellular localization. As expected, β-catenin nuclear accumulation was over 6-fold higher in cells transfected with the siRNA control and treated with Wnt3a. Importantly, β-catenin failed to accumulate in the nuclei of Wnt3a stimulated cells in which MEF2 proteins were depleted ($p \leq 0.0001$, Fig. 10B). These data provide

unequivocal evidence that, in this cellular context, MEF2 is absolutely required for canonical Wnt mediated β -catenin nuclear accumulation.

As we had observed in earlier experiments illustrated in Fig. 8, loss of nuclear β -catenin localization results in a corresponding loss of β -catenin reporter gene activity, and we were interested to know if this was also the case with MEF2 gene silencing. Previous studies in C2C12 myoblasts have shown that shRNA mediated silencing of MEF2A results in a significant reduction in TOPflash activity in high serum conditions (191), so we decided to assess the effects of MEF2 depletion in Wnt3a stimulated A10 cells. We observed that Wnt3a treatment greatly enhances TOPflash activity, and depletion of MEF2A by siRNA significantly reduces this activity in Wnt3a stimulated cells, thus underscoring that the presence of MEF2 is essential for Wnt-mediated β -catenin *transactivation* properties ($p \leq 0.0001$; Fig. 10C, left panel). Confirmation of MEF2A silencing by Western analysis is shown in Fig. 10C, right panel, and this experiment was repeated using a different siRNA targeting MEF2A, shown in Fig. S12 with similar results.

Affinity of MEF2: β -catenin interaction and β -catenin transactivation is potently regulated p38 MAPK phosphorylation of MEF2

Based on the indirect effect of activated p38 MAPK on β -catenin localization, coupled with the interaction of β -catenin with MEF2, a direct p38 MAPK substrate, we hypothesized that the MEF2: β -catenin interaction might be phospho-MEF2 dependent and was responsible for the enhancement in Wnt reporter activity by p38 MAPK. This would establish a direct connection between β -catenin, p38 MAPK, and MEF2. To test this idea, we employed a MEF2A mutation that is incapable of being phosphorylated by

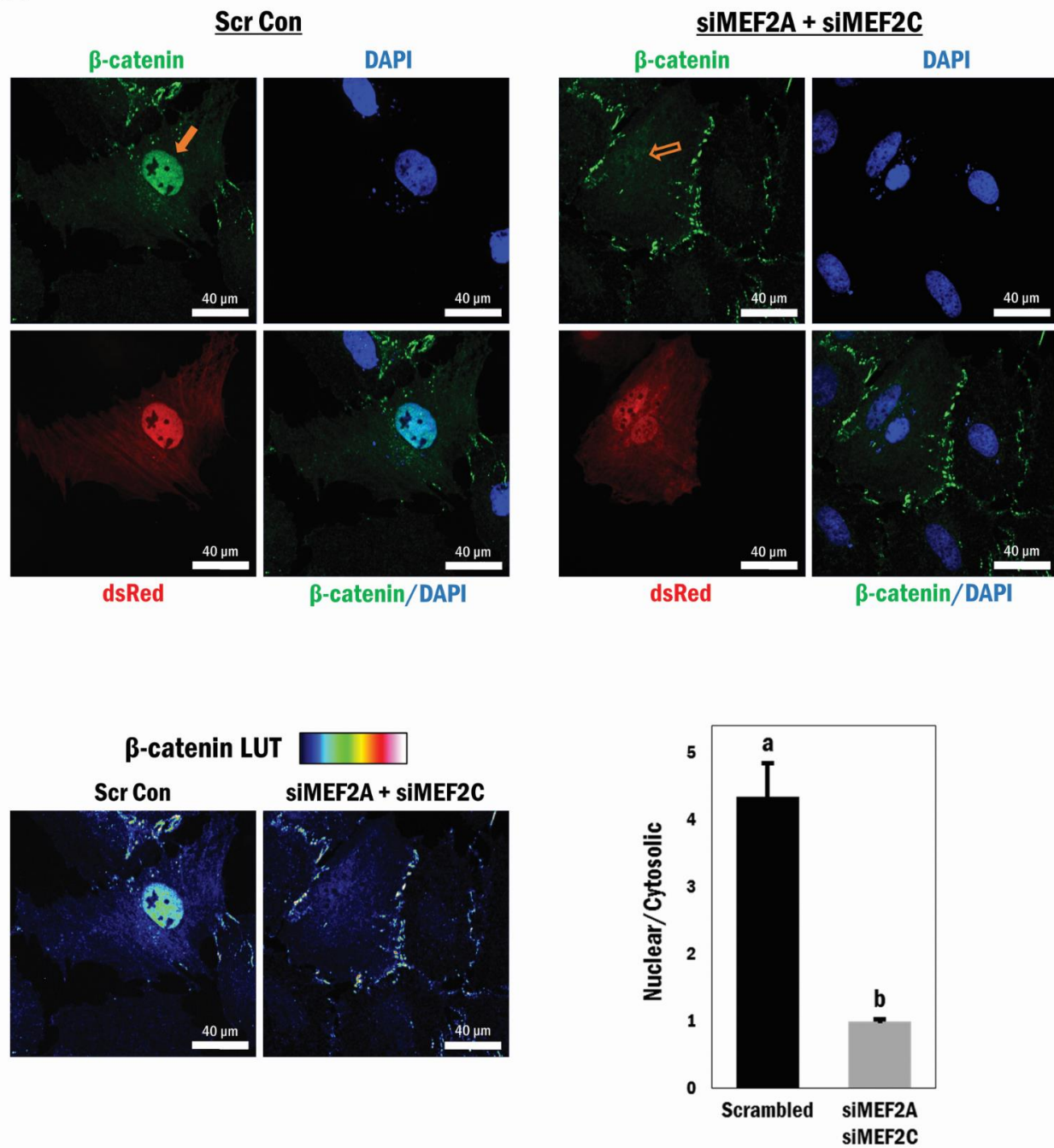
p38 MAPK (T312A/T319A) in a TOPflash assay using COS7 cells, which do not contain any appreciable endogenous MEF2 proteins or activity. Wild-type MEF2A was co-expressed with MKK6 (EE) and p38 MAPK, which increased TOPflash activity 9-fold. However, when the MEF2A mutant was co-expressed in a similar manner, there was no apparent change in TOPflash activity, thereby indicating that the mechanism of activation of the Wnt reporter gene is through phosphorylation of MEF2 by p38 MAPK ($p \leq 0.0001$; Fig. 10D, left panel). Western blot analysis show the expression patterns of the COS7 cell lysates (Fig. 10D, right panel).

We subsequently assessed the affinity of the interaction of p38 MAPK phosphorylated and unphosphorylated forms of MEF2C with purified β -catenin. These data clearly show that phosphorylation of purified MEF2C by p38 MAPK greatly enhances the MEF2C: β -catenin interaction *in vitro* (Fig. 10E).

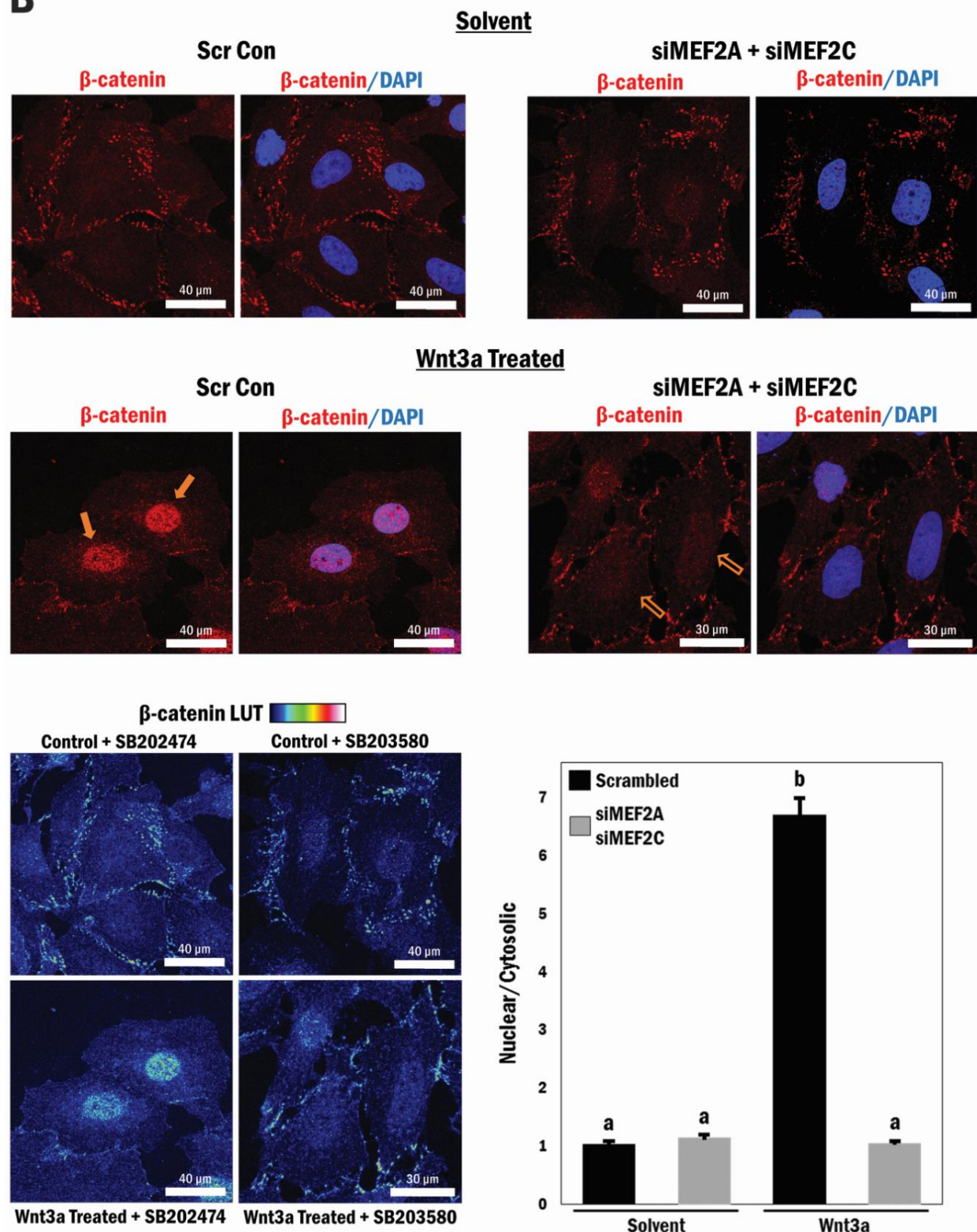
To probe the phospho-dependency of the MEF2: β -catenin interaction further, we incubated A10 whole cell lysates with calf-intestinal alkaline phosphatase (CIP), that catalyzes the removal of phosphate groups on peptides. Cell lysates were incubated with CIP at 37°C (10 units enzyme in 1 mg lysate for 30 min) during immunoprecipitation using MEF2A antibody. When compared with control cells that were incubated at 37°C without the addition of CIP, there was a marked reduction of corresponding β -catenin protein (Fig. 10F, top panels). Western blot analysis of MEF2A immunoprecipitates and input protein confirm the loss of an upper band in MEF2A with CIP treatment, indicating successful MEF2A de-phosphorylation which is correlated with the reduction in the interaction with β -catenin (Fig. 10F, bottom panels). Based on these experiments, we conclude that p38 MAPK mediated phosphorylation of MEF2 promotes the association of MEF2 and β -catenin.

Figure 10

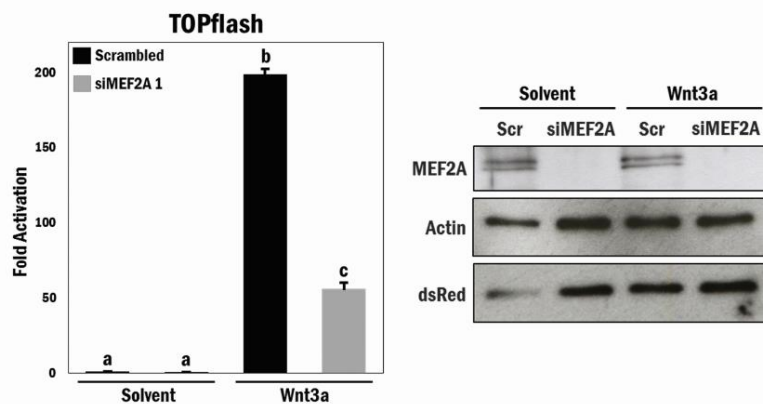
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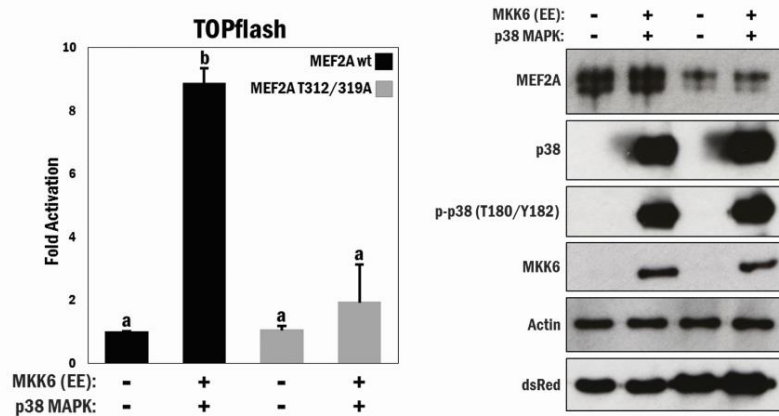
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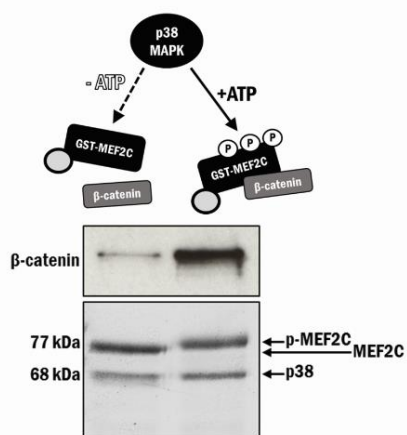
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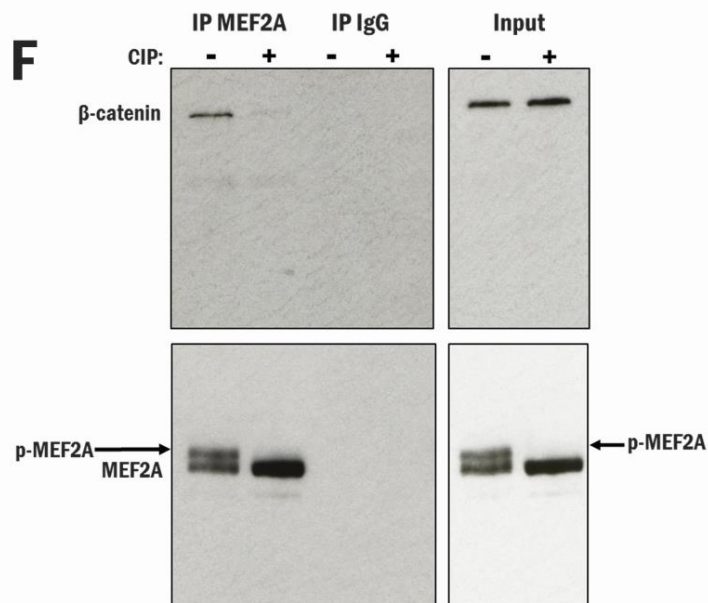


Figure 10: MEF2 protein expression is required for β -catenin nuclear retention and enhanced by p38 MAPK mediated phosphorylation of MEF2.

A. (Top panel) CMV-driven dsRed was co-transfected with either scrambled (control) or siMEF2A/C RNA, followed by immunofluorescent staining of β -catenin (FITC) and nucleus (DAPI), in A10 cells. (Bottom left panel) A rainbow LUT was used to indicate relative β -catenin intensity. (Bottom right panel) Relative nuclear to cytosolic fluorescence levels were measured and quantified ($n=11, 13$ from left to right); $b=p\leq 0.0001$. **B.** (Top panel) Scrambled or siMEF2A/C RNA transfected A10 cells were serum withdrawn for 12 hr and treated with either Wnt3a (200 ng/mL) or solvent (PBS, control) for 4 hr, followed by immunofluorescent staining of β -catenin (TRITC) and nucleus (DAPI). (Bottom left panel) A rainbow LUT was used to indicate relative β -catenin intensity. (Bottom right panel) Relative nuclear to cytosolic fluorescence levels were measured and quantified ($n=28, 27, 27, 28$ from left to right); $b=p\leq 0.0001$. **C.** (Left panel) A10 cells were transfected with TOPflash reporter, scrambled or siMEF2A RNA for 3 hr, cultured in serum conditions for 24 hr followed by treatment with either Wnt3a (200 ng/mL) or solvent (PBS) for 16 hr in serum free media; $b,c=p\leq 0.0001$. (Right panel) Corresponding Western blot analysis of cell lysates to confirm siRNA-mediated MEF2A depletion. **D.** (Left panel) COS7 cells were transfected with TOPflash reporter gene and different combinations of wild-type MEF2A, mutated MEF2A (T312/319A), MKK6 (EE), and p38 MAPK, as indicated; $b=p\leq 0.0001$. (Right panel) Corresponding Western blot analysis. **E.** Purified GST-MEF2C and GST-p38 MAPK were incubated at 30°C for 3 hr, with or without ATP as indicated in the figure, followed by addition of purified 6xHis- β -catenin and GST-agarose beads for 1 hr at room temperature. Upper panel: Immunoblot indicating relative amounts of β -catenin bound to MEF2C following precipitation. Lower panel: representation of total MEF2C phosphorylation following 3 hr incubation with p38 MAPK and ATP. **F.** A10 cell lysates (1 mg) were incubated with or without 10 units calf intestinal alkaline phosphatase (CIP) for 30 min at 37°C during immunoprecipitation using MEF2A antibody. Beads were then washed and analyzed for corresponding β -catenin protein by Western blot. Lysates without antibody conjugated beads were similarly incubated as input samples.

MEF2 and β -catenin associate and co-operate in the activation of the *c-jun* promoter

The effects observed with the TOPflash reporter gene illustrate that p38 MAPK/MEF2 signalling can co-operate with β -catenin in transcriptional activation. We used a known MEF2 target gene to determine co-operation between MEF2 and β -catenin, *c-jun*. To investigate potential selectivity with MEF2, we used the *c-jun* promoter region which has been shown by us and others to be potently regulated by a single MEF2 consensus site (C/T)TA(A/T)₄TA(G/C) (163, 192). In order to test the effects of β -catenin co-operativity on MEF2-dependent *c-jun* expression, we ectopically expressed the pCJ6 reporter gene (*c-jun* promoter based reporter gene), MKK6 (EE), and p38 MAPK in A10 cells. As expected, a pronounced increase in *c-jun* promoter activity was observed upon MKK6/p38 MAPK activation ($p \leq 0.0001$, Fig. 11A). Strikingly, short hairpin RNA (shRNA) mediated silencing of β -catenin expression resulted in a significant decrease in *c-jun* promoter activation ($p \leq 0.0001$), leading us to conclude that β -catenin can synergistically contribute to the activation of *c-jun* through MEF2. c-Jun fulfills a prominent cellular role as part of the activator protein-1 (AP-1) complex, that is known to promote cell proliferation by its induction of cyclin D1 (193) and down-regulation of p53 (194). This experiment was repeated using an siRNA targeting β -catenin at a different sequence with similar results, as shown in Fig. S13.

In view of this, we further investigated the cellular implications of the co-operativity between MEF2 and β -catenin on *c-jun* expression. Ectopically expressed combinations of MEF2C and degradation resistant β -catenin (act) with the pCJ6 reporter revealed that co-expression of MEF2C and β -catenin (act) results in a significant and synergistic increase in *c-jun* promoter activity ($p \leq 0.001$; Fig. 11B, black bars) in A10 cells, an effect not

observed when the MEF2 site is mutated (pJSX reporter gene; Fig. 11B, grey bars). We further analyzed c-Jun protein levels since c-Jun expression has been shown to be regulated by canonical Wnt signalling through a TCF/LEF binding motif on its endogenous promoter, which is truncated in the pJC6 reporter (172). Based on reports in the literature, inducing quiescence results in a reduction of c-Jun (195, 196), which was observed even with co-expression of both MEF2C and β -catenin (act; Fig. 11C, left panel). However, under serum stimulated conditions, which we would predict to allow co-operativity, co-expression of MEF2C and β -catenin (act) resulted in increased c-Jun protein levels (Fig. 11C, right panel). Conversely, in a loss-of-function approach, shRNA mediated knockdown of β -catenin resulted in decreased c-Jun protein levels (Fig. 11D, left panel). Under the same conditions, *c-jun* promoter activity was significantly decreased following depletion of β -catenin, but not in the pJSX reporter ($p \leq 0.01$; Fig. 11D, middle and right panels). The experiments in Fig. 11D were repeated using an siRNA targeting β -catenin to confirm the effect was target specific (Fig. S14). To assess the functional implications of this co-operativity, we performed MTT cell proliferation assays. In both A10 and HEK 293T cell types, co-expression of MEF2C and β -catenin (act) results in a significant increase in cell proliferation, when compared to either MEF2C or β -catenin (act) transfected alone ($p \leq 0.01$ and $p \leq 0.0001$, respectively; Fig. 11E).

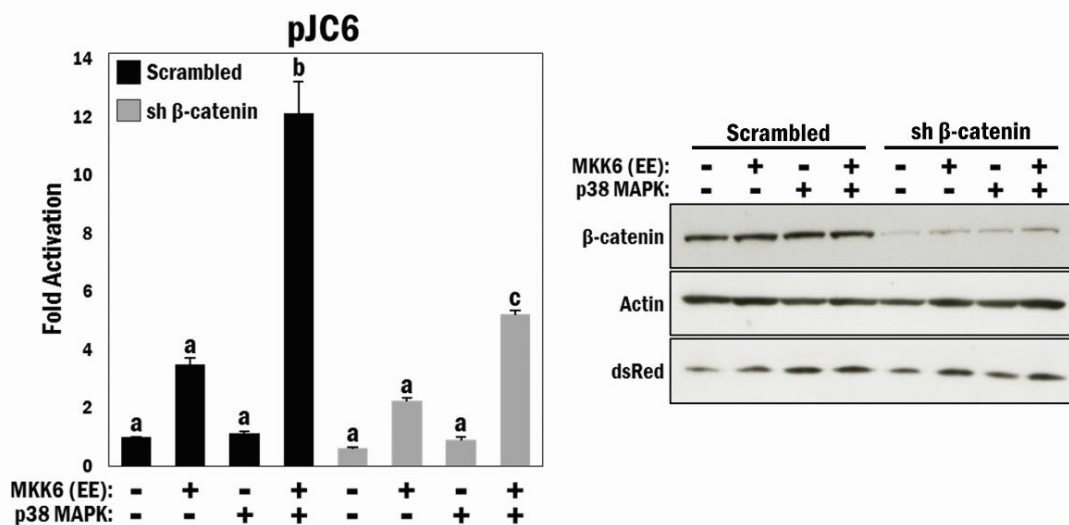
To establish a role for p38 MAPK regulation of Wnt-mediated cell proliferation, we performed another MTT assay with primary VSMCs that were cultured in serum free media then stimulated with Wnt3a in the presence of active or inactive p38 MAPK inhibitor. The results show a significant increase in cell proliferation following Wnt3a stimulation, which is completely abolished with p38 MAPK inhibition ($p \leq 0.0001$; Fig. 11F). Additionally, Wnt3a stimulation increased the promoter activity of c-Jun (≤ 0.001 ;

Fig. 11G, left panel), as well as the protein levels of c-Jun and another Wnt target gene, c-Myc, which were all reduced in the presence of p38 MAPK inhibitor (Fig. 11G, right panel). Since our data regarding PDGF stimulation showed increased nuclear β -catenin localization and Wnt reporter gene activity, we determined what the effect was on other Wnt targets. c-Jun reporter gene activity is increased with PDGF stimulation ($p \leq 0.01$; Fig. 11H, left panel), as well as c-Jun protein levels. Furthermore, this treatment also caused an increase in the protein levels of other Wnt target genes (Cyclin D1 (75), c-Myc (114), and Axin2 (171), Fig. 11H, right panel).

