5-2011

Mechanisms Regulating The P120-Catenin/Kaiso Pathway

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A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

And

The University of Texas

M.D. Anderson Cancer Center

Graduate School of Biomedical sciences

In Partial Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

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Houston, Texas

May, 2011

Dedication

I would like to dedicate this work to my family, Sung Hoon and Andrew. Additionally, I dedicate this work to my parents, Young Kyu Hong and Soon Hae Lee and my brother, Tae Won Hong who support my study and my whole life.

Acknowledgements

I would like to acknowledge my mentor, Pierre D. McCrea, Ph.D. Dr. McCrea has been very considerate and patience in guiding my ideas and hypothesis. He has always encouraged me to think critically on my project accompanied with consideration of the big picture. I have learned with regards to looking at things as a scientist from him. His utmost consideration and patience has facilitated my studies in this great environment in Houston.

I would also like to thank the members of my current and past committees: Warren S. Liao, Ph.D., Lei Li, Ph.D., Amy Sater, Ph.D., Margozata Kloc, Ph.D., Andreas Bergmann, Ph.D. and Richard Behringer, Ph.D. Further, I thank my collaborators for their assistance with providing data to strengthes and complement my work, and for kindly offering their helpful constructs, reagents and advice prior to publication: Jae-il Park, Ph.D. (UT MD Anderson Cancer Center, Houston), Juliet Daniel (McMaster U., Canada), Randall T. Moon, Ph.D. (U. of Wasington, Seattle), Peter Klein (U. of Pennsylvania), Sergei Y. Sokol (Mount Sinai School of Medicine), Dihua Yu (UT MD Anderson Cancer Center) and Xi He (Children's hospital, Boston). I also thank Mireia Dunach and Antonio Garcia de Herreros for sharing work before publication.

I would like to thank the Genes and Development Graduate Program, particularly Elisabeth Lindheim, the coordinator, as well as the Department of

Biochemistry and Molecular Biology for providing me with an exceptional environment and support.

I also thank all members of the McCrea lab, past and present, who have provided technical assistant, advice and friendship during my training: Hong Ji, Rachel K. Miller, Jae-il Park, Dongmin Gu, William A. Munoz, Moon Sup Lee and Kyucheol Cho. I especially thank Rachel and William who gave me many comments on my projects during lunch and lab meetings. I also appreciate Hong Ji, who is the lab manager in our lab, and who provided most of the constructs that I used in my work, and has been a great friend of our lab members including myself. I thank Jodie L. Polan, M.S. to teach me how to use the departmental microscopes and how to improve my images. I would like to thank some of Dr. Barton's and Dent's lab members, Svitlana Kutinna, Sabrina Stratton and Eunah Kim for their kind help to perform real-time PCR and Chip-sequencing.

Especially, I thank my husband, Sung Hoon Jeong, for his support and patience over 5 years and my precious Andrew Y. Jeong. Also, I am grateful to my parents, Young Kyu Hong and Soon Hae Lee, and my brother, Tae Won Hong, for their endless support to my study.

This publication was in part reproduced/ adapted with permission in writing from the *Journal of Cell Science*. (Hong JY, Park JI, Cho K, Gu D, Ji H, Artandi SE, McCrea PD "Shared molecular mechanisms regulate multiple catenin proteins:

canonical Wnt signals and components modulate p120-catenin isoform-1 and additional p120 subfamily members" *Journal of Cell Science* 123:4351-4365 (2010))

http://jcs.biologists.org/cgi/content/short/123/24/4351

This work was supported by a NIH (RO1GM52112) and a Texas Advanced Research Project Grant (003657-0008-2006). DNA sequencing and other core facilities were supported by a University of Texas M.D. Anderson Cancer Center National Cancer Institute Core Grant (CA-16672). I was supported from a William Randolf Hearst Foundation Student Research Award, and a Developmental Award from the UT SPORE in Lung Cancer (P50 CA 070907).

MECHANISMS REGULATING THE P120-CATENIN/KAISO PATHWAY

Publication No._____

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Supervisory Professor: Pierre D. McCrea, Ph.D.

The Wnt pathways contribute to many processes in cancer and developmental biology, with β-catenin being a key canonical component. P120-catenin, which is structurally similar to β-catenin, regulates the expression of certain Wnt target genes, relieving repression conferred by the POZ/ zinc-finger transcription factor Kaiso. In my first project, employing *Xenopus* embryos and mammalian cell lines, I found that the degradation machinery of the canonical Wnt pathway modulates p120-catenin protein stability, especially p120 isoform-1, through mechanisms shared with β-catenin. Exogenous expression of destruction-complex components such as GSK3β or Axin promotes p120-catenin degradation, and consequently, is able to rescue developmental phenotypes resulting from p120 over-expression during early *Xenopus* embryonic development. Conversely, as predicted, the in vivo depletion of either Axin or GSK3β coordinately increased p120 and β-catenin levels, while p120 levels decreased upon LRP5/6 depletion, which are positive modulators in the canonical Wnt pathway. At the primary sequence level, I resolved conserved GSK3β phosphorylation sites in p120's (isoform 1) amino-terminal region. Point-mutagenesis of these residues inhibited the association of destruction complex proteins including those involved in ubiquitination, resulting in p120-catenin

stabilization. Importantly, we found that two additional p120-catenin family members, ARVCF-catenin and δ-catenin, in common with β-catenin and p120, associate with Axin, and are degraded in Axin's presence. Thus, by similar means, it appears that canonical Wnt signals coordinately modulate multiple catenin proteins having roles in development and conceivably disease states.

In my second project, I found that the Dyrk1A kinase exhibits a *positive* effect upon p120-catenin levels. That is, unlike the negative regulator GSK3β kinase, a candidate screen revealed that Dyrk1A kinase enhances p120-catenin protein levels via increased half-life. Dyrk1A is encoded by a gene located within the trisomy of chromosome 21, which contributes to mental retardation in Down Syndrome patients. I found that Dyrk1A expression results in increased p120 protein levels, and that Dyrk1A specifically associates with p120 as opposed to other p120-catenin family members or β-catenin. Consistently, Dyrk1A depletion in mammalian cell lines and Xenopus embryos decreased p120-catenin levels. I further confirmed that Dyrk overexpression and knock-down modulates both *Siamois* and *Wnt11* gene expression in the expected manner based upon the resulting latered levels of p120-catenin. I determined that Dyrk expression rescues Kaiso depletion effects (gastrulation failure; increased endogenous Wnt11 expression), and vice versa. I then identified a putative Dyrk phosphorylation region within the N-terminus of p120-catenin, which may also be responsible for Dyrk1A association. I went on to make a phosphomimic mutant, which when over-expressed, had the predicted enhanced capacity to positively modulate endogenous

Wnt11 and *Siamois* expression, and thereby generate gastrulation defects. Given that Dyrk1A modulates *Siamois* expression through stabilization of p120-catenin, I further observed that ectopic expression of Dyrk can positively influence β-catenin's capacity to generate ectopic dorsal axes when ventrally expressed in early *Xenopus* embryos. Future work will investigate how Dyrk1A modulates the Wnt signaling pathway through p120-catenin, and possibly begin to address how dysfunction of Dyrk1A with respect to p120-catenin might relate to aspects of Down syndrome. In summary, the second phase of my graduate work appears to have revealed a novel aspect of Dyrk1A/p120-catenin action in embryonic development, with a functional linkage to canonical Wnt signaling. What I have identified as a "Dyrk1A/p120-catenin/Kaiso pathway" may conceivably assist in our larger understanding of the impact of Dyrk1A dosage imbalance in Down syndrome.

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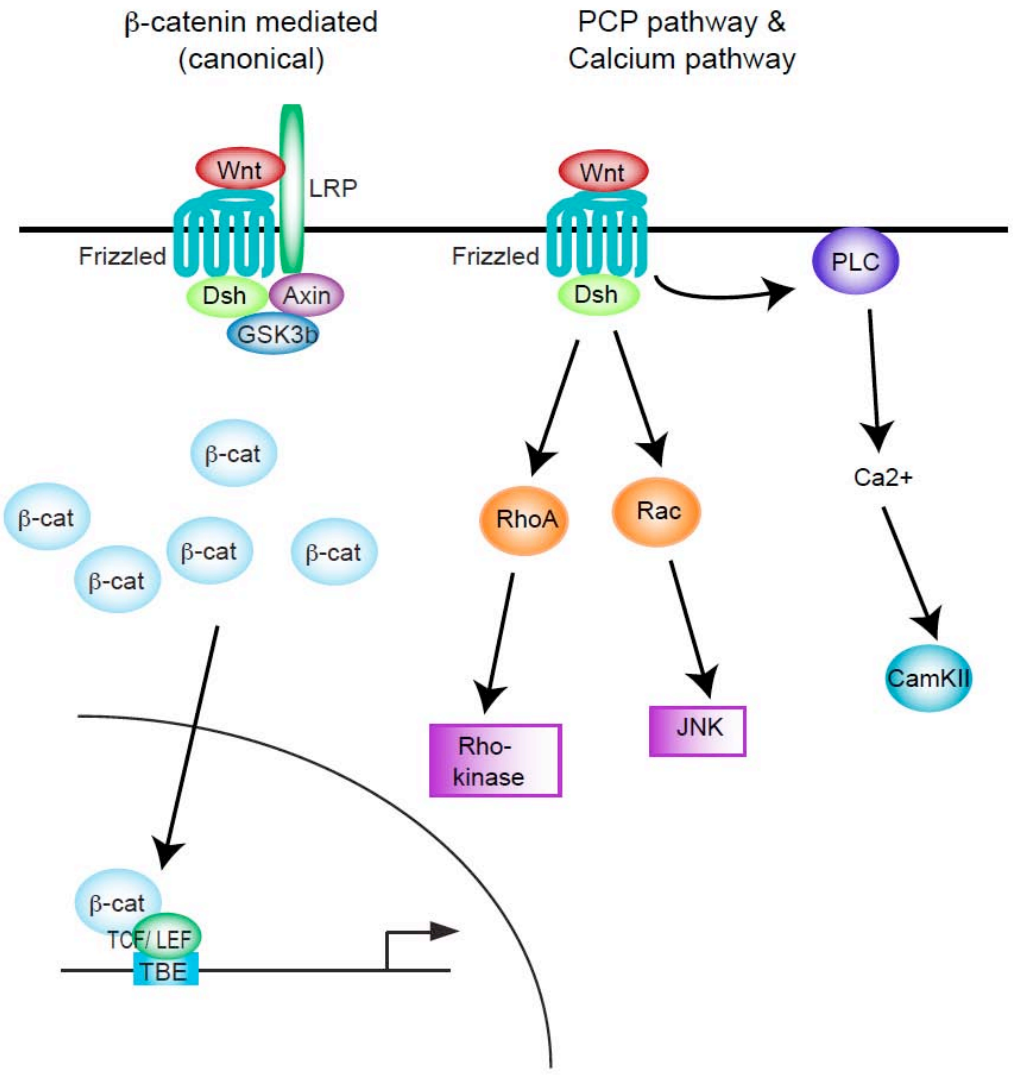
Chapter I

Introduction and Background

Wnt signaling pathways

Wnt signaling pathways are involved in multiple aspects of development and tumorigenesis (1-5). The Wnt family of ligands possesses at least 19 members, shown to be present in human and mice. Each of the Wnt ligands has unique expression patterns, some overlapping, and distinct functions during development. The Wnt pathways are roughly categorized into the canonical, and non-canonical pathway. Both are initiated by the binding of Wnt ligands to Frizzled receptors. In what is defined as canonical Wnt signaling, β-catenin is a key element transmitting Wnt signals into the nucleus via high mobility group (HMG) box transcriptional repressor LEF/TCF, to activate multiple Wnt target genes (1, 6, 7). Non-canonical Wnt signals are defined as other than that of the β-catenin-mediated pathway. Non-canonical Wnt pathways are mediated through Rho GTPases, Ca2+/PKC or JNK (Figure 1). Recent evidence indicates that non-canonical Wnt signals antagonize canonical Wnt pathways in certain contexts, although the precise mechanisms are still under study (8, 9). Mutations in Wnt pathway components result in varied developmental defects and human diseases including cancer.

Figure 1. A schematic diagram of selected components of the canonical and non-canonical Wnt pathway. Wnt ligands bind to the Frizzled receptor and LRP5/6 co-receptor, which recruits Dishevelled (Dvl). Upon activation of Dvl, Axin and GSK3β are recruited to the plasma membrane, leading to β-catenin's stabilization and accumulation in the cytoplasm. Increased levels of β-catenin enter the nucleus to activate multiple Wnt target genes. The non-canonical Wnt pathways include the PCP and Calcium pathways, amongst others. Upon the binding of Wnt ligands to Frizzled receptors, activated Dvl signals through small GTPases and/ or C-Jun N-terminal kinase (JNK). Calcium signaling is mediated in part via Phospholipase-C (PLC), leading to calcium release and Cam Kinase II (CamKII) activation.


β-catenin mediated
(canonical)
PCP pathway &
Calcium pathway
Wnt
LRP
Frizzled
Dsh
Axin
GSK3b
Wnt
Frizzled
Dsh
PLC
Ca2+
CamKII
RhoA
Rac
Rho-
kinase
JNK
β-cat
β-cat
β-cat
β-cat
β-cat
β-cat
TCF/ LEF
TBE

The regulation of β-catenin's stability in canonical Wnt signaling

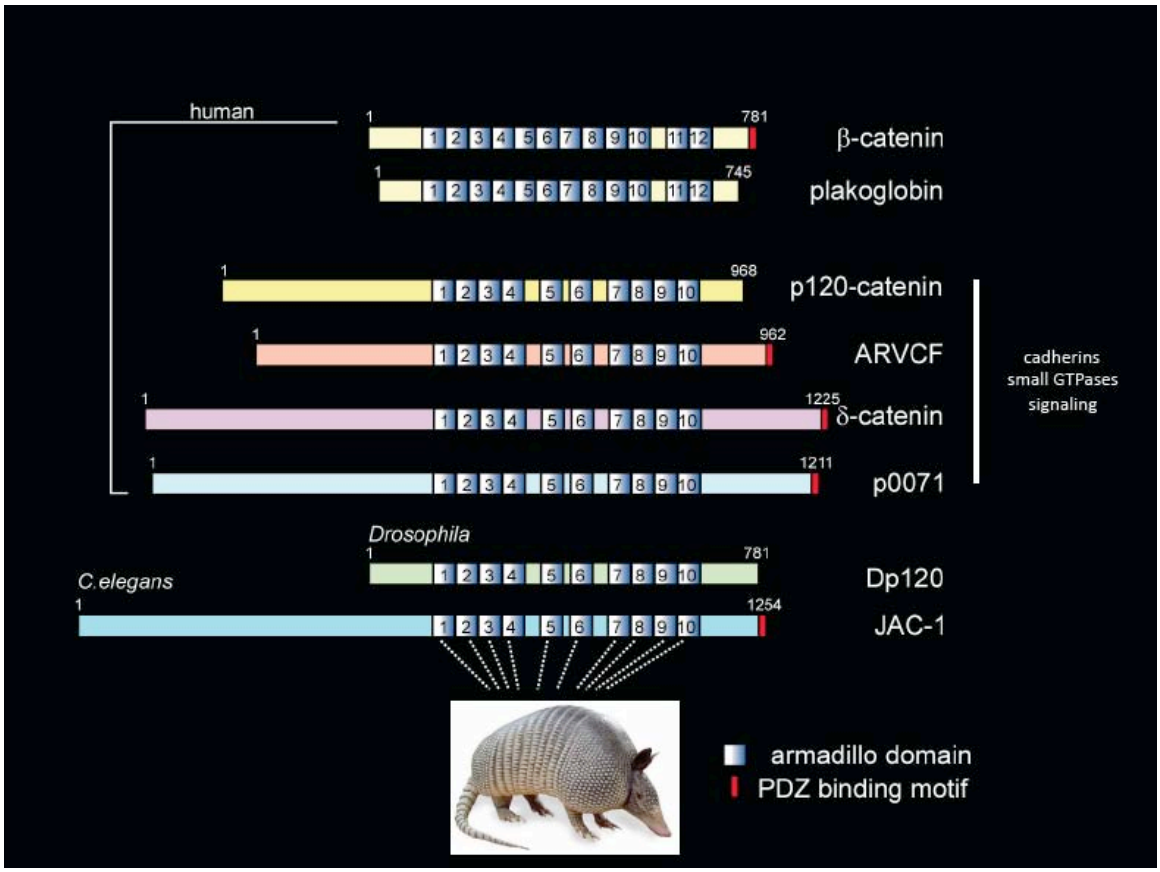
The metabolic stability of β-catenin is regulated by destruction complex components including GSK3β (Glycogen Synthase Kinase-3β), CK1α (Caseine Kinase-1α), Axin and APC (Adenomatus polyposis coli) (10-14). In the absence of Wnt signals, β-catenin is targeted for phosphorylation by CK1α and GSK3β in association with a larger scaffolding complex including Axin and APC. β-catenin is then ubiquitinated via β-TrCP (β-transducin repeat-containing protein), a substrate recognition / E3 subunit of ubiquitin ligase, resulting in β-catenin's degradation through the ubiquitin-mediated proteasome pathway (15-17). On the other hand, when extracellular Wnt ligands bind to the transmembrane receptor:coreceptor complex of Fz (Frizzled) and LRP (Low density lipoprotein receptor-Related Protein), intracellular Dvl (Dishevelled) becomes activated by unknown mechanisms (18-21). Among other possibilities, activated Dsh together with LRP is thought to sequester Axin and GSK3β to the inner plasma membrane and inhibit GSK3β's ability to phophorylate β-catenin, thereby promoting β-catenin stabilization (18, 19, 22-28). This pool of β-catenin responds to additional signals before accessing the nucleoplasmic space (29), where β-catenin relieves repression otherwise conferred by the HMG (High Mobility Group) proteins LEF (Leukocyte Enhancing Factor) or TCF (29-32) (Figure 1). Genes activated by the β-catenin/LEF/TCF complex (canonical-Wnt target genes) are numerous, with well-known examples including *Siamois* (*Xenopus*), as well as *c-Myc* and *Cyclin-D1* (33-36). In addition to the Wnt

pathway's extensive engagement in embryogenesis and adult-tissue homeostasis, pathological pathway activation is linked to multiple human diseases such as those characterized by bone abnormalities and cancer (37, 38).

P120-catenin subfamily members

Members of the p120-catenin subfamily include p120-catenin, ARVCF-catenin, δ-catenin, p0071, and plakophilin (39, 40) (Figure 2). The p120 subfamily members bear limited structural resemblance to the β-catenin subfamily members, β-catenin and plakoglobin (γ-catenin). The most obvious similarity is that each contains a central Arm (Armadillo) domain comprised of either 9 (p120 and plakophilin subfamily members) (41) or 12 (β-catenin & plakoglobin/ γ-catenin) Arm repeats. Through such Arm domains, members of each catenin subfamily were first observed to bind the cytoplasmic tails of cadherin cell-cell adhesion proteins. However, while the p120 subfamily members competitively associate with membrane-proximal tail regions where they contribute to cadherin stabilization (42, 43), β-catenin or plakoglobin bind more distally and confer other attributes to the complex, such as indirect cytoskeletal association (44-46). In addition to binding and modulating cadherin functions, and engaging in nuclear activities, p120 subfamily members have now been well recognized to modulate small GTPases, such as inhibiting RhoA and activating Rac (39, 47-53).

Figure 2. Linear structural representation of catenin family proteins. Distinct from β-catenin, p120-catenin contains 9 repeats within its Armadillo domain. PDZ binding motifs exist at the carboxyl-terminal end of p120 subfamily members, except p120-catenin itself. This figure was modified/reproduced from a published paper, Park et al, BBA, 2007 (39).


human
β-catenin
plakoglobin
p120-catenin
ARVCF
δ-catenin
p0071
cadherins
small GTPases
signaling
Drosophila
C.elegans
Dp120
JAC-1
armadillo domain
PDZ binding motif

In addition to the central Arm domains, which share only modest homology, among catenins the amino- or carboxyl-terminal regions bear yet less resemblance even within a catenin subfamily. These end domains engage in inter-protein associations and also may modulate Arm-domain interaction in an intra-molecular manner (30, 54-57). In the context of the canonical Wnt pathway, β-catenin's amino-terminal domain is well known to encompass conserved GSK3β and CK1α phosphorylation sites that when phosphorylated permit β-TrCP recognition (15-17). Thus, this domain participates in determining the extent and activity of β-catenin's cytoplasmic / nuclear signaling pool. In addition, the β-catenin sub-family member plakoglobin contains a similarly positioned destruction box, and its protein stability is likewise modulated by canonical Wnt signaling (58, 59).

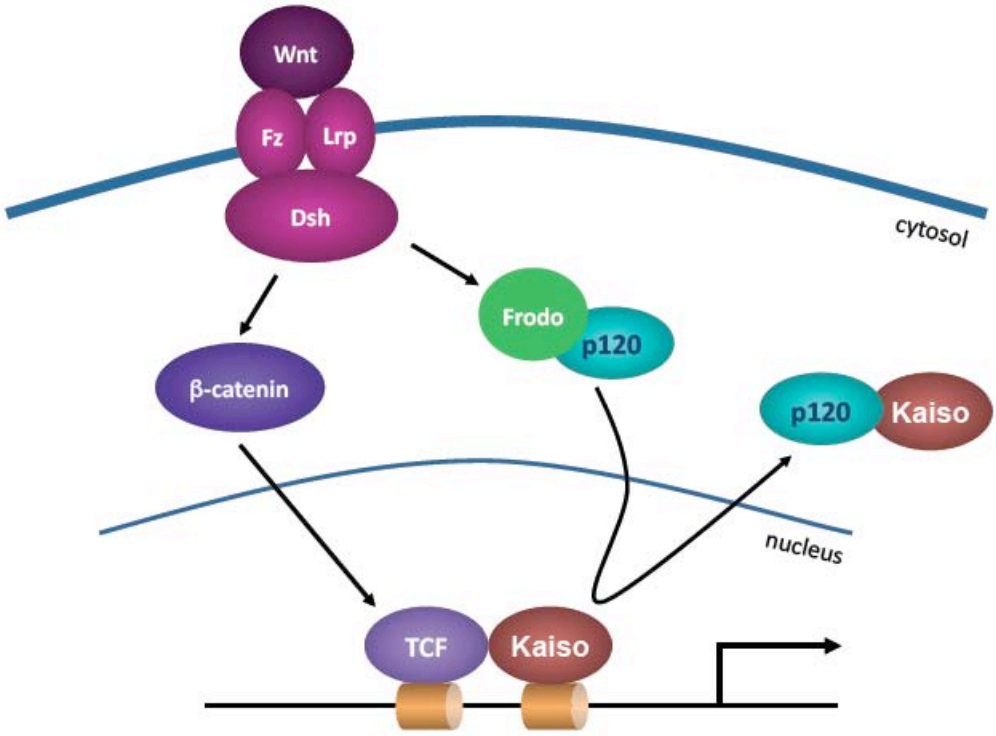
P120-catenin's nuclear signaling

Previous work arising from our group and other researchers has outlined a new role for p120-catenin in nuclear signaling. One context examined has been p120's response to canonical Wnt signals, where together with β-catenin, p120 modulates expression of select Wnt gene targets such as *Siamois* (*Xenopus*), *Cyclin-D1*, and *Matrilysin (60-62)*. At such promoters, p120 recognizes and associates with Kaiso, a transcriptional repressor of the BTB/POZ zinc-finger family that binds a Kaiso consensus sequences (KCS) in DNA (60, 61). Once formed, the p120:Kaiso complex is thought to dissociate from the gene promoter. Kaiso-directed gene repression is then relieved, and enhanced transcriptional activity ensues (Figure 3).

In addition to Kaiso: KCS (DNA) interactions, for which conflicting reports have arisen in certain gene contexts (63-65), Kaiso further recognizes methyl-CpG islands present in gene control regions that are associated with repressive states (66-69). However, at these sites in particular, no indications have yet arisen that p120-catenin acts to relieve Kaiso-mediated repression, and furthermore, the relationship between Kaiso's roles at CpG-island versus sequence-specific DNA binding sites is still unclear.