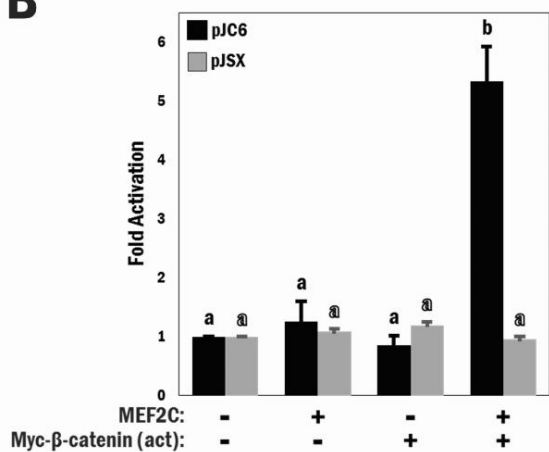
Based on the collective data, we conclude that p38 MAPK is a key regulator of canonical Wnt signalling by promoting a phospho-dependent interaction between MEF2 and β -catenin to enhance co-operative transcriptional activity and cell proliferation.

Figure 11

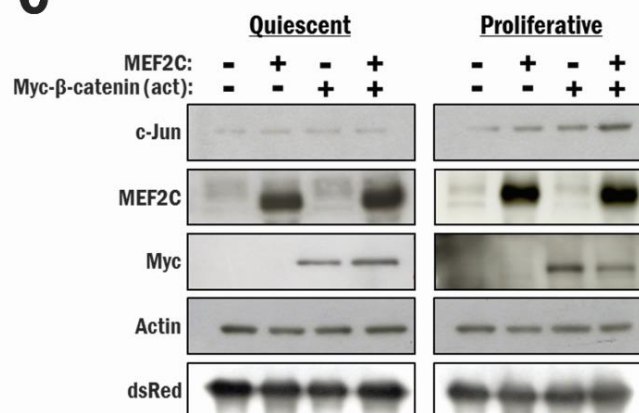
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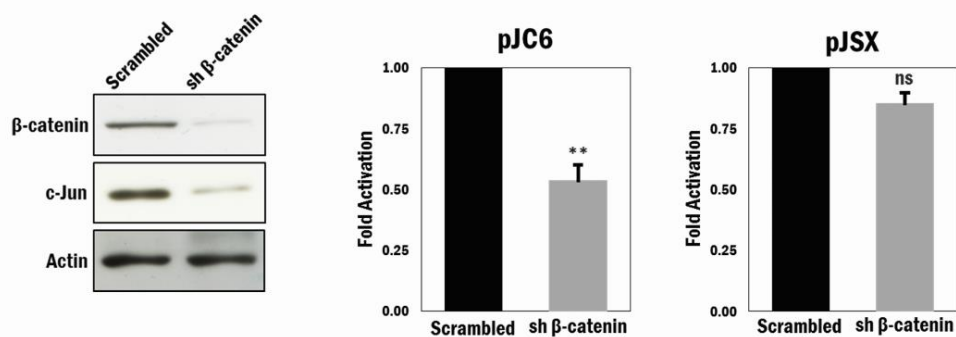
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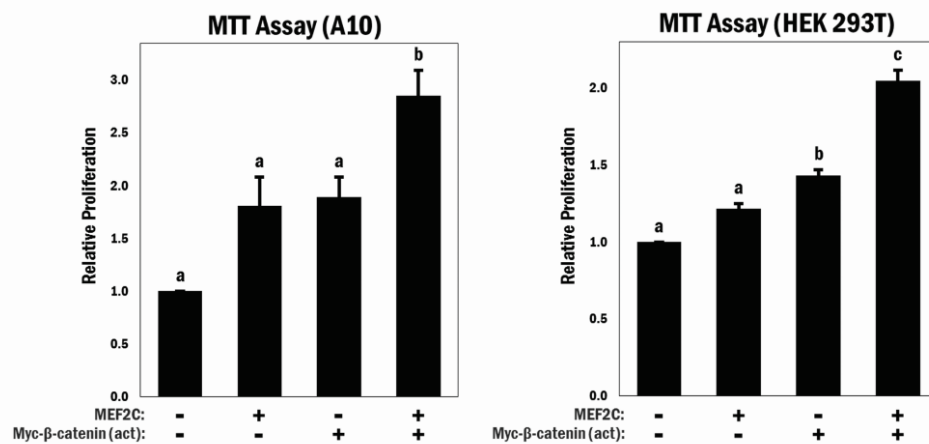
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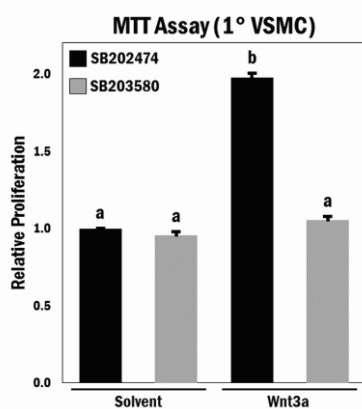
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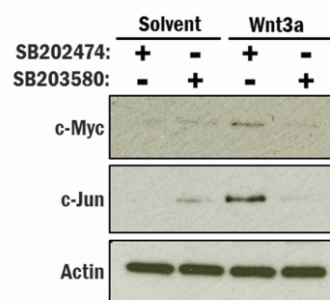
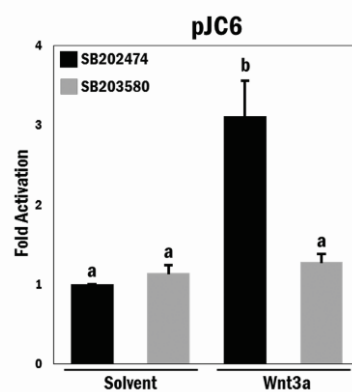
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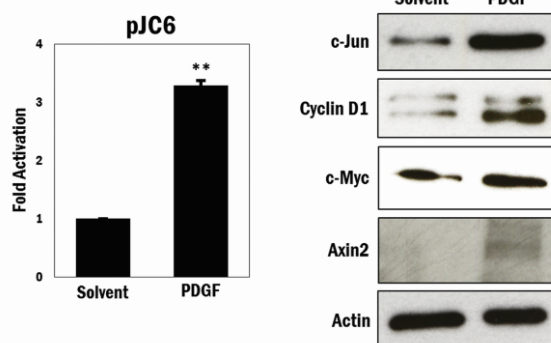


Figure 11: MEF2 and β -catenin cooperate to promote cell proliferation in a p38 MAPK-dependent manner, and p38 MAPK regulates Wnt target gene protein levels. **A.** (Left panel) c-Jun reporter gene (pJC6) activity was measured in A10 cells under various combinations of ectopically expressed MKK6 (EE) and p38 MAPK, and either scrambled shRNA (control) or shRNA β -catenin, as indicated in the figure; $b, c = p \leq 0.0001$. (Right panel) Corresponding Western blot analysis of cell lysates. **B.** A10 cells were transfected with different combinations of MEF2C and Myc- β -catenin (act), and co-transfected with either pJC6 or pJSX (analogous promoter region containing mutations in the MEF2 binding site), as indicated; $b = p \leq 0.001$. **C.** Western blot analysis of either quiescent (serum withdrawn, 12 hr) or proliferative (10% FBS maintained) A10 cells that have been transfected with different combinations of MEF2C and Myc- β -catenin (act), as indicated. **D.** Western blot analysis of A10 cells that have been transfected with scrambled shRNA (control) or shRNA β -catenin with comparative pJC6 and pJSX reporter activities; $** = p \leq 0.01$. **E.** MTT cell proliferation assay in A10 ($b = p \leq 0.01$) and HEK 293T ($b, c = p \leq 0.01$) cells that have been transfected with different combinations of MEF2C and Myc- β -catenin (act), as indicated. **F.** MTT cell proliferation assay in primary VSMCs that were serum withdrawn (12 hr) and pre-treated with SB203580 or SB202474 (10 μ M, 45 min) then stimulated with Wnt3a (200 ng/mL) or solvent (PBS) for 4 hr; $b = p \leq 0.0001$. **G.** (Left panel) A10 cells were transfected with the pJC6 reporter for 3 hr followed by treatment with either Wnt3a (200 ng/mL), solvent (PBS), SB203580 (10 μ M), or SB202474 (10 μ M) for 16 hr in serum-free media, as indicated; $b = p \leq 0.001$. (Right panel) Primary VSMCs were treated similar to F and lysates subjected to Western blot analysis, as indicated. **H.** (Left panel) pJC6 reporter assay in A10 cells that were transfected for 3 hr followed by PDGF treatment (20 ng/mL) or solvent (0.1% BSA in 4 mM HCl) for 16 hr in serum-free media; $b = p \leq 0.01$. (Right panel) Western blot analysis of primary VSMCs that were serum withdrawn for 12 hr and treated with PDGF (20 ng/mL) or solvent (0.1% BSA in 4 mM HCl) for 4 hr.

Discussion

A prolific literature concerning the genetic, developmental and biochemical basis of canonical Wnt signalling has positioned the β -catenin transcriptional regulatory protein as an indispensable determinant of embryonic development, stem cell biology, and also post-natal physiology and pathophysiology in a variety of organisms ranging from *C. elegans* to humans (169, 170, 197). Here we report a novel, critical step in the nuclear function of β -catenin mediated transcriptional control that results from a protein-protein interaction between β -catenin and MEF2, which is potently enhanced by p38 MAPK signalling. This phospho-dependent interaction consolidates β -catenin accumulation in the nucleus, leading to a substantial activation of Wnt dependent target genes (Fig. 12).

There is ample evidence of kinase mediated phosphorylation modulating the affinity of protein-protein interactions in the canonical Wnt pathway, resulting in important outcomes for Wnt dependent target gene activation. Interestingly, while it is widely acknowledged that CKI/GSK3 phosphorylation of β -catenin leads to the conformational formation of a binding site for β -TrCP, the E3 ligase that mediates ubiquitination and subsequent degradation of β -catenin, it is perhaps not as well recognized that these kinases also phosphorylate Axin and APC, leading to their tighter association with β -catenin (198, 199). Thus, phospho-dependent protein interactions are a signature of β -catenin function, and the association with MEF2 that we report here falls into this category. Moreover, it may be more than an interesting coincidence that two serine/threonine phosphatases, PP1 and PP2A, that target the β -catenin destruction complex (108, 200) to reduce β -catenin degradation, have also been characterized by us

and others as MEF2 phosphatases (189, 201). Whether MEF2 can recruit PP1 or PP2A to the β -catenin complex remains to be investigated.

While the prevalent dogma in the literature is that β -catenin stabilization results in enhanced nuclear levels, how β -catenin is retained in the nucleus is unclear (38, 174). One study reported that Wnt-induced β -catenin stabilization is insufficient for its nuclear accumulation, and further proposed that Wnt activation of the Rac1 GTPase is also necessary to promote nuclear localization (66). Previous studies indicated that β -catenin translocates into the nucleus in an NLS and importin-independent manner, possibly by direct interaction with nuclear pore proteins. Conversely, β -catenin nuclear extrusion has been linked to its association with APC (38), Axin (40), and Ran binding protein 3 (RanBP3) (39). It is of interest to consider the possibility that p38 MAPK/MEF2 signalling could alter the balance of the cell adhesive and transcriptional properties of β -catenin. In our studies, the dramatically enhanced levels of nuclear β -catenin with p38 MAPK activation appear to concomitantly reduce β -catenin amounts within the plasma membrane associated adhesion complex. As is the case with many proteins that partition between the cytoplasm and nucleus, β -catenin localization is dynamic and probably the product of several processes involving both shuttling and nuclear retention. Here we present evidence suggesting that a p38 MAPK dependent interaction with the MEF2 transcription factors enhances β -catenin nuclear retention in a number of cell lineages and in primary VSMCs.

An implication of the above discussion is that the p38 MAPK/MEF2 signalling pathway will dynamically intersect with the Wnt pathway in MEF2 dependent target tissues. To date, MEF2 has been found to play important roles in cardiac (202, 203), skeletal (204-206) and smooth muscle (164, 207), neurons (208), B cells (209, 210), and osteoblasts (211). Many of these tissues are also profoundly influenced by Wnt signalling. For example, in C2C12 myoblasts, Wnt4, generally recognized as a non-canonical Wnt, has been shown to increase myogenesis by activation of canonical Wnt/ β -catenin signalling and up-regulation of the muscle regulatory factors (MRFs) Myf5, myogenin, and muscle regulatory factor 4 (MRF4) (212). MEF2 is concomitantly required in these tissues during skeletal myogenesis by its direct interaction with the aforementioned MRF myogenin and MyoD to co-operatively activate the myogenic program (205). Moreover, the *mef2c* gene is controlled by these MRFs through an E-box in its regulatory region, whose expression is essential for the maintenance of the myogenic program (213). In view of the profound role that MEF2 plays in both embryonic skeletal muscle and in adult muscle stem (satellite) cells, it is tempting to speculate that p38 MAPK/MEF2 may intersect with Wnt signalling in these contexts. It will be interesting to observe if, in MEF2 dependent tissues, Wnt pathway modulation is a component of MEF2 function or whether, in fact, both pathways are mutually co-dependent.

A litany of highly insightful studies since the early eighties have implicated β -catenin and its associated regulators as a driver of various human cancers (214). Moreover, identification of several β -catenin interacting transcriptional regulators has provided a mechanism by which proliferative growth promoting genes are regulated (11, 215). Aberrations in components of this pathway of growth control have been directly linked to

colorectal cancers, Wilm's tumours in paediatric renal cancer, metastasis in some lung cancers, and a variety of others (214). In addition, Wnt pathway hyperactivity has been reported in certain breast cancer types without any apparent mutations in components of the pathway (216, 217). These observations raise the question as to whether "drugging" the canonical Wnt pathway may be efficacious in certain cancers (214). Moreover, our observations suggest that pharmacological targeting of the p38 MAPK pathway might serve as an unconventional way to blunt Wnt/ β -catenin signalling in certain cancer cells. Of course the efficacy of targeting a kinase that plays a role in other cellular processes is unclear at this point, but, the existence of a number of well characterized cell permeable small molecule inhibitors of p38 MAPK should make this a tractable question to address in the near future.

Here, we report a previously uncharacterized aspect of canonical Wnt/ β -catenin signalling through cross talk with the p38 MAPK/MEF2 signalling pathway, which may have implications in all muscle types, neurons, B cells, and osteogenic cells, as well as in Wnt dependent cancer cells. These observations raise the possibility of therapeutic intervention to target canonical Wnt signalling in a variety of pathologies.

Figure 12

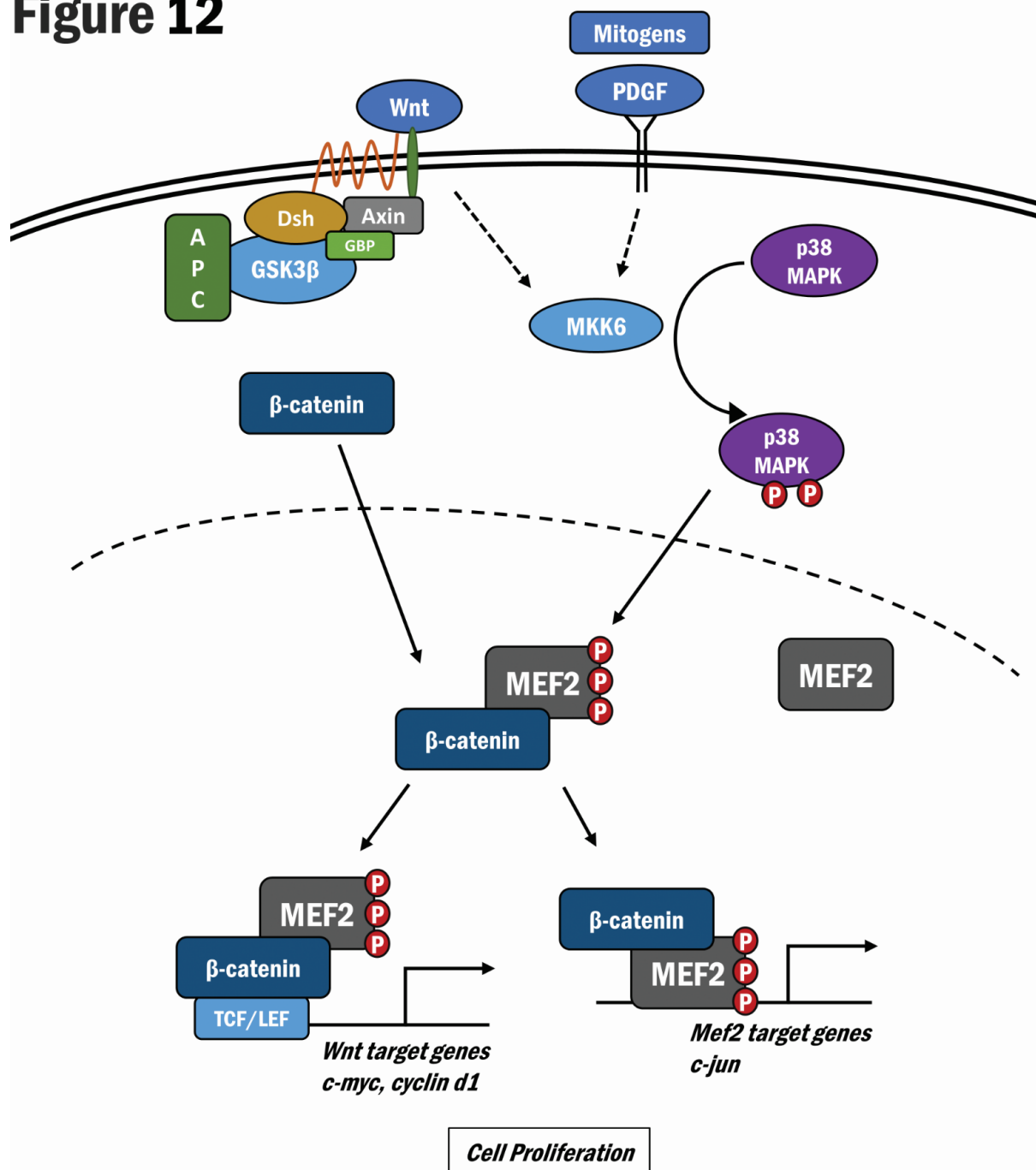


Figure 12: Schematic illustrating the cross-talk between Wnt/ β -catenin and p38 MAPK/MEF2 signalling. Wnt-mediated inactivation of GSK3- β -catenin destruction complex results in suppressed degradation of β -catenin. Activation of p38 MAPK signalling results in phosphorylation and activation of MEF2 in the nucleus, which enhances its physical interaction with nuclear β -catenin. This results in nuclear β -catenin retention, synergistic activation of Wnt target genes, *c-myc* and *c-jun*, and increased cell proliferation.

Materials and Methods

Cell culture: A10, COS7, HEK 293T, and C3H/10T1/2 cells were obtained from American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose (HyClone), supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Invitrogen), according to ATCC recommendations. Primary vascular smooth muscle cells were isolated and cultured from mice aortae as previously described (218). Cells were grown in serum free DMEM containing no FBS, for the time specified in each experiment. Cells were maintained in an incubator at 95% humidity, 5% CO₂, and 37°C. Drug and protein treatments were completed for the indicated times at the indicated concentrations.

Transfections: For reporter gene assays and protein over-expression, cells were transfected using TurboFect (Fisher Scientific) using recommended instructions. siRNA was transfected according to TurboFect recommendations. Cells were transfected for 3 hr followed by media replacement, and were harvested or serum withdrawn 24-48 hr later, unless otherwise indicated. Myc(N)- β -catenin was N-terminally truncated by 89 amino acids prior to tagging to prevent GSK3 β phosphorylation and degradation, named β -catenin act (activated).

Gene silencing: shRNA β -catenin (target sequence AACATGCAGTTGTCAATTTGA) or its scrambled control (target sequence GCAATTCTGAAGTCTGATATA) was inserted into the pSilencer 3.0 H1 vector, as per the manufacturer's instructions. siRNA (Sigma-Aldrich) for MEF2A (siRNA #1 SASI_Mm01_00120788, siRNA #2

SASI_Mm01_00120789), MEF2C (siRNA SASI_Mm01_00092891), β -catenin (siRNA SASI_Rn01_00099923), and universal scrambled RNA were used at a concentration of 25 nM. Double siRNA experiments used 25 nM of each siRNA (50 nM total), and equivalent controls used 50 nM of scrambled RNA.

Antibodies and reagents: Rabbit polyclonal MEF2A and MEF2C antibody has been previously described (219, 220). Actin, c-Jun, and dsRed were purchased from Santa Cruz. β -catenin antibody was purchased from Cell Signalling Technologies. MEF2D antibody was purchased from BD Biosciences. c-Myc antibody was purchased from the developmental studies hybridoma bank. Flag, Glutathione-S-transferase, and actin (α -smooth muscle) were purchased from Sigma-Aldrich.

Purified recombinant mouse Wnt3a protein was purchased from Abcam. p38 MAPK inhibitor (SB203580) and its inactive analog (SB202474) were purchased from Calbiochem. Purified recombinant human platelet-derived growth factor-BB was purchased from Sigma-Aldrich.

***In vitro* kinase assay and mass spectrometric analysis for protein phosphorylation:** 3 μ g of either purified recombinant GST, GST-MEF2C, or 6xHis- β -catenin was mixed with either 0.5 μ g purified recombinant GST-p38 MAPK (Millipore) and with [γ - 32 P]ATP, and incubated at 30°C for 30 min. Samples were denatured in SDS loading buffer at 95°C for 5 min, proteins separated by 10% SDS-PAGE, and exposed on X-ray film (GE Healthcare) for 16 hr at -80°C to detect 32 P incorporation.

In order to observe protein phosphorylation by liquid chromatography-mass spectrometry (LC-MS), *in vitro* p38 MAPK treatment was repeated with ATP in place of [γ - 32 P]ATP. The protein mixture was separated by SDS-PAGE. Coomassie blue stained gel bands whose molecular weights corresponded to ATF2 and β -catenin were cut away then destained with acetonitrile (Fisher Scientific). The gel bands were treated with 9 mM dithiothreitol in 100 mM ammonium bicarbonate (Sigma-Aldrich) at 60°C for 15 min, then alkylated with iodoacetamide (Sigma-Aldrich) in the dark for 30 min at room temp. Each band was treated with 1 μ g of porcine trypsin (Promega) at 37°C for 16 hr. Tryptic peptides were resolved by nanoflow-HPLC over 60 min acetonitrile gradients on a NanoLC-Ultra 2D-Nanoflex cHiPLC system (AB Sciex). A 0.5 mm long, 200 μ m diameter trap and a 150 mm long, 75 μ m diameter analytical column were used. The trap and column were both packed with 3 μ m diameter C18 particles having 120Å pores. Mobile phase A was 0.1% formic acid in water (v/v) and mobile phase B was 0.1% formic acid in acetonitrile (v/v) (Honeywell). The pump was operated at 300 nL/min with the following mobile phase B contents: 2% (v/v) at 0 min, 35% at 35 min, 80% from 35.5 to 38.5 min, 2% from 39 to 60 min. Peptides were detected on an Orbitrap Elite mass spectrometer (Thermo Scientific).

The mass spectrometer was operated in data-dependent acquisition mode with survey scans having resolution fixed at 60,000. The data acquisition method incorporated an inclusion list whose m/z values corresponded to fully tryptic β -catenin peptide and phosphopeptide ions containing putative p38 MAPK phosphosites based on the kinase consensus sequence. Predicted phosphosites S191, S246, and S605 were represented in the sequences SPQMVSAIVR, MLGSPVDSVLFYAITTLHNLLLHQEGAK, and GLNTIPLFVQLLYSPIENIQR, respectively.

Protein extraction and Western blot analysis: Cells were collected using NP-40 lysis buffer [0.5% v/v Nonidet P-40, 50 mM Tris-HCl (pH 8), 150 mM NaCl, 10 mM sodium pyrophosphate, 1 mM EDTA (pH 8), and 0.1 M NaF] containing 10 µg/mL leupeptin and aprotinin, 5 µg/mL pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM sodium orthovanadate. Protein extracts were denatured in SDS loading buffer at 95°C for 5 min and run on 10% SDS-PAGE, transferred to a PVDF membrane (Millipore), and blocked in 5% skim milk for 1 hr prior to antibody incubation.

Co-immunoprecipitation and CIP treatment: For co-immunoprecipitation, cells were harvested and protein extracted as described above. Immunoprecipitation was performed using the ImmunoCruz Optima kit (Santa Cruz Biotechnology), according to the manufacturer's instructions. Eluates were analyzed by Western blot, as described above.

For CIP treatment, 10 units of CIP was added to 1 mg cell lysate and incubated for 30 min at 37°C using the ImmunoCruz Optima kit.

GST pull down: For GST pull downs, GST-MEF2C and 6xHis-β-catenin fusion proteins were obtained using standard protocols. Briefly, cloned pGex2T (MEF2C) or pET28a (β-catenin) vectors were transformed in BL21 *E. coli*, and protein production induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6 hr. GST-MEF2C and 6xHis-β-catenin expressing cells were sonicated and the proteins purified with glutathione-agarose (Sigma-Aldrich) or nickel-agarose (Qiagen) beads, respectively. Protein concentrations were estimated by SDS-PAGE and Coomassie blue staining, using bovine

serum albumin (BSA) for comparative estimation. 5 µg GST-full length MEF2C protein (or the molar equivalent of the smaller GST, GST-N-terminus MEFC, or GST-C-terminus MEF2C), 5 µg 6xHis-β-catenin, and 25 µL glutathione-agarose beads (50% slurry) were incubated in 600 µL NETN buffer (100 mM NaCl, 20 mM Tris-HCl (pH 8), 0.5 mM EDTA, 0.5% v/v NP-40) overnight at 4°C. Beads were washed three times with PBS and eluates analyzed by Western blot.

Reporter gene assays: Transcriptional assays were performed using luciferase reporter plasmids. Cells were harvested using 20 mM Tris-HCl (pH 7.4) and 0.1% Triton X-100. Values obtained using a Lumat LB (Berthold) luminometer were normalized with Renilla expression in the same cells, and values expressed as fold activation by arbitrarily setting the control condition to 1. Bars represent the mean (n=3) and error bars represent the standard error of the mean (n=3). Independent samples t-tests were performed on experiments with two conditions, while a one-way analysis of variance (ANOVA) followed by a Tukey's HSD post-hoc analysis was performed on experiments with greater than two conditions (SPSS software). P-values are shown with respect to controls, unless otherwise indicated.

Immunofluorescence: Cells were fixed in 4% paraformaldehyde in PBS for 10 min and then permeabilized with 0.3% Triton X-100 in PBS for 5 min, at room temperature. Cells were blocked using 10% goat serum in PBS for 30 min at 37°C, and incubated overnight with β-catenin antibody diluted (1:500) in 1.5% goat serum in PBS at 4°C. Cells were washed three times with PBS (10 min each) and incubated with specified

tetramethylrhodamine (TRITC) or fluorescein isothiocyanate (FITC) antibody (Sigma-Aldrich) diluted (1:500) in 1.5% goat serum for 2 hr at room temperature, with 4',6-diamidino-2-phenylindole (DAPI) nuclear staining (Sigma-Aldrich, 1:10,000) in the last 15 min. Cells were washed three times with PBS (10 min each) and left in PBS at room temperature for confocal microscopy imaging. Images were captured using either a Carl Zeiss Axio Observer.Z1 (Photometrics Evolve 512 EMCCD Camera) or LSM 700 AxioObserver microscope. Cells were imaged under 40x (EC Plan-Neofluar, 1.30 NA in Oil), 63x (Plan-Apochromat, 1.40 NA in Oil), or 100x (alpha Plan-Apochromat, 1.46 NA in Oil) objectives using ZEN image acquisition software. Images were taken as a Z-stack and projected orthogonally by maximum intensity. Relative β -catenin nuclear/cytosolic fluorescence was quantified using ImageJ with the Fiji image processing package. The nucleus was traced using DAPI or Hoechst 33342 for reference and the following procedure and calculations were performed: the fluorescent intensity of 3 areas in the field with no cells were averaged and multiplied by the nuclear area to calculate background fluorescence. The integrated density of the nucleus was subtracted by this value and divided by the nuclear area to calculate the average nuclear intensity. 3 areas of cytosol that were evenly spaced around the nucleus were measured and background subtracted as above, and divided by the area to calculate average cytosolic intensity. This was divided into the average nuclear intensity to calculate the nuclear/cytosolic ratio of the cell.

***In vitro* binding assay:** 2 μ g of purified recombinant GST-MEF2C (Abnova) was incubated with 0.4 μ g of purified recombinant GST-p38 MAPK (Millipore) in kinase buffer (NEB) with 200 μ M ATP (NEB), as specified, for 3 hr at 30°C. 5 μ g of purified

recombinant 6xHis- β -catenin was added with 30 μ L glutathione-agarose beads (50% slurry) and 600 μ L NETN buffer (100 mM NaCl, 20 mM Tris-HCl (pH 8), 0.5 mM EDTA, 0.5% NP-40), and incubated for 1 hr at room temperature. Beads were washed three times with TBS and heated with SDS loading buffer for 5 min at 95°C, run on a 10% SDS-PAGE gel, and subjected to Western blotting.

MTT cell proliferation assay: Vybrant® MTT cell proliferation assay kit was purchased from Life Technologies, and used according to manufacturer's instructions. Cells were transfected for 3 hr and recovered for 24 hr prior to the MTT assay procedure. Bars represent the mean (n=3) and error bars represent the standard error of the mean (n=3). A one-way ANOVA followed by a Tukey's HSD post-hoc analysis was performed (SPSS software). P-values are shown with respect to controls, unless otherwise indicated.

Chapter 3

A Wnt-dependent β -catenin:FMRP interaction regulates translation

by

Saviz Ehyai¹, Tetsuaki Miyake², Declan Williams³, and John C. McDermott⁴

Rationale:

Based on the conclusions from Chapter 2, and from what we already know about the importance of β -catenin interacting partners, we were compelled to devise a strategy in which we could ascertain a large set of potential proteins interactions with β -catenin in VSMCs, a so-called interactome. We hypothesized that while many interacting partners have been discovered and characterized, there remains many more that have yet to be found, any one of which may play unexpected and novel roles in altering β -catenin function. To address this, we performed tandem LC MS/MS mass spectrometric analysis of A10 cell lysates using β -catenin immunoprecipitates to identify protein interactions. This was followed by biochemical analysis on a particular interacting partner, the fragile X mental retardation protein.

¹Experimental design, manuscript draft, figures 13A, C, 14-17, and supplementary S16-S18, S21.

²Experimental design, manuscript draft, and supplementary S19, S20.

³Experimental design, manuscript draft, figure 13B, and supplementary S15.

⁴Experimental design and manuscript draft.

Summary

Wnt/ β -catenin signalling is an essential and evolutionarily conserved pathway regulating various cellular and organismal functions throughout embryonic development and adulthood. Aberrant Wnt/ β -catenin signalling also contributes to various pathologies including cancer, necessitating a comprehensive understanding of the context dependent molecular mechanisms regulating this pathway. Since protein-protein interactions underpin β -catenin function and localization, we sought to identify novel β -catenin interacting partners by affinity purification coupled to tandem mass spectrometry (AP-MS/MS) in vascular smooth muscle cells (VSMC's.) where Wnt/ β -catenin is involved in both physiological and pathological control of cell proliferation. Using this AP-MS/MS approach, we report novel components of the VSMC β -catenin interactome. Bioinformatic analysis of the β -catenin interacting protein network(s) implied novel functions for β -catenin, particularly in protein translation where a direct interaction between β -catenin and the fragile X mental retardation protein (FMRP) was observed and confirmed. Biochemical and loss of function studies further revealed a basal recruitment of both proteins to the translational pre-initiation complex fulfilling a translational repressor function. Wnt stimulation reversed this translational repressor function by sequestering β -catenin away from the pre-initiation complex into the nucleus. Here, we present evidence that dissociation from FMRP and subsequent de-repression of translation is a previously unrecognized function of Wnt mediated β -catenin signalling.

Introduction

The β -catenin protein fulfills essential roles in a variety of cellular processes, both at the cell membrane and in the nucleus, and its malfunction has been linked to developmental disorders and diseases, including cancer (36, 124, 166, 168). Underlying β -catenin's pleiotropic functions are its complex domain structure, consisting of an N-terminal domain which is regulated by glycogen synthase kinase 3 (GSK3) (21), a middle region made up of 12 armadillo repeats for various protein-protein interactions, an adjacent "helix C" which packs together the armadillo repeats (1), and a C-terminal transactivation domain (7).

In basal conditions, β -catenin interacts with α -catenin and cadherins to promote cell adhesion, while activation of the canonical Wnt signalling pathway results in its nuclear translocation and interaction with various transcription factors to activate target gene expression and cell proliferation (170, 221). Thus, it is generally recognized that there are two main subcellular locations at which β -catenin functions: cell adhesion at the membrane, and gene transcription in the nucleus. These seemingly disparate roles of β -catenin are tightly regulated by signalling pathways which regulate protein-protein interactions to finely balance β -catenin stability and subcellular localization to provide diverse phenotypic responses (222, 223). Since the control of Wnt/ β -catenin signalling is a potential target for reversing pathological conditions by restricting proliferation, migration, and apoptosis, key β -catenin interactors are under investigation for potential therapeutic treatments (155, 157, 214). Although the regulation of β -catenin function by a number of different protein-protein interactions have been documented (44, 224), the diverse roles of β -catenin in a myriad of different cell types and tissues suggest that

multiple context dependent protein networks are involved in β -catenin function and are still at large.

One particular context of interest is in vascular smooth muscle cells (VSMCs). Since VSMC's are, unlike other muscle types, uniquely able to maintain a high degree of phenotypic plasticity without terminal differentiation, β -catenin signalling and localization are crucial determinants of the VSMC phenotype in response to external stimuli (155, 157, 222). VSMC phenotype changes have been implicated in a variety of vascular pathologies such as atherosclerosis and restenosis following angioplasty, we therefore sought to further elucidate β -catenin function in VSMC's.

Since a primary determinant of β -catenin function relies on protein interaction networks, our initial goal was to characterize the unique β -catenin interactome in VSMCs. To address this, we utilized immunoaffinity chromatography coupled with LC-MS/MS and identified 131 potential β -catenin interacting partners in VSMCs. The majority of the interacting partners were novel and uncharacterized, while known interactors such as α -catenin were also identified. Gene ontology (GO) enrichment analysis revealed a strong putative involvement in protein translation, a previously unknown function of β -catenin. Due to the potential connection with translation, we were interested in identification of the fragile X mental retardation protein (FMRP), a known translational repressor, as a β -catenin interacting protein. Further experiments supported the idea that β -catenin interacts with FMRP at the translational pre-initiation complex, and thus, we present evidence suggesting a non-canonical and potentially crucial role for β -catenin in translational repression. These observations have implications for our understanding of Wnt/ β -catenin signalling in various cellular processes and in disease states.

Results

Characterization of β -catenin interacting partners

β -catenin is a multifunctional protein and has been found at a variety of sub-cellular locations where it interacts with specific binding partners in a spatiotemporal manner (224). However, identification of interacting partners with β -catenin in a VSMC context has not been systematically carried out. Therefore, we sought to document the β -catenin interactome using an affinity purification approach coupled to tandem LC MS/MS (summarized in Figure 13A). The β -catenin containing protein complexes were immunoprecipitated with a polyclonal β -catenin antibody or an unprogrammed rabbit serum was used as control. The co-precipitated proteins with the antibodies were subsequently identified by LC- MS/MS analysis. The criteria used for the inclusion of a protein as a potential interactor was the presence of at least a single unique peptide in two of the five β -catenin IP replicates and none in any of the five unprogrammed rabbit serum control replicates. This approach was justified by the absence of β -catenin in all rabbit serum column eluates and its appearance in anti- β -catenin column eluates from all biological replicates. Protein identifications from anti- β -catenin column eluates were confirmed to be sample-specific based on their absence in SEQUEST results from the preceding blank runs and therefore did not result from HPLC column carryover. Those proteins found only in anti- β -catenin column eluates were similarly confirmed to be absent in the preceding blanks.

Using this pipeline (depicted in Figure 13A) we identified 131 potential β -catenin interacting proteins. We ranked the interactors according to the number of replicates in

which they were present and then by the average peptide score among those replicates (defined in *materials and methods*). The top 50 interacting partners are displayed in Figure 13B, and the full list is shown in Supplementary S15. Of the top 50 interactors, 88% were found in 3 or more of the biological replicates. Also, well known interactors such as α -catenin (225) and Mediator 12 (226) were present in the list of interactors indicating that the remainder might thus contain novel bona fide β -catenin interactors.

To categorize the β -catenin interactome, we initially performed a top level gene ontology (GO) term analysis of these interactors for the “Cellular Component” domain. This analysis ascertained that β -catenin interacting partners were present in various cellular compartments, for instance in the cytoplasm (19%) or nucleus (19%) (Figure 13C). What we found particularly interesting was the presence of the ribosome as a cellular component of a number of β -catenin interactors.

In order to further interrogate the identified interactors, we initially performed a preliminary analysis on a candidate protein that was ranked similarly to and shared comparable properties with the known interactor α -catenin. We chose the intermediate filament binding protein Plectin, which is a cytoplasmic protein that anchors intermediate filaments and microtubules to specific locations such as cell junctions, intracellular structures, and various organelles (227). Immunofluorescence of endogenous β -catenin and Plectin in A10 cells revealed distinct co-localization throughout and exclusively in the cytoplasm but not in the nucleus (Supplementary S16). Immunoprecipitation of Plectin and Western blot analysis for corresponding β -catenin confirmed co-precipitation in the same complex (Supplementary S17). Interestingly, these proteins also co-localized at cytoskeletal fiber structures in some cells

(Supplementary S18). This follow up biochemical analysis was utilized to confirm or reject the AP-MS identified interactors as interactions of interest for further study.

Gene ontology enrichment analysis

To further categorize and analyze the β -catenin interactome, we performed a Gene Ontology (GO) Biological Process enrichment analysis to determine biological pathways that were over-represented among the list of interacting proteins (Figure 14A). Terms that were shared among a set of common genes were evaluated using the Kappa statistic, and interrelatedness among nodes exceeding a Kappa score of 0.5 were connected to each other. A variety of processes were depicted here, and we confirmed several terms were previously associated with canonical β -catenin function, including tube morphogenesis (228), actin filament organization (229), and cell growth (35). Interestingly, we also noted that several terms that were not previously associated with β -catenin function such as mRNA regulation and translation. In addition, the largest number of genes and the lowest p-values within the network were associated with these terms, in comparison with canonical β -catenin related terms (Figure 14B). Since the terms “translation” and “mRNA processing” were novel for β -catenin and their p-values suggested that these terms were not coincidental, we decided to focus on characterizing a β -catenin interaction with proteins involved in these processes.

Figure 13

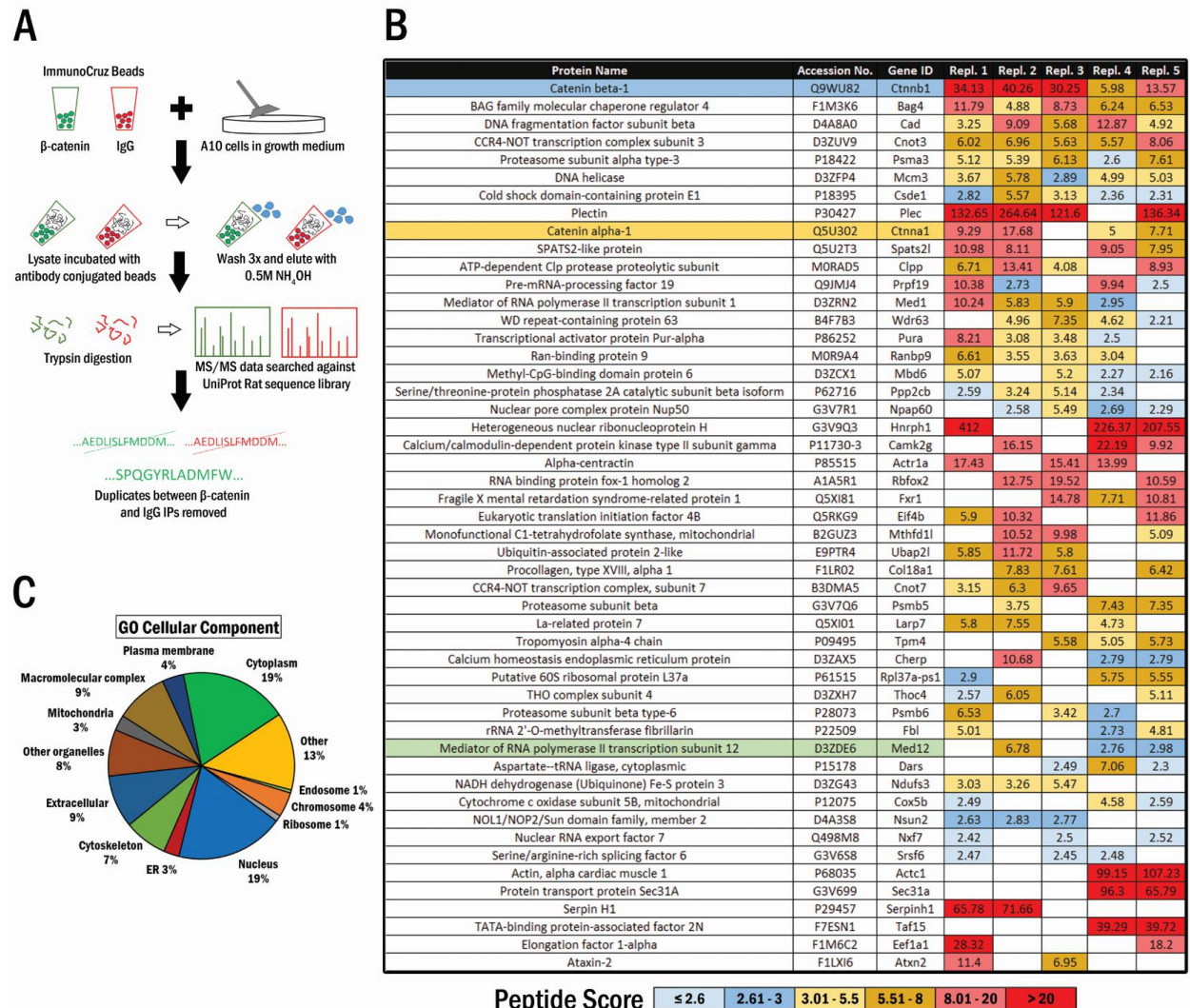
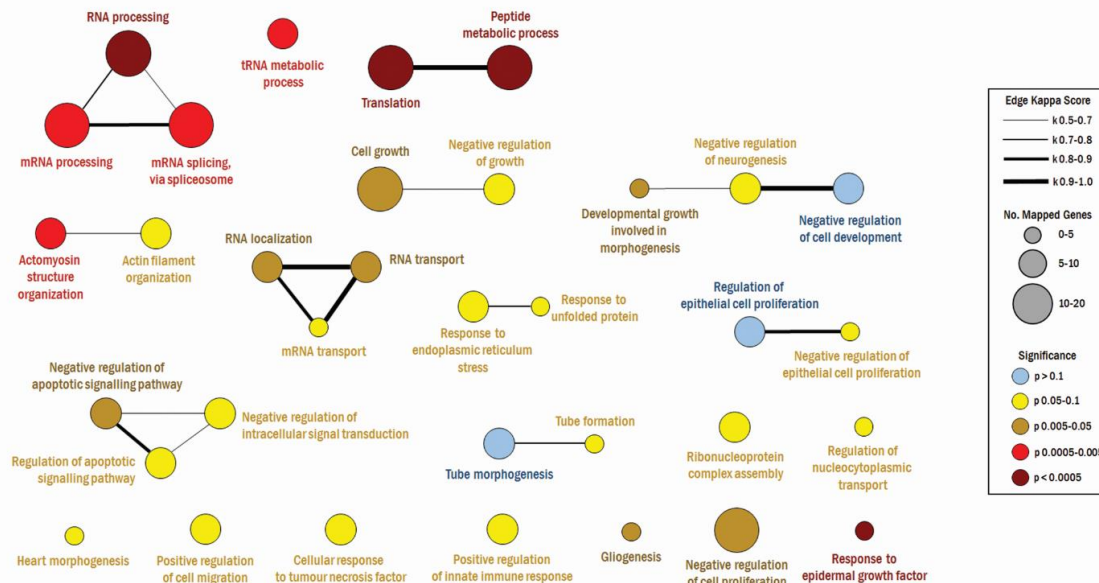


Figure 13: Experimental workflow and top 50 sorted β -catenin interactors shown with GO cellular component analysis. **A.** A10 cells were harvested and lysates mixed with agarose beads conjugated to either β -catenin antibody or rabbit serum overnight at 4°C. Beads were washed three times and bound proteins were eluted with 0.5M NH_4OH for 20 min at room temp. Elutions were dried by vacuum spinning and pellets were resuspended in PBS then trypsin digested overnight. A list of interacting partners was created using the MS/MS data searched against the UniProt database (> 95% peptide confidence). Proteins common to both β -catenin and rabbit serum eluates were removed from the list. **B.** The list of potential β -catenin interacting partners were sorted first by number of replicates present then by average score among replicates. β -catenin itself, present in all replicates, is highlighted in blue. Known cytoplasmic and nuclear interacting partners, α -catenin and Mediator 12, are highlighted in yellow and

green, respectively. **C.** A first level Gene Ontology (GO) domain analysis of the top 50 candidates was performed to identify the GO terms that are associated with the candidate proteins in the GO domain “cellular component”. The percentage of proteins associated with each GO term of the respective domain is represented in the pie chart.