Recent evidence shows that Frodo (Functional regulator of Dishevelled in ontogenesis) and the closely related Dapper functionally and physically interact with Dsh (70-72), indicating Frodo's involvement in the Wnt signaling pathway. Frodo and Dapper act as positive or negative modulators of the canonical and non-canonical Wnt pathway in a context dependent manner (70, 73-76). Previous investigation from our lab determined a new molecular mechanism of Frodo with respect to the p120/Kaiso pathway. In that report, Frodo was shown to promote p120's stabilization upon their association. The depletion of Frodo decreased p120's level in *Xenopus* embryos, and thereby, some canonical Wnt gene targets of p120/Kaiso pathway were affected (those having both TCF/ LEF & Kaiso binding sites) (70) (Figure 3). Relating to the identification of potential molecular mechanisms that modulate p120's level, my work shows that upstream Wnt pathway components stabilize p120-catenin protein levels, and that Frodo selectively associates with, and stabilizes p120-catenin (not β–catenin).

Figure 3. A schematic pathway diagram of p120-catenin's signaling to the nucleus. In the presence of Wnt ligands, β-catenin is stabilized by defined mechanisms. P120-catenin is stabilized by analogous means as β-catenin (shown in my work), and by the association with Frodo. Increased levels of p120 leads to the relocalization of the repressor Kaiso from the nucleus to the cytoplasm, promoting increased transcription from certain Wnt target genes.


Wnt
Fz
Lrp
Dsh
cytosol
Frodo
p120
β-catenin
p120
Kaiso
nucleus
TCF
Kaiso

P120-catenin was originally identified as a Src tyrosine kinase substrates. Since its amino-terminal domain includes multiple serine/threonine and tyrosine phosphorylation sites, this domain may act as a regulatory region (77). The phosphorylation of p120-catenin has been proposed to be relevant to the association of p120-catenin with cadherin or the stability of cadherin (78-83). Despite considerable efforts to understand p120-catenin's phosphorylation events, the relationship of varied kinases to p120's function *in vivo* have remained elusive. Even the phosphorylation of p120 relating to cadherin function leaves the specific phosphorylation sites to be determined. My recent findings have pointed to the role of two different kinases in p120's nuclear function, particularly GSK3β and Dyrk1A. I have examined their contributions in the context of functional outcomes in *Xenopus* embryos and mammalian cell lines.

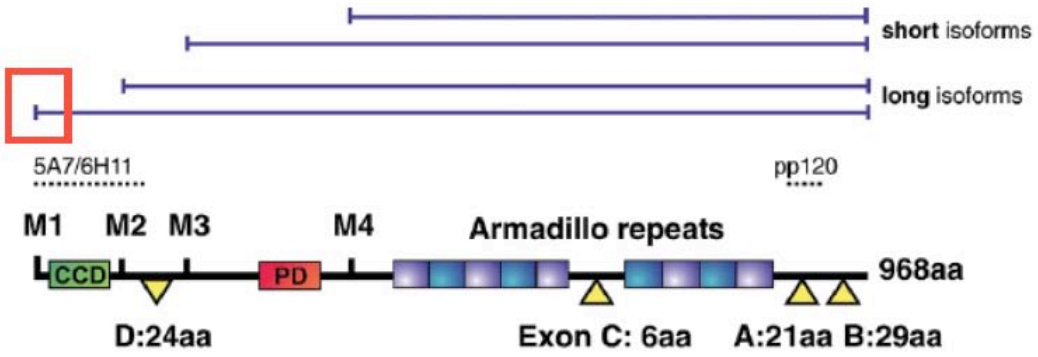
P120-catenin isoforms

P120 subfamily members can further be distinguished from β-catenin/ plakoglobin in that each transcript bears multiple potential translational start sites and arises from differential splicing events. These characteristics have added layers of regulatory complexity to studies of p120 subfamily proteins (84, 85). Human p120-catenin has potentially up to 48 isoforms due to the use of four alternative start codons, and RNA-splicing events (Figure 4). Several domains have been identified in p120, including coiled-coil domain found only in p120 isoform-1. The

regulatory domain, which includes multiple phosphorylation sites, exists in all isoforms except isoform-4. P120-catenin is ubiquitously expressed in both adult and fetal tissues. However the expression pattern of distinct p120 isoforms varies in abundance between cell types and tissues. Its expression pattern was monitored employing antibodies that specifically recognize the N-terminal domain of p120 (6H11) or p120's C-terminal region (pp120) (85-88). Studies have shown that motile cells such as fibroblasts and macrophages preferentially express the longest isoforms (p120 isoform-1), while epithelial cells preferentially express short isoforms (isoform-3 or -4) that associate with E-cadherin at cell-cell junction. Some non-adherent cells do not express detectable levels of p120. In human adult and fetal tissues, the longest p120 isoform is mainly expressed in brain, heart, liver, lung, spleen, small intestine, testis and ovary, while short isoforms are mainly expressed in liver, lung, pancreas, colon, small intestine, prostate and ovary, and weakly in heart, kidney, spleen and thymus (86). P120-catenin transcripts are also present in multiple tumor tissues and cell types, with its pattern differing from the corresponding normal tissue. For example, in pancreatic adenocarcinoma and lung carcinoma, the level of the long p120 isoform is increased, whereas the level of the long isoform is decreased in ovarian carcinoma (85). In *Xenopus*, there are two isoforms identified. The longest isoform is xp120 isoform-1, thought to be analogous to human p120 isoform-1. Xp120 isoform-2 is most homologous to human p120 isoform-3 (89). Recent evidence indicates that p120 isoforms have distinct functions, perhaps including functions in tumor invasion and metastasis (84, 87). In lung cancer, p120-catenin isoform-1 is highly expressed in the cytoplasm of highly

metastatic lung cancer tissues, and it co-localizes with Kaiso in the cytoplasm (90-92). This suggests that p120-catenin isoform-1 may be associated with more aggressive cellular phenotypes. Therefore, defining the molecular mechanisms that govern distinct p120-catenin isoforms is likely to contribute to our knowledge on catenin biology.

Figure 4. Schematic representation of the major p120-catenin isoforms. P120-catenin has multiple isoforms, in humans up to a theoretical limit of 48 if considering the four splicing variants and four alternative translational start sites. This figure was modified/reproduced from a published paper, van Hengel, 2007 (87).


short isoforms
long isoforms
5A7/6H11
pp120
M1
M2
M3
M4
Armadillo repeats
CCD
PD
968aa
D:24aa
Exon C: 6aa
A:21aa B:29aa

The molecular mechanisms of Dyrk1A

Dual-specificity tyrosine-regulated kinase 1A (Dyrk1A) belongs to a novel subfamily of protein kinases that engage in autophosphorylation on tyrosine residues, while instead phosphorylating serine / threonine residues of other substrates (93, 94). In mouse, Dyrk1A-/- null mutants display a general growth delay and die during mid-gestation. Mice heterozygous for the mutation (Dyrk1A+/-) show decreased neonatal viability and brain size in a region-specific manner, and increased neuronal densities in some brain regions (95). Mouse and chick Dyrk1A mRNA is expressed in neuroepithelial cells beginning at early CNS stages and is asymmetrically distributed during mitoses of neuronal progenitor cells. A series of studies have suggested Dyrk1A's involvement in neuronal differentiation (96-98).

The human Dyrk1A genes maps to chromosome 21, falling into what is referred to as the Down Syndrome critical region (DSCR) 21q22.2 (99). In the fetal brain of Down syndrome patients, Dyrk1A is over-expressed and expected to contribute to the phenotype induced by trisomy 21(100, 101). Interestingly, Down syndrome is known to increase the risk of Alzheimer disease by three-to-five fold. Dyrk1A was recently found to phosphorylate the microtubule binding protein tau, a component of Alzheimer neurofibrillary tangles. Indeed, hyper-phosphorylation of tau is associated with Alzheimer neurofibrillary tangles, and in both Alzheimer and Down syndrome patients, Dyrk1A itself further accumulates in such tangles (102-105).

Thus, consistent with being expressed in neural progenitor cells and its participation in dendrite differentiation, Dyrk1A is a candidate contributor in both Down syndrome and Alzheimer disease. However, despite its implication in human genetic disease, the molecular mechanisms underlying the impact of Dyrk1A gene dosage imbalance *in vivo* remain largely unknown.

Human and rodent Dyrk1A are also highly expressed in the developing heart, yet little is known of Dyrk1A's roles in heart tissue. Intriguingly, relative to the normal population, Down syndrome patients have increased chances of having congenital heart defects (atrial, atrioventricular or ventricular septal defects, or patent ductus arteriosus). One report found that Dyrk1A over-expression inhibits cardiomyocyte hypertrophy, while Dyrk1A knockdown or inhibition had the opposing effect (106).

In brain tissue, p120-catenin appears to regulate dendritic spine and synapse development (52). P120-catenin depletion in mouse forebrain resulted in dramatic decreases in spine and synapse density. In rat brain, p120-catenin is highly expressed and is potentially involved in morphogenetic events and plasticity of the CNS (107, 108). In a small-scale screen to identify kinases that positively or negatively regulate p120-catenin levels, I uncovered a new relationship between Dyrk1A and p120-catenin. In my dissertation, I identify Dyrk1A as a positive modulator of p120-catenin in both *Xenopus* embryos and mammalian cell lines. These results suggest that one means by which Dyrk1A participates in embryogenesis is through modulation of the Wnt/ p120/ Kaiso pathway, occurring

through the stabilization of p120 to relieve Kaiso-mediated repression. These findings will conceivably contribute to establishing a new bridge between catenin biology, especially p120-catenin, and Down syndrome pathology. Upstream control of Dyrk1A remains an area of uncertainty, with questions including if it is responsive to Wnt or other ligands. Therefore, more precise examination of the molecular mechanisms relating to Dyrk in the content of Wnt signaling resides ahead.

Chapter II

Materials and Methods

cDNA constructs

Standard recombinant DNA techniques were used to construct pCS2-based plasmids harboring *Xenopus laevis*: Myc-p120-catenin, HA-p120-catenin (49), Myc-β-catenin, HA-β-catenin, Myc-ARVCF, HA-ARVCF (49), Myc-δ-catenin and HA-δ-catenin (109). Myc-GSK3β-pXT7 and catalytically inactive mutant (K85R) (S. Sokol, Mount Sinai School of Medicine) (110), HA-CK1α-pCS2 (Xi He, Harvard Medical School) (111), Myc-Axin-pCS2 (P.S. Klein, U. of Pennsylvania) (12), xWnt8 (S. Sokol, Mount Sinai Scholl of Medicine) (112), xWnt11 (R. Keller, University of Virginia) (113, 114) and xWnt5a (R. Harland, University of California, Berkley) (115), were kindly provided as indicated. GSK3β was subcloned into the pCS2-HA vector for co-immunoprecipitation. Generated previously were pCS2 plasmids harboring Myc-p120 deletion constructs, Myc-Frodo and HA-Frodo (70). The quadruple p120-catenin point mutant (S6, S8, S11 & S15 →4SA) was generated by PCR amplification of HA-p120-catenin (in vector pCS2) as a template with 5' mutated primer as follows: $P120^{4SA}$-F, 5'-GGAATTCATGGATGAGCCAGAGGCTGAAGCTCCGGCCGCTATATTGGCCGCAGTGAGAGCT-3'; $P120^{4SA}$ -R, 5'-

AAGGCCTGACACGCTGATCTTCAGCATCACCAAGATTCAGTGATCCTCCAGCA CTTACGGA-3'.

Embryo culture, microinjections, in vitro transcription and antisense oligonucleotides

Fertilization, embryo culture and microinjections were conducted in accordance with a standard methods (49). Embryos were microinjected with capped mRNA synthesized in vitro (mMessage mMachine, Ambion). All pCS2-based constructs were linearlized by using Not I restriction enzyme prior to in vitro transcription. Gastrulation phenotypes, principally failure of blastopore closure, were visually scored at embryonic stages 11-12. I employed an xDyrk1A-morpholino (xDyrk1A-MO), and standard morpholino (STD MO, Gene Tool). xDyrk1A-MO sequences: 5'-ATGAGACTTGAA AGAGGACGATGCA-3'.

Analysis and Gene Expression Using Real-time RT-PCR

Total RNA was prepared from stage 10.5 or 11.5 embryos that had been earlier injected with morpholino or mRNA. Preparations employed Trizol followed by RNase-Free DNase as instructed by Promega (RQ1 RNase-Free DNase). cDNA was made using the Superscript first-strand synthesis system (Invitrogen), followed by real-time RT-PCR and quantitation (Applied Biosystem, 7500 Real-time PCR). Primers for RT-PCR were: Siamois, 5'- CCCAACTTCAGAAGGACCTAGATC-3' and 5'-TGGGTAGGGCTGTGTATTTGA-3'; Wnt11, 5'-TGACAGCTGCAACCTCATGT-3'

and 5'-ACAGAGGGCTGTCAGTGCTT-3'; ODC, 5'-CGAGCGGATTATCTATGCA-3' and 5'-GCGTATTTGATCTGGGAAA-3'.

Immunoprecipitation and immunoblot analysis

Immunoprecipitation and immunoblotting employed monoclonal antibodies directed against Myc (9E10), HA (12CA5), p120-catenin (mouse/ human pp120, BD Transduction; mouse/ human 6H11, Santa Cruz), and GSK3β (mouse, BD Transduction). Polyclonal antibodies included those directed against β-catenin and p120-catenin, that were raised in our lab (70). Antibodies directed against Frodo were provided by Dr. Sergei Sokol's lab, while rabbit polyclonal antibodies directed against Dyrk1A were commercially obtained (Abcam). For immunoprecipitations, procedures were largely performed as described (49). Whole-embryo lysates were prepared using 0.5% Triton X-100 buffer (0.5% Triton X-100, 10 mM HEPES [pH 7.4], 150 mM NaCl, 2 mM EDTA, 2 mM EGTA), inclusive of a proteinase inhibitor cocktail (Sigma). Interference from the IgG heavy chain in immunoblot analyses was reduced by employing TrueBlot™ anti-mouse IgG IP Beads, and Mouse TrueBlot™ ULTRA:Horseradish Peroxidase (HRP) anti-mouse IgG (eBioscience). Embryos were lysed in 0.5% Triton X-100 buffer (20 μl per embryo), in the presence of a proteinase inhibitor cocktail (Sigma). After centrifugation (14000 rpm, 20 min), the supernatant fraction was denatured in 5x SDS sample buffer (200 mM Tris-Cl [pH 6.8], 40% glycerol, 8%SDS, 0.08% Bromophenol Blue), followed by incubation at 95°C for 5 min. Half-embryo equivalents were resolved by SDS-PAGE and

transferred onto nitrocellulose membranes. Immunoblotting and antibody incubations took place in 2% bovine serum albumin-TBST (25 mM Tris-HCl [pH 7.8], 125 mM NaCl, 0.5% Tween 20). SuperSignal WestPico (Pierce Biotechnology, Inc.) reagents were utilized to detect HRP-conjugated secondary antibodies.

Mammalian cell culture and immunofluorescence staining

HeLa, 293T and MDA-MB-435 cells were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. MDA-MB-231 cells were kindly provided by Dihua Yu (UT MD Anderson Cancer Center). Using Lipofectamine 2000, cells were transiently co-transfected with DNA constructs such as Myc-Kaiso and HA-Dyrk1A. 24 hours after transfection or the indicated time after treatment, cells were fixed with 4% PFA (paraformaldehyde) for 10min, blocked with 5% goat serum in PBS and immunostained with anti-Myc antibody (9E10). Fluorescence images (Nikon ECLIPSE E800 microscope) were recorded using SPOT advanced software.

Transfection and RNA interference

siRNA oligonucleotide sequences directed against the transcripts of Axin1&2 or LRP5&6 were gathered from published reports (20), and synthesized by Applied Biosystems. The proprietary duplex negative control siRNA was purchased from Ambion. Duplex oligonulceotides were directed against the target sequences: Axin1

5’-GGCGAGAGCCATCTACCG AAA-3’; Axin2 5’-GCAGACGATACTGGACGATCA-3’; LRP5 5’-CCAACGACCTCACCATTGTCT-3’; and LRP6 5’-AGACATTGTTCTGCAGTTAGA-3’ ; Dyrk1A-1 5’-TTAAGGATGCTTGATTATGAC-3’; Dyrk1A-2 5’-AAACTCGAATTCAACCTTATT-3’. siRNA oligonucleotide sequences of Dyrk1A were gathered from published reports (116, 117). For transfection of siRNA alone into 6-well plates, Lipofectamine^{TM} RNAiMax (Invitrogen) was employed (50pmol/well). When DNA plasmids were transfected along with siRNA, Lipofectamine 2000 (Invitrogen) was used. After transfection and incubation for 48-72 hours, cell lysates were collected using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific Inc.), and proteins detected by immunoblotting.

Pulse-Chase Analysis

HeLa-S3 cells were transiently transfected with (HA epitope tagged) pCS2-HA-p120 or pCS2-HA-p120^{4SA}. After 24 hours, cells were washed and preincubated for 1 hour with Met/Cys-free DMEM made 10% in FBS. The medium was then replaced with fresh Met/Cys-free medium containing 40 μCi/μl of [^{35}S]-Met/Cys, and incubated for 1 hour. The cells were washed and incubated in complete medium, and 2 mg of cell lysates were immunoprecipitated with anti-HA-7 agarose (Sigma). The samples were subjected to 8% SDS-PAGE followed by autoradiography.

In Vitro Kinase Assay

For in vitro kinase assays, flag-tagged p120 N-term wild-type or 4SA-mutant (amino acids 1-280), and flag-tagged GSK3β or HA-tagged Dyrk1A wild-type and Kinase Dead (KD) were synthesized in vitro (TnT system, Promega). Each p120 construct was incubated with kinases or kinase-dead enzyme at 30°C for 90 minutes in the presence of [32P]γ-ATP. Substrate proteins were then immunoprecipitated, with isotope labeling resolved by SDS-PAGE/ autoradiography.

Chapter III

Negative regulator of p120-catenin and p120 subfamily members: canonical Wnt signals/components modulate p120-catenin isoform-1, ARVCF and δ-catenin

Result

(Chapter III)

GSK3β modulates p120-catenin stability

Given that our prior work suggesting similarities in p120-catenin and β-catenin regulation (70), I directly investigated the involvement of established components of β-catenin's destruction machinery in p120 regulation. Studies were initially performed to examine the impact of expressing exogenous GSK3β on p120's level in early *Xenopus* embryos. Based upon our prior findings to show that inhibiting GSK3 via LiCl increased endogenous p120-catenin levels, it was speculated that GSK3β ectopic expression should decrease p120-catenin levels (70). Indeed, wild-type GSK3β and CK1α reproducibly reduced the level of Myc-tagged xp120 isoform-1 (longest isoform; translation beginning at p120's most upstream ATG site), while CK1ε or a kinase-dead form of GSK3β (KD) (negative-control), did not have statistically significant impact upon p120 (Figure 5A, and data not shown). Unexpectedly, lower as opposed to intermediate or sometimes even high GSK3β expression (5 pg versus 10pg, or in some cases 100 pg mRNA), produced modestly greater reductions in ectopic or endogenous p120-catenin isoform-1 levels and functional consequences in *Xenopus* embryos (Figure 5A, 5B and 5C), as discussed later in relation to other findings (Figure 10 & Discussion). Ectopically expressed p120-catenin was employed in these initial studies given that a large

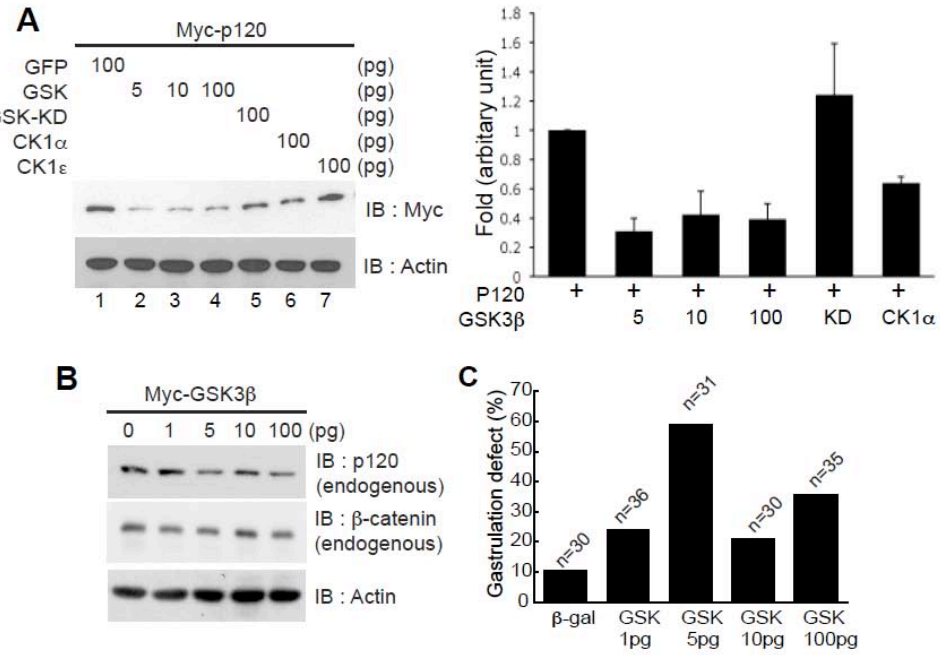
proportion of endogenous p120 is bound to cadherin, which as established for β-catenin, would be expected to exhibit less sensitivity to Wnt-pathway regulation.

I next tested for a functional relationship between p120 and GSK3β using a phenotypic assay in vivo. As predicted, p120-catenin over-expression resulted in gastrulation failures in a significant fraction of embryos (49). However, when a carefully titrated dose of GSK3β was co-injected with p120, I observed significant rescue of the gastrulation phenotypes (Figure 5D; compare the first two bars). Kinase-dead GSK3β did not produce such rescue effects (GSK3β KD, negative control), while a titrated dose of Axin appeared to have modest rescuing activity. To complement over-expression assays, I next used loss-of-function experiments. As morpholinos to deplete GSK3 have not been well characterized for work in *Xenopus*, we instead used a proven short-hairpin/ shRNA to block GSK3 function in HeLa cells (118). As anticipated, p120-catenin protein levels were elevated upon the depletion of endogenous GSK3, as assessed using immunofluorescence analysis as well as immunoblotting (Figure 5E). Noteworthy in our immunofluorescence images is p120's increased presence in both the cytoplasmic and nuclear compartments, relative to negative control shRNA transfected cells. Collectively, these data (Figure 5) propose that p120 is subject to modulation by GSK3β.

Figure 5. GSK3β modulates p120-catenin protein levels.

(A) Myc-p120-catenin mRNA (0.5ng) was microinjected into each blastomere of 2-cell stage embryos with the indicated levels (in vitro transcribed mRNA) of GSK3β kinase, GSK3β kinase-dead mutant (KD), CK1α or CK1ε. Embryos were harvested at stages 10-11 for immunoblotting with anti-Myc antibody, with actin serving as an internal loading control. The right panel quantities Myc-p120 protein levels normalized to actin, using data from four independent experiments. (B) Increasing total doses of Myc-GSK3β were microinjected into both blastomeres at the 2-cell stage. Embryos were collected at late-gastrula stage 12, and endogenous p120-catenin and β-catenin levels respectively visualized via immunoblotting. (C) Gross gastrulation effects following exogenous GSK3β expression. Although many direct/ indirect GSK3β targets are likely to have been affected, it is interesting that the dose-dependent effects on gastrulation mirrored the dose-dependent GSK3β effects upon p120 and β-catenin levels in panel (B). (D) Gastrulation (blastopore closure) failure following exogenous p120-catenin expression (0.5 ng mRNA injection), versus rescue upon co-expression with GSK3β mRNA (5 pg), or more partial rescue using co-expressed Axin (5pg). (E) Either Venus-sh-random or Venus sh-GSK3 and Myc-xp120-catenin were co-transfected into HeLa cells as indicated. 48 hours after transfection, cells were assayed for Myc-p120 using immunofluorescence or immunoblotting (left versus right panels). For immunofluorescence, cells were fixed with 4% PFA and probed for Myc-p120 followed by Texas red-conjugated anti-mouse visualization. For immunoblotting, tubulin served as an internal loading control. This figure was modified/reproduced

with permission from the Journal of Cell Science (119).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GFP 100 (pg)
GSK 5 10 100 (pg)
GSK-KD 100 (pg)
CK1α 100 (pg)
CK1ε 100 (pg)
IB : Myc
IB : Actin
1 2 3 4 5 6 7
Fold (arbitary unit)
P120
GSK3β
KD
CK1α
B
Myc-GSK3β
0 1 5 10 100 (pg)
IB : p120 (endogenous)
IB : β-catenin (endogenous)
IB : Actin
C
Gastrulation defect (%)
n=30
n=36
n=31
n=30
n=35
β-gal
GSK 1pg
GSK 5pg
GSK 10pg
GSK 100pg
D
Gastrulation failure (%)
n=53
n=44
n=42
n=36
n=31
n=47
P120
GSK3β
Axin
GFP
KD
P120
P120 + GSK5pg
Stg. 12
E
sh-random
sh-GSK3
Myc-p120
Venus
DAPI
Merge
sh-random
sh-GSK3
IB : Myc-p120
IB : Tubulin

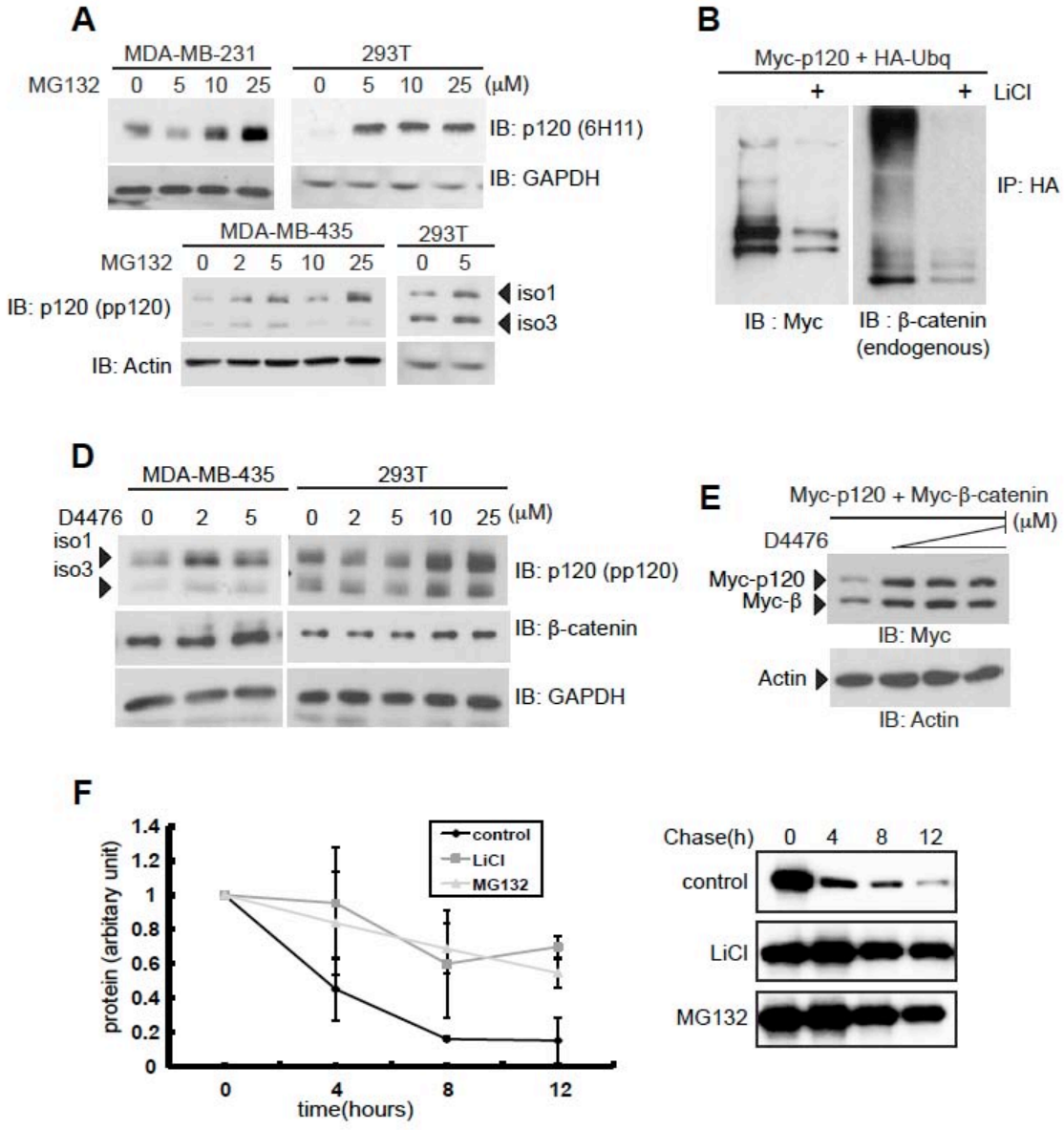
P120-catenin is regulated by the ubiquitin-proteasome pathway

Given β-catenin's phosphorylation by CK1α/ GSK3β, followed by degradation via the proteasomal pathway, I examined whether p120 is regulated in a manner similar to that of β-catenin. To better resolve the p120-catenin signaling pool, we employed MDA-MB-231 and MDA-MB-435 cells, which are largely E-cadherin deficient, in addition to 293T cells, which express E-cadherin. MDA-MB-231, MDA-MB-435 and 293T cells were incubated with the proteasome inhibitor MG132, and endogenous p120 was monitored via immunoblotting. As expected, p120-catenin was stabilized by MG132 in a dose-dependent manner in all cell lines tested (Figure 2A). To detect endogenous p120-catenin, we used two previously characterized anti-p120 monoclonal antibodies, pp120 and 6H11 (120). 6H11 is directed against an amino-terminal epitope, which selectively resolves the isoform-1 generated from the most upstream translational start site. pp120 antibody detects all p120 isoforms, because it recognizes a carboxyl-terminal epitope present across most p120 translation products. In the cell lines employed here, pp120 detects two predominantly expressed products thought to be isoforms-1 and -3. Inhibition of the proteasome pathway via dose-dependent titration of MG132 elevated p120 isoform-1 levels in all cell lines tested, with an observable yet lesser impact upon isoform-3, as was evident in MDA-435 cells in which E-cadherin is deficient (Figure 6A). Further analogous to β-catenin (17), p120 ubiquitination was clearly evident upon its co-expression with HA-ubiquitin in HeLa cells. Likewise as expected, being dependent upon GSK3β, such p120 ubiquitination dropped when cells were

incubated with the GSK inhibitor LiCl (Figure 6B). Since CK1α is a priming kinase for GSK3β in the β-catenin degradation pathway, I examined the effect of incubating embryos in the presence of the CK1 inhibitor D4476. Consistently, D4476 treatment raised expressed p120 levels, as was predicted and clearly observed for β-catenin (positive control) (Figure 6E). Consistent with our Xenopus embryo and exogenous expression data, endogenous p120 isoform-1's level in mammalian cells was also increased when MDA-435 or 293T cells were exposed to D4476 (Figure 6D). Additionally, p120's sensitivity to GSK3 was supported in pulse-chase data, where LiCl or MG132 treatment prolonged the half-life of both endogenous and exogenous p120-catenin (Figure 6F and data not shown).

Figure 6. Proteasome mediated degradation appears to modulate p120-catenin levels.

(A) MDA-231, MDA-435 and 293T cells were treated with varying doses of the proteasome inhibitor MG132 (0, 2, 5, 10, 25 μM). After 6 hours, cells were harvested for immunoblotting with anti-p120 antibody (6H11 & pp120). (B) Myc-p120-catenin was co-transfected into HeLa cells with HA-ubiquitin. After 24 hours, cells were treated with LiCl (25mM) for 4 hours to block GSK3β function. Cells were harvested and HA-ubiquitinated proteins immunoprecipitated, followed by Myc-p120 immunoblotting. Endogenous β-catenin was used as a positive ubiquitination control. (D) MDA-435 and 293T cells were treated with varying doses of the CK1 inhibitor D4476 (0, 2, 5, 10, 25 μM). P120-catenin was visualized using an antibody directed against it (pp120), and endogenous β-catenin served as an endogenous positive control. (E) Single cells of 2-cell embryos were injected with Myc-p120-catenin and Myc-β-catenin mRNA (0.1ng mRNA each), and treated for 24 hours with varying concentration of the CK1 inhibitor D4476 (10, 50, 200 μM) in the presence of Fugene6. Anti-myc-antibody was employed to detect both p120-catenin and β-catenin. (F) HA-p120-catenin was expressed in 293T cells. Following a 1 hour incubation with 40 μCi/μl of ^{35}S-Met/Cys, cells were treated at the indicated times with 0.5% NP-40 lysis buffer. HA-p120 was immunoprecipitated using anti-HA antibodies, and resolved by SDS-PAGE followed by autoradiography (band densities quantitated using ImageJ). This data is collected from two independent experiments. This figure was modified/reproduced with permission from the Journal of Cell Science (119).


A
MDA-MB-231
293T
MG132 0 5 10 25 0 5 10 25 (μM)
IB: p120 (6H11)
IB: GAPDH
MDA-MB-435
293T
MG132 0 2 5 10 25 0 5
IB: p120 (pp120)
iso1
iso3
IB: Actin
B
Myc-p120 + HA-Ubq
+ + LiCl
IP: HA
IB : Myc
IB : β-catenin
(endogenous)
D
MDA-MB-435
293T
D4476 0 2 5 0 2 5 10 25 (μM)
iso1
iso3
IB: p120 (pp120)
IB: β-catenin
IB: GAPDH
E
Myc-p120 + Myc-β-catenin
(μM)
D4476
Myc-p120
Myc-β
IB: Myc
Actin
IB: Actin
F
control
LiCl
MG132
protein (arbitary unit)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
4
8
12
time(hours)
Chase(h) 0 4 8 12
control
LiCl
MG132

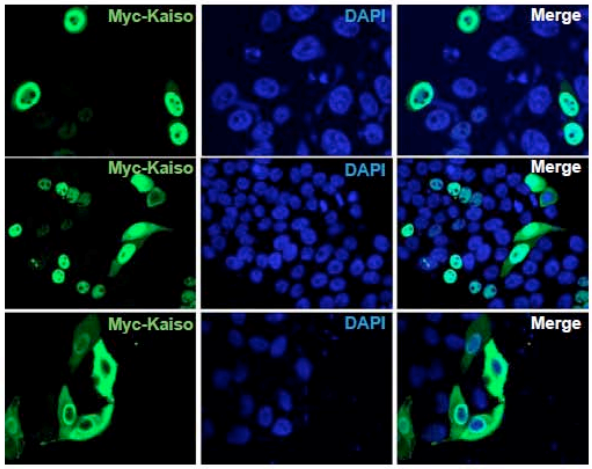
Inhibition of the destruction machinery recruits Kaiso to the cytoplasm

Since expression of exogenous p120 recruits the transcription repressor Kaiso from the nucleus to the cytoplasm (60, 121), we next examined whether endogenous p120 stabilization correlates with a similar outcome. As predicted, Kaiso relocalized to the cytoplasm in the coordinate presence of proteasome inhibitor (MG132) and GSK chemical inhibitor (LiCl) (Figure 7A), potentially through known effects upon p120 stabilization (Figure 5E&6A). LiCl or MG132 alone produced a reproducible but considerably subtler effect on Kaiso's relocalization, consistent with its lesser protection of p120 relative to the combined treatment (data not shown). To ask if endogenous Kaiso displays a similar outcome as exogenous Kaiso, I employed MDA-435 cells, which express neither E-cadherin RNA nor protein. Endogenous Kaiso appears to relocalize from the nucleus to the cytoplasm in MDA-435 cells, when employing a monoclonal antibody directed against Kaiso (6F) (Figure 7B). Together, the data from Figures (5, 6 & 7) suggest that endogenous p120-catenin is subject to some of the same regulatory processes established for β-catenin in the context of the canonical Wnt signaling pathway.

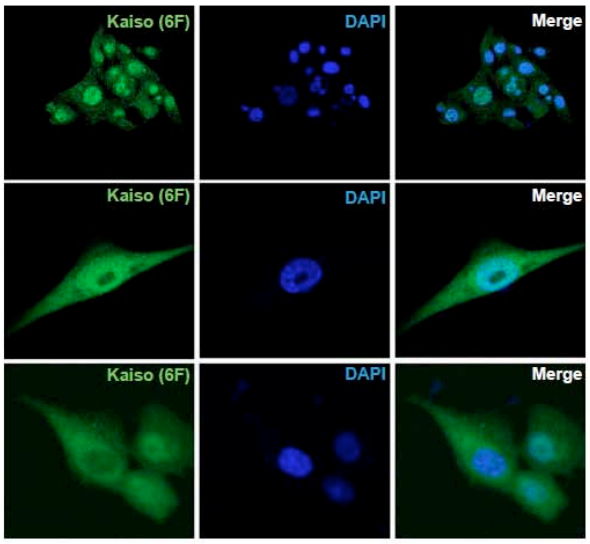
Figure 7. P120 stabilization via inhibition of the proteasome pathway promotes Kaiso's relocalization.

(A) HeLa cells were grown on cover slips, transiently transfected with Myc-Kaiso and treated with MG132 (10μM) and LiCl (50μM). Cells were fixed with 4% PFA for 10 min, blocked with 5% goat serum in PBS and immunostained with anti-Myc antibody. (B) MDA-435 cells were treated with MD132 and LiCl as indicated in (A), then fixed and immunostained with anti-Kaiso antibody (6F). This figure was modified/reproduced with permission from the Journal of Cell Science (119).

A


Myc-Kaiso
DAPI
Merge
Myc-Kaiso
DAPI
Merge
+ LiCl
Myc-Kaiso
DAPI
Merge
+ MG132
+ LiCl

B

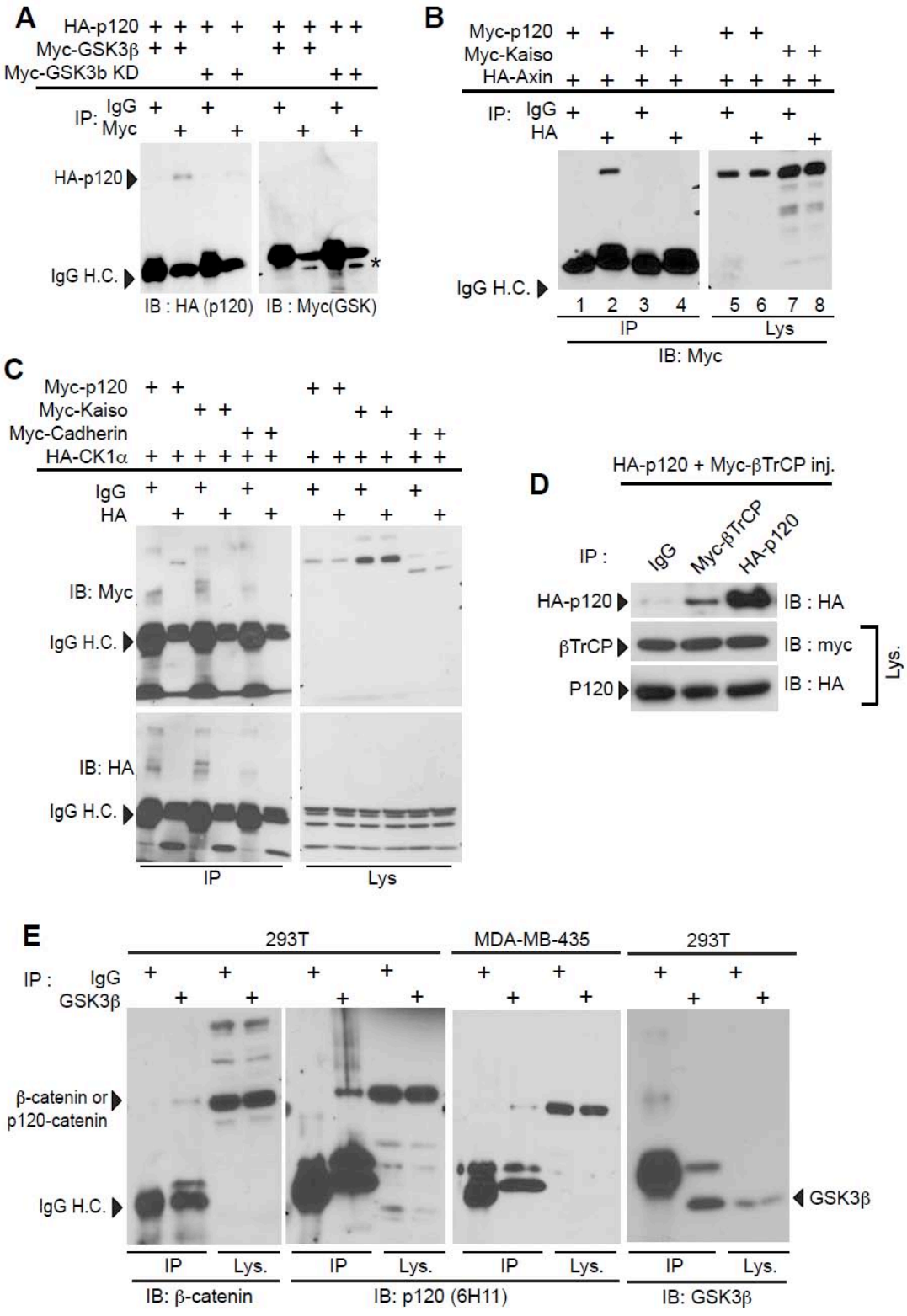

Kaiso (6F)
DAPI
Merge
Kaiso (6F)
DAPI
Merge
+ LiCl
Kaiso (6F)
DAPI
Merge
+ MG132
+ LiCl

Association of destruction complex components with p120-catenin

Several in vivo and in vitro reports show that β-catenin associates with Axin, β-TrCP, GSK3β and CK1α (10, 15, 111). Given that p120-catenin's levels and functions outcome in vivo are modulated by the destruction complex (Figure 5 & 6), we assayed if p120 might likewise engage in such associations. Since a significant fraction of endogenous p120 is in complex with cadherins at the membrane as opposed to being within an accessible signaling pool in the cytoplasm, co-immunoprecipitations were performed from *Xenopus* embryo extracts using ectopically expressed proteins. We first tested for the association of p120 with GSK3β. While weakly apparent in immunoblots, the p120:GSK3β complex was reproducibly resolved relative to GSK3β KD, serving as a negative control (Figure 8A, see also Figure 9B). We next examined Axin, which is known as a scaffolding protein, and thus interacts with multiple components of β-catenin's destruction complex. In precipitations conducted in either direction (reverse precipitation not shown), Axin interacted with p120 as resolved in immunoblotting (Figure 8B; Kaiso negative control). P120 was also detected in complex with CK1α, the priming kinase that acts upon β-catenin immediately prior to GSK3β (Figure 8C; Kaiso and C-cadherin serving as negative controls). I also resolved association of the E3 ubiquitin ligase β-TrCP with p120, relative to negative control IgG immunoprecipitates (Figure 8D). Given the data presented here with ectopically expressed proteins, I finally asked if endogenous p120 associates with endogenous GSK3β. Employing established GSK3β, p120 and β-catenin antibodies, I

immunoprecipitated endogenous GSK3β and resolved the p120:GSK3β complex in 293T and MDA-435 cells (Figure 8E). Thus, in an in vivo context, p120-catenin directly or indirectly interacts with some of the key proteins known to regulate β-catenin stability in the canonical Wnt pathway.

Figure 8. P120-catenin associates with destruction complex components. (A) HA-p120-catenin (1ng) was co-injected with either Myc-GSK3β or Myc-GSK3β KD (0.5ng) into both blastomeres of 2-cell embryos. Myc-GSK3β immunoprecipitates were immunoblotted with anti-HA antibody to detect p120. (B) HA-Axin (0.5ng) was microinjected with Myc-p120-catenin (1ng) or Myc-Kaiso (1ng) into both blastomeres of 2-cell embryos, subsequently harvested at gastrulation. Anti-HA antibody (Axin) immunoprecipitates were blotted with anti-Myc-antibody to detect p120-catenin versus Kaiso (negative control). (C) HA-CK1α was injected into both blastomere of 2-cell embryos along with Myc-p120, Myc-C-cadherin or Myc-Kaiso, and embryos harvested at early-mid gastrulation (stage 10-11). This was followed by anti-HA immunoprecipiation (CK1α), and then anti-Myc (p120, or negative controls C-cadherin or Kaiso), or anti-HA immunoblotting (CK1α). (D) The association of Myc-β-TrCP (0.5ng) and HA-p120-catenin (1ng) was resolved using methods analogous to those used in (C). (E) 293T and MDA-435 cells were grown in 10cm dishes, and lysates immunoprecipitated for endogenous GSK3β (BD Transduction). The association of endogenous p120-catenin (6H11) with GSK3β was resolved by immunoblotting (β-catenin positive control). This figure was modified/reproduced with permission from the Journal of Cell Science (119).


A
HA-p120
Myc-GSK3β
Myc-GSK3b KD
IP: IgG
Myc
HA-p120
IgG H.C.
IB : HA (p120)
IB : Myc(GSK)
B
Myc-p120
Myc-Kaiso
HA-Axin
IP: IgG
HA
IgG H.C.
1 2 3 4
5 6 7 8
IP
Lys
IB: Myc
C
Myc-p120
Myc-Kaiso
Myc-Cadherin
HA-CK1α
IgG
HA
IB: Myc
IgG H.C.
IB: HA
IgG H.C.
IP
Lys
D
HA-p120 + Myc-βTrCP inj.
IP :
IgG
Myc-βTrCP
HA-p120
HA-p120
βTrCP
P120
IB : HA
IB : myc
IB : HA
Lys.
E
293T
MDA-MB-435
293T
IP : IgG
GSK3β
β-catenin or
p120-catenin
IgG H.C.
GSK3β
IP
Lys.
IB: β-catenin
IB: p120 (6H11)
IB: GSK3β

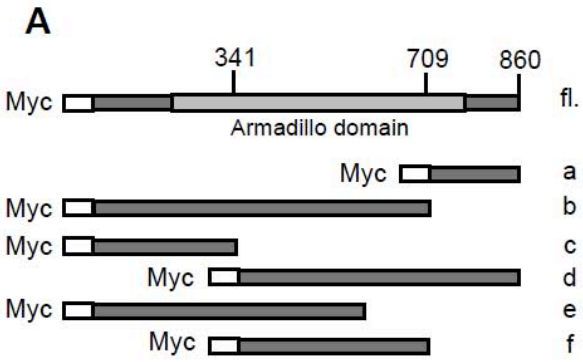
P120's amino-terminal domain associates with GSKβ and CK1α

The amino-terminal domain of β-catenin includes four serine residues phosphorylated by CK1α and GKS3β, and thus provides the platform for their association (15, 122). As a first step in assessing p120-catenin phosphorylation, we searched for potential GSK3β phosphorylation sites. Three evolutionally conserved GSK3β phosphorylation residues were identified, with one being amino-terminal and two residing in the Armadillo repeat domain. P120's amino-terminal region interestingly harbors a $^{3}DSX_3SX_2SX_3S^{15}$ motif comparable to the GSK3β-sensitive site existing in β-catenin (Figure 10A) (16). To map p120's interaction domains with GSK3β and CK1α, Hong Ji, one of our lab members, generated a series of p120 deletion constructs (a-f) (Figure 9A). Using these p120 deletion constructs, I performed co-immunoprecipitations from *Xenopus* embryo extracts after the microinjection of those constructs with either GSK3β or CK1α. Constructs containing p120's amino-terminal domain associated with GSK3β (fl, b, c & e), whereas p120 constructs lacking this region (a, d & f) did not (Figure 9B). I then tested if the p120 deletion mutant lacking the amino-terminal region is ubiquitinated. Consistent with the association of p120 amino-terminus with GSK3β, only the construct (d) lacking p120's amino-terminal region were negative for ubiquitination (Figure 9C). Based on such findings, I next examined if the CK1α priming kinase of β-catenin (primes for GSK3β), associates with the analogous region of p120-catenin. Employing a similar co-immunoprecipitation strategy, I resolved the

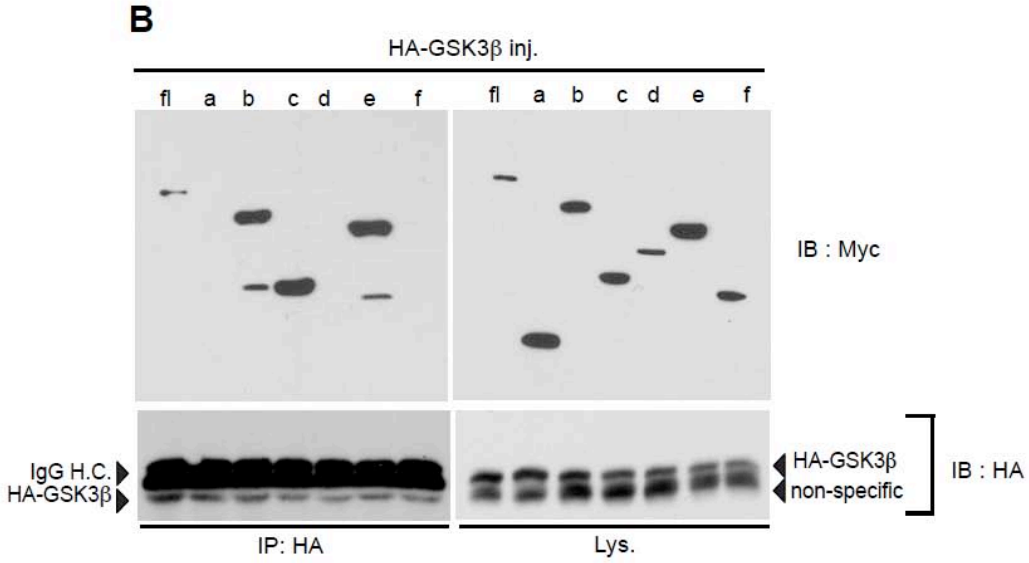
interaction between CK1α and p120's amino-terminal domain (c), but not with a p120 mutant construct (d) which lacks this region (Figure 9D). Thus, in keeping with our prior findings, this evidence suggests that p120 and β-catenin engage in shared protein interactions reflecting their similar biochemical and likely functional responses to Wnt signals.