Figure 14

A



B

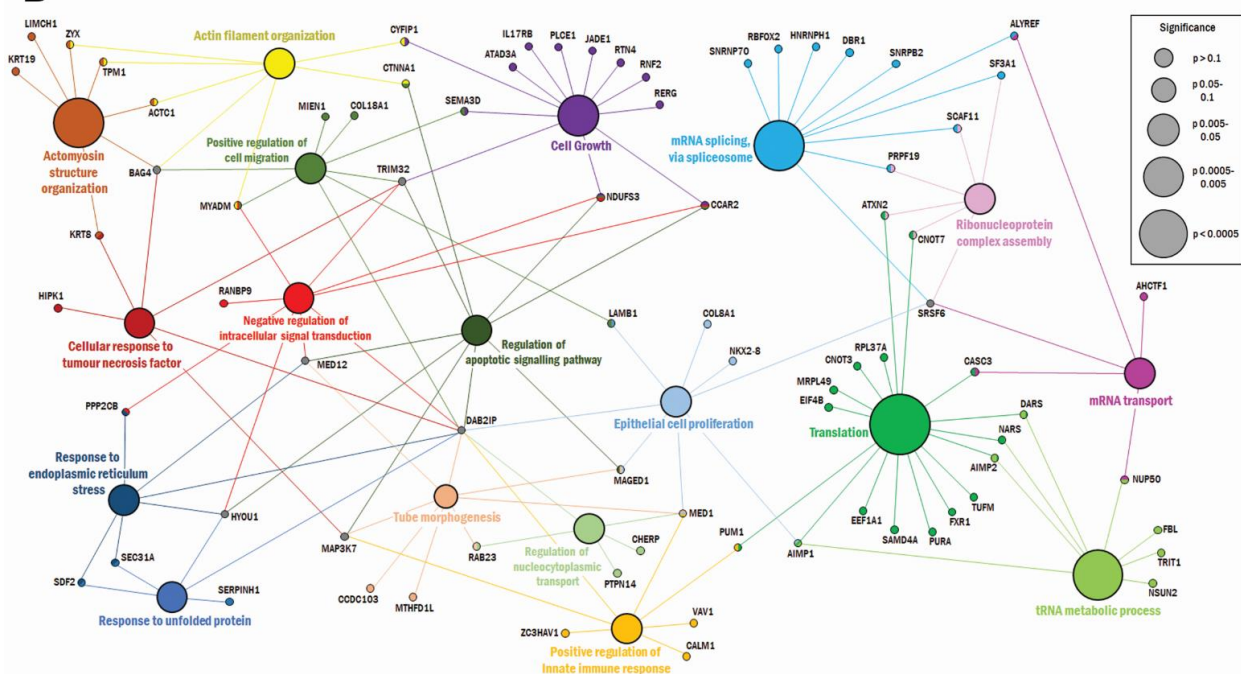


Figure 14: GO Biological Process enrichment analysis. A. All 131 candidate proteins were used to identify over-represented GO Biological Process terms, which are represented in the following network. The node size corresponds to the number of candidate proteins associated with a particular term. The node colour corresponds to the associated term's p-value. The line thickness corresponds to the kappa score value to show interrelatedness, as defined in the legend. Node position and line length were created arbitrarily. **B.** Leading terms from **(A)** are represented by their associated proteins. The node size corresponds to the p-value of a particular term, as defined in the legend. Node position and line length were created arbitrarily.

β -catenin colocalizes and directly interacts with FMRP in the cell

First, we sought to verify the interactions between β -catenin and a potential interactor involved in translational regulation – the fragile X mental retardation protein (FMRP). Our initial MS/MS analysis revealed that FMRP was present in five out of six biological replicates and correspondingly absent in IgG controls. FMRP was again detected in additional experimental pull-downs assays although we did also identify it in some IgG controls in the subsequent analyses. On balance, we reasoned that the data indicated that the interaction was worth pursuing and further biochemical and imaging data supported the FMRP: β -catenin interaction (see below). Immunofluorescence analysis of endogenous β -catenin and ectopically expressed Flag-FMRP in A10 cells revealed co-localization in distinct perinuclear puncta. A line scanned RGB profile revealed that FMRP and β -catenin fluorescence intensity peaks were matched, and an orthogonal projection showed co-localization in the XY, XZ, and YZ planes above and around the nucleus (Figure 15A). Furthermore, we confirmed endogenous β -catenin and ectopically expressed Flag-FMRP formed a protein complex by co-immunoprecipitation analysis (Figure 15B i). We also found when analyzing endogenous levels of the two proteins, that they interact in primary vascular smooth muscle cells by co-immunoprecipitation analysis (Figure 15B ii).

To further determine if this interaction is direct, we performed a series of GST pull down assays using bacterially expressed and purified GST- β -catenin fusion proteins expressed as bait and purified 6xHis-FMRP as prey. Since GST- β -catenin but not GST alone interacted with FMRP, these data indicate that the interaction is specific and direct.

Both the N-terminus (aa 1-106) and armadillo repeat region (aa 120-683) of β -catenin were sufficient to interact with FMRP independently (Figure 15C).

Next, we used an interaction trap in a cellular system using a 3-way fluorescence hybridization assay based on GFP-nanotrap technology to assess the robustness of the β -catenin/FMRP interaction (230). In this system, a GFP binding protein (GBP), a fragment of llama single chain antibody (13kDa) specific for GFP (231), is anchored to a distinctive sub-cellular site, and GFP/EYFP fusion proteins (of the bait protein of interest) are coordinately recruited to the same sub-cellular location due to the interaction of the GFP/EYFP moiety with the GBP. If a candidate interacting protein (labelled with mCherry or detected by immunofluorescence) interacts with the GFP/EYFP fusion protein (and not GFP/EYFP by itself), the interacting protein would be enriched at the specific anchor site thus providing a very robust assay of the protein-protein interaction in a cellular system. This interaction can be assessed by confocal fluorescence microscopy. We used two different anchor sites for the GBP: cytosolic F-actin and the nuclear lamina. First, to anchor GBP to F-actin, we used a 17 amino acid peptide fragment from the actin binding protein 140 (Abp140) of *S. Cerevisiae*, known as Lifeact (232). The Lifeact peptide has been previously shown to bind to F-actin with high affinity and selectivity while having minimal effect on actin and microtubule dynamics (233). This Lifeact peptide fragment was fused to GBP, and we also constructed a second GBP fusion using Lamin B1, an integral component of the nuclear lamina (234).

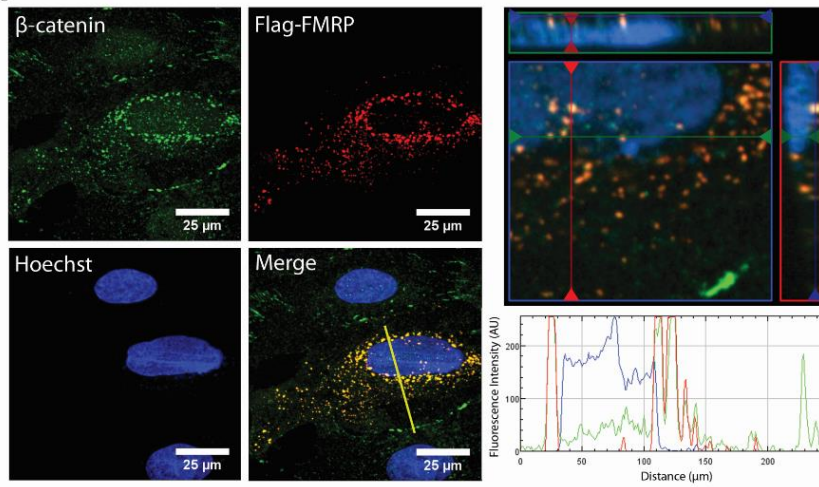
Next, we ectopically expressed GBP-Lifeact and EYFP- β -catenin, and checked for EYFP localization (green) to actin filaments. Lifeact did localize to the actin filaments and we also observed staining with anti- β -catenin indicating that it was recruited to the GBP-Lifeact (Alexa Fluor 546, red) and Alexa Fluor 633 tagged phalloidin (blue, F-actin

marker). A line scanned RGB profile reveals matching peaks for all three fluorescent signals (Supplementary S19). Consistent with this data, A10 cells transfected with GBP-Lifeact and EYFP- β -catenin resulted in distinct β -catenin localization to F-actin, while Flag-FMRP or mCherry- β -catenin was not recruited to the F-actin when transfected alone (Figure 15D, top panels). However, ectopic expression of all three factors together – GBP-Lifeact, EYFP- β -catenin, and Flag-FMRP – resulted in a readily observable FMRP localization to F-actin, and precise overlap with EYFP- β -catenin (Figure 15D, bottom panels). These data indicate a robust interaction between β -catenin and FMRP.

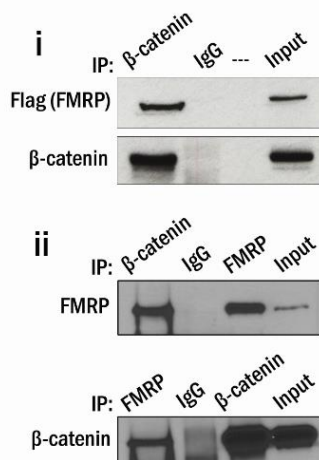
Next, we anchored EYFP- β -catenin to the inner nuclear membrane using GBP-Lamin B1. In C2C12 myoblasts expressing GBP-Lamin B1, EYFP- β -catenin, and mCherry showed a distinct nuclear localization of EYFP- β -catenin, but not mCherry alone (Supplementary S20). In A10 cells, EYFP- β -catenin was recruited to the nuclear envelope where GBP-Lamin B1 was anchored. Ectopic expression of GBP-Lamin B1 and Flag-FMRP or mCherry- β -catenin alone resulted in a dispersed localization (Figure 15E, top panels). However, similar to the results observed with Lifeact, when co-expressed together GBP-Lamin B1, EYFP- β -catenin, and Flag-FMRP resulted in a striking re-localization of Flag-FMRP to the nuclear membrane (Figure 15E, bottom panels). Taken together, these data indicate a robust interaction between β -catenin and FMRP.

Figure 15

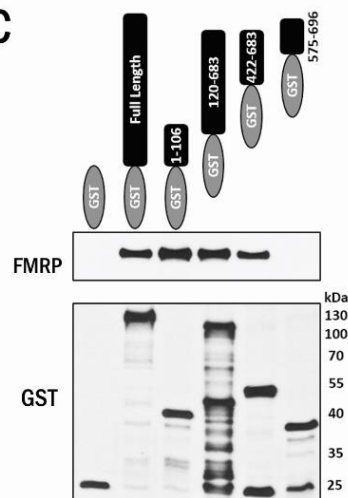
A



B

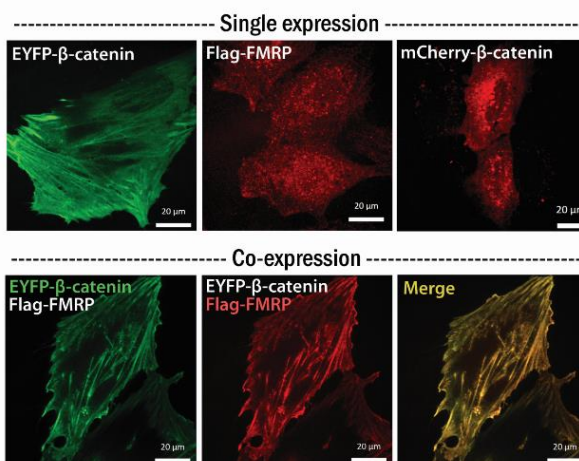


C



D

GBP-Lifeact



E

GBP-Lamin B1

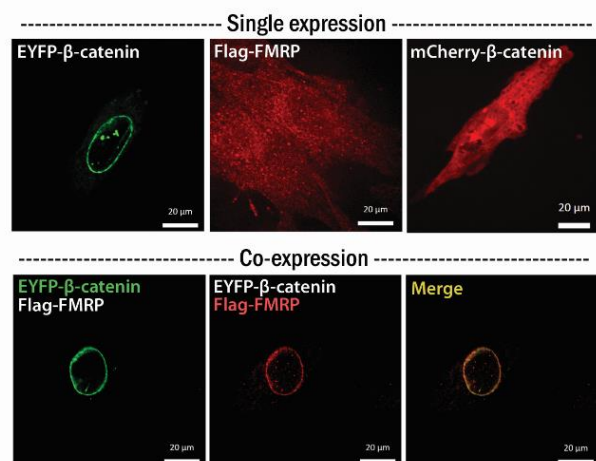


Figure 15: β -catenin co-localizes and directly interacts with FMRP. **A.** (Left panel) A10 cells were transfected with Flag-FMRP for 3 hr then cultured for 24 hr prior to fixation and staining for β -catenin (green), Flag (FMRP, red), and nucleus (blue). (Bottom right panel) A line was drawn through a representative cell to indicate relative intensity of RGB signals. (Top right panel) An orthogonal projection was generated by a Z-stack (240nm) image set using the ZEN program (Zeiss) depicting the region surrounding the nucleus in the XY, XZ, and YZ planes. **B.** (i) HEK 293T cells were transfected with Flag-FMRP as in A, and endogenous β -catenin protein complexes were immunoprecipitated with β -catenin antibody. Co-immunoprecipitated over-expressed Flag-FMRP protein was detected by Western blot analysis with the flag antibody. Rabbit IgG was used as a control. (ii) Primary VSMCs were harvested and subjected to an endogenous coIP analysis using either β -catenin or FMRP antibody. The corresponding protein was detected in the precipitated immunocomplex by Western blot. Rabbit IgG was used as a control. **C.** Interaction between FMRP and β -catenin was assessed by a GST pull down assay. Purified 6x-His-FMRP and indicated GST fused β -catenin proteins were mixed with glutathione-agarose beads. Purified GST or GST-fusion proteins were visualized by Western blotting using the GST antibody, and sizes of corresponding GST-fusion proteins were confirmed. Pulled-down FMRP proteins by GST fused β -catenin were detected by immunoblotting with the FMRP antibody. **D.** (Top panel) A10 cells were transfected with GBP-Lifeact and either only EYFP- β -catenin, Flag-FMRP, or mCherry- β -catenin for 3 hr then cultured for 24 hr prior to fixation and either fluorescence microscopy or immunofluorescent staining for Flag (red), as indicated. (Bottom panel) A10 cells were transfected with GBP-Lifeact similar to above, but with both EYFP- β -catenin and Flag-FMRP and subjected to fluorescent microscopy and immunofluorescent staining for Flag (red) **E.** A10 cells were transfected as in **(D)** but with GBP-Lamin B1 instead of GBP-Lifeact.

β -catenin represses translation and associates with the pre-initiation complex

Based on the FMRP: β -catenin interaction and co-localization data, we next tested the idea that β -catenin might be involved in FMRP function at the translation machinery. We therefore tested two possibilities: 1) whether global translation was affected by manipulation of β -catenin protein levels and 2) whether β -catenin is part of the pre-initiation complex with FMRP.

To address the first question, we utilized a technique known as surface sensing of translation (SUnSET) to measure global protein translation synthesis using puromycin incorporation into newly synthesized peptides coupled with monoclonal puromycin antibody detection (Figure 16A). This method of measuring translation is comparable to classical radioactive methods (235). We performed a series of β -catenin siRNA silencing experiments using two different siRNAs that target β -catenin in primary VSMCs, A10, and HEK 293T cells. We first depleted β -catenin protein by siRNA (controls were treated with a scrambled siRNA), and then the cells were subjected to a 15 minute pulse of puromycin treatment (1 μ M). Global translation was assessed by visualization of puromycin incorporated peptides by Western blot analysis. The results in each experiment indicated that a loss of β -catenin discernibly increased protein translation (Figure 16B). The observed increase in translation was apparent in all three cell types.

To answer the second question related to β -catenin's possible recruitment to the pre-initiation complex, we isolated the pre-initiation complex using a well characterized m⁷GTP-agarose bead pull-down assay. Since eIF4E interacts with the 7-methylguanylate cap (m⁷G) of mRNA with high affinity to initiate translation, cell lysates can be incubated with m⁷GTP-agarose beads to enrich for the eukaryotic initiation factor 4E (eIF4E)

complex and other associated proteins (Figure 16C) (236); this technique has previously been used to assess FMRP in the pre-initiation complex (237). In our analysis with A10 smooth muscle cells, endogenous β -catenin protein was detected in the pre-initiation complex along with FMRP. Moreover, in HEK 293T cells, forced expression of Flag-FMRP results in an increased association of endogenous β -catenin with the eIF4E pre-initiation complex (Figures 16D and E, respectively). These results indicate the possibility that β -catenin/FMRP recruitment to the pre-initiation complex inhibits protein translation.

Cycloheximide treatment disrupts cytoplasmic β -catenin and FMRP co-localization

Previous studies have linked FMRP to translation regulation by interaction with eIF4E through a unique 4E binding protein, cytoplasmic FMRP-interacting protein 1 (CYFIP1) (237). Interestingly, CYFIP1 was also identified in our list of β -catenin interacting proteins further supporting the idea that β -catenin might play a role in FMRP function (Supplementary S15). Next, we determined if pharmacological inhibition of translation would affect the β -catenin:FMRP interaction. In order to do this, we used the protein synthesis inhibitor cycloheximide, which reversibly prevents translational elongation in eukaryotes (238). Initially, we transfected A10 cells with Flag-FMRP and 24 hr later treated with cycloheximide (50 ng/ μ L) or solvent (DMSO) for a period of up to 16 hr. We then checked for relative protein levels of Flag-FMRP and β -catenin to determine at which time point translation was inhibited but existing ectopically expressed protein was not yet degraded (Supplementary S21). Based on these results, we incubated Flag-FMRP expressing cells with cycloheximide or its control (DMSO) for 4 hr, then performed an immunofluorescence assay to determine the localization of Flag-FMRP and β -catenin.

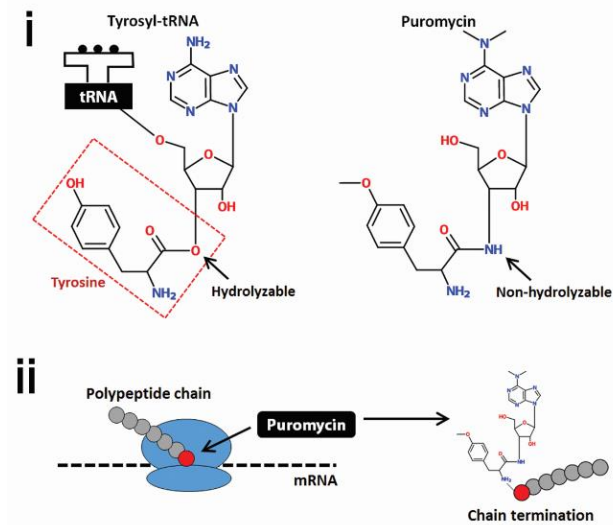
When compared to control conditions where Flag FMRP and β -catenin were co-localized, cells in which translation was blocked resulted in a loss of co-localization between endogenous β -catenin and Flag-FMRP (Figure 16F).

The β -catenin:FMRP interaction is modulated by mTOR signalling

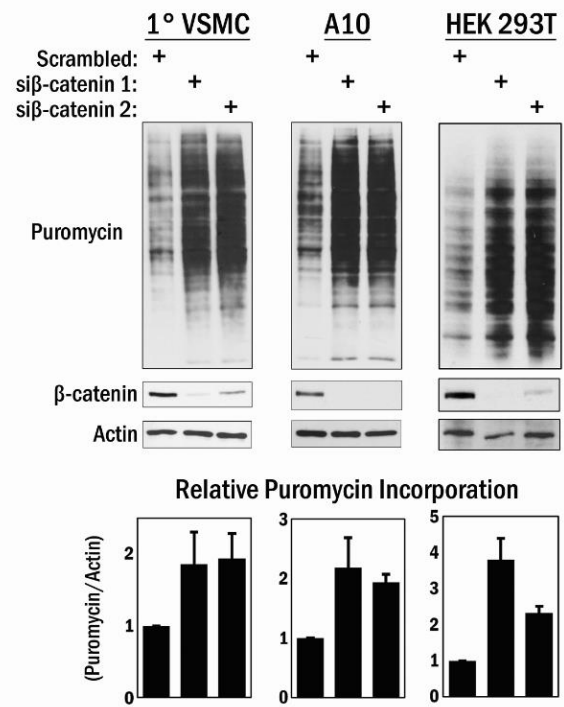
To address the issue from a different angle, we reasoned that the interaction between β -catenin and FMRP might be dependent on upstream mTOR signalling, which is known to regulate cap-dependent translation (Figure 16G) (239). Since FMRP forms the pre-initiation complex to regulate mRNA translation, and studies have suggested that mTOR signalling is hyperactive in *fmr1* deficient mice (240), we used the mTOR inhibitor rapamycin to determine if the β -catenin:FMRP interaction is altered when mTOR signalling is suppressed. Treatment with rapamycin resulted in a reduced interaction by co-immunoprecipitation, and also a loss of perinuclear localization of β -catenin and FMRP (Figure 16H and I, respectively).

Figure 16

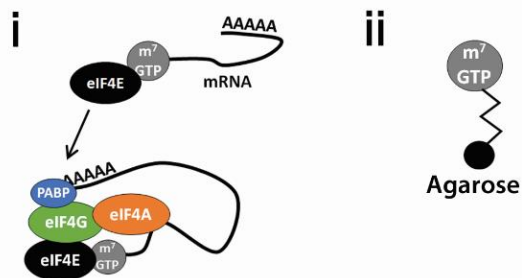
A



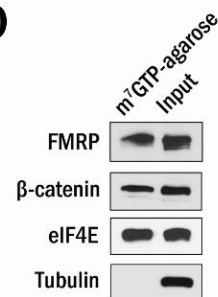
B



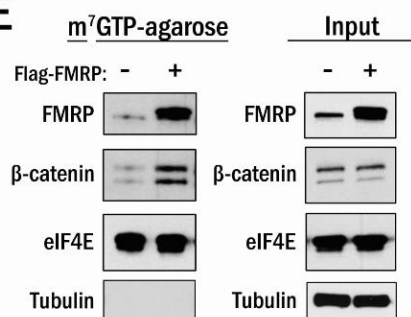
C



D



E



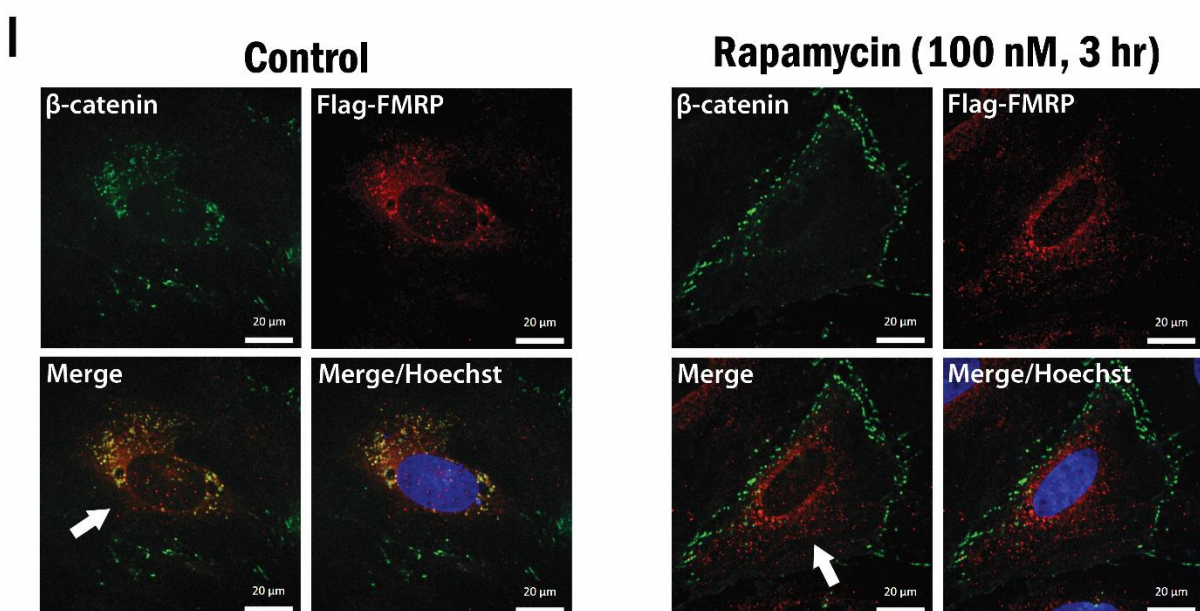
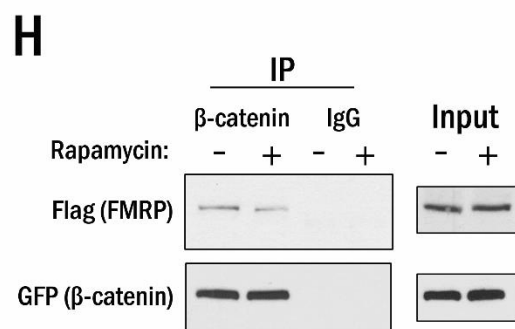
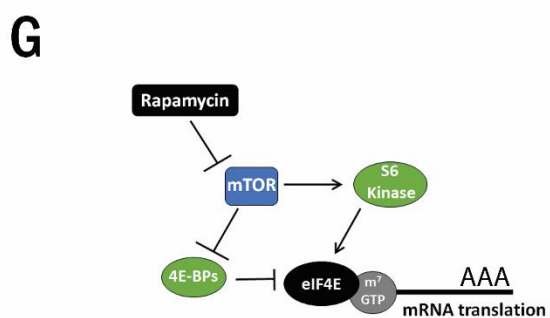
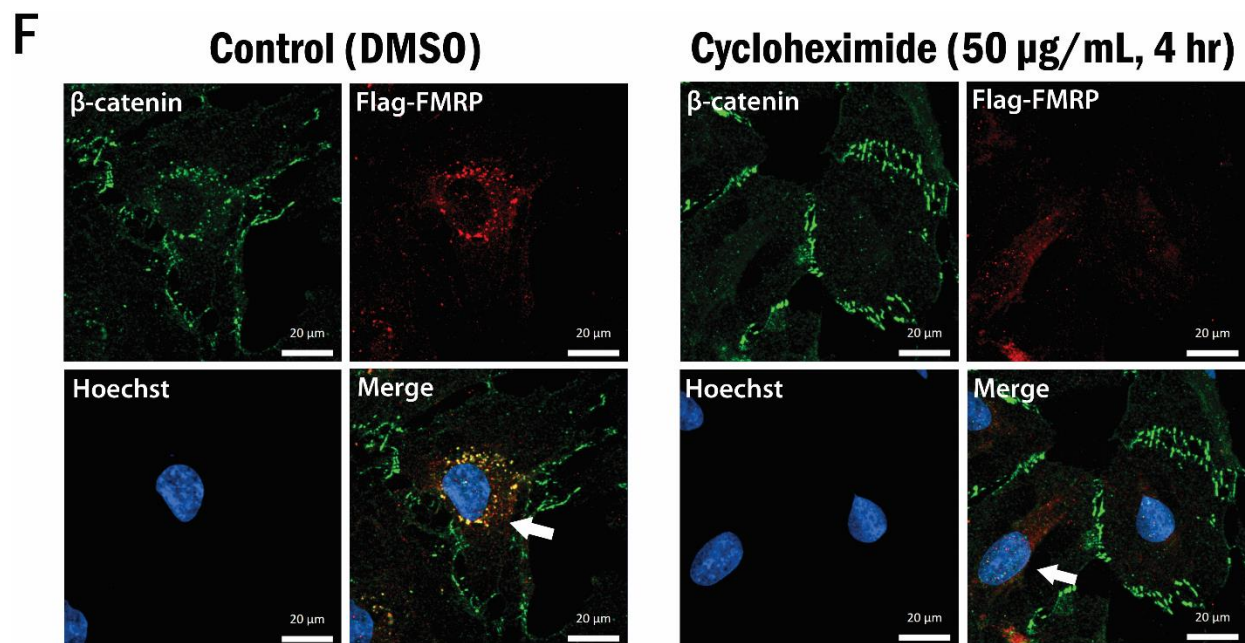


Figure 16: β -catenin and FMRP are co-localized in the pre-initiation complex to inhibit translation. **A.** Depiction of the SUnSET technique **i.** Puromycin is structurally similar to tyrosyl-tRNA, but contains a non-hydrolyzable amide bond where tyrosine is normally cleaved from the tRNA complex and incorporated into a polypeptide chain. **ii.** Puromycin is added to a growing polypeptide chain by chance. Since its amide bond cannot be broken, it terminates elongation of the peptide chain synthesis. **B.** Primary VSMCs (left panel), A10 (middle panel), and HEK 293T cells (right panel) were transfected with siRNAs to β -catenin and 36 hr later pulsed with 1 μ M puromycin for 15 min. Cells were then harvested and subjected to Western blot analysis with puromycin antibody as indicated; β -catenin blots indicated efficacy of siRNA, and actin was used as a loading control. The average puromycin/actin ratio is shown in the graphs below each cell type; the experiment was performed 3 times, and error bars indicate standard deviation. **C. i.** eIF4E preferentially binds to the 5'cap (m⁷GTP) of mRNAs and recruits the pre-initiation complex to that site. **ii.** m⁷GTP-agarose beads can be used to precipitate the pre-initiation complex from cell lysates. **D.** A10 cell lysates were incubated with m⁷GTP-agarose overnight at 4°C followed by washing, and precipitated proteins with beads were identified by Western blot analysis. eIF4E was used as a positive control for pull-down and tubulin as a control for non-specific interactions. Total lysates were used as input control. **E.** HEK 293T cells were transfected with empty vector or Flag-FMRP, and lysates were subjected to m⁷GTP-agarose pull-down as in **(C)**. eIF4E and tubulin were used as positive and negative controls, respectively. **F.** A10 cells were transfected with Flag-FMRP then treated with either cycloheximide or its solvent (DMSO) for 4 hr prior to fixation, and β -catenin (green), Flag (FMRP, red) were visualized by immunofluorescence. Nucleus (blue) was identified by DNA staining with Hoechst 33342. Arrows indicate a change in localization in the perinuclear space following treatment. **G.** Schematic depiction of rapamycin mediated inhibition of cap-dependent translation. **H.** A10 cells were transfected with Flag-FMRP as above then pre-treated with rapamycin or solvent (DMSO) for 1 hr in serum-free media, and serum was added for 2 hr. Cells were harvested and subjected to coIP analysis using β -catenin antibody for immunoprecipitation and Western blot analysis for corresponding Flag (FMRP). Rabbit IgG was used as a control. **I.** A10 cells were transfected with EYFP- β -catenin and Flag-FMRP followed by rapamycin treatment similar to **(H)**. Cells were fixed and stained for β -catenin (green), Flag (FMRP, red), and nucleus (blue).

Wnt-mediated nuclear translocation of β -catenin enhances translation

The initial data led us to posit that cytoplasmic β -catenin associates with the pre-initiation complex to inhibit translation. Therefore, we reasoned that sequestering β -catenin cellular away from the cytoplasm might de-repress translation. This is a tractable hypothesis since Wnt treatment promotes an extensive re-localization of β -catenin to the nucleus (221). Therefore, to test this idea, we used purified Wnt-3a to stimulate cells that were serum deprived and again assessed protein translation using puromycin incorporation. Upon activation of Wnt signalling, β -catenin translocated into the nucleus, which was confirmed by immunofluorescence analysis. In conjunction, global translation increased by greater than two-fold compared to control; this effect was consistent in both primary VSMCs and in A10 cells (Figures 17A and B, respectively). These results demonstrated that sequestration of cytoplasmic β -catenin into the nucleus by Wnt signaling de-represses translation. This may be an observation of some importance for understanding the cellular effects of Wnt signalling.

Figure 17

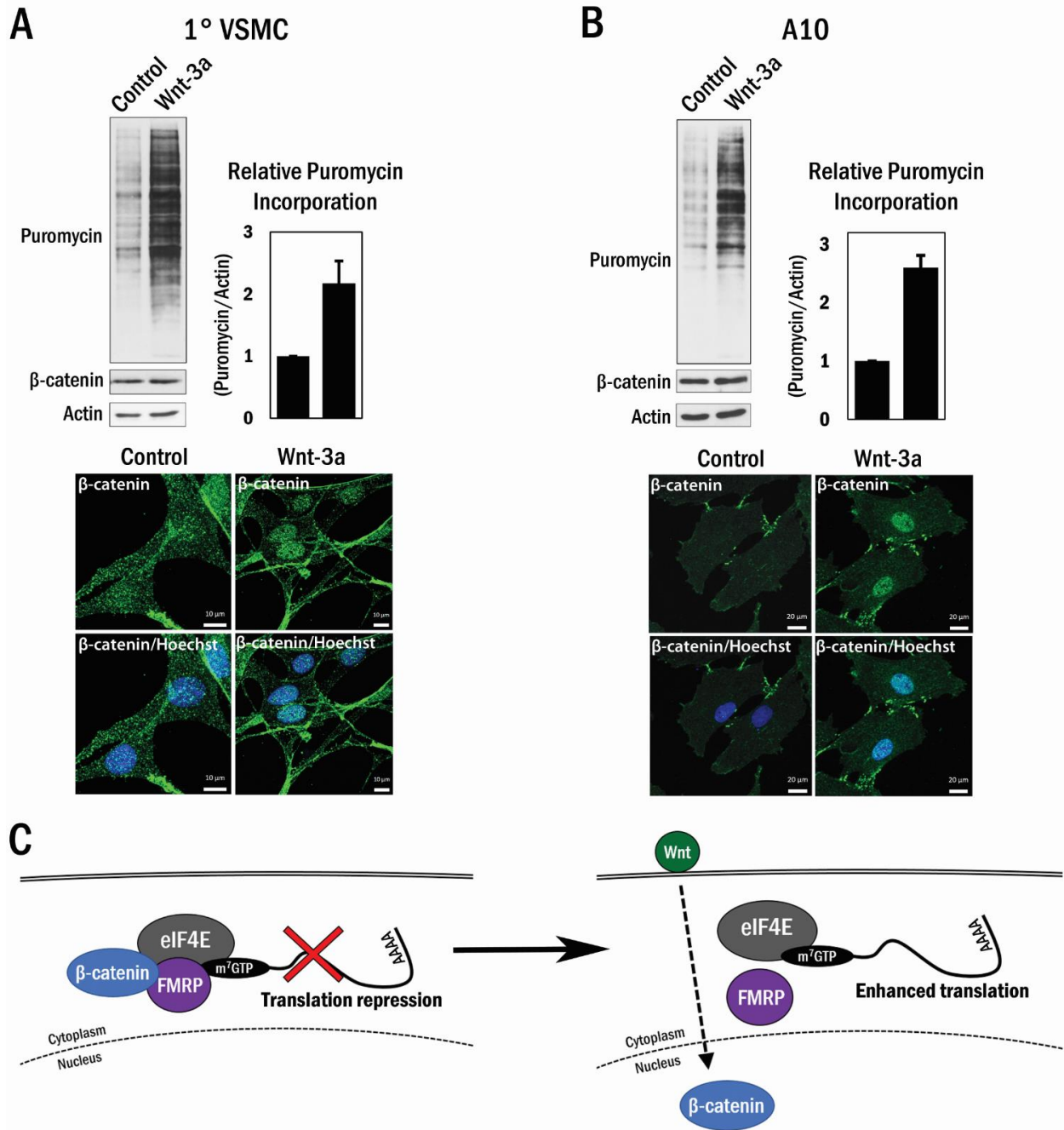


Figure 17: Wnt dependent cytoplasmic to nuclear translocation of β -catenin de-represses translation. **A.** (Top panel) Primary VSMCs were serum deprived overnight prior to stimulation with Wnt3a (100 ng/mL) or its solvent (0.1% BSA in PBS) for 4 hr, and the cells were treated with 1 μ M puromycin for 15 min. The cells were harvested and subjected to Western blot analysis. Three biological replicates were performed and the average puromycin/actin ratio was graphed. Error bars indicate standard deviation. (Bottom panel) Cells were treated similarly to above, followed by fixation and immunofluorescent staining for β -catenin (green) and nucleus (blue). **B.** A10 cells were treated similar to **(A)** and analyzed for puromycin incorporation and β -catenin localization by immunofluorescence (n=3). **C.** A working model; Cytoplasmic β -catenin inhibits translation. In the absence of Wnt stimulation, the cytoplasmic pool of β -catenin associates at the pre-initiation complex to facilitate the inhibition of translation. Upon Wnt stimulation, β -catenin leaves the cytoplasm and enters the nucleus, and translation is no longer repressed.

Discussion

In this study, we have identified and characterized a novel cytoplasmic β -catenin binding partner in the FMRP protein. Moreover, this interaction underpins a previously unknown function of β -catenin at the pre-initiation complex in repressing translation. β -catenin has been well characterized and established as an indispensable multi-functional protein that has no paralog (2). It is a key member of the cadherin cell adhesion complex and provides a link between the cytoplasmic tail of cadherin and the actin cytoskeleton (241). In addition, β -catenin's C-terminal transactivation domain makes it a critical nuclear transcriptional co-activator as a downstream effector of canonical Wnt signalling (21). The divergent roles and localization of β -catenin make it one of a few “jack-of-all-trades” proteins, and one whose function is exclusively determined by which proteins it interacts with at any juncture. Studies have suggested that the major mechanism of β -catenin localization is retention by protein interactions rather than by regulated shuttling, which is corroborated by the fact that β -catenin does not contain any well characterized localization signals in its primary sequence (40, 103, 221). Here, we present evidence indicating a hitherto unrecognized role for β -catenin, in association with FMRP, in translational repression that may have important implications for our mechanistic understanding of canonical Wnt signalling.

A hallmark of Wnt signalling is the dramatic nuclear translocation of β -catenin and subsequent transcription of a variety of Wnt dependent target genes. This results in substantive cellular phenotypic changes in many developmental systems as well as altered properties of some cancer cells (74, 114, 172). Our data suggest that in addition to

promoting the transcription of Wnt dependent target genes, β -catenin translocation into the nucleus may, in so doing, de-represses the translational machinery. Thus, we provide a revised model for β -catenin's function in baseline conditions and in response to Wnt signalling which involves translational de-repression in addition to its well-known role in transcriptional induction. In brief, we contend that in the absence of Wnt stimulation, β -catenin functions both at the membrane, in association with cadherins, to regulate cell adhesion and concomitantly at the translational machinery to repress translation in association with FMRP. Upon Wnt activation, β -catenin re-localizes to and accumulates in the nucleus to activate target gene transcription and, by its sequestration to the nucleus, simultaneously dissociates from the pre-initiation complex to de-repress translation (Figure 17C). Our model then purports a dual function on both transcriptional induction and translational de-repression that results in a compound impact on Wnt dependent gene expression. The subcellular localization of β -catenin is therefore an important determinant of its impact on protein translation. Thus, we conclude that the cytoplasmic pool of β -catenin/FMRP is primarily responsible for the regulation of translation. Of note, several studies have indicated that some neurodegenerative effects of fragile X syndrome (FXS), the genetic disease resulting from suppression of FMRP expression, can be ameliorated by pharmacological inhibition of GSK3, which ordinarily mediates the APC dependent degradation of β -catenin (242). Whether these observations are linked remains to be tested.

While the cytoplasmic accumulation of β -catenin in response to GSK3 inhibition by Wnt stimulation has been mechanistically implicated in the increased nuclear levels of β -catenin in which a “spillover” mechanism is proposed, we recently showed that this model is too simplistic in that β -catenin nuclear accumulation is dependent on a p38

MAPK dependent phospho-interaction with MEF2 in vascular smooth muscle cells (221). In the studies reported here, the mechanism by which β -catenin dissociates from the pre-initiation complex in response to Wnt stimulation remains to be determined and may be equally complex. Based on our previous studies with β -catenin, the dissociation of the β -catenin/FMRP complex may be dependent on post-translational modifications of β -catenin and/or FMRP that leads to disruption of the interaction interface. Furthermore, FMRP is thought to bind approximately 4% of mRNAs in the brain (243), and it may be relevant to know if the subset of mRNAs regulated by FMRP are also modulated by perturbations in β -catenin.

In summary, we have provided evidence of a protein-protein interaction between β -catenin and FMRP that may serve to regulate protein translation. In VSMCs, although the modulation from quiescent to proliferative phases is essential for wound repair, mis-regulation can also lead to improper stimulation. Since β -catenin plays an important role in this process, determining the mechanisms involved in this phenotypic switch could provide novel therapeutic targets in some vascular diseases such as atherosclerosis in which VSMC switching is important. Furthermore, these observations may not be limited to a specific cell type due to the relatively ubiquitous expression of both β -catenin and FMRP. Our data so far indicate that this interaction can occur in a number of cells of different lineage. Our observation that the β -catenin/FMRP complex can exert effects on translational control highlights a mechanistic advance in our understanding of Wnt/ β -catenin signaling and also extends possibilities for therapeutic targeting of both Wnt and FMRP dependent pathologies.

Materials and Methods

Cell culture and Immunoprecipitation for LC-MS/MS

Cell culture

A10 rat aorta vascular smooth muscle cells (ATCC CRL-1476) were grown at 37°C in 5% CO₂ and 95% humidity in 10 mL high glucose DMEM with 100 U/mL penicillin, 100 µg/mL streptomycin, 1.5 g/L sodium bicarbonate and 10% (v/v) fetal bovine serum. Cells were harvested by scraping after two washes in PBS and immediately lysed in 5 volumes of NP-40 lysis buffer (0.5% NP-40 v/v, 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 100 mM NaF, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM of Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin A). Lysates were centrifuged for 10 min at 14,000 rpm at 4°C and the supernatants collected. Cell culture, collection and lysis were performed in quintuplicate.

Antibody-bead conjugation

5 µg anti-β-catenin antibody (Cell Signaling #9562) was immobilized to 50 µL of ImmunoCruz IP/WB Optima F agarose bead substrate (Santa Cruz Biotechnology sc-45043) overnight at 4°C in 500 µL PBS with 0.1% Tween 20 (v/v). For negative controls, beads were incubated with rabbit serum (Sigma Aldrich R9133). Unbound antibody was washed away twice with 500 µL PBS.

Protein binding and elution

Antibody-conjugated beads were incubated with A10 cell lysates on a nutator overnight at 4°C, then the beads were washed twice with 500 µL NP-40 lysis buffer and then twice

with 500 µL of NP-40 lysis buffer without NP-40. The washes were kept for SDS-PAGE analysis to determine that washing effectively removed all unbound proteins (data not shown). Bound proteins were eluted for LC-MS analysis using 500 µl of 0.5 M NH₄OH on a nutator for 20 min at room temperature. Separate immunoprecipitation columns were used for each sample and parallel rabbit serum controls were generated from separate biological replicates of A10 cells. Eluates were dried by centrifugal concentration and then dissolved in 100 mM NH₄HCO₃, and treated with 5 mM dithiothreitol at 60 °C then 10 mM iodoacetamide at room temperature in the dark. The alkylated immunoprecipitates were incubated at 37°C overnight with 0.75 µg of trypsin each.

LC-MS/MS

Sample run

Trypsin digests were dried in a centrifugal concentrator, dissolved in aqueous 0.1% (v/v) formic acid and separated on a C18 column (15 cm long, 75 µm diameter internal diameter, 3 µm diameter stationary phase, 120Å pore size) by HPLC over a 60 minute 300 nL/minute acetonitrile gradient. The HPLC system consisted of a NanoLC-Ultra 2D pump with a Nanoflex cHiPLC system (AB Sciex, Concord ON, Canada). Mobile phases A and B were 0.1% formic acid (v/v) in water and acetonitrile respectively (Honeywell, Morristown, NJ, USA). The following mobile phase B concentrations were used: 2% (v/v) at 0 minutes to 35% at 35 minutes to 80% from 35.5 to 38.5 minutes to 2% from 39 to 60 minutes. Mass spectra of the HPLC eluate were collected on an Orbitrap Elite mass spectrometer (Thermo Scientific, Waltham, MA). Precursor ion scans were collected on the Orbitrap at a resolution of 30,000 and the linear ion trap was used for MS/MS.

Tandem mass spectrometry was performed using normalized collision energy of 35, activation Q of 0.250, 2 Da isolation width and a 10 mS activation time. Dynamic precursor ion exclusion with a repeat count of 1 and an exclusion mass width of 10 ppm was applied. A 10 second dynamic exclusion duration and 20 second repeat duration were used. Between each pair of samples, three blank runs were acquired under identical instrument parameters in order to control analyte carryover.

Peptide score

MS/MS data were searched against the UniProt *Rattus norvegicus* sequence library (September 18, 2013 edition downloaded from the UniProt Knowledge Base on September 26, 2013) with the SEQUEST algorithm navigated through Proteome Discoverer™ software version 1.3. Search settings were as follows: precursor mass range: 350-5000 Da; minimum peak count: 1; enzyme: trypsin; maximum missed cleavages: 1; precursor ion tolerance: 10 ppm, product ion tolerance: 0.4 Da, product ion series considered: b and y; dynamic modifications: methionine oxidation (+15.995 Da), phosphorylation (S, T and Y at +79.966 Da), glutamine deamidation (+0.984 Da) and tryptophan dioxidation (+31.990); static modification: cysteine carbamidomethylation (+57.021 Da). Cutoff scores at the peptide level were determined by automated false discovery rate (FDR) estimation.

Gene Ontology enrichment analysis

Proteins common to anti- β -catenin column eluates and rabbit serum controls were removed from potential β -catenin binding partners. Following subtraction of the rabbit

serum control data set from the experimental data set, we used the Software Tool for Researching Annotations of Proteins (STRAP, v1.5) program to summarize the interactome using each individual protein's annotation in the Gene Ontology (GO) Cellular Localization domain (244).

Next we performed an enrichment analysis of these proteins using the Cytoscape program (245) and ClueGO plug-in (246). We employed a right-sided hypergeometric statistical test, corrected using the Bonferroni step down method to determine enriched Biological Process GO terms from levels 3 to 8. We specified a minimum of 5 genes comprising at least 2% of a particular GO term to be the cutoff for its inclusion. The proportion of proteins that were shared among pairs of GO terms was determined using the Kappa statistic. This was visualized as edges connecting two nodes together and had a minimum Kappa score of 0.5. Thicker edges signified higher Kappa score values.

We utilized the Cluepedia plugin (247) to create a visual network of the enriched GO terms using the terms with the highest number of statistically significant proteins. We then used the most significant GO terms to create another network visualizing the specific proteins associated with particular term(s).

Protein extraction and Western blot analysis

Cells were collected as described above. A10 and HEK 293T cells were obtained from American Type Culture Collection (ATCC). Primary vascular smooth muscle cells were isolated and cultured from mice aortae as previously described (218). Cells were grown in serum free DMEM overnight for the specified experiments. Extracted proteins were denatured in SDS loading buffer at 95°C for 5 min then run on 10% SDS-PAGE,

transferred to a PVDF membrane (Millipore), and blocked in 5% skim milk for 1 hr prior to antibody incubation. After brief washes with TBS, the blot was incubated with corresponding HRP conjugated secondary antibody in 5% milk in TBS for 1hr at room temp. Expression levels of indicated proteins were recorded by exposing the blot to film (GE Healthcare).

Transfections

For protein over-expression and small interfering RNA (siRNA) experiments, cells were transfected with Lipofectamine 2000 (Life Technologies) using recommended instructions. Cells were transfected for 3 hr followed by media replacement, and were harvested or drug treated 24-48 hr later, unless otherwise indicated.

Gene silencing

MISSION siRNA (Sigma-Aldrich) for rat and mouse β -catenin (SASI_Rn01_00099923 and SASI_Rn01_00099924), human β -catenin (SASI_Hs01_00117960 and SASI_Hs01_00117958), and universal scrambled siRNA (SIC001) were used at a concentration of 25 nM.

Antibodies and reagents

Actin (sc-1616), GFP (sc-9996), Plectin (sc-33649), and dsRed (sc-33354) antibodies were purchased from Santa Cruz. β -catenin (#9562), FMRP (#4317), eIF4E (#9742), and Tubulin (#3873) antibodies were purchased from Cell Signaling Technology. Flag (F1804) and glutathione-S-transferase (GST) (G7781) antibodies were purchased from

Sigma-Aldrich. Puromycin antibody (PNY-2A4) was purchased from the developmental studies hybridoma bank (DSHB).

Cycloheximide (CYC003) and puromycin dihydrochloride (PUR333) were purchased from BioShop Canada. Rapamycin (#9904) was purchased from Cell Signaling Technologies. Purified Wnt-3a (315-20) was purchased from PeproTech.