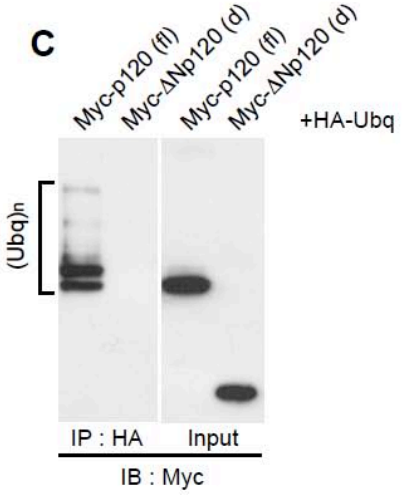
Figure 9. Mapping of p120 association with GSK3β, CK1α and ubiquitin.

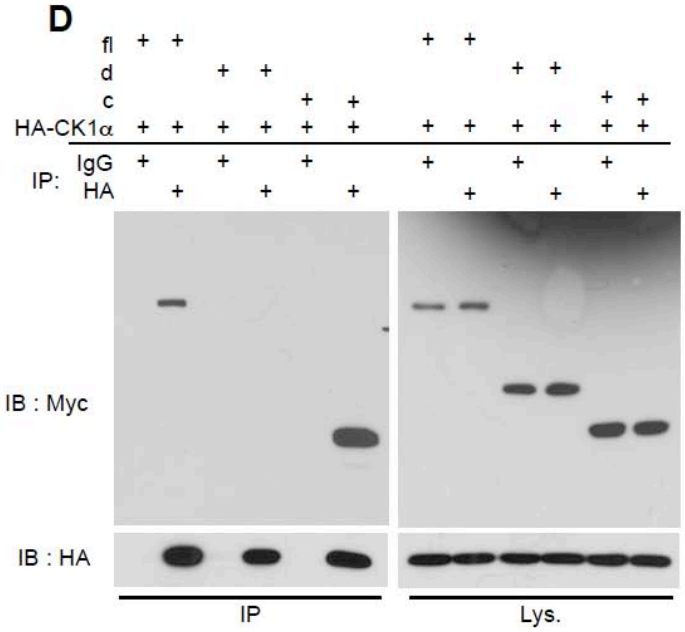
(A) Myc-tagged p120-catenin deletion constructs (a-f). The table summarizes the relative p120-construct:GSK3β association as shown in (B). (B) HA-GSK3β (0.5ng mRNA) was co-expressed with varying p120-catenin deletion constructs (a-f) (0.5ng), followed by HA-GSK3β immunoprecipitation and Myc-construct immunoblotting. (C) Either Myc-p120-catenin (fl) or Myc-ΔN-p120-catenin (construct d) was co-transfected with HA-tagged ubiquitin in HeLa cells. Following HA-ubiquitin immunoprecipitation, co-associated (versus not) Myc-p120 constructs were visualized via immunoblotting. (D) Myc-p120 constructs (fl, c & d) (0.5ng mRNA) were co-injected with HA-CK1α (0.5ng) in both blastomeres of 2-cell embryos. Gastrula embryo extracts were immunoprecipitated for HA-CK1α and blotted for Myc-p120. Right panel, lysate indicates Myc-p120 construct expression. This figure was modified/reproduced with permission from the Journal of Cell Science (119).



P120 constructs	GSK3β association
fl. (1:860)	+
a (709:860)	–
b (1:708)	+
c (1:340)	+
d (341:860)	–
e (1:614)	+
f (341:708)	–







P120 point mutant differs in destruction-complex responsiveness

To evaluate whether the potential GSK3β or CK1α sites identified in p120's amino-domain are in fact relevant to its sensitivity to destruction complex components, I designed a p120 compound point mutant in which four serines were mutated to alanines ($p120^{4SA}$/ Ser → Ala: Ser6, Ser8, Ser11, Ser15) (Figure 10A). P120-catenin full-length (fl, comparable to p120 isoform-1) versus $p120^{4SA}$ mutant were microinjected into *Xenopus* embryos, and their protein stability was monitored with immunoblotting in response to the presence of GSK3β. While p120-fl levels were consistently decreased upon GSK3β co-expression (Figure 10B compare lanes 1 & 2, see also Figures 1A & 10A), levels of the mutant were in contrast not lowered (Figure 10B compare lanes 3 & 4).

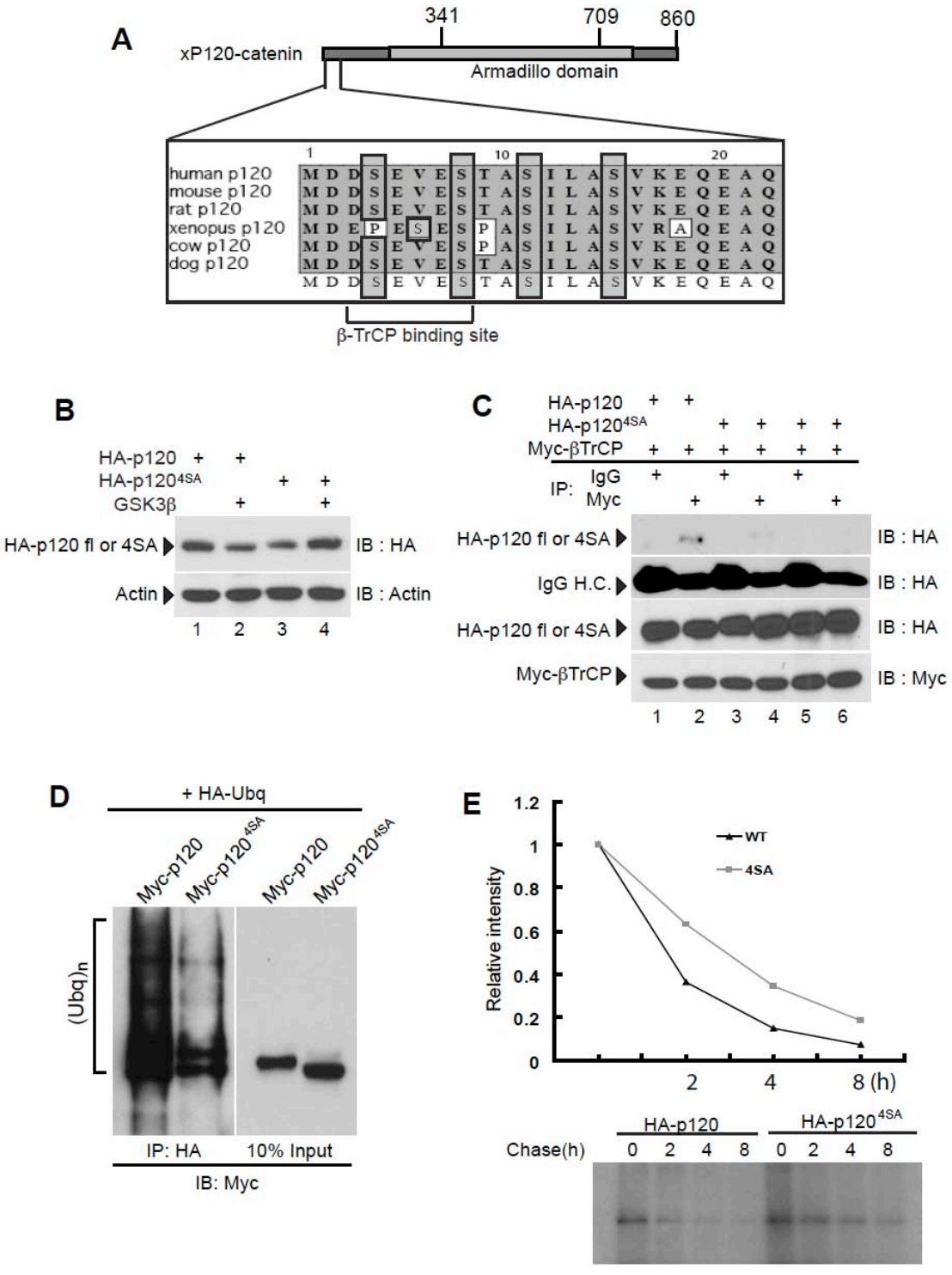
In fact, surprisingly, $p120^{4SA}$'s level was reproducibly increased upon co-expression with GSK3β in Xenopus embryos. Apparently, a direct or indirect protective effect of GSK3β was unmasked in producing the $p120^{4SA}$ mutant. This possibility may also have been resolved in the unexpected lesser impact upon p120 levels/ degradation of intermediate (10pg or in some cases higher) GSK3β levels relative to lower (5pg) or higher (100pg) GSK3β levels (Figure 5A & 5B). I hypothesize that at intermediate GSK3β levels, wild type p120 may become susceptible to direct or indirect GSK3β effects that are protective in nature (phosphorylation/ other), whereas lower or higher GSK3β activity largely acts at negative-regulatory sites that we resolved. Indeed, other potential but more distal

GSK sites have been identified in p120 such as the site just upstream of the Arm domain (Ser199 amino-acid) (123). Evidence supporting GSK3's modification of such additional predicted sites was not obvious, however, in our in vitro kinase assays (Figure 12, and data not shown). Alternative possibilities include GSK3β phosphorylation of LRP5/6, leading to destruction complex inhibition via recruitment of either Axin or GSK3β to the membrane, followed by β-catenin stabilization (18, 19, 24, 25). Whatever the underlying mechanistic explanation, mutation of p120's conserved four amino-terminal serines (p1204SA/ Ser → Ala: Ser6, Ser8, Ser11, Ser15) protects p120 from destruction complex-mediated degradation.

In addition to candidate kinase sites, study of p120's primary sequence pointed to a conserved potential β-TrCP recognition motif, DSEXXS (16, 122). In common with β-catenin, this motif resides adjacent to p120's putative amino-terminal CK1α/ GSK3β phosphorylation sites (Figure 10A). Indeed, employing a co-immunoprecipitation/ immunoblotting approach, the association of p120 with β-TrCP was resolved, whereas our phosphorylation mutant $p120^{4SA}$ exhibited significantly reduced β-TrCP co-association (Figure 10C, compare lane 2 with lanes 4 and 6). These observations are consistent with β-catenin's known regulation, wherein phosphorylation is required for β-TrCP recruitment (15). Given this outcome, I next tested if $p120^{4SA}$ would prove less susceptible to ubiquitination relative to wild-type, and tested this possibility in HeLa cells. Indeed, while p120 appears to have a strong ubiquitination signal, poly-ubiquitination upon $p120^{4SA}$ was greatly reduced. Interestingly, mono- or di-ubiquitinated forms showed lesser differences when

comparing p120 versus p120^{4SA} (Figure 10D). Consistent with reduced ubiquitination, pulse-chase data indicated that p120^{4SA} has a prolonged half-life relative to native p120 (approximately 3 versus 1.5 hours) (Figure 10E).

Figure 10. Phosphorylation dependent p120-catenin ubiquitination and proteasomal degradation. (A) Cross-species sequence alignment of p120-catenin regions harboring conserved predicted GSK3β phosphorylation and ubiquitination sites. Highlights shown in each region include conserved serine residues, and a DSEXXS motif for β-TrCP recognition. (B) HA-p120 (0.3ng mRNA), versus the HA-p120^{4SA} point mutant (4S→A, see Figure 6A, 0.3ng), was co-injected with HA-GSK3β (0.1ng) into both cells of 2-cell embryos. Gastrula embryo lysates (stages 11-12) were immunoblotted for the HA-p120 constructs, revealing a differing response to GSK3β. (C) HA-p120 versus HA-p120^{4SA} (0.5ng) were co-injected with Myc-β-TrCP (0.5ng) into one blastomere of 2-cell embryos. Gastrula embryo lysates were immunoprecipitated for Myc-β-TrCP and assayed for co-associated HA-p120 or HA-P120^{4SA}. (D) Myc-p120 or Myc-p120^{4SA} was co-transfected with HA-ubiquitin into HeLa cells. HA-ubiquitin was immunoprecipitated and immuno-blotting used to detect ubiquitinated Myc-p120 versus Myc-p120^{4SA}. (E) The half lives of HA-p120 versus HA-p120^{4SA} were monitored by pulse-chase analysis. Following 1hour pulse-chase with [^{35}S] Met/Cys, HeLa cells transfected with HA-p120 and HA-p120^{4SA} were harvested at the indicated times, and anti-HA immunoprecipitates were resolved by SDS-PAGE and visualized by autoradiography (quantitation of band intensities employed Image J, and was normalized to the zero time point). This data is representative of two independent experiments. This figure was modified/reproduced with permission from the Journal of Cell Science (119).


A
341
709
860
xP120-catenin
Armadillo domain
human p120
mouse p120
rat p120
xenopus p120
cow p120
dog p120
β-TrCP binding site
B
HA-p120
HA-p120 4SA
GSK3β
HA-p120 fl or 4SA
IB : HA
Actin
IB : Actin
C
HA-p120
HA-p120 4SA
Myc-βTrCP
IP: IgG Myc
HA-p120 fl or 4SA
IB : HA
IgG H.C.
Myc-βTrCP
IB : Myc
D
+ HA-Ubq
Myc-p120
Myc-p120 4SA
(Ubq)n
IP: HA
10% Input
IB: Myc
E
WT
4SA
Relative intensity
HA-p120
HA-p120 4SA
Chase(h)

Stabilized p120 point mutant exhibits an enhanced impact upon target gene expression

At the functional level, p120-catenin has a number of in vivo activities including the capacity to relieve Kaiso-mediated repression of target genes containing sequence-specific binding sites (KCS). Given that $p120^{4SA}$ shows an expanded half-life, we next asked if the transcriptional activity of $p120^{4SA}$ is elevated above that of wild-type p120, using *Wnt-11* and *Siamois* as direct endogenous gene readouts (62). Employing semi-quantitative RT-PCR (Figure 11A), and real-time PCR (Figure 11B), I observed induction of *Wnt-11* expression by $p120^{4SA}$ (relief of Kaiso-mediated repression) to a greater extent than wild-type p120-catenin, even though $p120^{4SA}$ was reproducibly expressed at lower levels relative to wild-type (Figure 11B, compare lanes 3 & 4). Furthermore, the $p120^{4SA}$ mutant proved more effective in promoting the expression of x*Siamois* (Figure 11C), another direct p120/ Kaiso (as well as β-catenin/ TCF/ LEF) gene target (61). These data indicate that stabilized p120 ($p120^{4SA}$) exhibits more potent gene regulatory outcomes than wild-type p120-catenin, analogous to the β-catenin context (124, 125). We next conducted in vitro kinase assays with p120, CK1 and GSK3β. As occurs for β-catenin (15), phosphorylation of p120 was faint but reproducible, suggesting it is a direct GSK3β/ CK1α target (Figure 12 & data not shown).

Figure 11. Stabilized p120-catenin mutant exhibits increased capacity to activate target gens. (A) Myc-GSK3β (5pg mRNA) or Myc-Kaiso (0.25ng) was microinjected with HA-p120 versus HA-p120^{4SA} (0.25ng) into one blastomere of 2-cell embryos. Total mRNA injection loads were equalized using β-galactosidase mRNA. Gastrula embryo cDNA (stage 10-12) was subject to RT-PCR to assay endogenous *xWnt-11* transcript levels. Band intensities are indicated relative to β-gal control (set at 1), following normalization to the *Histone H4* internal loading control. (B) Myc-Kaiso or β-gal (negative control) was injected alone (0.25ng), or Myc-Kaiso was co-injected with HA-p120 versus HA-p120^{4SA} (0.25ng), into one blastomere of 2-cell embryos. Gastrula embryo cDNA was assayed by real-time RT-PCR for x*Wnt-11* transcript levels. (C) Across varying doses, the stabilized p120^{4SA} mutant increases *xSiamois* luciferase activity to a greater extent than wild-type p120-catenin (p120^{4SA} exhibits greater relief of Kaiso-mediated repression of xSiamois). *xSiamois* luciferase reporter plasmid (0.25ng) was co-injected with the indicated amounts of HA-p120 versus HA-p120^{4SA}. Gastrula embryos (stage 12-13, eight embryos per condition) were collected to quantities luciferase activity. This figure was modified/reproduced with permission from the Journal of Cell Science (119).

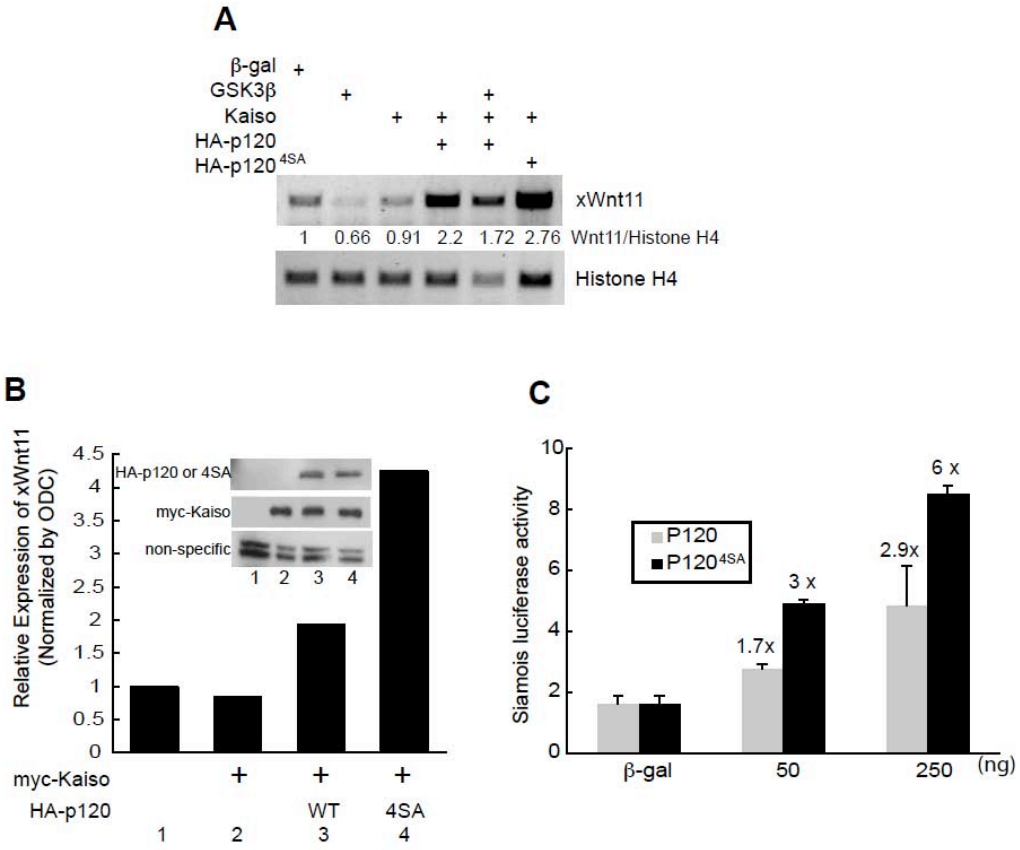
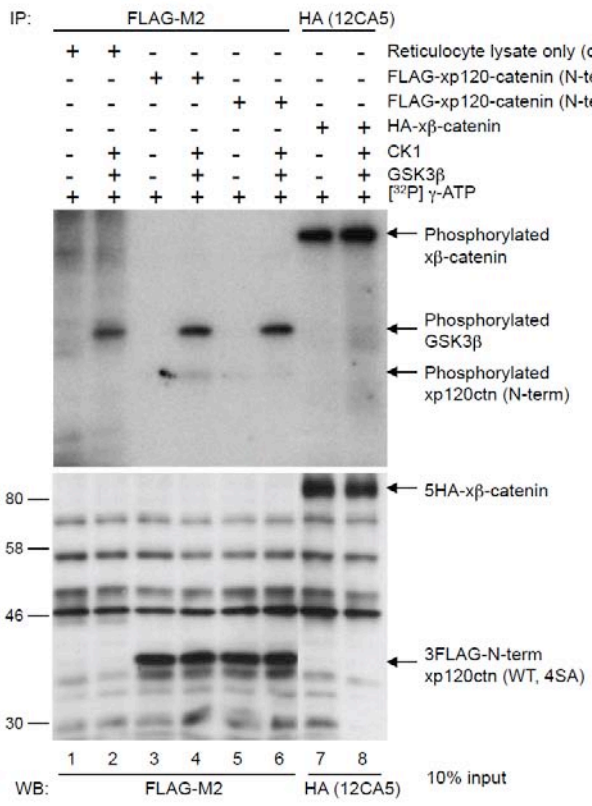

A
β-gal
GSK3β
Kaiso
HA-p120
HA-p120 4SA
xWnt11
1 0.66 0.91 2.2 1.72 2.76 Wnt11/Histone H4
Histone H4
B
Relative Expression of xWnt11 (Normalized by ODC)
HA-p120 or 4SA
myc-Kaiso
non-specific
1 2 3 4
myc-Kaiso
HA-p120
WT
4SA
C
Siamois luciferase activity
P120
P120 4SA
1.7x
3 x
2.9x
6 x
β-gal
50
250
(ng)

Figure 12. In vitro assay of GSK3β and CK1α kinase activity upon p120 For in vitro kinase assays, flag-tagged p120 N-term wild-type or 4SA-mutant (amino acids1-280), and flag-tagged GSK3β were synthesized in vitro (TnT system, Promega). Each p120 construct was incubated with flag-GSK3β and CK1 (NEB P6030) at 30°C for 90 minutes in the presence of [32P]γ-ATP. Substrate proteins were then immunoprecipitated, with isotope labeling resolved by SDS-PAGE/ autoradiography. Comparing lanes 4 & 6, wild-type p120-catenin is very modestly labeled in the presence of GSK3β and CK1, whereas the 4SA mutant shows no detectable phosphorylation. This is consistent with modest CK1/ GSK3β labeling observed upon β-catenin in vitro (15). Robust GSK3β auto-phosphorylation serves as a positive control (lane 3, 4, 5 and 6). This figure was modified/reproduced with permission from the Journal of Cell Science (119).


IP:
FLAG-M2
HA (12CA5)
Reticulocyte lysate only (control)
FLAG-xp120-catenin (N-term) WT
FLAG-xp120-catenin (N-term) 4SA
HA-xβ-catenin
CK1
GSK3β
[32P] γ-ATP
Phosphorylated xβ-catenin
Phosphorylated GSK3β
Phosphorylated xp120ctn (N-term)
5HA-xβ-catenin
3FLAG-N-term xp120ctn (WT, 4SA)
80
58
46
30
1 2 3 4 5 6 7 8
WB:
FLAG-M2
HA (12CA5)
10% input

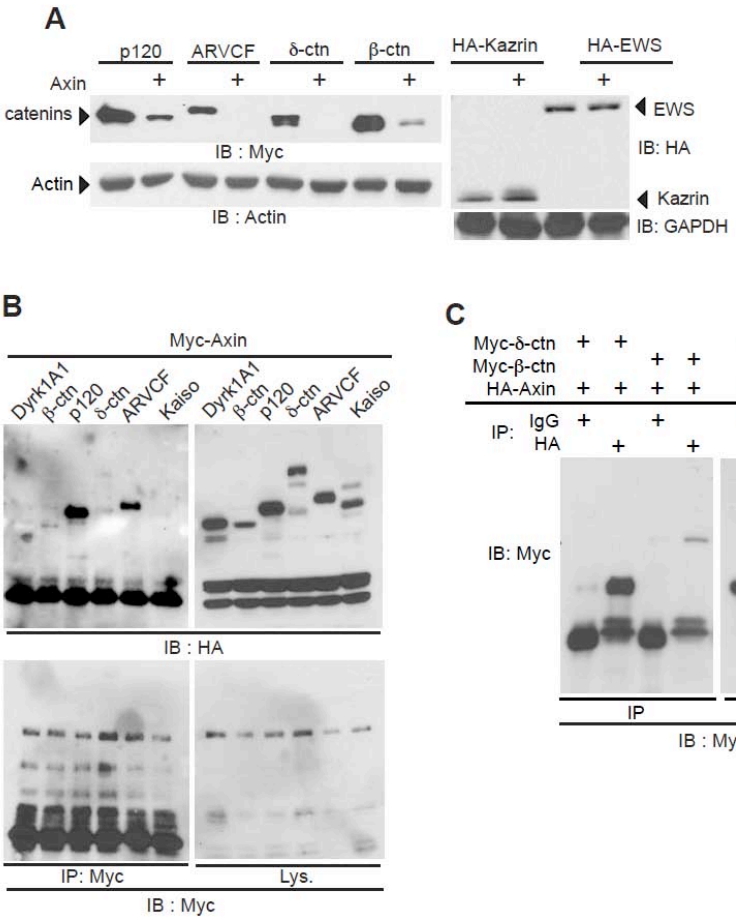
Shared mechanisms modulate the metabolic stability of multiple p120-catenin sub-family members

P120-catenin is the prototypical member of the p120-catenin sub-family consisting of p120-catenin itself, ARVCF-catenin, δ-catenin and p0071-catenin (39). These p120-catenin family members have certain shared features, such as a central Armadillo domain (9 repeats as opposed to the 12 in β-catenin/ plakoglobin), their interaction with cadherin membrane-proximal regions (β-catenin/ plakoglobin instead bind membrane-distal regions), and their modulation of small GTPases (β-catenin/ plakoglobin apparently lack this functionality). Based upon my findings on p120-catenin, I next asked if other p120 sub-family members might associate with and respond to destruction complex components of the Wnt pathway. Probing *Xenopus* embryo extracts, I first tested if one of the main components of the destruction complex, Axin, had an impact upon ARVCF and δ-catenin levels. Serving as positive controls, β-catenin and p120 levels were decreased in the presence of Axin (Figure 13A & 14A). Likewise, ARVCF and δ-catenin were considerably decreased upon Axin co-expression, whereas negative controls (xKazrin and EWS) produced no response to Axin (Figure 13A). I further asked whether ARVCF- and δ-catenin interact with Axin, as we demonstrated above for p120, and as known to occur for β-catenin. Indeed, in common with p120-catenin and β-catenin (positive controls), both ARVCF and δ-catenin associated with Axin, relative to negative controls such as xDyrk and xKaiso (Figure 13B & 13C). Although full-length δ-catenin exhibited less association with Axin than did β-catenin

or p120, shorter forms of δ-catenin, which likely arose from endogenous proteolytic processing or incomplete translation, showed strong association with Axin (Figure 13C).

Figure 13. Axin promotes the degradation of p120-catenin subfamily members.

(A) The indicated Myc-tagged p120 sub-family members (1ng mRNA each) were co-injected with Myc-Axin (0.1ng) into both blastomere of 2-cell embryos, and gastrula embryo lysates were Myc-immunoblotted. HA-Kazrin and HA-EWS (1ng each) were co-injected with Myc-Axin (0.5ng) as negative controls for Axin's effects, while Actin and GAPDH served as internal loading controls. (B) The HA-tagged p120 sub-family members (1ng each) were co-injected with Myc-Axin (1ng) into both blastomere of 2-cell embryos. Gastrula (stage 11-12) embryo lysates were immunoprecipitated for Myc-Axin, followed by immunoblot for HA-tagged p120 sub-family catenins. The bottom panel confirms Axin immunopreciptiations. HA-Kaiso and HA-Dyrk serve as negative controls. (C) HA-Axin was co-injected with either Myc-tagged β-catenin or δ-catenin into both blastomere of two-cell embryos. HA-Axin immunoprecipitates were immunoblotted with anti-Myc antibody to detect β-catenin or δ-catenin. This figure was modified/reproduced with permission from the Journal of Cell Science (119).


A
p120
ARVCF
δ-ctn
β-ctn
HA-Kazrin
HA-EWS
Axin
+
+
+
+
+
+
catenins
IB : Myc
Actin
IB : Actin
EWS
IB: HA
Kazrin
IB: GAPDH
B
Myc-Axin
Dyrk1A1
β-ctn
p120
δ-ctn
ARVCF
Kaiso
IB : HA
IP: Myc
Lys.
IB : Myc
C
Myc-δ-ctn
Myc-β-ctn
HA-Axin
IP:
IgG
HA
IB: Myc
IP
Lys.
IB : Myc

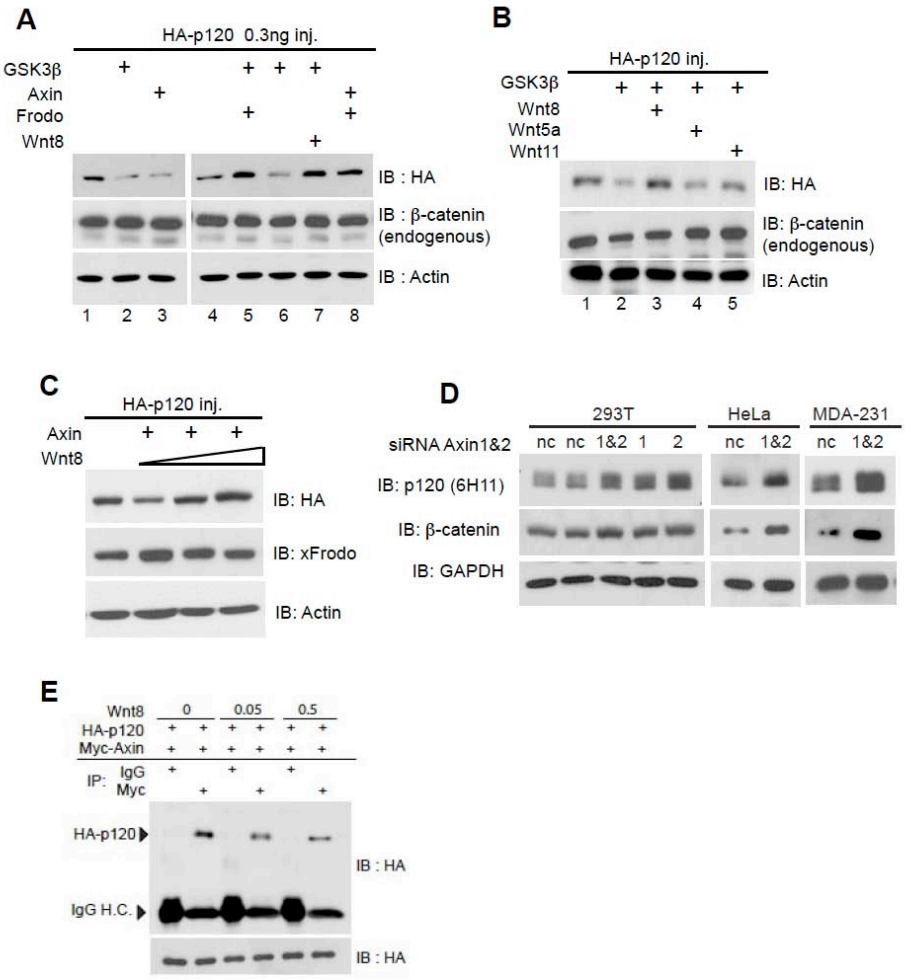
Canonical Wnt signals modulate p120-catenin

Generally, β-catenin destruction is inhibited upon activation of the canonical Wnt pathway. In addition to more recently reported mechanisms (29), canonical Wnt signals activate intracellular Dishevelled and LRP5/6, which block the association of β-catenin with the destruction complex by membrane recruitment of the core component Axin and GSK3β, followed by LRP phosphorylation by a dual-kinase mechanism (18, 19). Membrane recruitment of Axin, together with associated GSK3β, permits β-catenin's release to the cytoplasmic signaling pool by a mechanism that remains somewhat unclear. Released β-catenin can then accumulate in the cytoplasm and enter the nucleus, facilitating its activation of Wnt target genes such as x*Siamois*, *c-Myc* and *Cyclin-D1*. Given that p120's level is diminished by presence of the destruction machinery, I wished to extend this mechanism conceptually to p120-catenin. To confirm that Wnt signals promote p120 levels due to destruction complex inhibition, I co-injected p120-catenin with GSK3β in the presence versus absence of the Wnt8 ligand, one of the typical canonical Wnt-ligands in Xenopus (in some contexts it has non-canonical activity) (8, 9). I also assessed the impact of co-expressing Frodo with GSK3β. Although the role of Frodo in Wnt signaling remains unclear and somewhat controversial, it associates with Dsh and engages in Wnt signaling (72). We earlier reported its positive regulation of p120-catenin levels (70). As predicted, expression of Wnt8 or Frodo reproducibly protected p120-catenin from the negative effects of GSK3β (Figure 14A, compare lanes 5 & 7 with lane 2). Further, in keeping with prior data

showing the partial capacity of Axin to rescue developmental phenotypes produced by ectopically expressed p120 (Figure 5D), we found that Axin facilitates p120-catenin's degradation (Figure 14A, compare lane 3 with 1). In a dose dependent manner, Wnt8 countered Axin mediated destruction of p120 (Figure 14C). Frodo likewise protected p120 from the negative effects of Axin (Figure 14A, compare lane 8 with 3), as if also countered co-expressed GSK3β (Figure 14A, compare lane 5 with 2). At the low levels of GSK3β and Axin exogenously expressed, I was not able to monitor the effects upon endogenous β-catenin. However, higher GSK3β expression caused endogenous β-catenin's degradation (Figure 14B). To evaluate the protective effects of distinct Wnt ligands, I micro-injected Wnt8, Wnt 11 or Wnt5a together with GSK3β and p120-catenin into Xenopus embryos, and then monitored p120's level by immunoblotting. Wnt8 as anticipated inhibited GSK3β-facilitated destruction of p120 (Figure 14B). Intriguingly, Wnt11 and Wnt5a showed subtle protective effects upon p120 as well as β-catenin levels. Wnt8 is generally thought to activate the canonical Wnt/ β-catenin pathway, while Wnt11 and Wnt5a are portrayed as non-canonical in most contexts. However, Wnt11 and Wnt5a have also been shown to activate the canonical pathway in axis formation in *Xenopus* embryos (126, 127). Indeed, upon ectopical expression, p120 and even endogenous β-catenin experienced a modest protective benefit when Wnt11 or Wnt5a was co-expressed with GSK3β (Figure 11B). As anticipated, relative to Wnt8 and Wnt11, the non-canonical Wnt5a had lesser capacity to protect HA-p120 from the effect of GSK3β (Figure 14B). Due to the complexity of reported Wnt ligand and Frizzled receptor effects in *Xenopus* embryos during axis specification (etc.), I

performed a knock-down approach in conjunction with a cell line system to evaluate p120-catenin's response to other Wnt signaling components such as Axin. MDA-MB-231, HeLa and 293T cells were employed to examine the effect of knocking-down Axin 1&2 using proven siRNAs (20). Complimenting our over-expression experiments conducted in Xenopus embryos, Axin1&2 depletion increased endogenous p120-catenin's stabilization in all tested cell lines, reflecting the role of the destruction machinery in p120's regulation (Figure 14D). Finally, I considered existing models wherein canonical pathway activation leads to Dsh- and LRP-mediated recruitment of Axin to the plasma membrane and degradation of Axin, such that β-catenin escapes phosphorylation, ubiquitination and degradation. In keeping with this model devised originally for β-catenin, I found that when Wnt8 expression levels increased, reduced amounts of p120 co-precipitated with Axin (Figure 14E). Collectively, our data indicate that p120 and β-catenin are subject to similar regulatory mechanisms in response to Wnt signals.

Figure 14. Upstream Wnt pathway components modulate p120-catenin levels. (A) Co-injection of Frodo or Wnt8 counter the effectiveness of Myc-GSK3β (5 pg) or Myc-Axin (0.1 ng) to reduce HA-p120 (0.5 ng) levels. Embryos expressing up to two exogenous constructs (as noted) in addition to HA-p120 were harvested at gastrulation and corresponding lysates immunoblotted with anti-HA antibody to detect p120. Actin was used as an internal loading control. Total mRNA injection loads were equalized using β-galactosidase. (B) Exogenous Wnts (0.5 ng of Wnt8, Wnt11 or Wnt5a mRNA) were co-injected with both HA-GSK3β (0.1ng) and HA-p120 (0.25ng) into both blastomere of 2-cell embryos. At stage 12, Wnt protection (versus not) from GSK3β impact upon HA-p120 was assessed by immunoblot. Actin served as an internal loading control. (C) An increasing dose of Wnt8 mRNA was co-injected with both HA-p120 (0.5ng) and Myc-Axin (0.5ng) into two blastomeres of 2-cell embryos that were later collected at gastrulation (stage 11, with 20 embryos per condition). P120-catenin's protein levels were assessed by immunoblotting. (D) MDA-MB-231, HeLa and 293T cells were transfected with siRNAs directed against Axin1&2 (50pmol), as indicated, for 48 hours. Endogenous p120-catenin levels were monitored via anti-p120 immunoblotting (6H11). Each experiment was repeated at least three times. (E) HA-p120 and Myc-Axin were co-injected into both blastomeres of 2-cell stage embryos with the indicated amount of Wnt 8 ligand (0, 0.05 and 0.5ng). Gastrula stage embryos were harvested for immunoprecipitation/ immunoblotting. Myc-Axin immunoprecipitates were immunoblotted with anti-HA antibody to detect the association of p120 with Axin. This figure was modified/reproduced with from the permission Journal of Cell Science (119).

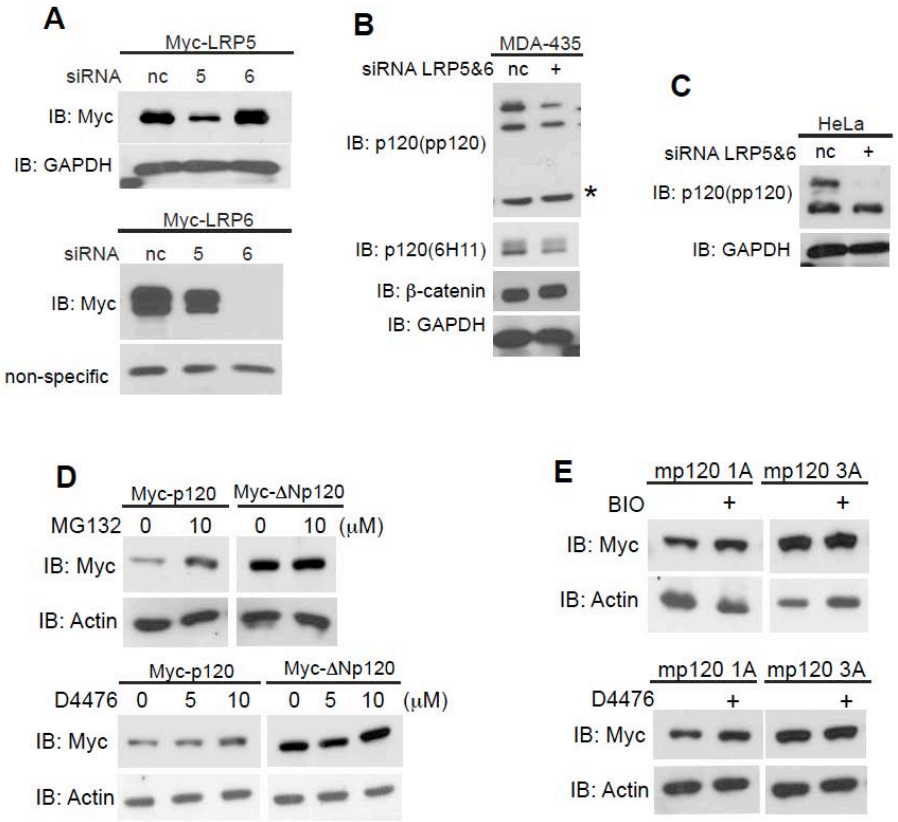

A
HA-p120 0.3ng inj.
GSK3β
Axin
Frodo
Wnt8
IB : HA
IB : β-catenin (endogenous)
IB : Actin
1 2 3 4 5 6 7 8
B
HA-p120 inj.
GSK3β
Wnt8
Wnt5a
Wnt11
IB: HA
IB: β-catenin (endogenous)
IB: Actin
1 2 3 4 5
C
HA-p120 inj.
Axin
Wnt8
IB: HA
IB: xFrodo
IB: Actin
D
293T
HeLa
MDA-231
siRNA Axin1&2
nc nc 1&2 1 2
nc 1&2
nc 1&2
IB: p120 (6H11)
IB: β-catenin
IB: GAPDH
E
Wnt8
0
0.05
0.5
HA-p120
Myc-Axin
IP: IgG
Myc
HA-p120
IgG H.C.
IB : HA
IB : HA

The inhibition of upstream Wnt components reduces the level of p120-catenin isoform-1

I further tested the effect of knocking down LRP5&6. Being positive modulators of canonical Wnt signaling, I would expect that the knock down of LRP5&6 to produce an impact opposite to Axin depletion. I employed a previously characterized siRNA directed against LRP5&6 and confirmed its activity against exogenous LRP5&6 (Figure 15A) (20), and indeed found that p120 levels were diminished upon LRP5&6 depletion in MDA-MB-435 and HeLa cells (Figure 15B&C). Intriguingly, the pp120 antibody, which detects the C-terminal region of p120, and thus detects most p120-catenin isoforms, revealed that the longer isoform of p120-catenin, presumably p120-catenin isoform-1, was more responsive to LRP5&6 depletion. Since isoform-2 and higher initiate translation at a primary sequence position following p120's destruction motif, this endogenous data appears to support the view that p120's most amino-terminal region, present in isoform-1, is required for its regulation by the Wnt pathway. This view was additionally supported via use of proteasome and CK1 inhibitors (D4476), which resulted in increased p120 isoform-1 levels (Figure 6). Consistently, lesser effects on isoform-3 were resolved upon proteasome (MG132) or CK1 inhibition (Figure 6A&6D). Although p120 isoform-3 showed a lesser response to inhibition of the upstream Wnt pathway or the destruction complex, it still exhibited some response to Wnt signaling in the endogenous context. I thus assayed further possible for differences between p120 isoforms using exogenous constructs. I transfected full-length xp120 (similar to

human isoform-1) or an N-terminal deletion mutant (ΔNp120; similar to human isoform-4) into 293T cells. Based upon RNA expression, prior reports indicate occurrence of two Xenopus p120 isoforms (iso1 similar to hp120 isoform-1; and iso2 similar to hp120 isoform-3) upon RNA expression. However, the Xenopus iso2 (similar to hp120 isoform-3) is not yet functionally characterized, while Xenopus isoforms similar to hp120 isoform-2 or -4 are not yet reported. As noted earlier, ΔNp120 lacks the amino-terminal destruction box modulated by the Wnt pathway as well as another potential GSK phosphorylation site (S199). ΔNp120 also associate with neither GSK or CK1 (Figure 9B&D), nor evidence of ubiquitination (Figure 9C). Thus, while human isoforms-3 and -4 are functionally distinct (84, 128), we largely used ΔNp120 (Xenopus surrogate isoform-4) to compare Wnt pathway responses with isoform-1. Relative to Xenopus isoform-1 (full-length), ΔNp120 did not respond to CK1 or proteasome inhibition (Figure 15D). To complement our Xenopus experiments, I compared mouse isoform-1 versus -3 upon GSK3 inhibition using another agent, BIO (inhibition of GSK), in 293T cells. Isoform-1 became modestly but reproducibly stabilized, whereas isoform-3 appeared unresponsive upon BIO treatment (Figure 15E). Inhibition of CK1 reproducibly showed a modest response by isoform-1, but not by isoform-3 (Figure 15E). Collectively, our data point to the view that p120 and β-catenin, and likely additional p120 subfamily members, share regulatory mechanisms responsive to Wnt signaling activity. Further, with respect to p120, it is isoform-1 that is most clearly modulated, consistent with its capacity to associate with destruction complex components and to be phosphorylated by CK1/GSK3β.

Figure 15. P120-catenin isoform-1 is most responsive to canonical Wnt signals. (A) HeLa cells were transiently transfected with siRNA for LRP5 or LRP6 (50pmol), along with either Myc-tagged LRP5 or LRP6, and effects assessed via Myc-immunoblot. (B) MDA-435 cells were seeded in 6-well plates followed by transfection with LRP5&6 siRNAs. The effect of LRP5&6 depletion on p120-catenin was monitored using distinct antibodies directed against p120 (pp120 or 6H11). The asterisk indicates a non-specific band serving as an additional negative control. (C) The stability of p120-catenin in HeLa cells was resolved as described for (B). (D) Myc-p120 full-length (isoform-1) or ΔN-p120 were transiently transfected into 293T cells for 24 hours, followed by MG132 or D4476 treatment (6 hours) at the indicated doses. The levels of p120 were monitored via Myc-immunoblot. (E) 293T cells were transiently transfected with mouse p120 isoform-1A or -3A. P120-catenin isoforms were monitored in the presence of BIO or D4476 (6 hours), employing anti-Myc antibody. This figure was modified/reproduced with permission from the Journal of Cell Science (119).

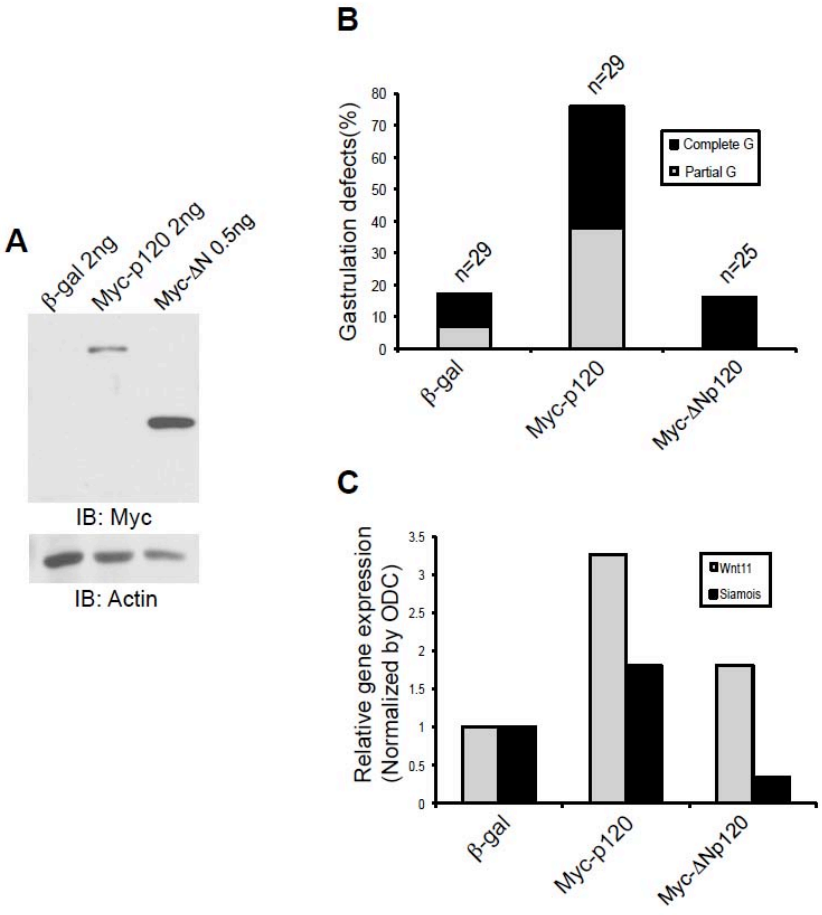

A
Myc-LRP5
siRNA nc 5 6
IB: Myc
IB: GAPDH
Myc-LRP6
siRNA nc 5 6
IB: Myc
non-specific
B
MDA-435
siRNA LRP5&6 nc +
IB: p120(pp120)
*
IB: p120(6H11)
IB: β-catenin
IB: GAPDH
C
HeLa
siRNA LRP5&6 nc +
IB: p120(pp120)
IB: GAPDH
D
Myc-p120 Myc-ΔNp120
MG132 0 10 0 10 (μM)
IB: Myc
IB: Actin
Myc-p120 Myc-ΔNp120
D4476 0 5 10 0 5 10 (μM)
IB: Myc
IB: Actin
E
mp120 1A mp120 3A
BIO + +
IB: Myc
IB: Actin
mp120 1A mp120 3A
D4476 + +
IB: Myc
IB: Actin

The amino-terminal portion of p120-catenin is critical for its functional activity

Given that ΔNp120 (similar to hp120 isoform-4) and mp120 isoform-3 appears to be less responsive to the Wnt pathway (Figure 15D&E), I next questioned how this isoform selectivity acts in whole Xenopus embryos. While we had previously tested only Xenopus isoform-1 expression in Xenopus, there are at least two (likely more) Xenopus isoforms (89). Since this second Xenopus isoform is not yet isolated (equivalent to human isoform-3), we compared Xenopus p120-catenin isoform-1 with an xp120 amino-terminal deletion construct similar in structure to human p120 isoform-4 (ΔN-p120; removal of amino acids 1-341). ΔN-p120 lacks the amino-terminal destruction motif, so it was initially expected to be more stable and to increase target-gene expression more robustly than Xenopus p120 isoform-1 (more effective at relieving Kaiso-mediated repression of target genes). While it exhibited higher protein expression levels as anticipated (Figure 16A), our preliminary results indicate that ΔN-p120 does not produce gastrulation failures above negative-control levels, and further, it exhibits less impact than p120 isoform-1 upon Siamois and Wnt11 gene expression (Figure 16B&C). P120 has multiple roles that extend to the modulation of RhoA and Rac (48-50), and as supported by recently published work (84, 129), we were reminded that ΔN-p120 expression may have produced lesser gastrulation phenotypes due to its reduced capacity to regulate small GTPases. Further, ΔN-p120's reduced activation of Wnt-11 expression (weaker relief of Kaiso-mediated repression of Wnt-11; functional underpinnings are unclear) would be expected to result in lesser ectopic activation of PCP signaling (62). We speculate

that under physiologic conditions, p120 isoform-1 plays more prominent roles in cell and morphogenic (eg. gastrulation) movements than shorter isoforms.