Co-immunoprecipitation

For co-immunoprecipitation, cells were harvested and protein extracted as described above. Immunoprecipitation was performed using the ImmunoCruz Optima kit (Santa Cruz Biotechnology), according to the manufacturer's instructions. Eluates were analyzed by Western blot, as described above.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde in PBS for 5 min on ice then 10 min at room temp, and then permeabilized with ice cold 90% methanol in water for 1 min on ice. Cells were blocked using 10% goat serum in PBS for 30 min at room temp, and incubated overnight with primary antibody diluted (1:100-1:500) in 10% goat serum in PBS at 4°C. Cells were washed three times with PBS (10 min each) and incubated with specified tetramethylrhodamine (TRITC) or fluorescein isothiocyanate (FITC) (Sigma-Aldrich) conjugated corresponding secondary antibody diluted (1:500) in 10% goat serum for 2 hr at room temperature. Nucleus was visualized with Hoechst 33342 DNA staining (Thermo-Fisher, 1 µg/mL) in the last 15 min. Cells were washed three times with PBS (10 min each) and left in PBS at room temperature for confocal microscopy imaging. Images were

captured using a Carl Zeiss Axio Observer Z1 with Photometrics Evolve 512 EMCCD Camera. Fluorescence images were acquired under 40x (EC Plan-Neofluar, 1.30 NA in Oil) or 63x (Plan-Apochromat, 1.40 NA in Oil) objectives using ZEN imaging software (Zeiss). Images were taken as a Z-stack and projected orthogonally by maximum intensity using ZEN.

m⁷GTP-agarose pull down

50 μ L immobilized γ -aminophenyl-m⁷GTP(C₁₀-spacer)-agarose slurry (Jena Bioscience AC-155S) was washed 3 times in TBS and incubated with prepared cell lysates overnight at 4°C with gentle agitation. Beads were washed once with TBST (0.1% Tween-20) then twice with TBS. Samples were heated with SDS loading buffer at 95°C for 5 min to elute from the beads and run on a 10% SDS-PAGE, and subjected to Western blot analysis.

GST pull down

For GST pull downs, GST- β -catenin and 6xHis-FMRP fusion proteins were obtained using standard protocols. Briefly, cloned pGEX (β -catenin) or pET28a (FMRP) vectors were transformed in BL21 *E. coli*, and protein production induced with 0.4 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) overnight at 16°C. GST- β -catenin and 6xHis-FMRP expressing bacteria were sonicated and the proteins purified with glutathione-agarose (Sigma-Aldrich) or nickel-agarose (Qiagen) beads, respectively. Protein concentrations were estimated by SDS-PAGE and Coomassie blue staining, using bovine serum albumin (BSA) for comparative estimation. 1 μ g GST-full length β -catenin protein (or the molar equivalent of the smaller fragments), 2 μ g 6xHis-FMRP, and 25 μ L glutathione-agarose

beads (50% slurry) were incubated in 600 μ L NETN buffer (100 mM NaCl, 20 mM Tris-HCl (pH 8), 0.5 mM EDTA, 0.5% v/v NP-40) overnight at 4°C. Beads were washed once TBST and twice with TBS, and eluates analyzed by Western blot.

Protein synthesis rates by puromycin incorporation and quantification

Cells were initially treated as described in the figures, then were incubated with 1 μ M puromycin for 15 min prior to harvesting or subjected to immunofluorescence analysis. Puromycin incorporation was visualized by Western blot or immunofluorescence using puromycin antibody. Relative puromycin incorporation by Western blot was quantified using ImageJ with the Fiji image processing package. The puromycin bands of a particular condition was traced in their entirety along with the corresponding actin band and the following was performed: the integrated density of the area directly below the traced lanes was multiplied by the traced area of the protein of interest to calculate background intensity. The integrated density of the protein of interest was subtracted by the background intensity calculate absolute protein intensity. The puromycin protein intensity was divided by the actin protein intensity to calculate normalized puromycin intensity. Three biological replicates were performed and the average puromycin intensity was graphed with error bars representing standard deviation.

Plasmid construction

GBP ORF was amplified with F-primer (ATAAGATTATGGCCGACGTCCAG) and Rev-primer (ATGGATCCACCTCCACCTCCACCTC) using pCGA-GBP1-10gly-Gal4DBD vector (Addgene #49438) as a template by PCR. The amplified DNA was digested with HindIII

and BamHI and ligated into the pcDNA3 vector (pcDNA3-GBP) (Invitrogen). Lamin B1 ORF was PCR-amplified with F-primer (ATGGATCCGCGACTGCGACCCCCGTGCCGCCGCGGATG) and Rev-primer (ATCTCGAGTTACATAATTGCACAGCTTCTATTG) using a C2C12 cDNA library constructed by RT with oligodT. The amplified DNA fragments were digested with BamHI and XhoI and ligated into pcDNA3-GBP in a same reading frame for GBP-Lamin B1 fusion protein expression. Lifeact-GBP was constructed by a 3-way ligation technique with synthesized two double stranded DNAs (#1 reading strand; CTAGCATGGGTGTCGCTGACCTG ATCAAAAAG with #1 complimentary strand; CGAACTTTTTGATCAGGTCAGCGACACCGATG), (#2 reading strand; TTCGAGTCCATCTCGAAGGAAGAGC with #2 complimentary strand; TCGAGCTCTTCCTTCGAGATGGACT) and digested pcDNA3 (NheI / XhoI), and PCR-amplified GBP-ORF (Xho I / EcoR I) was further ligated for pcDNA3-Lifeact-GBP.

pFRT-TODestFLAGHAhFMROiso1 (Flag-FMRP) was a gift from Thomas Tuschl (Addgene plasmid #48690). For pet28a-FMRP, Flag-FMRP was used as a template and amplified using Fwd primer (CGCGGAATTCATGGAGGAGCTGGTGGTGAAGTGCGGGGCT) and Rev primer (CGCGGCGGCCGCTTAGGGTACTCCATTCACGAGTGGTTGCTG), which was digested with EcoRI and NotI then ligated into pet28a vector (Invitrogen).

Chapter 4

Discussion and concluding statements

Vascular smooth muscle cells maintain the ability to switch between a quiescent or active phenotype based on external cues, and is an important feature for vascular repair. However, since this same evolutionary feature allows VSMCs to be affected by improper signalling, understanding the molecular mechanisms responsible for these phenotypes is greatly important (248). Our lab and others have published extensively on the role of MEF2 in regulating striated and non-striated muscle phenotypes. Previous work in our lab has shown that MEF2 activity increases in response to vascular injury, in conjunction with c-Jun protein levels whose promoter contains a MEF2 DNA binding sequence. However, MEF2 over-expression alone was unable to potentiate c-Jun promoter activity in cultured VSMCs, which prompted us to suggest that other factors may also be in play *in vivo* (163). The results of a Yeast two-hybrid screen using the N-terminal domain of MEF2D as bait (188) suggested that β -catenin may be a MEF2 interacting partner.

Wnt/ β -catenin signalling plays an important role in a variety of cellular processes, such as embryonic development, cell differentiation and proliferation, and is prominently dysregulated in a variety of cancers (124, 165, 167). In addition, many studies have shown that p38 MAPK signalling converges with Wnt signalling at different levels (139, 143). Since p38 MAPK signalling is known to be a potent activator of MEF2 activity by direct phosphorylation (184, 249), and since β -catenin typically acts in the nucleus as a co-activator of proliferative genes, we hypothesized that these two signalling pathways intersected at MEF2 and β -catenin. Based on our experimental data, we concluded that

p38 MAPK signalling directly promotes β -catenin nuclear retention by phosphorylating MEF2 and enhancing a nuclear MEF2: β -catenin interaction that in turn increases Wnt target gene activation, c-Jun expression, and cell proliferation. The significance of this discovery is further highlighted by the fact that this phenomenon was not limited to VSMCs, but rather a variety of cell types. Also, pharmacological p38 MAPK inhibition using two different inhibitors reversed these effects.

The ability of p38 MAPK inhibitors SB203580 and SB202190 to inhibit both Wnt3a mediated TOPflash activity and nuclear β -catenin translocation is a significant result, as it points to a level of cross-talk not seen before. It also opens the door to new approaches of drug therapy. Therapeutic targeting of p38 MAPK signalling using small molecule inhibitors has been a topic of extensive research and development in the pharmaceutical industry for a number of years. Inhibition of p38 MAPK has been pursued to treat a variety of diseases such as chronic obstructive pulmonary disease (250, 251), atherosclerosis (252), cardiovascular disease (253), and rheumatoid arthritis (254). Based on the data published in my first manuscript, I have determined that this interaction is essential for Wnt signalling dependent β -catenin transactivation potential, and that p38 MAPK promotes the interaction by phosphorylating MEF2. There seems to be a likely therapeutic target of p38 MAPK signalling by inhibition of the MEF2: β -catenin interaction, which may prove more beneficial than upstream targeting of p38 MAPK and would reduce off-target side effects.

While the studies performed in Chapter 2 extensively characterized the relationship between p38 MAPK and Wnt signalling, they also underscored an important feature controlling β -catenin activity: its protein binding partners. While in the past the use of

small molecule inhibitors to disrupt protein-protein interactions seemed daunting, new understanding of how proteins interact with one another have allowed researchers to devise drugs and small peptides to disrupt protein interactions. Their designs are based on the understanding of “hot spot” residues that are small regions contributing most of the free energy of binding for various interactors (255). Computational approaches have made targeting these hot spots viable (256), and so having a larger domain of protein-protein interactions would prove beneficial for creating new drug inhibitors.

The identification of a β -catenin interactome has provided us with an opportunity to study previously unknown and potentially important mechanisms of β -catenin regulation. Many of these novel interacting partners are also involved in translational regulation, a process previously thought to be outside the realm of β -catenin function. β -catenin has long been known to be absolutely critical at the membrane to regulate cell adhesion, and in the nucleus to regulate target gene expression. In fact, β -catenin is indispensable at these two locations for proper cellular behaviour, both during development and in adult tissues. The data presented in Chapter 3 provides compelling evidence that β -catenin is directly involved in a third major cellular process. The role of β -catenin in inhibiting translation also provides a novel mechanism of Wnt target gene activation. Cytoplasmic to nuclear shuttling of β -catenin can de-repress translation and thus may act in tandem with β -catenin’s role in gene transcription to allow rapid protein synthesis of its target genes and cellular phenotype change. Lack of FMRP results in the mental disorder FXS (257), and studies have shown that it is an important regulator of mRNA translation (237, 258). The inclusion of β -catenin in this complex is enticing since it adds a new dimension in the role of FMRP-mediated translation inhibition that seems to be regulated by Wnt signalling.

Based on its function at the membrane, it is possible that β -catenin may be acting to stabilize an FMRP containing protein complex that represses cap-dependent translation. Loss of β -catenin protein may alter the ability of FMRP to bind its mRNA targets at the pre-initiation complex. Interestingly, in FXS mice, use of the GSK3 inhibitor lithium chloride can alleviate FXS related behaviours to a small degree (259). This leads us to hypothesize that perhaps β -catenin can play a role independent of FMRP in regulating translation, or that it may be able to compensate loss of FMRP to an extent in FXS patients. Lastly, characterization of specific mRNAs that are regulated by β -catenin at the pre-initiation complex would be significant. Aberrant Wnt stimulation and over-abundant β -catenin is found in many cancers and diseases. Knowledge of specific mRNA targets could provide novel therapeutic targets in these systems and would open a field of β -catenin study that was previously unknown.

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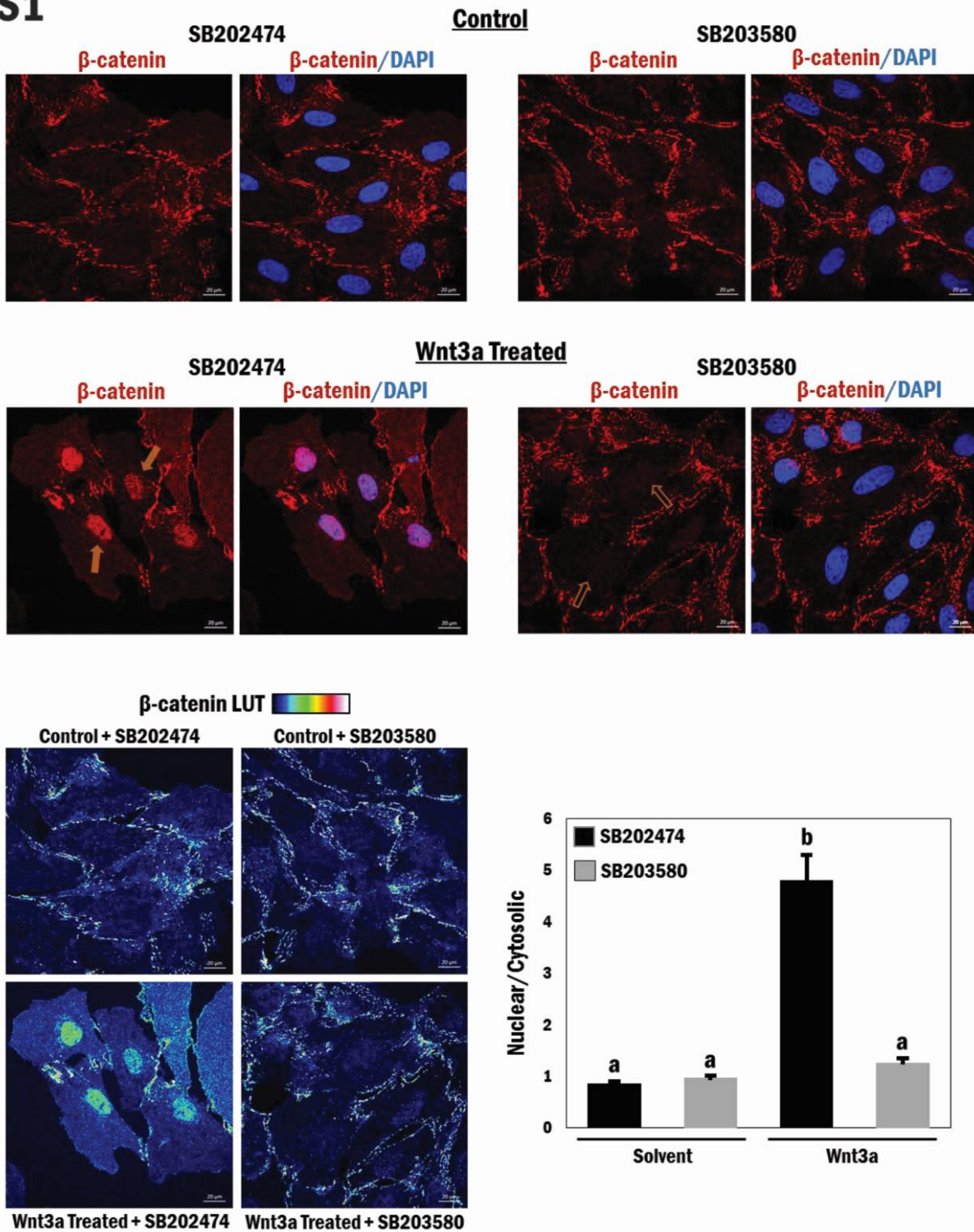
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Appendix

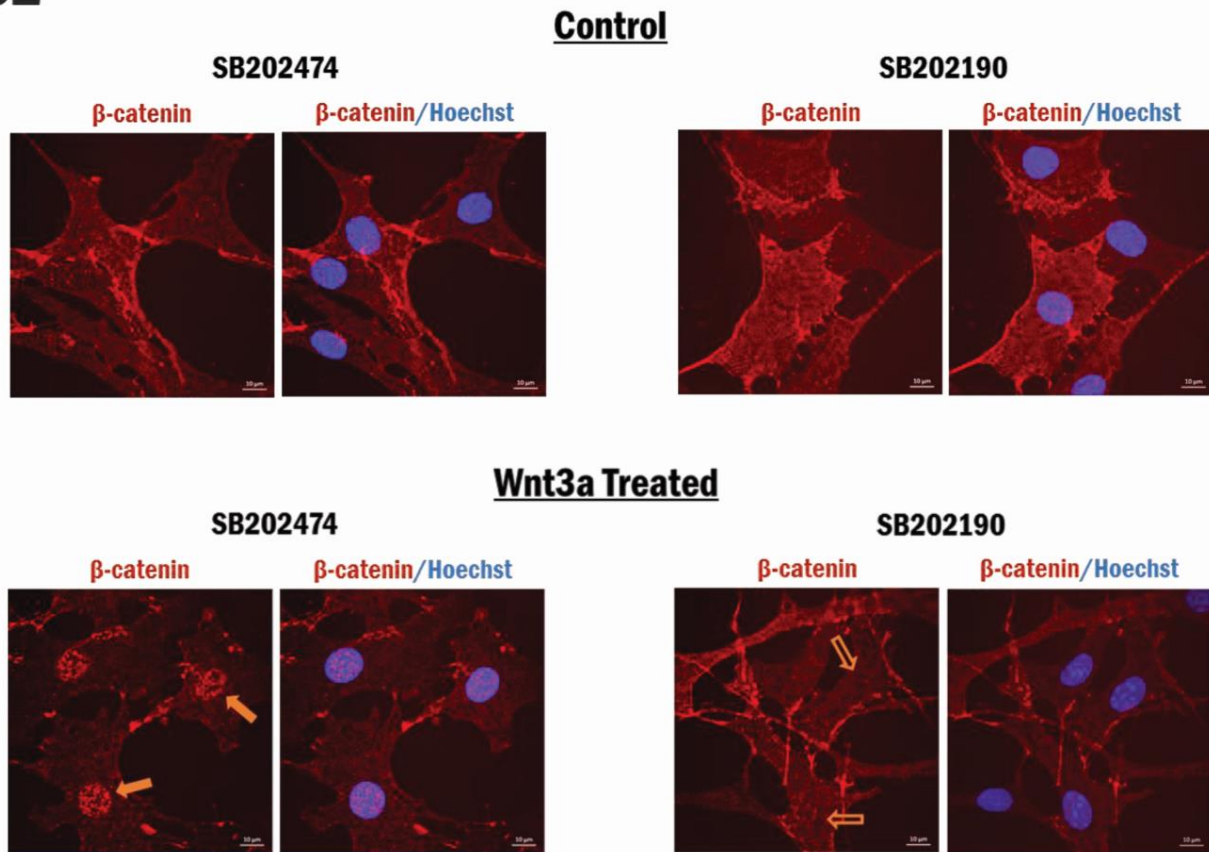
A: Supplementary figures

S1

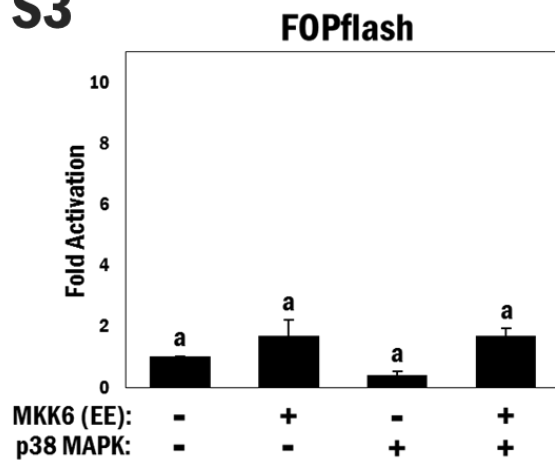


S1. Modulation of β -catenin localization with Wnt3a stimulation and p38 MAPK inhibition (SB203580). A10 cells were serum starved for 12 hr and pre-treated with SB203580 or SB202474 (10 μ M, 45 min) then stimulated with Wnt3a (200 ng/mL) or solvent (PBS, control) for 4 hr. Cells were fixed and stained with DAPI (blue, nucleus) and TRITC (red, β -catenin). Filled arrows indicate presence of nuclear β -catenin, empty arrows indicate lack thereof. (Bottom left panel) A rainbow (blue to red) lookup table (LUT) was used to indicate relative β -catenin intensity. (Bottom right panel) Relative nuclear to cytosolic fluorescence levels were measured and quantified (n=28, 25, 21, 27 from left to right); b=p $\leq$ 0.0001.

S2

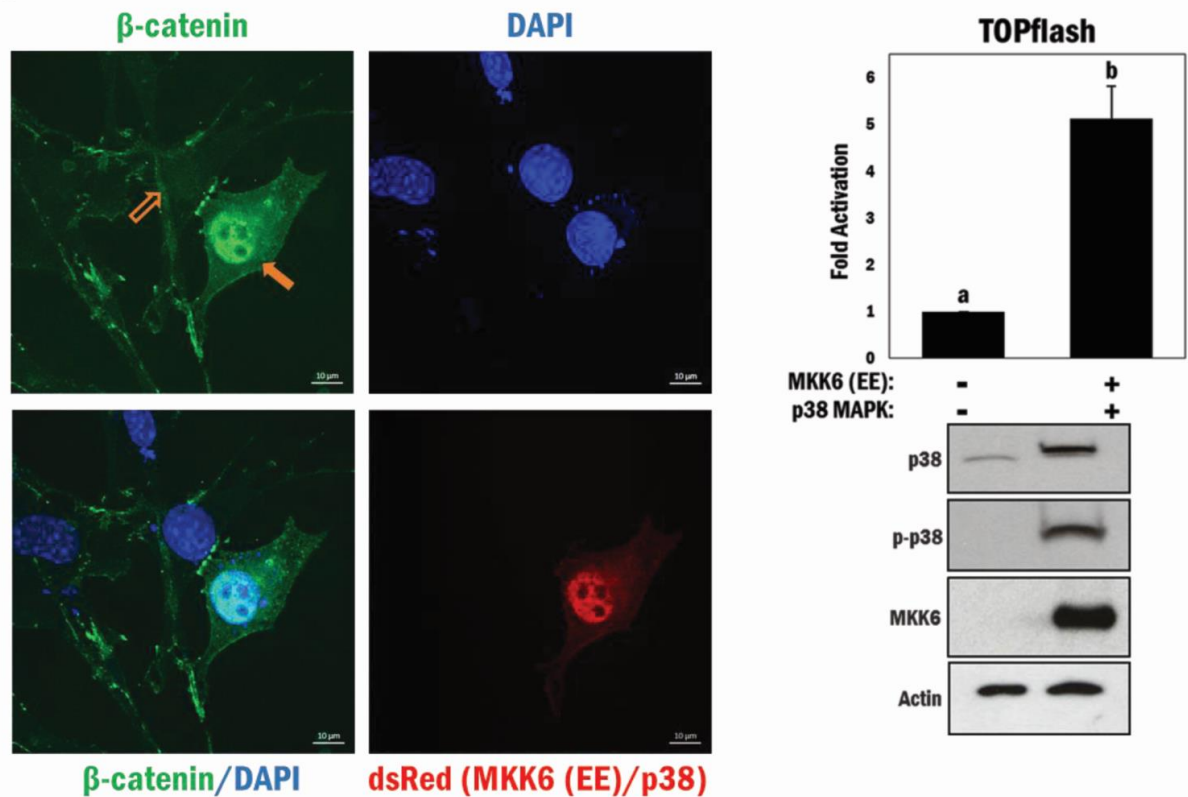


S2. Modulation of β -catenin localization with Wnt3a stimulation and p38 MAPK inhibition (SB202190). Primary VSMCs were serum starved for 12 hr and pre-treated with SB202190 or SB202474 (10 μ M, 45 min) then stimulated with Wnt3a (200 ng/mL) or solvent (PBS, control) for 4 hr. Cells were fixed and stained with DAPI (blue, nucleus) and TRITC (red, β -catenin). Filled arrows indicate presence of nuclear β -catenin, empty arrows indicate lack thereof.

S3

S3. FOPflash reporter gene activity in response to active p38 MAPK signalling. FOPflash reporter gene activity was measured in A10 cells that were transfected with different combinations of p38 MAPK and active MKK6 (EE) as indicated.