Figure 16. The amino-terminal region of p120-catenin isoform-1 contributes to its functional developmental effects in Xenopus embryos. (A) mRNA encoding β-galactosidase (2 ng) (negative control), Myc-p120 (2 ng) or Myc-ΔN-p120 mRNA (0.5 ng) were injected into one-cell stage embryos, harvested at gastrula stage 10.5 and Myc immunoblotted. Actin serves as a loading/ negative control. (B) Gastrulation failures, scored grossly at embryonic stage 12, following expression of full-length versus DN-p120. The gray bar indicates the fraction of partial gastrulation failure, while the black bar reflects complete gastrulation failure. (C) β-galactosidase (negative control) (2ng), Myc-p120 (2ng) or Myc-ΔN p120 (0.5 ng) were injected into one-cell embryos. Gastrula-stage (91) cDNA was assayed by real-time RT-PCR to assess the levels of x*Wnt-11* or *xSiamois* transcripts. Unexpectedly, ΔN-p120 did not produce greater gastrulation failures relative to full-length p120.

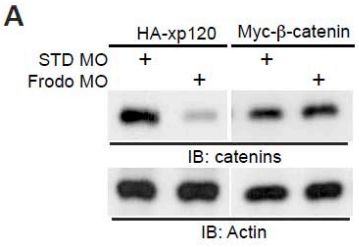

A
β-gal 2ng
Myc-p120 2ng
Myc-ΔN 0.5ng
IB: Myc
IB: Actin
B
Gastrulation defects(%)
n=29
n=29
n=25
Complete G
Partial G
β-gal
Myc-p120
Myc-ΔNp120
C
Relative gene expression
(Normalized by ODC)
Wnt11
Siamois
β-gal
Myc-p120
Myc-ΔNp120

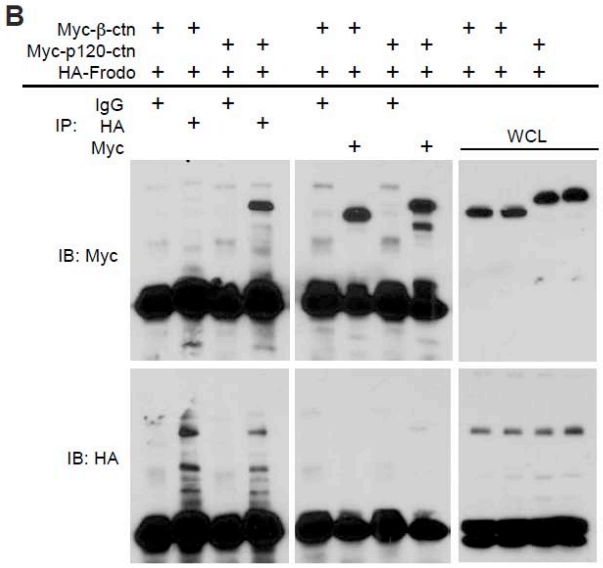
Frodo has a selective impact upon p120-catenin

Our previous work showed that p120-catenin binds and is stabilized by the intracellular protein Frodo, allowing the p120-catenin/ Kaiso pathway to modulate certain Wnt target genes (70). We thus tested if Frodo depletion might also have an impact upon β-catenin, but found that while p120 levels became lowered as expected, β-catenin levels were maintained (Figure 17A). This was consistent with our subsequent finding that Frodo does not appear to bind β-catenin, in contrast to its known interaction with p120, suggesting that the selective role of Frodo in regulating the canonical Wnt pathway may occur specifically through p120-catenin (Figure 17B).

Figure 17. Frodo associates with and stabilizes p120- but not β-catenin.

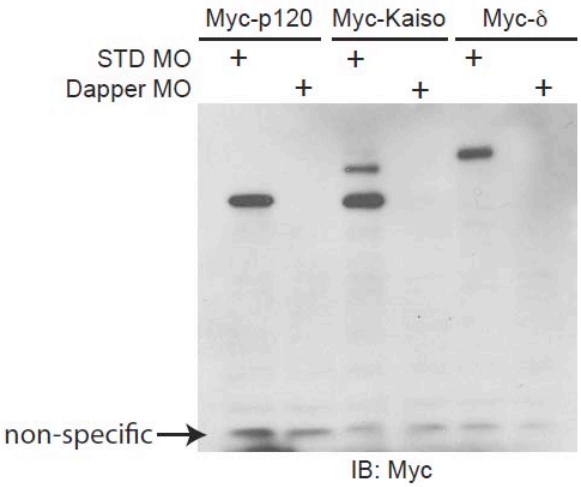
(A) Either HA-p120-catenin (0.25 ng) or Myc-β-catenin mRNA (0.25 ng) were co-injected with Frodo morpholino (10 ng, versus STD-MO/ negative control), into both blastomeres of 2-cell embryos. Embryos were harvested at stage 11-12 for immunoblotting with anti-Myc (β-catenin) or anti-HA antibody (p120). Actin serves as a loading/ negative control. (B) Either Myc-β-catenin (0.5ng) or Myc-p120-catenin (0.5ng) was co-injected with HA-Frodo (0.5ng) into both blastomere of embryos at the 2-cell stage. Embryos were harvested at early-mid gastrula stages (10-11) and lysates immuno-precipitated for HA-Frodo followed by Western blotting to test for Myc-p120-catenin or Myc-β-catenin association (anti-HA antibody was used to detect HA-Frodo). This figure was modified/reproduced with permission from the Journal of Cell Science (119).


A
HA-xp120
Myc-β-catenin
STD MO
Frodo MO
IB: catenins
IB: Actin


B
Myc-β-ctn
Myc-p120-ctn
HA-Frodo
IP:
IgG
HA
Myc
WCL
IB: Myc
IB: HA

Dapper, a close relative of Frodo, were previously reported to have an impact upon p120-catenin's stability (70). We thus tested whether Dapper has an impact upon other p120 subfamily members. Strikingly, the depletion of Dapper in early Xenopus embryos dramatically reduced the level of ectopically expressed δ-catenin and Kaiso, as well as p120-catenin (positive control), suggesting its larger role in modulating p120 subfamily biology (Figure 18). In summary, our results indicate that components of the destruction complex known to act upon β-catenin are further involved in regulating the level of p120 sub-family catenins, and that Frodo has a more obvious impact upon the p120- than the β-catenin trajectory of the Wnt pathway. I speculate that there may be additional currently unknown, but selective modulators of the p120 subfamily, that allows for distinct catenin outputs (e.g. gene regulation) in response to activation by different Wnt ligand on Wnt receptors.

Figure 18. Dapper appears to modulate p120-catenin subfamily members. Either Myc-p120-catenin (0.25 ng), Myc-Kaiso or Myc-δ-catenin mRNA (0.25 ng) were co-injected with Dapper morpholino (10 ng, versus STD-MO/ negative control), into both blastomeres of 2-cell embryos. Embryos were harvested at stage 11-12 for immunoblotting with anti-Myc (β-catenin) or anti-HA antibody (p120).


Myc-p120
Myc-Kaiso
Myc-δ
STD MO
Dapper MO
+
+
+
+
+
+
non-specific
IB: Myc

Discussion

(Chapter III)

The canonical Wnt pathway has been viewed as having β-catenin as the primary signal transduction mediator in response to Wnt pathway activation. While plakoglobin/ γ-catenin, a member of the β-catenin sub-family, has been involved in context-dependent Wnt gene regulation (58, 130), little emphasis has been directed towards the possible roles of p120-catenin subfamily members.

P120 isoform-1 protein levels are subject to destruction complex regulation

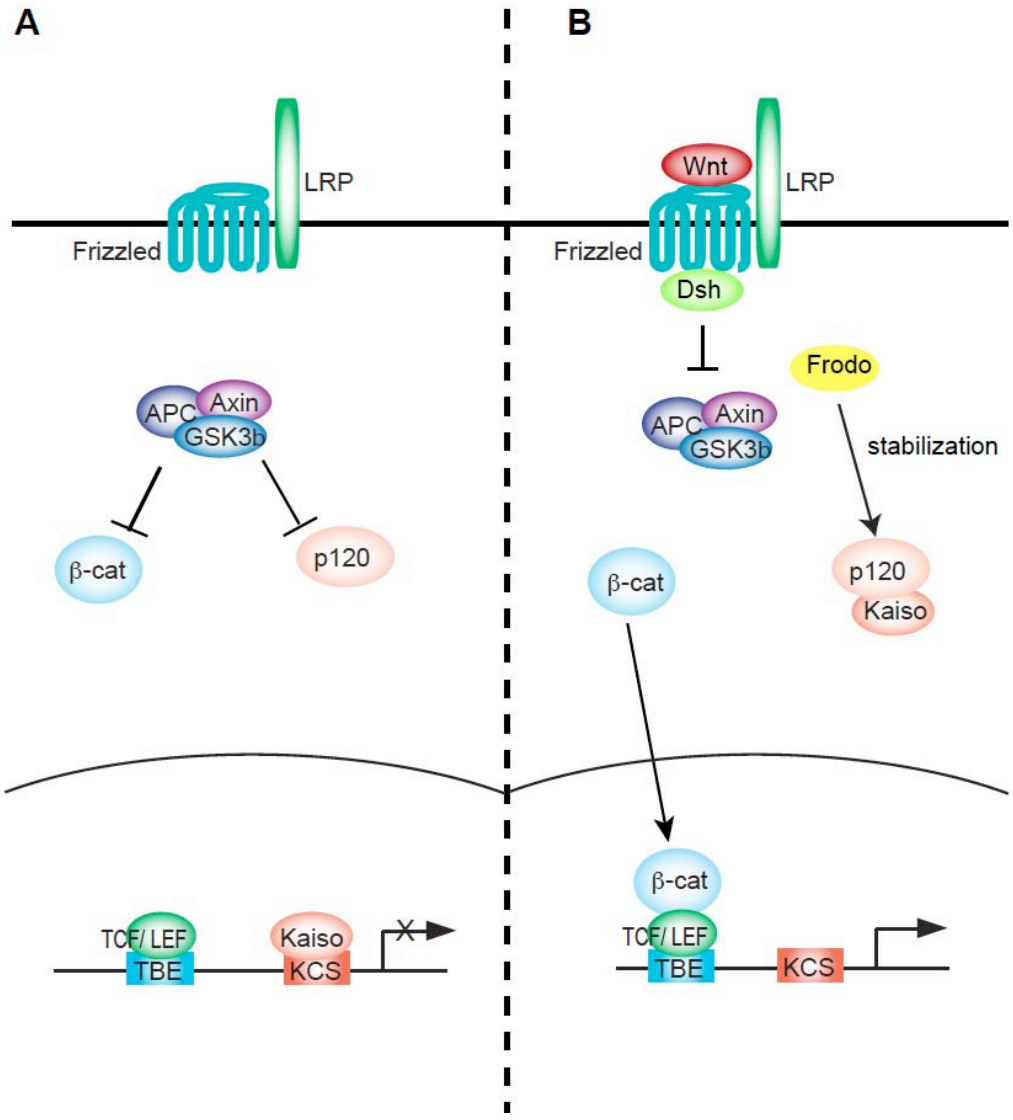
Previous study from our group has pointed to the regulation of p120's level in response to the canonical Wnt signaling pathway via Frodo, resulting in the modulation of certain Wnt/ β-catenin target genes harboring both Kaiso- and TCF-binding sites (61, 70). Based upon our prior findings, I hypothesized that additional pathway components of the Wnt pathway might act upon p120-catenin, prompting us to address potential mechanisms. In my first graduate work, I found that multiple protein components involved with β-catenin's destruction, also facilitate p120-catenin degradation - with the prime examples being Axin and GSK3β. Employing mapping studies with several deletion constructs, the amino-terminal region of p120-catenin was determined to be responsible for rendering sensitivity to, and for association with, destruction complex components (CK1α and GSK3β). Importantly,

I found that the degradation mechanism applies mainly to p120 isoform-1, which encompasses amino-terminal GSK3 and CK1 sites that are not present in isoforms arising from more carboxyl-terminal translational initiations. I next mutated four potential phospho-residues (Serine) predicted to harbor a CK1α/ GSK3β consensus region and β-TrCP sites similar to that of β-catenin. In contrast to wild type, the levels of this mutant construct when co-expressed with GSK3β were no longer lowered. I further identified that the p120 point-mutant lost most of its capacity to associate with β-TrCP and was largely free of ubiquitination. Expanding the molecular mechanisms acting upon β-catenin's stability to p120-catenin, I tested if upstream components of the canonical Wnt pathway such as certain Wnt ligands and LRP5&6, in addition to the little-understood component Frodo, produce protective effects upon p120. While a core component of the destruction complex, Axin, negatively modulates p120 in both Xenopus and cell line systems, the upstream Wnt component, LRP5&6 protected p120 isoform-1 from degradation, likely through recruiting the destruction complex to the plasma membrane to enable its inactivation. My results point to the view that p120 protein levels are modulated via mechanisms analogous to β-catenin in vivo. Interestingly, other members of p120-catenin-subfamily, namely δ-catenin and ARVCF-catenin, were also responsive (apparent substrates) of the destruction complex. While I did not examine these latter catenins in depth, I note that in common with β-catenin and p120, each contains a number of conserved potential GSK3β sites. Indeed, independent evidence from the laboratory of K. Kim supports δ-catenin's responsiveness to GSK3β and ubiquitination (131). In their work, GSK3 depletion

(via siRNA) or chemical inhibition increased δ-catenin's levels. Consistent with my findings centered upon p120, δ-catenin likewise became ubiquitinated in the presence of MG132, with ubiquitination being reduced upon GSK inhibition. Mutation of a potential GSK3 phosphorylation site (Thr^{1078} residue) resulted in less ubiquitination, although interestingly, the site was located in δ-catenin's carboxyl as opposed to the amino-terminal region of p120-catenin. Together with our data here and that earlier published (132), I envisage that the destruction complex of the canonical Wnt pathway modulates multiple members of the catenin family, with these catenins forming a Wnt responsive network extending considerably beyond β-catenin alone.

Intriguingly, an independent group recently reported p120-catenin's involvement in canonical Wnt signaling in a manner distinct from what we resolved (53). In their report, p120 was found to promote Dishevelled phosphorylation through p120's interaction with CK1ε and E-cadherin in response to canonical Wnt ligand. This was shown to increase β-catenin stability. This study complements our own, since cadherin-dissociated p120 (perhaps more than one isoform) would represent a distinct signaling-pool origin, whereas that which I describe arises from Wnt-pathway inhibition of the destruction complex, which exhibits selectivity towards isoform-1. In all cases, my collective work supports the concept that p120-catenin participates in canonical Wnt signaling (Figure 19).

Figure 19. Model of the Wnt/p120/Kaiso signaling module in relation to Wnt signaling activation. (A) In the absence of Wnt ligands, p120-catenin as well as β-catenin are degraded by the destruction complex via the proteasome-mediated pathway. Thus, target genes are repressed more efficiently by TCF/LEF and Kaiso transcriptional repressors. (B) In the presence of Wnt activation, Wnt ligands associate with transmembrane proteins including LRP5/6 and Frizzled(s). Wnt activation enables Dishevelled (Dsh) to recruit Axin and GSK3β to the plasma membrane, which allows for β-catenin and p120-catenin stabilization. Stabilized β-catenin is more likely enter the nucleus to relieve repression conferred by TCF/LEF. Concurrently, stabilized p120-catenin recruits Kaiso from the nucleus to the cytoplasm by an unknown mechanism, presumably via p120's entering the nucleus to dissociate Kaiso from DNA. This results in de-repression of Kaiso target genes, some of which are shared with TCF/LEF. This figure was adapted/modified from a published paper, Park et al, Dev Cell, 2006 (70).


A
B
LRP
Frizzled
Wnt
LRP
Frizzled
Dsh
Frodo
APC
Axin
GSK3b
stabilization
β-cat
p120
β-cat
p120
Kaiso
β-cat
TCF/ LEF
TBE
Kaiso
KCS
X
TCF/ LEF
TBE
KCS

Although our results indicate p120 (likely also ARVCF- and δ-catenin) responsiveness to canonical Wnt pathway components/ signals, central questions remain to be addressed. First is the issue of why, in addition to β-catenin, p120 and other sub-family members would be coordinately regulated. In the case of p120-catenin, evidence is accumulating to show that p120 acts in combination with β-catenin at shared and developmentally significant gene promoters, such as *Xenopus Siamois* and to a lesser extent *Wnt11*. In the case of *Siamois*, for example, gene activation occurs to an additively larger extent when β-catenin's de-repression of TCF/ LEF occurs in combination with p120-mediated de-repression of the Kaiso transcriptional repressor (61, 62). Thus, the Wnt/ p120-catenin and Wnt/ β-catenin pathways could be required for coordinate regulation of Wnt signaling in context dependent manners. Future study is required, especially as an independent group has questioned if *Siamois* and *Wnt11* are gene targets of Kaiso (63, 65), Another independent study has supported the responsiveness of *Siamois* to Kaiso depletion in the presence of weak Wnt pathway stimulation (64). Whatever the outcome of this discussion, my data here while directed towards upstream p120 regulation, continue to be consistent with *Siamois* and *Wnt11* gene responsiveness to p120 and Kaiso (Figure 11). I thus expect that the Wnt/p120-catenin pathway, and possibly the Wnt/ ARVCF and Wnt/ δ-catenin pathways, act in parallel with the Wnt/β-catenin signaling trajectory to modulate certain canonical Wnt gene targets (or cytoplasmic downstream effectors) in context dependent manners. Interestingly, δ-catenin has also been reported to modulate Kaiso function at gene promoters (133, 134), and ARVCF appears present in some cell/ tissue nuclei (135). While

there is no prior report on the function of ARVCF in the nucleus, we have recently resolved its interaction with Kazrin, a structurally novel and incompletely characterized cortical/ junctional protein, which shuttles into the nucleus (136-138). Collectively, a coordinate response of catenins could be imagined to lead a networked gene regulatory readout downstream of Wnt signals. With respect to potential upstream modulators of catenins, Frodo and the closely related Dapper, which are known to functionally and physically interact with Dishevelled, will need to be evaluated in the Wnt/p120 pathway context. Frodo and Dapper appear to positively or negatively modulate Wnt signals in context-dependent manners (71, 72, 139, 140). In this study, I could not resolve a β-catenin:Frodo complex, although I confirmed the expected interaction of p120-catenin:Frodo (70). Since Frodo binds Dishevelled, we conjecture that Frodo exhibits some selectivity for the p120 signaling trajectory in upstream Wnt contexts, and that analogous but presently undefined proteins may work together with Dishevelled to regulate the trajectory of other catenins, especially p120-catenin subfamily members. Intriguingly, my data presented here indicates that the depletion of Dapper diminished δ-catenin's levels in addition to p120. This suggests the possibility of Dapper being a more general modulator of the Wnt pathway.

As noted earlier, the initial p120-catenin transcript (pre-RNA) is subject to inter- and intra-exonic splicing events that allow for the generation of multiple isoforms of p120, as does the presence and use of four alternative translation start sites in humans (87). Recent evidence indicates that differing isoforms confer distinct or

even opposing functions, with regard to cell adhesion and invasion (84, 92, 141). P120 isoform-1, transcribed from the first translational start site, includes the destruction motif subject to Wnt-pathway regulation, and correspondingly is more responsive to LRP5&6 depletion, or the inhibition of proteasomes, GSK3 or CK1. In the context of exogenous p120 isoforms, isoform-1 was considerably more sensitive than p120 isoform-3 or an N-terminal deletion mutant of p120, ΔNp120 (latter similar to human isoform-4). Endogenous Xenopus isoforms equivalent to human isoform-2 and -4 have not yet been reported/ characterized. However, in certain mammalian cell lines, I observed an additional response of isoform-3, which might conceivably arise from its association with isoform-1, indirect effects upon the cadherin-catenin complex, or the existence of additional destruction motifs within p120. Although exogenous isoform-3 and ΔNp120 (similar to human isoform-4) produced similarly absent or mild responses to the inhibition of GSK, CK1 or the proteasome, these isoforms have distinctive features, including their most apparent intracellular distributions (128). Relative of other p120 isoforms, expression of isoform-1 is associated with cells having mesenchymal characteristics, and includes epithelial cells that have undergone progression towards invasive and transformed cell states (84). I thus envisage that regulatory events determining the choice of translational initiation sites would provide another layer of control for p120's responsiveness to canonical Wnt signals. At present, little is known concerning how a particular translational initiation site in the p120 transcript is selected, or similarly, what governs the differential splicing decisions of the p120 transcript, which together produce multiple p120 isoforms.

Besides its nuclear functions, p120 sub-family members play additional key roles at cell-cell junctions and other locations, modulating cadherin stability and trafficking (120, 142, 143), as well as regulating small GTPases (eg. inhibiting RhoA and activating Rac) (see Introduction). Thus, in considering the potential outcome of Wnt signaling upon p120 sub-family members, we must include possible effects that are not transcriptional in nature, but rather represent more immediate effects upon cell adhesion, motility or cytoskeletal activity.

Chapter IV

Signaling components having positive impact upon p120-catenin levels:

Down-syndrome related kinase Dyrk1A acts upon the p120/Kaiso trajectory of the Wnt signaling pathway

Results

(Chapter IV)

Dyrk1A has an essential role in early embryonic development

To gain insight into the mechanisms by which p120-catenin is regulated, I performed a small-scale kinase screen. Varied kinases were transfected into 293T cells, and p120 levels monitored by immunoblotting (data not shown). Dyrk1A was selected due to its robust impact on p120-catenin levels (see below). Although mammalian Dyrk1A has been examined in embryonic development, *Xenopus* Dyrk1A had yet to be evaluated. To address the molecular relationship of p120-catenin with Dyrk1A, I designed a morpholino to deplete Dyrk1A in Xenopus embryos. The characterization of the Dyrk1A morpholino is summarized in Figure 20. Employing an antibody directed against mouse, rabbit and human Dyrk1A, endogenous Dyrk1A was detected in *Xenopus* embryo extracts. The level of Dyrk1A was diminished in the presence of the Dyrk1A morpholino, suggesting specificity of the Dyrk1A antibody and morpholino (Figure 20 A&B). Given that the mouse knock-out of Dyrk1A is embryonic lethal (144), it was expected that Dyrk1A depletion would result in severe defects during embryonic development. Indeed, Dyrk1A depletion resulted in gastrulation failure at stage 12 in a significant fraction of embryos (Figure 20C). Since Dyrk1A is a candidate gene for some of the

neurobiological alterations observed in Down syndrome, I microinjected Dyrk1A morpholino into the dorsal region of embryos at the 4-cell stage. Intriguingly, Dyrk1A depletion in the dorsal-animal region resulted in embryos bearing kinks or shortened axes (a and b), small heads (a and b), skin fragility (c and data not shown), and very interestingly, developmental arrest (c) at neurulation stages (Stage 19-20) (Figure 21A). These phenotypes were reproducibly observed. Although the detailed functions of Dyrk1A in early embryonic development requires further evaluation, these data indicate that Dyrk1A has pivotal roles in amphibian development consistent with knock-out or over-expression phenotypes defined in mouse, rat and Drosophila.

Figure 20. The characterization of Dyrk1A morpholino (A) Dyrk1A antisense morpholino was designed to overlap the translational start site, and was microinjected into 1-cell stage embryos. Endogenous Dyrk1A was detected with polyclonoal Dyrk1A antibody (Abcam). Actin serves as a negative control. (B) Increasing total doses of Dyrk1A morpholino were microinjected into Xenopus embryos, and embryos harvested at late-gastrula stage 12. Endogenous p120-catenin, β-catenin, C-cadherin and actin levels were respectively visualized via immunoblotting. (C) Gross gastrulation effects following Dyrk1A depletion. In a dose dependent manner, Dyrk1A depletion resulted in gastrulation failure. This data reflects combined results from three independent experiments.

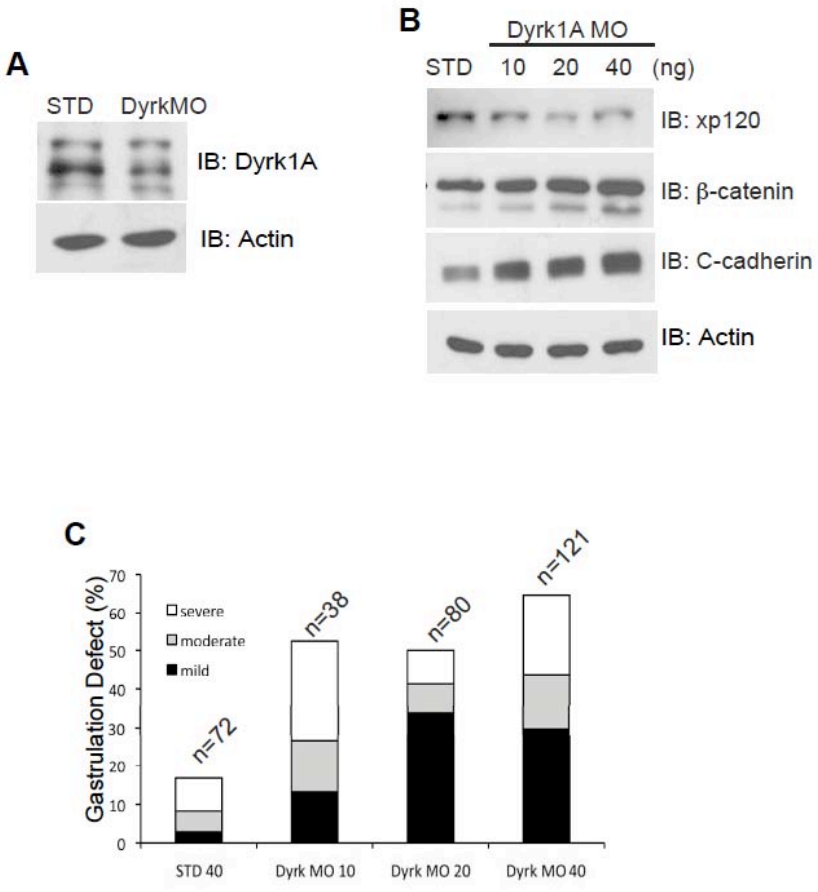
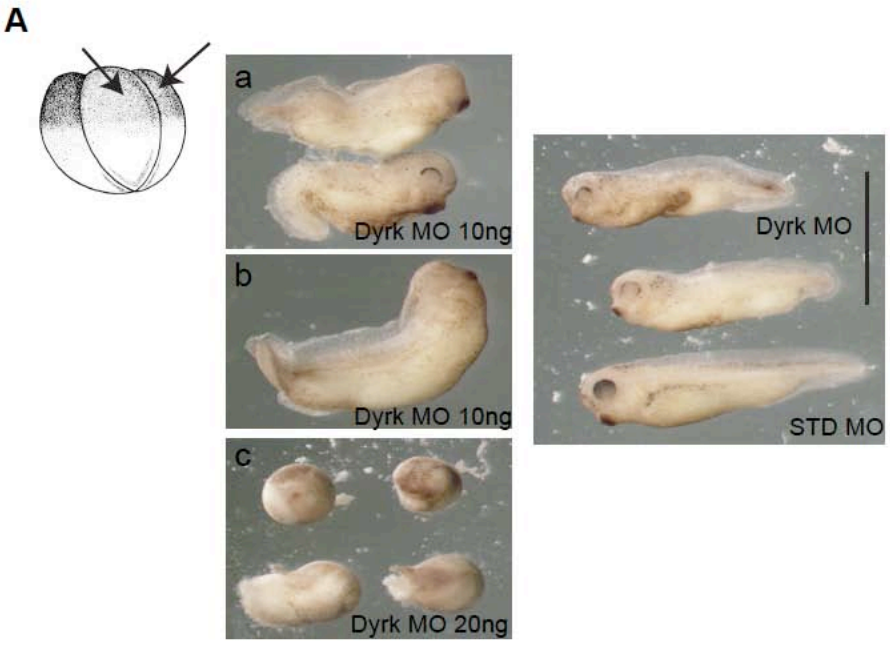
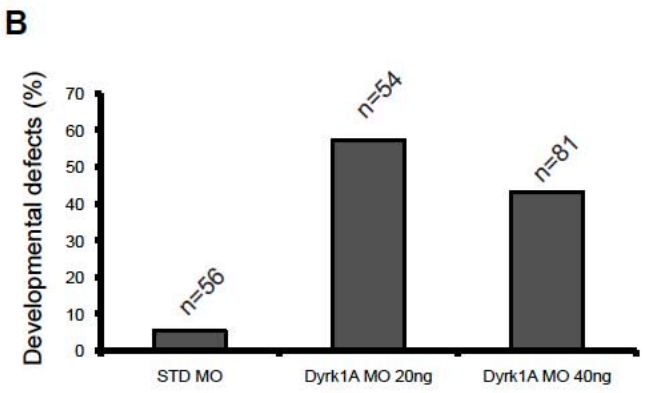

A
STD
DyrkMO
IB: Dyrk1A
IB: Actin
B
Dyrk1A MO
STD
10
20
40
(ng)
IB: xp120
IB: β-catenin
IB: C-cadherin
IB: Actin
C
Gastrulation Defect (%)
severe
moderate
mild
n=72
n=38
n=80
n=121
STD 40
Dyrk MO 10
Dyrk MO 20
Dyrk MO 40

Figure 21. Dyrk1A is essential to early embryonic development. (A) Dyrk1A morpholino was microinjected at the indicated levels into each dorsal-animal blastomere of 4-cell stage embryos. Embryos were monitored from stage 15-38 to score for gross developmental defects. Data from two independent experiments are represented in (B).


A
a
Dyrk MO 10ng
b
Dyrk MO 10ng
c
Dyrk MO 20ng
Dyrk MO
STD MO

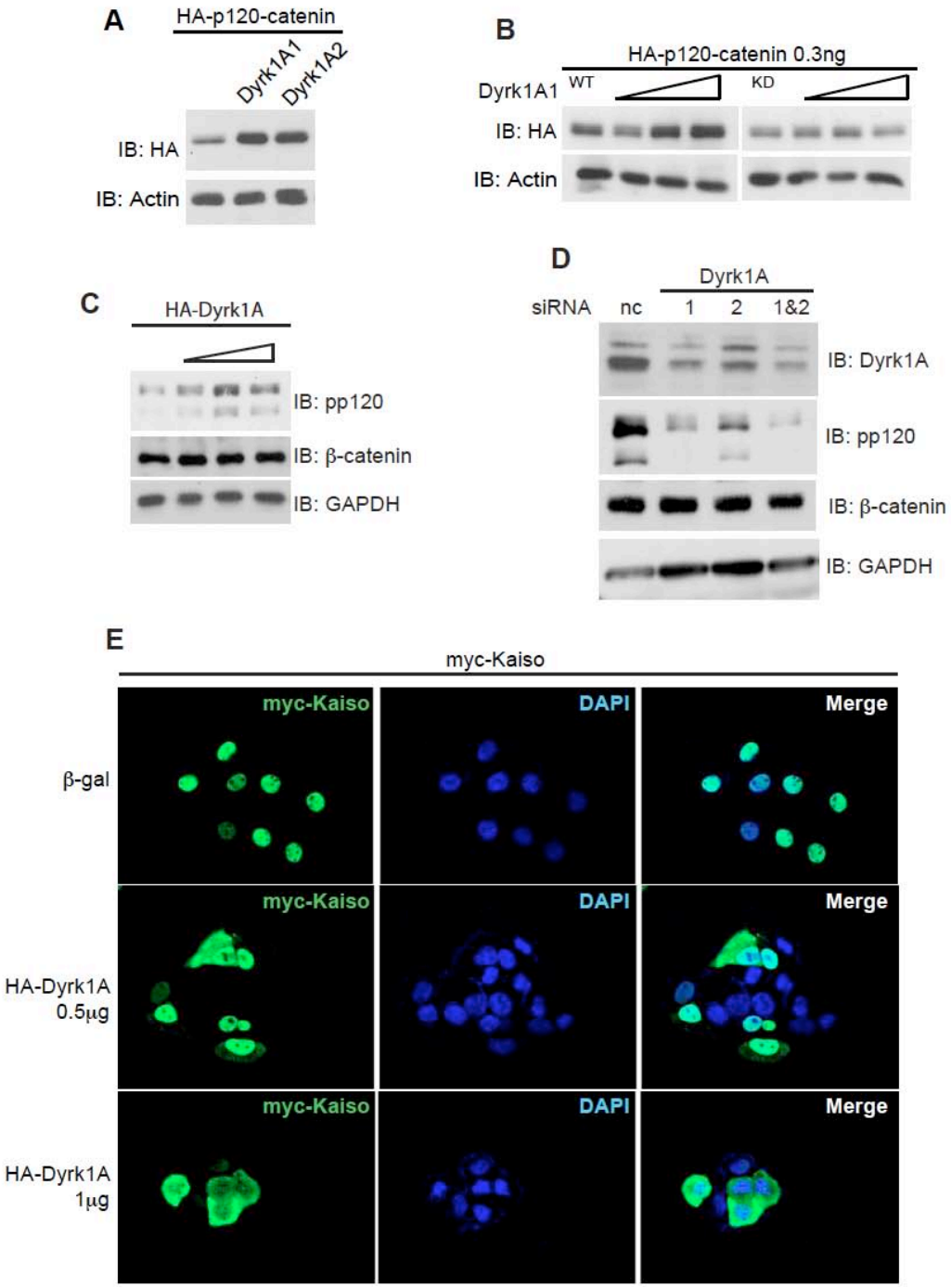

B
Developmental defects (%)
70
60
50
40
30
20
10
0
n=56
n=54
n=81
STD MO
Dyrk1A MO 20ng
Dyrk1A MO 40ng

Dyrk1A expression specifically increases p120-catenin protein levels

To validate Dyrk1A's effects in vivo, in vitro transcribed mRNA encoding HA-tagged xDyrk1A1 or 1A2 were co-injected with that for HA-p120 into *Xenopus* early embryos. Co-expression of either Dyrk1A1 or 1A2 with p120 resulted in heightened p120 protein levels (Figure 22A). This was in contrast to Dyrk1A Kinase Dead (KD), which had no such effect (Figure 22B). Human Dyrk1A is encoded within the Down Syndrome Critical (DSC) chromosomal region, which with increased gene copy number contributes to human phenotypes including cognitive deficits (99, 145-147). P120-catenin is likewise tied to brain cognitive functions, with gene knock-out pointing to roles in dendritic spine and synapse formation (52, 148), and potentially, morphogenetic events and plasticity of the CNS (107, 108). Consistent with our Xenopus data, Dyrk1A transfected into 293T cells increased endogenous p120-catenin levels, while β-catenin remained unaffected (Figure 22C). Likewise, we determined that depletion of Dyrk1A, through the use of two independent Dyrk1A siRNAs, strikingly lowered endogenous p120 but not β-catenin levels (Figure 22D). Previous reports from our lab and others have shown that p120-catenin relieves Kaiso-mediated repression of certain Wnt target genes such as *Siamois* and *Wnt-11* (61, 62, 119). Upon binding, p120 is thought to somehow displace or compete Kaiso from its sequence specific sites (KCS), and facilitate Kaiso's departure from the nucleus. We thus tested whether Dyrk1A expression likewise recruits Kaiso from the nucleus. Consistent with a model where Dyrk1A stabilizes p120-catenin and is positively involved in p120/Kaiso pathway function, exogenous Dyrk1A expression

resulted in a significantly greater proportion of Kaiso appearing in the cytoplasm (Figure 22E).

Figure 22. Dyrk1A modulates p120-catenin levels and the intracellular localization of Kaiso (A) Either Dyrk1A1 or 1A2 was co-injected with HA-tagged p120-catenin (0.25 ng) into Xenopus embryos at the 1-cell stage, followed by immunoblotting for HA-p120 or actin (negative control). (B) HA-p120 (0.25ng) was microinjected into in 1-cell Xenopus embryos with wild-type or Kinase Dead (KD) Dyrk1A. Embryos were harvested at early gastrula stages (10-11) and immunoblotted for HA-p120, with actin serving as an internal loading control. (C) Increasing doses of HA-tagged Dyrk1A were transfected into 293T cells, and endogenous p120, β-catenin and GAPDH monitored via immunoblotting. (D) 293T cells were transfected with one or both Dyrk1A siRNAs (50 pmol), as indicated, for 48 hours. Endogenous p120-catenin, β-catenin, Dyrk1A and GAPDH levels were monitored via immunoblotting (pp120, BD Transduction; Dyrk1A, ab71464, Abcam). The experiments were repeated three or more times. (E) HeLa cells were grown on glass cover slips, and transiently transfected with Myc-Kaiso or Myc-Kaiso plus HA-Dyrk1A as indicated. Cells were fixed with 4% PFA for 10 min, blocked with 5% goat serum in PBS and immunostained with anti-Myc antibody.

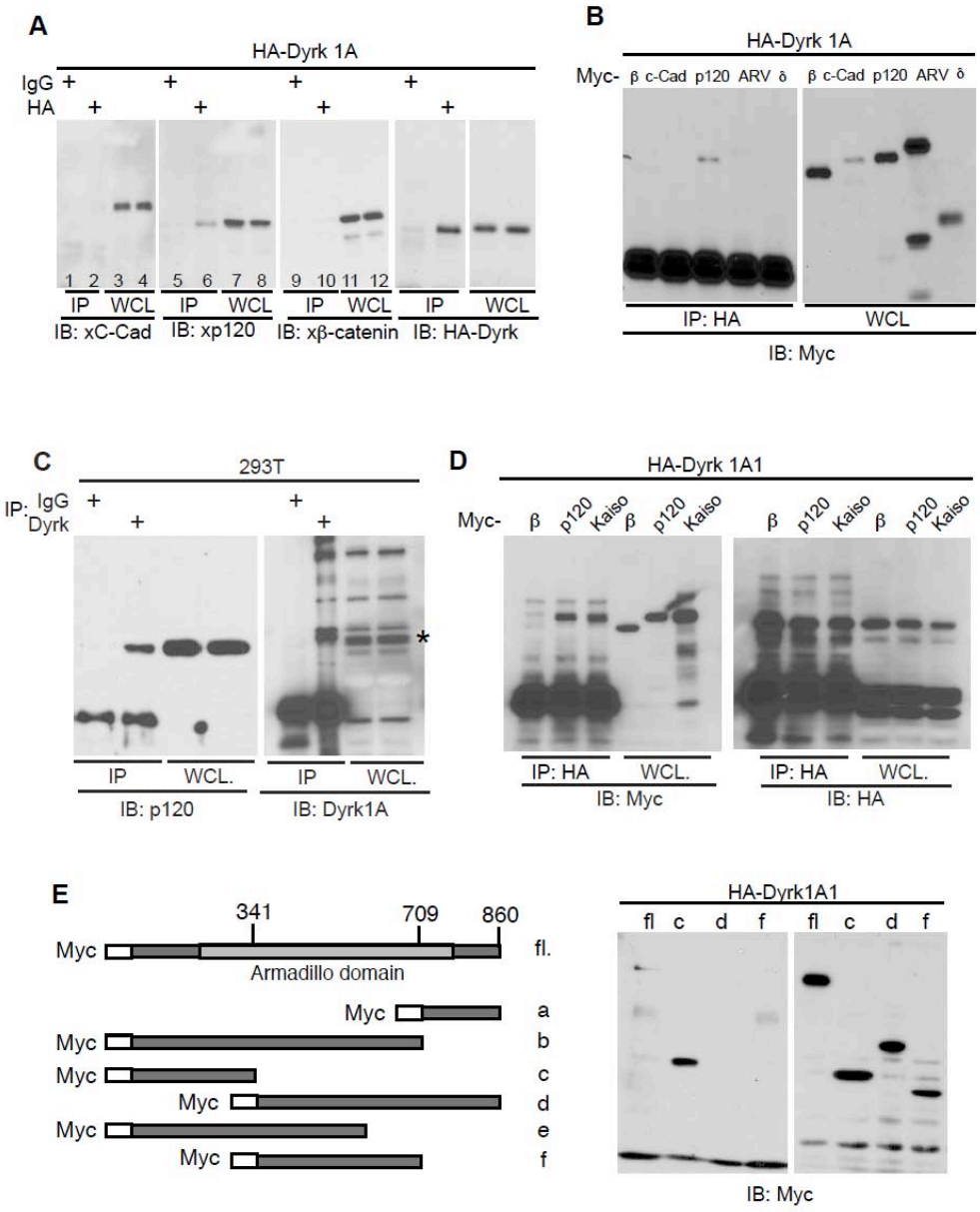

A
HA-p120-catenin
Dyrk1A1
Dyrk1A2
IB: HA
IB: Actin
B
HA-p120-catenin 0.3ng
Dyrk1A1
WT
KD
IB: HA
IB: Actin
C
HA-Dyrk1A
IB: pp120
IB: β-catenin
IB: GAPDH
D
Dyrk1A
siRNA
nc
1
2
1&2
IB: Dyrk1A
IB: pp120
IB: β-catenin
IB: GAPDH
E
myc-Kaiso
myc-Kaiso
DAPI
Merge
β-gal
HA-Dyrk1A 0.5μg
HA-Dyrk1A 1μg

Association of Dyrk1A with p120-catenin

Given the effect of Dyrk1A on p120, we next examined whether Dyrk1A interacts with p120-catenin. A Dyrk:p120 association was modestly but reproducibly resolved (lane 6, Figure 23A), in contrast to C-Cadherin or β-catenin that did not co-precipitate (negative control lanes 2 & 10). Since p120-catenin belongs to the p120-subfamily, other members such as ARVCF- and δ-catenin were examined. Interestingly, only p120 associated with Dyrk1A (lane 3, Figure 23B), suggesting that Dyrk1A might be a selective modulator of the p120/ Kaiso trajectory of the Wnt pathway. To assess the interaction of endogenous Dyrk1A with endogenous p120, we immunoprecipitated endogenous Dyrk1A from 293T-cell extracts, and resolved p120 (lane 2, Figure 23C). Interestingly, an association of Dyrk1A with Kaiso, presumably bridged through p120-catenin, was resolved when HA-Dyrk1A was immunoprecipitated from Xenopus embryo extracts (lane 3, Figure 23D). To map p120's interaction region with Dyrk1A, we expressed p120 deletion constructs (a-f) generated previously (119), and performed co-immunoprecipitations. Full-length (fl), but more prominently the isolated amino-terminal region of p120 (c) associated with Dyrk1A (lane 2, Figure 23E), while p120 constructs lacking this region (d&f), did not. These results indicate that the Dyrk1A kinase is capable of associating with p120's N-terminal region, presumably accounting for Dyrk1A's noted impact upon p120 protein levels (Figure 22).

Figure 23. Association of Dyrk1A with p120-catenin

(A) Embryos were injected with HA-Dyrk1A and harvested at early gastrula (stage 10.5). Lysates were immunoprecipitated for HA-Dyrk1A, and the association with endogenous p120-catenin resolved by immunoblotting. β-catenin and C-cadherin served as negative controls. (B) HA-Dyrk1A (0.5ng) was co-injected with Myc-tagged β-catenin, C-cadherin, p120-catenin, ARVCF or δ-catenin (1ng) into both blastomeres of 2-cell embryos. HA-Dyrk1A immunoprecipitates were immunoblotted with anti-Myc antibody to detect co-associating proteins. (C) Endogenous Dyrk1A was immunoprecipitated from 293T cells, and endogenous p120 monitored using anti-p120 antibody (6H11). Asterisk indicates prospective Dyrk bands. (D) HA-Dyrk1A was co-injected with Myc-p120 or Myc-Kaiso into early Xenopus embryos. Embryos were harvested at gastrulation (stage 12), and lysates immunoprecipitated for HA-Dyrk1A. Anti-Myc or -HA immunoblotting was used to test association of β-catenin, p120-catenin or Kaiso. (E) Depiction of Myc-p120-catenin deletion constructs (a-f). Panels to the right show immunoblotting of the Myc-p120-catenin constructs (a-f; 0.5 ng mRNA), co-precipitating (versus not) with co-injected HA-Dyrk1A1 (0.5 ng mRNA).


A
HA-Dyrk 1A
IgG + + + +
HA + + + +
1 2 3 4 5 6 7 8 9 10 11 12
IP WCL IP WCL IP WCL IP WCL
IB: xC-Cad
IB: xp120
IB: xβ-catenin
IB: HA-Dyrk
B
HA-Dyrk 1A
Myc- β c-Cad p120 ARV δ β c-Cad p120 ARV δ
IP: HA
WCL
IB: Myc
C
293T
IP: IgG + +
Dyrk + +
*
IP WCL. IP WCL.
IB: p120
IB: Dyrk1A
D
HA-Dyrk 1A1
Myc- β p120 Kaiso β p120 Kaiso β p120 Kaiso β p120 Kaiso
IP: HA WCL.
IB: Myc
IP: HA WCL.
IB: HA
E
341 709 860
Myc
Armadillo domain
fl.
a
b
c
d
e
f
HA-Dyrk1A1
fl c d f fl c d f
IB: Myc

Acting through p120-catenin, Dyrk1A relieves Kaiso repressor activity

At the functional level, p120-catenin has a number of in vivo activities including the capacity to relieve Kaiso-mediated repression of target genes containing sequence-specific Kaiso consensus sites (KCS). Given that Dyrk1A associates with, and by some mechanism results in increased p120 protein levels (we expect via kinase-mediated stabilization, Figure 22B & see below), we tested if Dyrk1A expression has an impact upon established endogenous p120/ Kaiso target genes such as *Wnt11* and *Siamois*. Indeed, as reflected via real-time PCR, the expression of exogenous Dyrk1A in early Xenopus embryos (resulting in p120 stabilization and thus relief of Kaiso-mediated repression) enhanced *Wnt-11* and *Siamois* gene transcription (Figure 24B). Complementing such over-expression data, the morpholino-directed knock-down of Dyrk1A dramatically decreased both *Wnt11* and *Siamois* expression (Figure 24A). A previous report from our lab showed that the Kaiso repressor is required for *Xenopus* gastrulation, with its depletion or over-expression resulting in respectively heightened or lowered expression of Wnt11, and thus failed gastrulation movements (62). Based upon such findings, we tested if Kaiso over-expression phenotypes could be rescued via co-expression of Dyrk1A. As anticipated, exogenous Kaiso alone resulted in significant gastrulation-failure. However, when Kaiso was co-expressed with a minimal amount of Dyrk1A (previously titrated to exhibit little phenotypic consequence alone), significant rescues were observed (Figure 24C&D). Consistent with such phenotypic rescues, real-time PCR showed at the molecular level that exogenous Dyrk1A relieved gene

repression due to exogenous Kaiso acting upon *Wnt11* and *Siamois* (Figure 24E). To further test the functional link between Dyrk1A and Kaiso (presumably taking place via p120-catenin), we conversely made note of the fact that exogenous Dyrk1A produces gastrulation delays in Xenopus embryos (data not shown). Thus, we next tested if exogenous expression of Kaiso, the down-stream component, could rescue Dyrk1A gastrulation delays. Although gastrulation delays produced by Dyrk1A were subtle, I observed significant rescues with the appropriately titrated expression of Kaiso (Figure 25A). Gene expression data upon *Wnt11* and *Siamois* likewise supported our model of the functional role of Dyrk1A on the p120/ Kaiso pathway (Figure 25B). We next tested if p120 over-expression could interfere with Kaiso's rescue of Dyrk1 expression. Indeed, the co-injection of p120-catenin with Kaiso and Dyrk1A re-elevated the level of gastrulation failures, as well as the level of Wnt11 and Siamois (Figure 25C). These data suggest that Dyrk1A transmits an apparently significant fraction of its biological activity through the p120/Kaiso pathway.