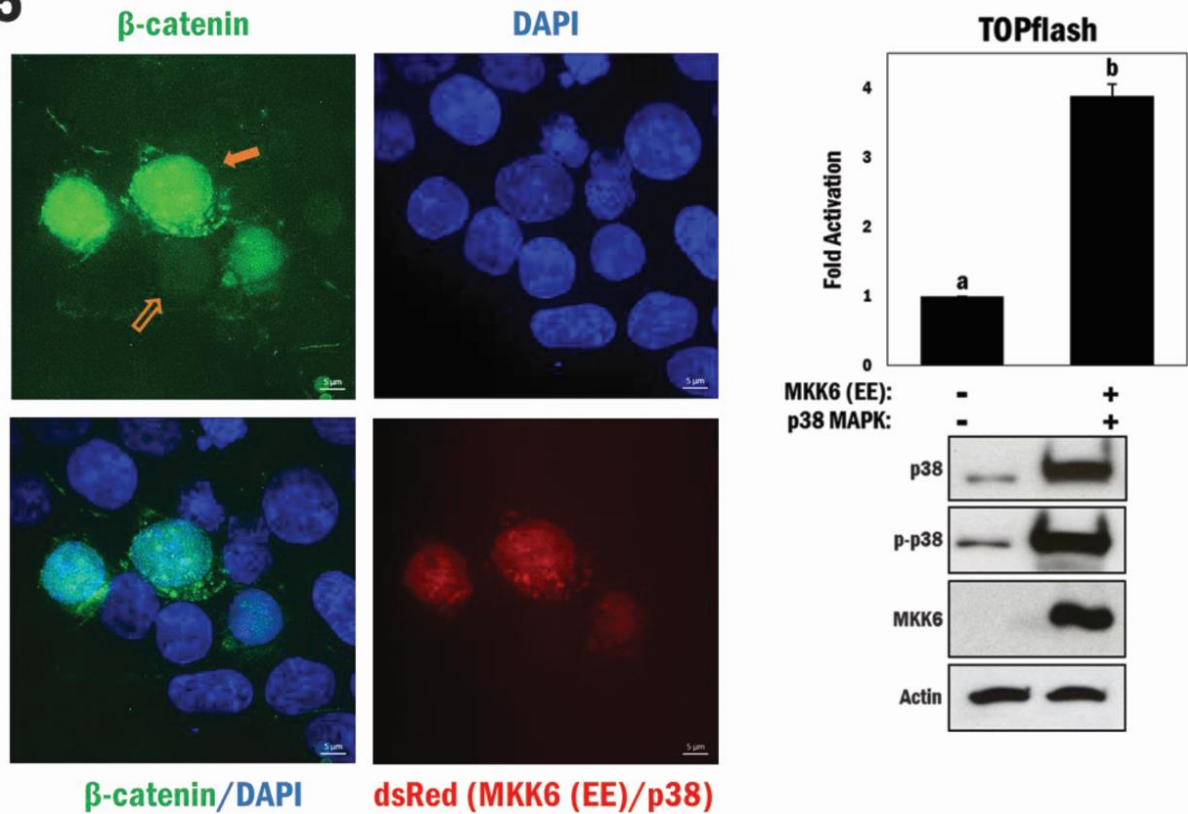
S4



S4. β-catenin localization and *transactivation* in response to active p38

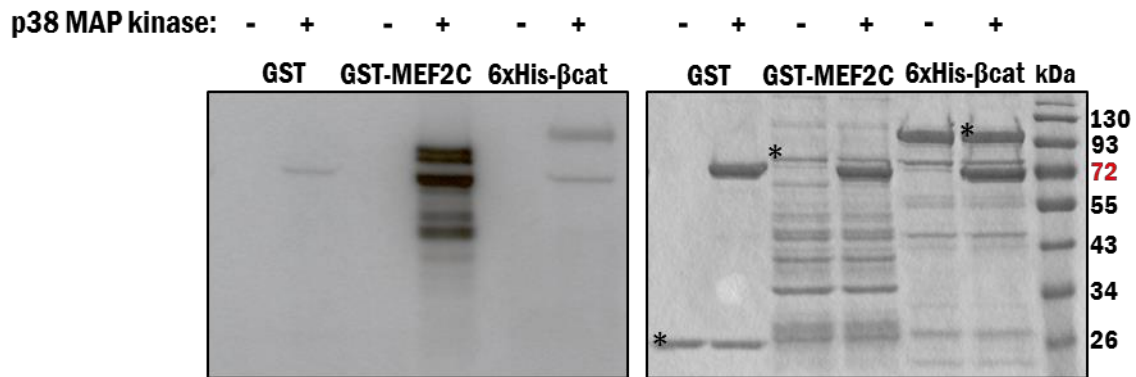
MAPK. (Left panel) 10T1/2 cells were co-transfected with MKK6 (EE) and p38 MAPK and marked for ectopic expression (dsRed, red), DAPI was used for nuclear staining and FITC was used for β-catenin staining (green); (Top right panel) TOPflash reporter gene activity was measured in 10T1/2 cells that were transfected with different combinations of p38 MAPK and active MKK6 (EE) as indicated; $b=p\leq 0.05$. (Bottom right panel) Western blot analysis to show activation of p38 MAPK.

S5



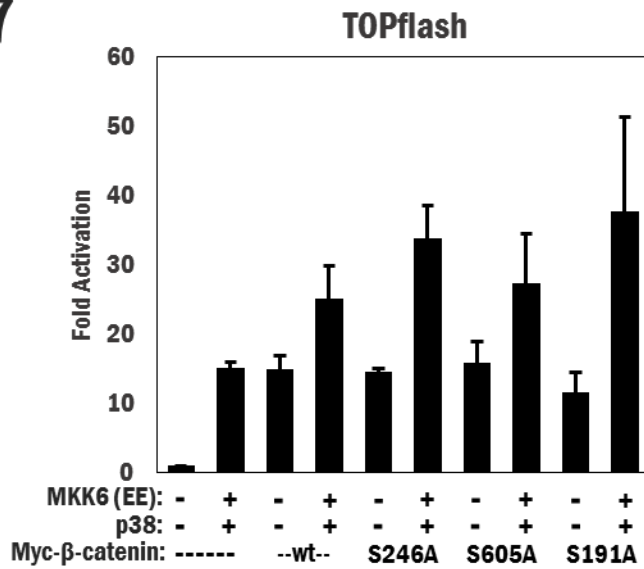
S5. β-catenin localization and *transactivation* in response to active p38 MAPK. (Left panel) HEK 293T cells were co-transfected with MKK6 (EE) and p38 MAPK and marked for ectopic expression (dsRed, red), DAPI was used for nuclear staining and FITC was used for β-catenin staining (green); (Top right panel) TOPflash reporter gene activity was measured in HEK 293T cells that were transfected with different combinations of p38 MAPK and active MKK6 (EE) as indicated; $b=p\leq 0.01$. (Bottom right panel) Western blot analysis to show activation of p38 MAPK.

S6

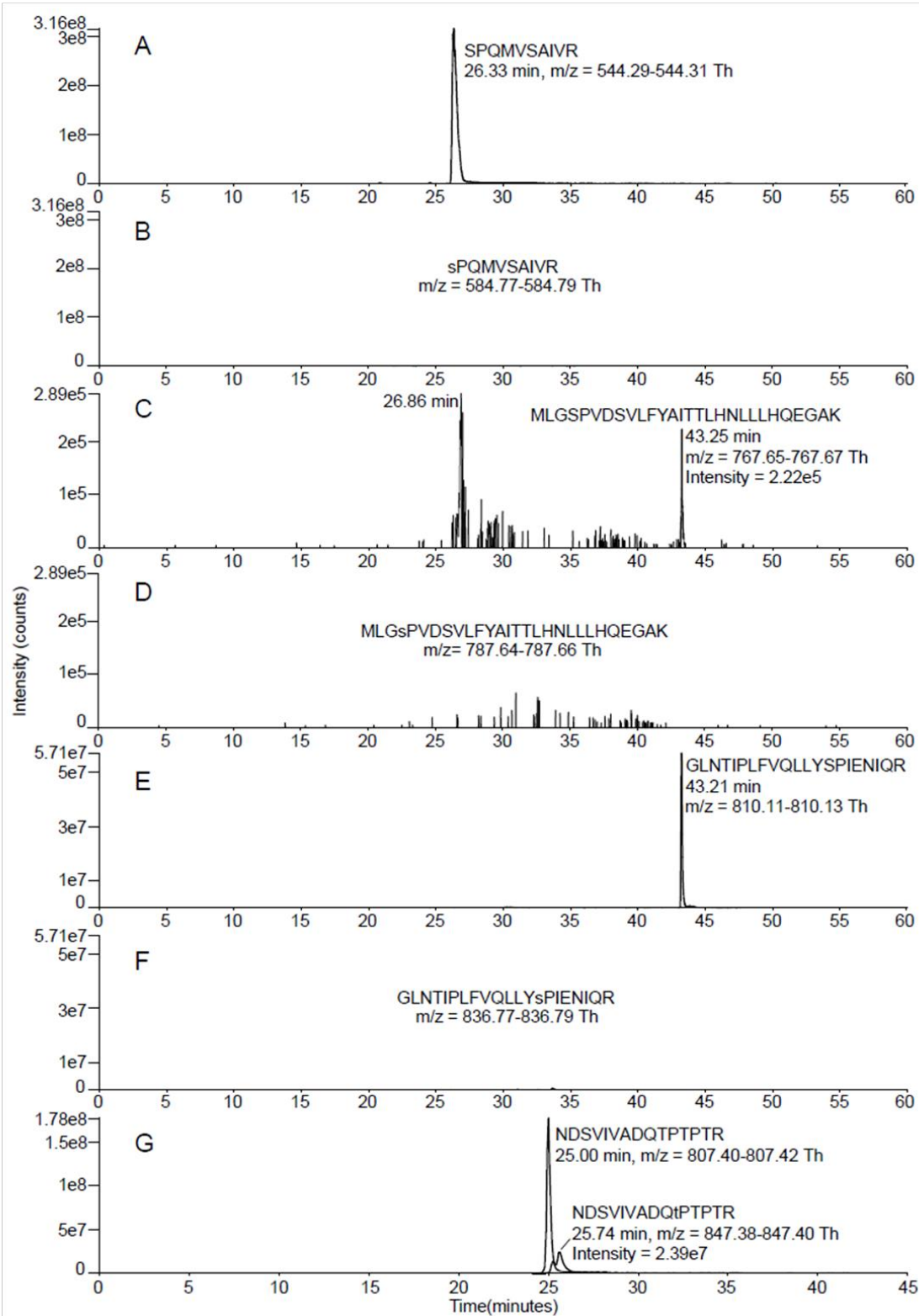


S6. Determination of β -catenin as a potential substrate of p38 MAPK by *in vitro* kinase assay. Purified p38 MAPK was incubated with [γ - 32 P]ATP, and either GST (negative control), GST-MEF2C (positive control), or 6xHis- β -catenin, as indicated, for 30 min at 30°C. The subsequent SDS-PAGE was exposed to X-ray film to determine 32 P incorporation. Stars indicate location of substrate proteins. (This figure was contributed by Mathew Dionyssiou).

S7

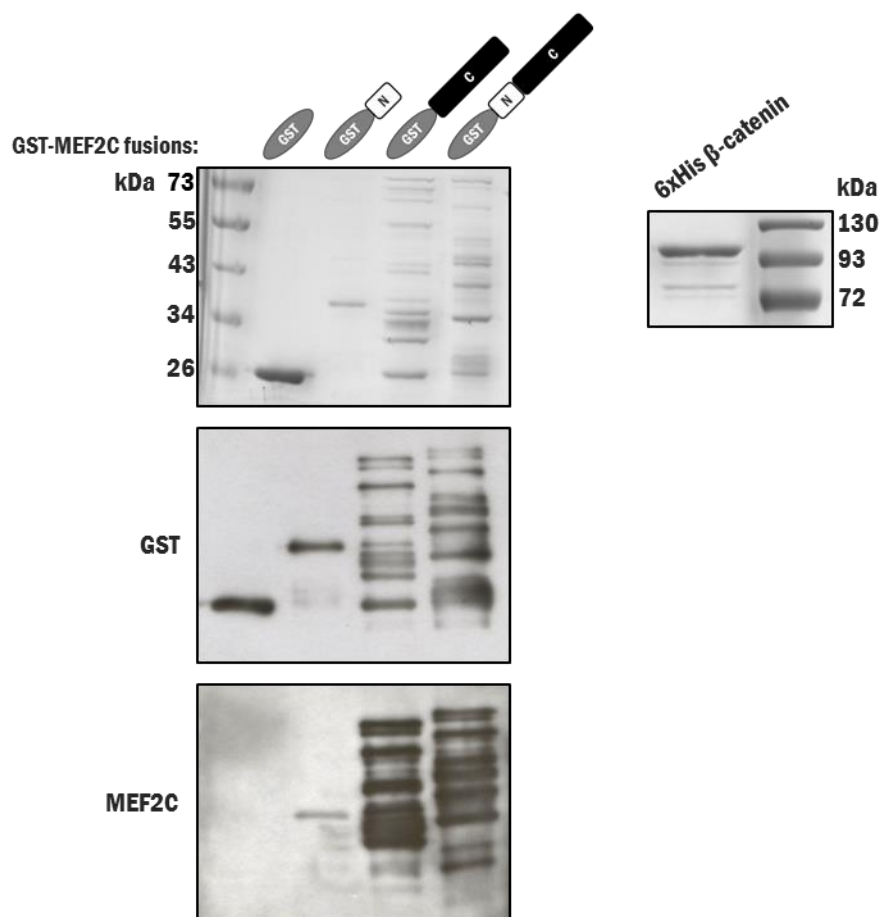


S7. β-catenin transactivation properties following Ser to Ala mutations of putative MAPK phospho-sites in response to p38 MAPK signalling. Wild-type or mutated Myc-tagged β-catenin was over-expressed with MKK6 (EE) and p38 MAPK in a TOPflash reporter assay, as indicated.



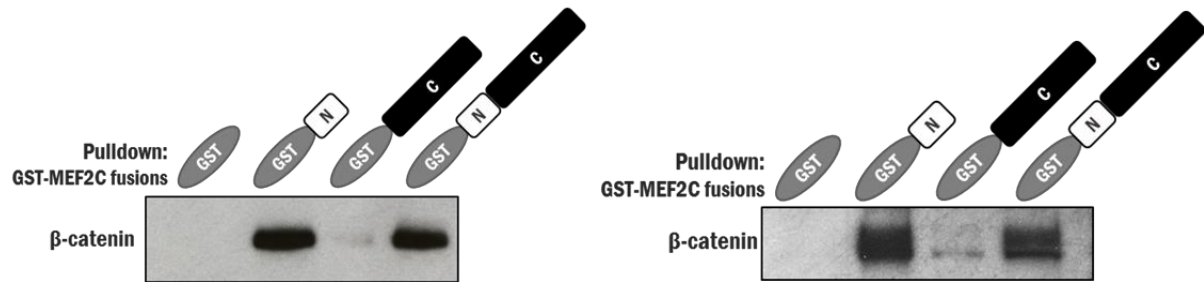
S8. Ion chromatograms of tryptic β -catenin peptides containing candidate p38 MAPK phosphosites and a tryptic ATF2 peptide and phosphopeptide containing known p38 MAPK phosphosites. Phosphorylated residues are shown as lower case letters. Doubly protonated SPQMVSAIVR (A) from β -catenin gave a prominent chromatographic peak while the corresponding phosphopeptide (B) did not. The quadruply protonated peptide MLGSPVDSVLFYAITTLHNLLLHQEGAK (C) was confirmed by MS/MS at 43.25 minutes while the ion at 26.86 minutes was an isobaric species. No chromatographic peak from phosphorylated MLGSPVDSVLFYAITTLHNLLLHQEGAK was present (D). Doubly protonated GLNTIPLFVQLLYSPIENIQR (E) was observed at 43.21 minutes while the extracted ion chromatogram of the phosphorylated counterpart (F) had no signal. The ATF2 peptide NDSVIVADQTPTPTR is shown in both its unmodified and phosphorylated state in panel G. (This figure was contributed by Declan Williams).

S9



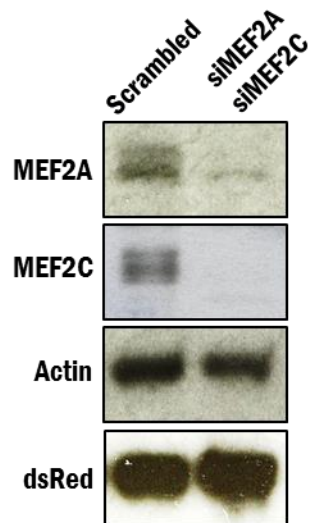
S9. Purification of GST-MEF2C and 6xHis- β -catenin. GST-MEF2C or 6xHis- β -catenin fusion proteins were purified from *E. coli* using GST-agarose or Nickel-agarose columns, respectively. Representative Coomassie blue SDS-PAGE and Western blots are shown to confirm purification.

S10



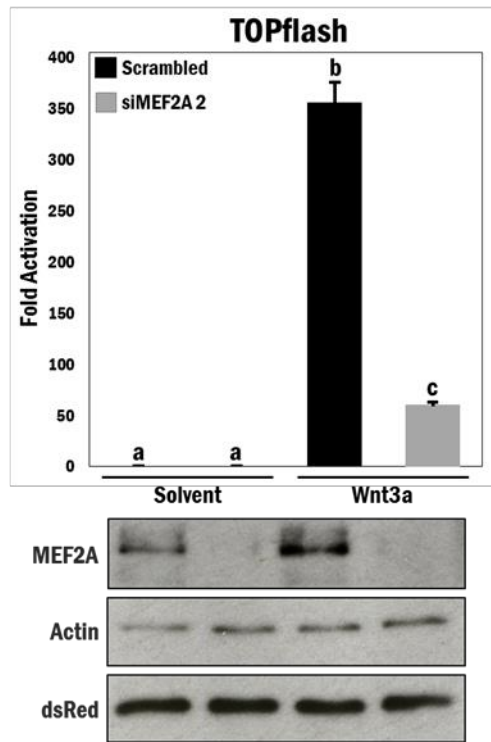
S10. *In vitro* binding assay. An *in vitro* GST pull down assay was performed using purified 6x-His-β-catenin and either GST (control), the N-terminal (aa 1-86), C-terminal (aa 87-465), or full length (aa 1-465) GST-MEF2C fusion proteins.

S11



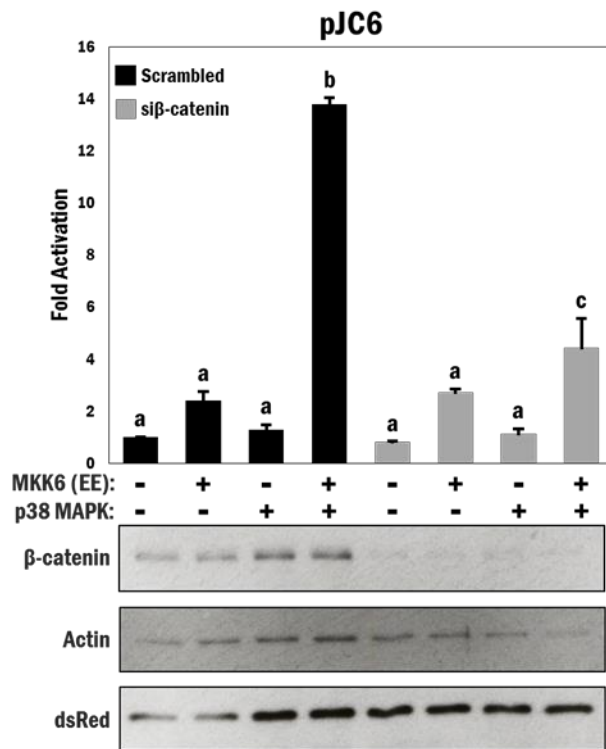
S11. Confirmation of siRNA mediated silencing of MEF2A/C. A10 cells were transfected with either scrambled RNA control or siRNA for MEF2A and MEF2C, and confirmed by Western analysis.

S12



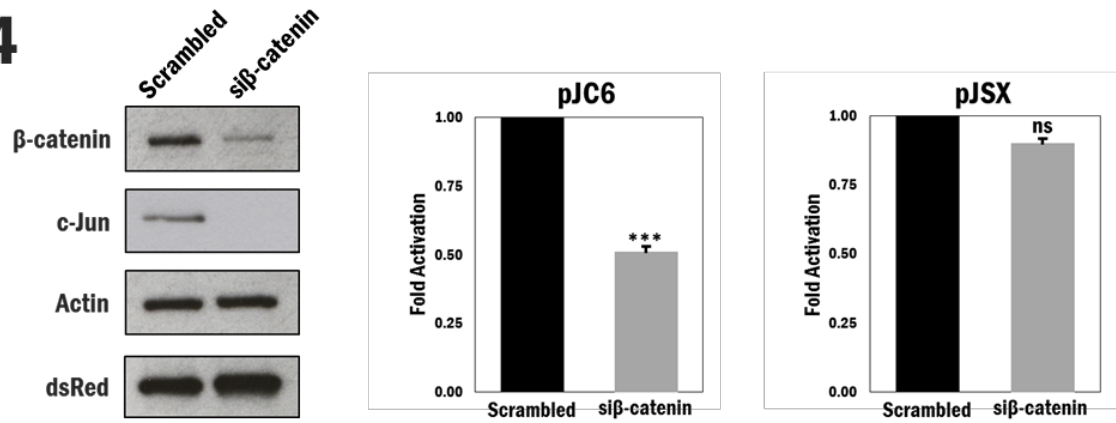
S12. TOPflash assay during Wnt stimulation using siRNA to MEF2A. (Top panel) A10 cells were transfected with TOPflash reporter, scrambled or siMEF2A RNA for 3 hr, cultured in serum conditions for 24 hr followed by treatment with either Wnt3a (200 ng/mL) or solvent (PBS) for 16 hr in serum free media; $b, c = p \leq 0.0001$. (Bottom panel) Corresponding Western blot analysis of cell lysates to confirm siRNA-mediated MEF2A depletion.

S13



S13. c-Jun reporter assay with active p38 MAPK and β-catenin gene silencing. c-Jun reporter gene (pJC6) activity was measured in A10 cells under various combinations of ectopically expressed MKK6 (EE) and p38 MAPK, and either scrambled RNA (control) or siRNA β-catenin, as indicated in the figure; b,c=p≤0.0001.

S14



S14. Effect of β-catenin gene silencing on c-Jun protein and promoter activity. Western blot analysis of A10 cells that have been transfected with scrambled shRNA (control) or shRNA β-catenin with comparative pJC6 and pJSX reporter activities; ***= $p \leq 0.001$.

S15

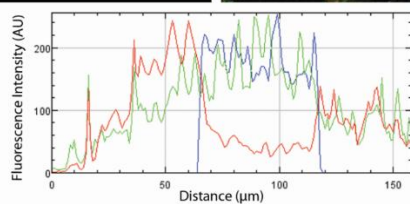
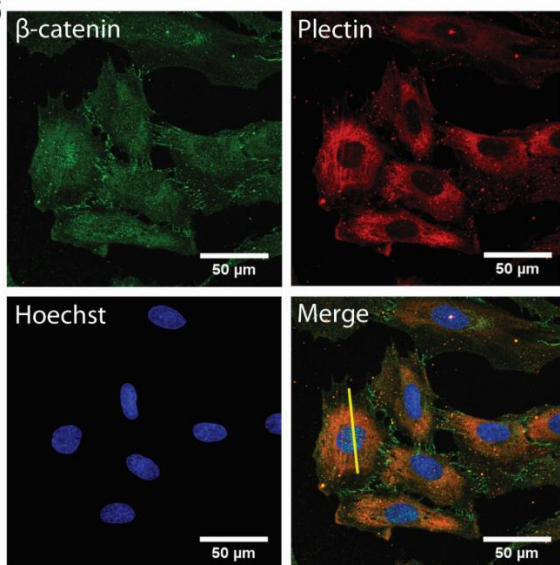
Protein Name	Accession No.	Gene ID	Repl. 1	Repl. 2	Repl. 3	Repl. 4	Repl. 5
Catenin beta-1	Q9WU82	Ctnnb1	34.13	40.26	30.25	5.98	13.57
BAG family molecular chaperone regulator 4	F1M3K6	Bag4	11.79	4.88	8.73	6.24	6.53
DNA fragmentation factor subunit beta	D4A8A0	Cad	3.25	9.09	5.68	12.87	4.92
CCR4-NOT transcription complex subunit 3	D3ZUV9	Cnot3	6.02	6.96	5.63	5.57	8.06
Proteasome subunit alpha type-3	P18422	Psma3	5.12	5.39	6.13	2.6	7.61
DNA helicase	D3ZFP4	Mcm3	3.67	5.78	2.89	4.99	5.03
Cold shock domain-containing protein E1	P18395	Csde1	2.82	5.57	3.13	2.36	2.31
Plectin	P30427	Plec	132.65	264.64	121.6		136.34
Catenin alpha-1	Q5U302	Ctnna1	9.29	17.68		5	7.71
SPATS2-like protein	Q5U2T3	Spats2l	10.98	8.11		9.05	7.95
ATP-dependent Clp protease proteolytic subunit	M0RAD5	Clpp	6.71	13.41	4.08		8.93
Pre-mRNA-processing factor 19	Q9JMJ4	Prpf19	10.38	2.73		9.94	2.5
Mediator of RNA polymerase II transcription subunit 1	D3ZRN2	Med1	10.24	5.83	5.9	2.95	
WD repeat-containing protein 63	B4F7B3	Wdr63		4.96	7.35	4.62	2.21
Transcriptional activator protein Pur-alpha	P86252	Pura	8.21	3.08	3.48	2.5	
Ran-binding protein 9	M0R9A4	Ranbp9	6.61	3.55	3.63	3.04	
Methyl-CpG-binding domain protein 6	D3ZCX1	Mbd6	5.07		5.2	2.27	2.16
Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform	P62716	Ppp2cb	2.59	3.24	5.14	2.34	
Nuclear pore complex protein Nup50	G3V7R1	Npap60		2.58	5.49	2.69	2.29
Heterogeneous nuclear ribonucleoprotein H	G3V9Q3	Hnrph1	412			226.37	207.55
Calcium/calmodulin-dependent protein kinase type II subunit gamma	P11730-3	Camk2g		16.15		22.19	9.92
Alpha-centractin	P85515	Actr1a	17.43		15.41	13.99	
RNA binding protein fox-1 homolog 2	A1A5R1	Rbfox2		12.75	19.52		10.59
Fragile X mental retardation syndrome-related protein 1	Q5XI81	Fxr1			14.78	7.71	10.81
Eukaryotic translation initiation factor 4B	Q5RKG9	Eif4b	5.9	10.32			11.86
Monofunctional C1-tetrahydrofolate synthase, mitochondrial	B2GUZ3	Mthfd1l		10.52	9.98		5.09
Ubiquitin-associated protein 2-like	E9PTR4	Ubap2l	5.85	11.72	5.8		
Procollagen, type XVIII, alpha 1	F1LR02	Col18a1		7.83	7.61		6.42
CCR4-NOT transcription complex, subunit 7	B3DMA5	Cnot7	3.15	6.3	9.65		
Proteasome subunit beta	G3V7Q6	Psmb5		3.75		7.43	7.35
La-related protein 7	Q5XI01	Larp7	5.8	7.55		4.73	
Tropomyosin alpha-4 chain	P09495	Tpm4			5.58	5.05	5.73
Calcium homeostasis endoplasmic reticulum protein	D3ZAX5	Cherp		10.68		2.79	2.79
Putative 60S ribosomal protein L37a	P61515	Rpl37a-ps1	2.9			5.75	5.55
THO complex subunit 4	D3ZXH7	Thoc4	2.57	6.05			5.11
Proteasome subunit beta type-6	P28073	Psmb6	6.53		3.42	2.7	
rRNA 2'-O-methyltransferase fibrillarin	P22509	Fbl	5.01			2.73	4.81
Mediator of RNA polymerase II transcription subunit 12	D3ZDE6	Med12		6.78		2.76	2.98
Aspartate--tRNA ligase, cytoplasmic	P15178	Dars			2.49	7.06	2.3
NADH dehydrogenase (Ubiquinone) Fe-S protein 3	D3ZG43	Ndufs3	3.03	3.26	5.47		
Cytochrome c oxidase subunit 5B, mitochondrial	P12075	Cox5b	2.49			4.58	2.59
NOL1/NOP2/Sun domain family, member 2	D4A3S8	Nsun2	2.63	2.83	2.77		
Nuclear RNA export factor 7	Q498M8	Nxf7	2.42		2.5		2.52
Serine/arginine-rich splicing factor 6	G3V6S8	Srsf6	2.47		2.45	2.48	
Actin, alpha cardiac muscle 1	P68035	Actc1				99.15	107.23
Protein transport protein Sec31A	G3V699	Sec31a				96.3	65.79
Serpin H1	P29457	Serpinh1	65.78	71.66			
TATA-binding protein-associated factor 2N	F7ESN1	Taf15				39.29	39.72
Elongation factor 1-alpha	F1M6C2	Eef1a1	28.32				18.2
Ataxin-2	F1LXI6	Atxn2	11.4		6.95		
Asparagine--tRNA ligase, cytoplasmic	F1LPV0	Nars	4.9			9.83	
DNA polymerase zeta catalytic subunit	F1M8G6	Rev3l	7.83		5.23		
Laminin subunit beta-1	D3ZQN7	Lamb1		3.75			9.16
U1 small nuclear ribonucleoprotein 70 kDa	B2RZ74	Snrnp70		4.46			7.93
Cell cycle and apoptosis regulator protein 2	F1LM55	Ccar2		8.25			2.39

Isoform RTN1-S of Reticulon-1	Q64548-2	Rtn1				5.19	4.99
39S ribosomal protein L49, mitochondrial	Q7TP77	Mrpl49	6.68		3.47		
Debranching enzyme homolog 1	B2RYJ2	Dbr1	6.67	3.33			
Protein ELYS	D3ZZZ0	Ahctf1	4.79			5.08	
Cytoplasmic FMR1 interacting protein 1	D4A8H8	Cyfp1				4.88	4.98
Pumilio homolog 1	D3Z8L5	Pum1			3.67	6.17	
Calmodulin	D4ABV5	Calm2				7.17	2.58
ATPase family AAA domain-containing protein 3	Q3KRE0	Atad3		2.88		6.79	
Elongation factor Tu, mitochondrial	P85834	Tufm				4.55	5.12
Myotubularin-related protein 1	D3ZKK5	Mtmr1		6.05	2.63		
Protein Scaf11	D4ABH1	Scaf11	2.54		5.69		
Keratin, type I cytoskeletal 19	Q63279	Krt19		5.88		2.3	
Isoform 2 of Dynactin subunit 4	Q9QUR2-2	Dctn4		5.58		2.48	
E3 ubiquitin-protein ligase RING2	Q4KLY4	Rnf2		2.68			5.38
Ras-related and estrogen-regulated growth inhibitor	F1M2G3	Rerg		5.48		2.45	
Mediator of RNA polymerase II transcription subunit 6	B0BNE1	Med6				5.12	2.7
Metastasis-associated protein MTA2	B2GV01	Mta2	2.44		5.19		
Synaptonemal complex protein SC65	Q64375	Leprel4	5.28				2.14
Acyl-Coenzyme A binding domain containing 3	G3V6E4	Acdb3	2.84				4.57
Melanoma-associated antigen D1	Q9E573	Maged1	5.16			2.22	
NK2 transcription factor related, locus 9	D4A7B5	Nkx2-8	2.39				4.83
Hypoxia up-regulated protein 1	F1LN18	Hyou1	2.46				4.53
Stromal cell-derived factor 2	D4A4H5	Sdf2	3.19		3.5		
E3 ubiquitin-protein ligase TRIM32	Q66H79	Trim32		3.62		2.94	
Anaphase-promoting complex subunit 5	A1L1K3	Anapc5		3.04	3.08		
Orexin	D3ZVN7	Ppox		3.18			2.75
Proteasome subunit alpha type-6	P60901	Psma6		3.35	2.54		
Cancer susceptibility candidate 3	G3V793	Casc3	2.75		3.11		
Splicing factor 3A subunit 1	D3ZQM0	Sf3a1	2.9			2.95	
Transcription elongation factor SPT4	B5DEM7	Supt4h1	3.01		2.83		
Myeloid-associated differentiation marker	Q05BA4	Myadm				2.96	2.87
Isoform RC6-IS of Proteasome subunit alpha type-7	P48004-2	Psma7				3.08	2.72
Zyxin	D4A7U1	Zyx	2.78		3		
Biglycan	P47853	Bgn		2.52			3.03
Zinc finger CCH-type antiviral protein 1	A2RRT6	Zc3hav1		2.8			2.75
Pecanex-like protein 3	D4A825	Pcnxl3	2.49		2.99		
Hemicentin-1	F1M4Q3	Hmcn1		3.14		2.28	
Ligand-dependent corepressor	D3ZAA6	Lcor			2.88	2.5	
Keratin, type II cytoskeletal 8	Q10758	Krt8	2.42	2.9			
EGF-containing fibulin-like extracellular matrix protein 2	Q5XI84	Efemp2		2.85	2.45		
CST complex subunit STN1	Q6AYD2	Obfc1	2.96				2.3
Catalase	P04762	Cat	2.78			2.48	
Semaphorin-3D	F1MAG8	Sema3d		2.72	2.54		
Activating signal cointegrator 1 complex subunit 3	F1LPQ2	Ascc3	2.36			2.88	
Protocadherin Fat 3 (Fragment)	F1LMF4	Fat3		2.52	2.72		
Migration and invasion enhancer 1	D3ZSU7	Mien1		2.57	2.67		
PHD finger protein 17 (Predicted), isoform CRA_a	D3ZM64	Phf17		2.7	2.51		
Kinesin-like protein KIF7	D4A9P0	Kif7		2.61			2.6
Disabled homolog 2-interacting protein	Q6P730	Dab2ip	2.72	2.44			
Tyrosine-protein phosphatase non-receptor type 14	D3ZSL5	Ptpn14	2.44	2.71			
LIM and calponin homology domains-containing protein 1	F1M392	Limch1	2.65		2.49		
LIM domain only protein 7	E9PTK8	Lmo7	2.54	2.6			
Cytochrome P450 3A9	P51538	Cyp3a9	2.66				2.47
Protocadherin-7	Q68HB6	Pcdh7	2.51				2.6
Isoform 5 of Tropomyosin alpha-1 chain	P04692-5	Tpm1	2.47	2.6			
Protein Smaug homolog 1	B5DF21	Samd4a	2.5	2.56			
Procollagen, type VIII, alpha 1 (Predicted)	D4AC70	Col8a1				2.44	2.6
Isoform 2 of Homeodomain-interacting protein kinase 1	A4L9P5-2	Hipk1		2.49		2.51	

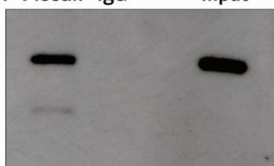
Aminoacyl tRNA synthase complex-interacting multifunctional protein 1	Q4G079	Aimp1		2.54		2.44	
Ras-associating and dilute domain-containing protein	D4A1Z8	Radil			2.76		2.21
Interleukin 17 receptor B	B5DF15	Il17rb			2.59	2.32	
Ras-related protein Rab-23	D3ZRM5	Rab23	2.37	2.51			
Myelin expression factor 2	D4AEI5	Myef2			2.4	2.48	
A disintegrin and metalloproteinase with thrombospondin motifs 6	D4A065	Adams6			2.53		2.34
Mitogen-activated protein kinase kinase kinase 7	P0C8E4	Map3k7			2.48		2.38
Proto-oncogene vav	P54100	Vav1			2.4	2.4	
1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase epsilon-1	G3V9X9	Plce1	2.36				2.37
U2 small nuclear ribonucleoprotein B''	B5DEQ4	Snrpb2	2.46				2.26
Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	Q32PX2	Aimp2	2.36			2.33	
HEAT repeat-containing protein 5A	F1LSK5	Heatr5a		2.48			2.15
Proteasome subunit alpha type-4	P21670	Psm4	2.39				2.22
tRNA dimethylallyltransferase, mitochondrial	D4A175	Trit1				2.29	2.32
Insulin-degrading enzyme	P35559	Ide				2.26	2.33
Calcium-binding mitochondrial carrier protein SCaMC-2	Q8K3P6	Slc25a25				2.37	2.2
Coiled-coil domain-containing protein 103	D3Z8H9	Ccdc103				2.25	2.31
Beta-ureidopropionase	D3Z8J1	Upb1				2.23	2.28
Forkhead-associated domain-containing protein 1	Q4V7C3	Fhad1				2.29	2.21

Peptide Score ≤ 2.6 2.61 - 3 3.01 - 5.5 5.51 - 8 8.01 - 20 > 20

S16

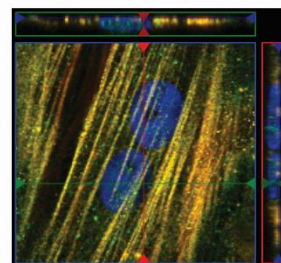
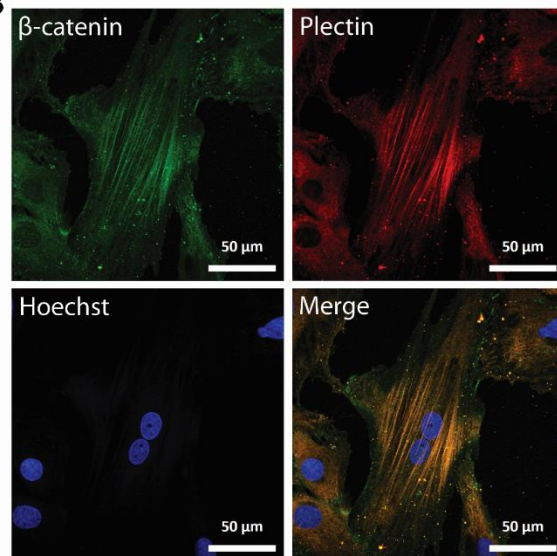


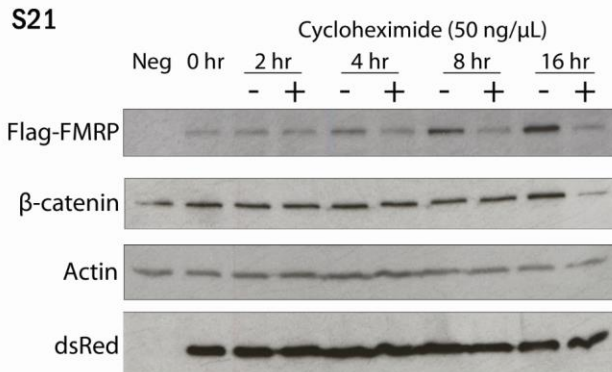
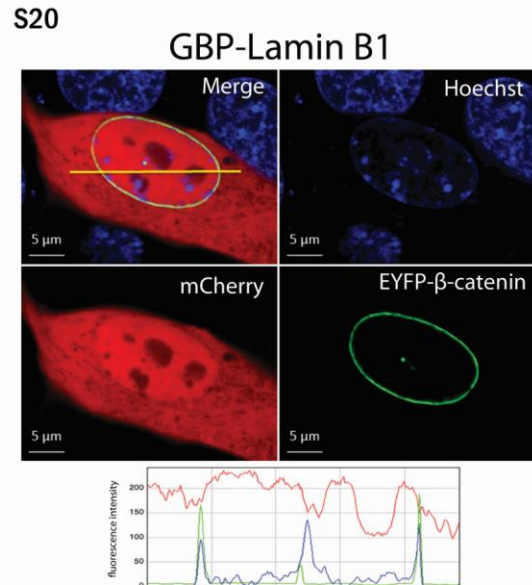
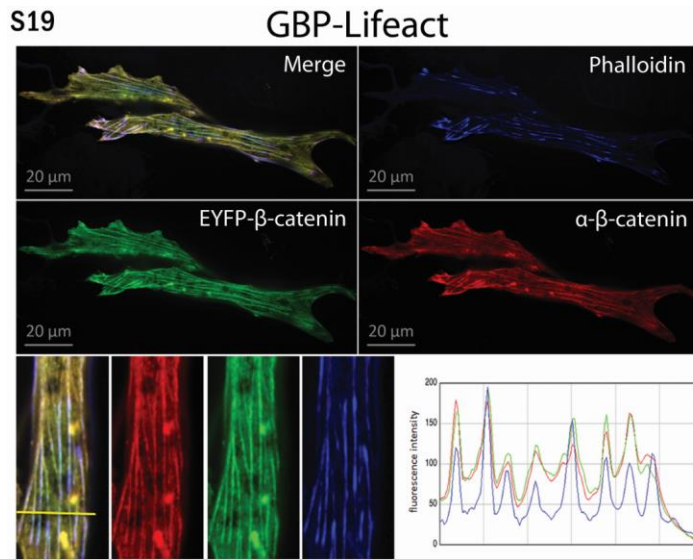
S17 IP: Plectin IgG ---- Input



IB β-catenin

S18





S15. Full list of 131 interacting partners identified by mass spectrometry. **S16.** A10 cells were fixed and subjected to immunofluorescent staining for β -catenin (green), Plectin (red), and nucleus (blue). Co-localization of these proteins was analysed by an RGB plot. **S17.** A10 cell lysates were subjected to coIP analysis using Plectin for immunoprecipitation and Western blot for corresponding β -catenin. IgG was used to determine non-specific binding. **S18.** A10 cells were treated similarly to (**S16**) and a set of Z-stack images (240nm) was used to generate orthogonal projection images. **S19.** C2C12 myoblasts were transfected with GBP-Lifeact and EYFP- β -catenin and subjected to fluorescent and immunofluorescent analysis, as indicated. Co-localization of F-actin (blue), EYFP- β -catenin (green), and β -catenin (red) were confirmed by an RGB plot. **S20.** C2C12 myoblasts were transfected with GBP-Lamin B1, mCherry, and EYFP- β -catenin then subjected to fluorescent and immunofluorescent analysis, as indicated. mCherry did not co-localize with GBP-lamin B1 and EYFP- β -catenin was confirmed by an RGB plot. **S21.** A10 cells were transfected with Flag-FMRP and treated with cycloheximide (50 ng/ μ L) or solvent (DMSO) for the indicated times and subjected to

Western blot analysis for expression levels of indicated proteins. Actin was used as a loading control and dsRed as a measure of transfection efficiency. (Figures S5 and S6 were contributed by Tetsuaki Miyake).

B: Selected materials and methods

m⁷GTP-agarose pull down assay

1. Grow approximately 2000-3000 µg of cell lysate and harvest using normal NP-40 extraction techniques.
2. Equilibrate 50 µL m⁷GTP-agarose beads by washing 3 times with TBS and add cell lysate. Incubate overnight with gentle rocking at 4°C.
3. Wash beads once with TBST and twice with TBS.
4. Add 50 µL of 3xSDS loading buffer and boil for 5 min.
5. Load for target proteins and for eIF4E and tubulin controls.
6. Perform normal Western blot analysis.

Puromycin incorporation for determining protein translation

1. Grow/treat/transfect cells as normal.
2. Prior to harvesting or fixation for immunofluorescence, add 1 µM Puromycin (0.544 µg/mL, resuspended in ddH₂O) to cells and incubate for 15 min in CO₂ incubator at 37°C.
3. Proceed with normal harvesting/fixation.
4. Use puromycin antibody (mouse monoclonal from DSHB, PMY-2A4) at 1:100 dilution for Western blot (0.5 µg/mL), or at 1:20 for immunofluorescence (2.5 µg/mL).

6xHis protein purification

1. Grow transformed bacteria (BL21 or Rosetta blue) in LB broth at 37°C until they reach 0.6 OD₆₀₀.
2. Add IPTG to a final concentration of 0.4 mM and incubate bacteria in shaker at 24°C for 4 hours and then at 16°C overnight (can skip 4 hours if necessary).
3. Centrifuge bacteria at 6000 g for 15 min at 4°C.
4. Re-suspend pellet in **common buffer** (20 mM Tris pH 8, 500 mM NaCl, 10% glycerol in ddH₂O) with protease inhibitors (1 mM PMSF, 1 mM benzamidine), 0.5 mM BME, 0.05% NP-40, and 10 mM imidazole pH 8. Use about 25-30 mL for a 500 mL culture pellet.
5. Sonicate for 10 min ON time (10 sec pulse, 15 sec rest, 35% amplitude).
6. Centrifuge at 17 000 g for 30 min at 4°C. Collect supernatant.
7. Equilibrate 5 mL of 50% slurry nickel beads by washing 3 times with TBS.
8. Place beads in column and add supernatant. Rock gently for 30 min at 4°C.
9. Wash beads with about 100 mL **wash buffer** (common buffer + protease inhibitors, 0.5 mM BME, and 20 mM imidazole).
10. Elute with **elution buffer** (common buffer + 250 mM imidazole). Usually two rounds of 2 mL is enough. Check elutions using Bradford dye until colour no longer turns blue.
11. Dialyze into 250 mL common buffer for 1 hour at 4°C, then in another 250 mL common buffer overnight at 4°C.
12. Check concentration and purification by running an SDS-PAGE with BSA comparison, and store at -80°C. Avoid multiple freeze thaws (aliquot).

GST protein purification

This protocol is identical to 6xHis purification except for the **elution buffer** (common buffer + 30 mM reduced glutathione) used and that **no imidazole** is added to any buffer. Glutathione is acidic so remember to pH this buffer back to 8 before using.

Cell lines and culture

A10: Grow in modified DMEM media (same as normal DMEM, except only 1.5 g sodium bicarbonate and pH to 7.25). This is a powder that needs to be mixed with ddH₂O manually and does not contain any bicarbonate (Gibco 12800-017). Add the bicarbonate, pH to 7.25, and then filter sterilize into a blue capped bottle. FBS, L-glutamine, and Pen/Strep is added as normal prior to use.

A10 cells are passaged 1:3 every 2-4 days (usually 3) and **must** be centrifuged each time they are passaged. Modified trypsin must be used that contains **no EDTA** (0.125% in PBS instead). Cells can be passaged approximately 10 times and transfected with Lipofectamine 2000 as normal. A10 cells are frozen using 5% DMSO v/v.

HEK 293T: Grow in normal DMEM. Passage 1:10 every other day (they will overgrow if you wait 3 days). Cells do not need to be centrifuged when passaged and normal trypsin-EDTA can be used. Cells can be passaged 30-40 times.

COS7: Same as HEK293T.

10T1/2: Same as HEK293T, except can only be passaged approximately 10 times.
Do not allow them to become confluent as they may start differentiating.

Western blot analysis

1. Grow/transfect/treat cells as normal in a 6-well dish.
2. Wash cells twice with cold PBS on ice.
3. Add 150 μ L lysis buffer (20 mM Tris pH 8, 150 mM NaCl, 0.1% Triton-X100) and scrape.
4. Collect lysate in eppendorf tube and directly add 75 μ L of 3xSDS loading buffer. Pipette slowly to mix.
5. Boil samples for 5 min.
6. Load between 5-20 μ L of sample and perform Western blot analysis as normal (use 5-10 μ L for high density cells such as HEK 293T and COS7 and 15-20 μ L for low density cells such as A10. Adjust where necessary). **Actin is absolutely required** for this protocol since protein concentrations were not calculated after harvesting.
7. Note: if a particular transfection or treatment results in different confluence prior to harvesting, adjust the loading volume accordingly (e.g. using a p38 inhibitor overnight kills up to 50% of A10 cells, therefore load 20 μ L of this condition and 10 μ L of the control condition to account for this).