Figure 24. Dyrk1A modulates p120/ Kaiso-dependent gene expression

(A) The indicated amounts of Dyrk1A morpholino were injected into embryos at the 1-cell stage. Gastrula cDNA was assayed by real-time RT-PCR for Wnt11 and Siamois transcript levels. Gene expression levels were normalized to ODC (ornithine decarboxylase). (B) Dyrk1A (5pg) was injected into embryos at the 1-cell stage. cDNA was isolated from gastrulation stage embryo lysates and analyzed by real-time PCR for Wnt11 and Siamois transcript levels, normalized to ODC. (C&D) Gastrulation (blastopore closure) failures followed the expression of Kaiso (0.5ng), whereas the co-expression of Dyrk1A (5pg or 10pg), partially rescued Kaiso's effects. (E) Under similar experimental conditions, gastrula cDNA was assayed by real-time RT-PCR for Wnt11 and Siamois transcript levels, normalized to ODC (right panel).

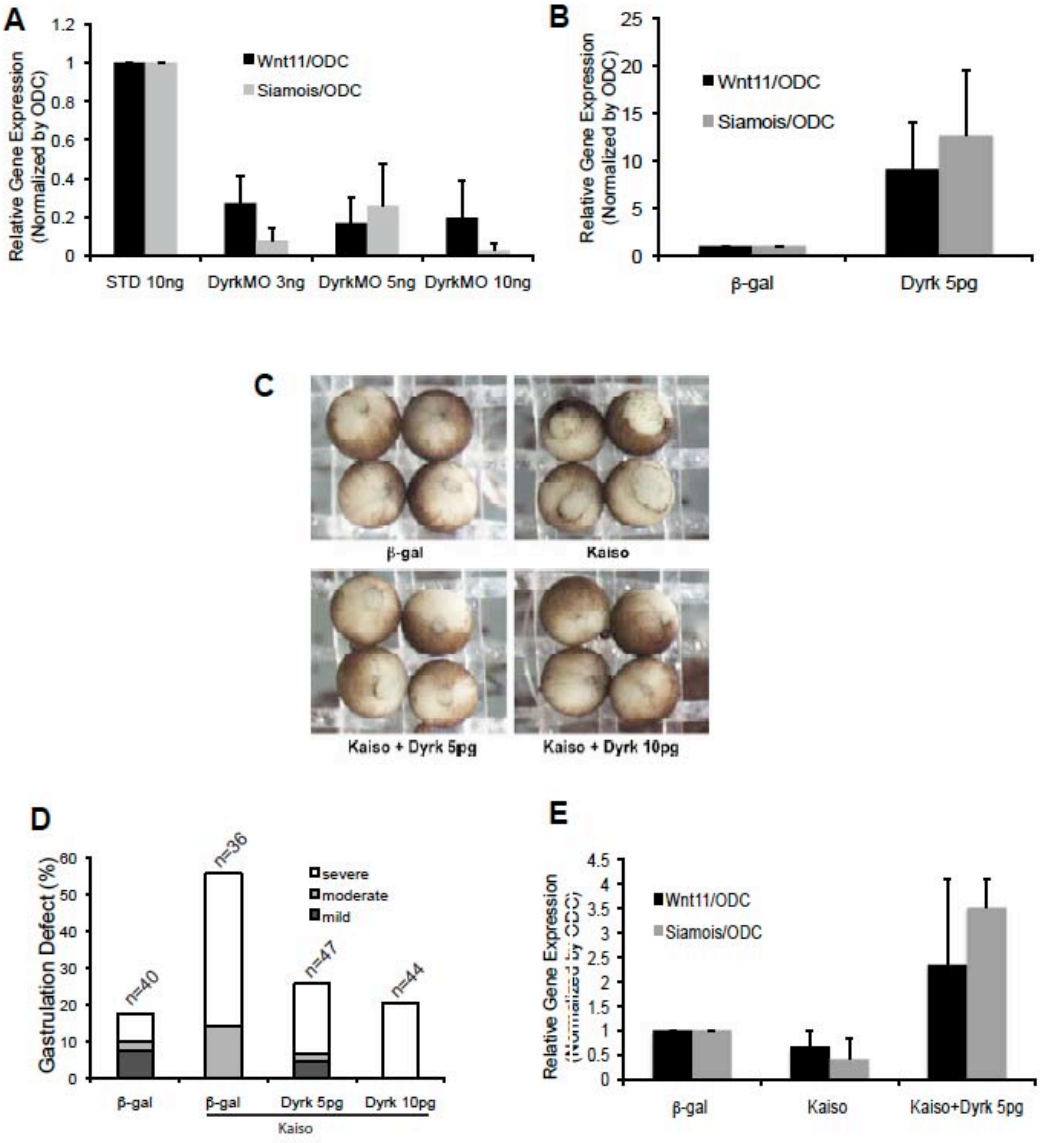
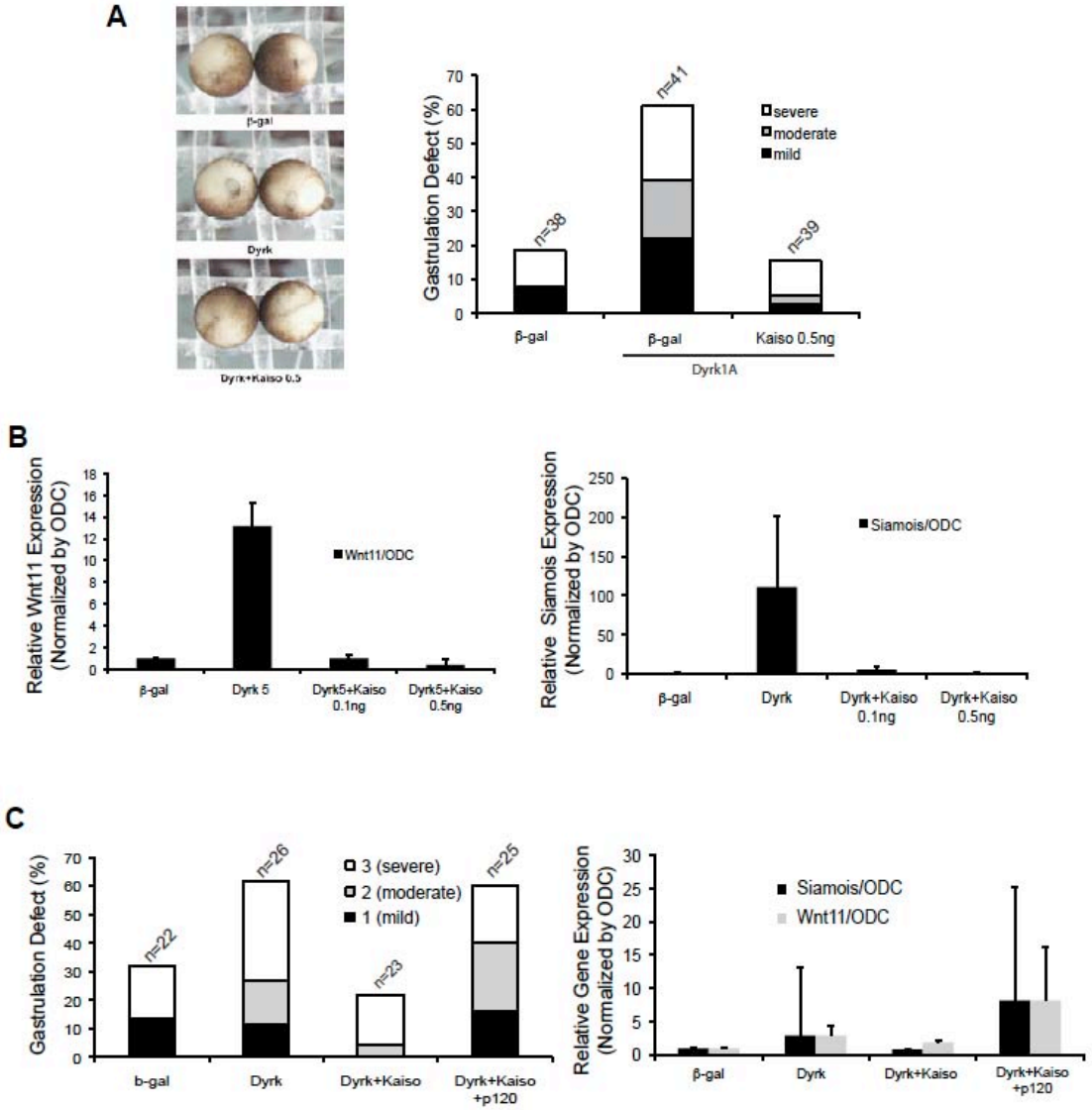

A
Relative Gene Expression (Normalized by ODC)
1.2
1
0.8
0.6
0.4
0.2
0
Wnt11/ODC
Siamois/ODC
STD 10ng
DyrkMO 3ng
DyrkMO 5ng
DyrkMO 10ng
B
Relative Gene Expression (Normalized by ODC)
25
20
15
10
5
0
Wnt11/ODC
Siamois/ODC
β-gal
Dyrk 5pg
C
β-gal
Kaiso
Kaiso + Dyrk 5pg
Kaiso + Dyrk 10pg
D
Gastrulation Defect (%)
60
50
40
30
20
10
0
severe
moderate
mild
n=40
n=36
n=47
n=44
β-gal
β-gal
Dyrk 5pg
Dyrk 10pg
Kaiso
E
Relative Gene Expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Wnt11/ODC
Siamois/ODC
β-gal
Kaiso
Kaiso+Dyrk 5pg

Figure 25. P120/Kaiso pathway rescues Dyrk1A-mediated effect in Xenopus embryos (A) Kaiso expression rescues the gastrulation delays resulting from Dyrk1A expression (left panels). (B) At the molecular level, *Wnt11* and *Siamois* transcript levels reflect the rescue effected by Kaiso. (C) Gastrulation failures resulting from Dyrk1A expression are rescued by co-expression of Kaiso. The co-expression of a third component, p120-catenin, once again results in increased gastrulation failures, presumably in part by relieving the repression/ rescue conferred by Kaiso.

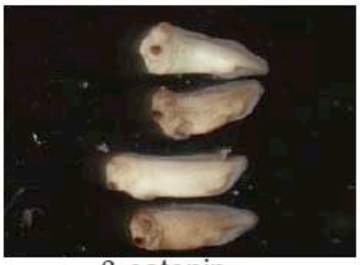

A
β-gal
Dyrk
Dyrk+Kaiso 0.5
Gastrulation Defect (%)
n=38
n=41
n=39
severe
moderate
mild
β-gal
β-gal
Kaiso 0.5ng
Dyrk1A
B
Relative Wnt11 Expression (Normalized by ODC)
Wnt11/ODC
β-gal
Dyrk 5
Dyrk5+Kaiso 0.1ng
Dyrk5+Kaiso 0.5ng
Relative Siamois Expression (Normalized by ODC)
Siamois/ODC
β-gal
Dyrk
Dyrk+Kaiso 0.1ng
Dyrk+Kaiso 0.5ng
C
Gastrulation Defect (%)
n=22
n=26
n=23
n=25
3 (severe)
2 (moderate)
1 (mild)
b-gal
Dyrk
Dyrk+Kaiso
Dyrk+Kaiso +p120
Relative Gene Expression (Normalized by ODC)
Siamois/ODC
Wnt11/ODC
β-gal
Dyrk
Dyrk+Kaiso
Dyrk+Kaiso +p120

xDyrk1A over-expression facilitates canonical Wnt signaling

Previously, our group determined that exogenous Kaiso represses canonical Wnt signaling as evaluated using a classic in vivo assay, the suppression of ectopic β-catenin-dependent axis duplication (61). Given that Dyrk1A stabilizes p120-catenin, that in turn transcriptionally modulates a key gene product involved in axis specification, *Siamois*, we tested if Dyrk1A has an impact upon β-catenin-mediated axis duplication. By co-injecting mRNA encoding Dyrk1A and β-catenin into one cell of 4-cell (as opposed to 1-cell) embryos (ventral-vegetal region), we largely avoided gastrulation delays or failures resulting from Dyrk1A over-expression. As predicted, co-expression of Dyrk1A with an intentionally modest (almost sub-phenotypic) dose of β-catenin enhanced secondary axis formation in a dose-dependent manner (Figure 26A). Kinase-dead Dyrk1A did not produce such effects (Dyrk1A KD, negative control).

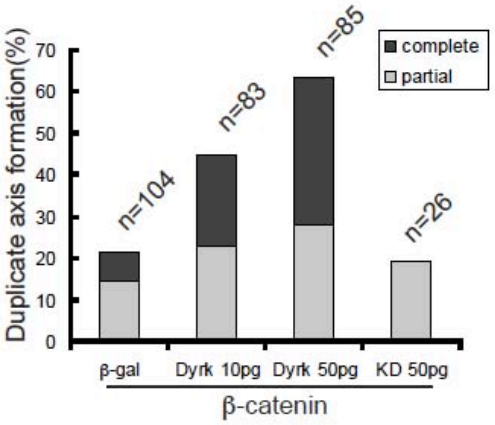
Figure 26. Dyrk1A's involvement in the canonical Wnt pathway (A) Duplicate axis formation following the expression of a sub-maximal dose of β-catenin, is enhanced upon the co-expression of the indicated doses of Dyrk1A, as evaluated in tailbud embryos (stage 27-29). Kinase-dead (KD) Dyrk1A serves as a negative control.

A



β-catenin

β-catenin+Dyrk1A



Phospho-mimic mutant of p120 enhances target gene expression

As a first step in assessing the mechanism of Dryk1A's effects, we searched for potential Dyrk phosphorylation sites in p120. Consistent with our earlier Dyrk1A:p120 association results (Figure 23), one evolutionary conserved Dyrk1A phosphorylation site was resolved in the amino-terminal region of p120. This ^{45}RVSP^{48} motif was comparable to the Dyrk canonical consensus (RPX(S/T)P; Fig. 27A), satisfying the requirement of proline-directed kinases for a proline immediately trailing a serine/ threonine residue and the requirement of arginine for substrate recognition (149). To approximate the impact of Dyrk1A phosphorylation at this site, we generated a phospho-mimic point mutant (p120-T47D). P120-catenin wild-type versus p120-T47D was expressed in Xenopus embryos (Fig. 27B). As wild-type p120 over-expression relieves Kaiso-mediated gene repression and thus contributes to failed gastrulation (p120 effects upon small-GTPases also contribute to the observed phenotypes), we suspected that p120-T47D might be yet more active in relieving Kaiso-mediated repression, possibly resulting from its increased protein levels. As expected, p120-T47D proved more effective than wild-type in promoting expression of the known p120/ Kaiso gene targets *Siamois* and *Wnt11* (Fig. 27C). Correspondingly, p120-T47D produced greater levels of gastrulation failure than corresponding doses of wild-type (Fig. 27D). The half-life of p120 wild-type (WT) versus the p120-T47D mutant was then tested in the presence of cyclohexamide (CHX; Fig. 27E). As anticipated, p120-T47D exhibited a notably prolonged half-life. To assess if threonine 47 of p120 is directly phosphorylated by

Dyrk1A, we conducted an in vitro kinase assay using the amino-terminal region of p120 versus the same region of p120-T47D. Phosphorylation by Dyrk1A of p120 was faint but reproducible, and did not appear upon p120-T47D, nor when kinase-dead Dyrk1A was employed (Figure 28). These data together suggest that p120-catenin phosphorylation by Dyrk1A is direct, and that such modification is relevant to its functional role in embryonic development.

Figure 27. Phosphorylation-dependent Dyrk1A in embryonic development

(A) Cross-species p120-catenin sequence alignment of the conserved predicted Dyrk1A phosphorylation-site region (red box). (B) Expression of HA-p120 wild-type, versus the phospho-mimic mutant (T47D), was detected via immuno-blotting of Xenopus embryo lysates. (C) P120 wild-type (WT), or the p120 point-mutant (T47D), were injected into Xenopus embryos at the 1-cell stage, followed later by cDNA isolation and real-time PCR, to monitor increased Wnt11 and Siamois transcript levels. (D) Gastrulation failures were more severe in embryos expressing p120-T47D relative to p120-WT. (E) HA-p120 WT or T47D was expressed in HeLa cells. Cells transfected with each construct were treated with cyclohexamide (CHX) for the times indicated. Each HA-tagged construct was detected via immuno-blotting of the corresponding cell extracts (right panel), followed by densitometer quantitation of the band intensities (left panel). These data were collected from two independent experiments.

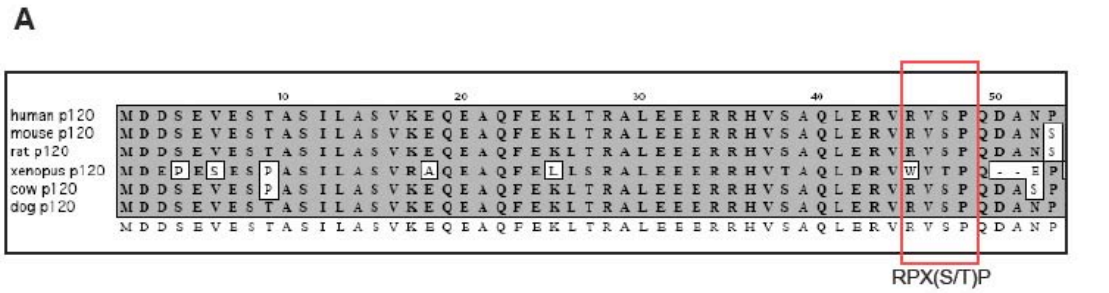
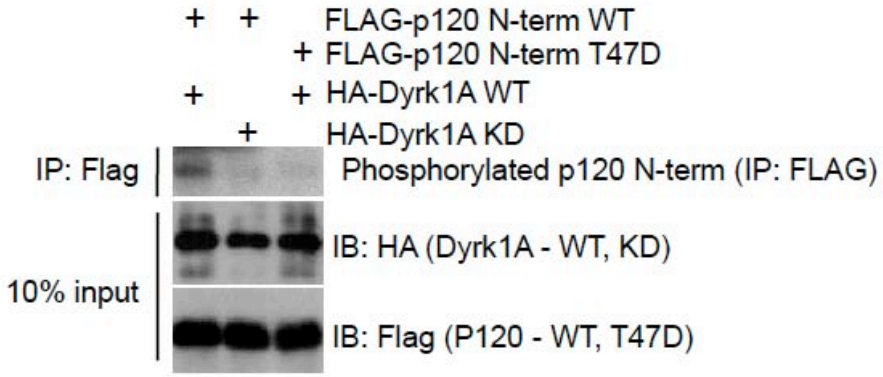

A
human p120
mouse p120
rat p120
xenopus p120
cow p120
dog p120
RPX(S/T)P
B
Non
HA-p120
HA-p120-T47D
IB: HA
C
Relative Expression (Normalized by ODC)
Wnt11/ODC
Siamois/ODC
β-gal
p120
T47D
D
Gastrulation defect (%)
severe
moderate
mild
n=68
n=89
n=99
β-gal
p120
T47D
E
p120 WT
p120 47TD
CHX(h) 0 2 4
p120 WT
p120 T47D

Figure 28. Dyrk1A phosphorylation of p120 in vitro. An amino-terminal region of xp120-catenin (flag-tagged wild-type or T47D mutant; amino acids 1-280) (Hong et al., *J Cell Sci* 2010), or full-length xDyrk1A (HA-tagged wild-type or kinase dead), were each in vitro transcribed and translated using a reticulocyte lysates system (Promega, L4611). Kinase (Dyrk1A) and substrate (xp120-catenin) were incubated for 30 minutes at 30°C as indicated in kinase reaction buffer (10 mM Hepes, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 10mM MnCl2, 1 mM EGTA, 1 mM dithiothreitol, 5 μM ATP, 10 mM NaF and 1 mM Na_3VO_4), along with 10 μCi γ-^{32}P-ATP. Samples were then immunoprecipitated for 1 hour at 4°C with anti-FLAG-M2 magnetic beads (Sigma) in the presence of 0.5% NP-40 Buffer (25mM HEPES pH 7.5, 150mM KCl, 0.5% NP-40, 1.5mM $MgCl_2$, 10% glycerol, 5mM β-mercaptoethanol). The precipitates were washed 3x and the samples then subjected to SDS-PAGE/ autoradiography. Anti-HA antibody (3F10) and anti-flag antibody (M2) were employed to detect Dyrk1A and p120-catenin, respectively.


+ + FLAG-p120 N-term WT
+ FLAG-p120 N-term T47D
+ + HA-Dyrk1A WT
+ HA-Dyrk1A KD
IP: Flag
Phosphorylated p120 N-term (IP: FLAG)
IB: HA (Dyrk1A - WT, KD)
10% input
IB: Flag (P120 - WT, T47D)

Discussion

(Chapter IV)

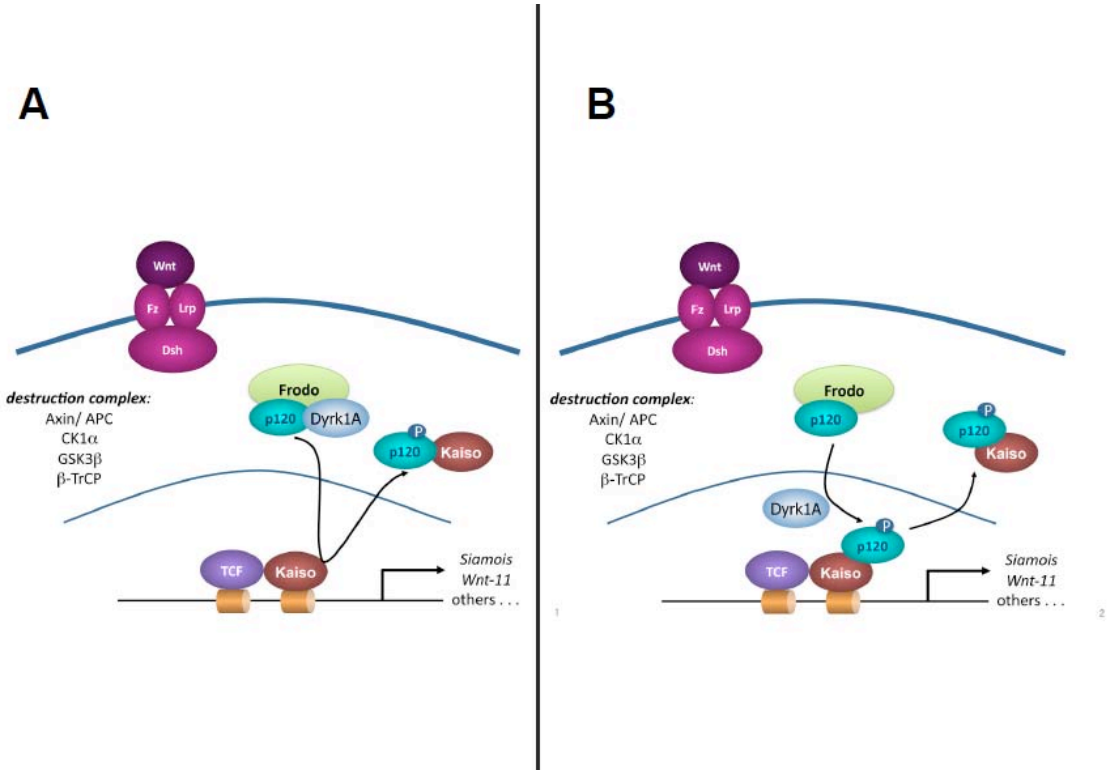
In summary, I report here that the Down-syndrome related kinase, Dyrk1A, associates with p120-catenin. This results in kinase-dependent increases in p120 levels, which in turn has an impact upon certain Wnt/ p120/ Kaiso target genes such as *Wnt11* and *Siamois*. I show that exogenous Kaiso expression rescues the effect of Dyrk1A on target genes, suggesting that Dyrk1A transmits signals through the p120/ Kaiso trajectory of the Wnt signaling pathway. The data presented here provides new thoughts regarding Dyrk1A in development and perhaps disease.

Dyrk1A in the canonical Wnt signaling pathway

Although Dyrk1A is recognized as a critical component of Down syndrome pathology, molecular analysis of Dyrk1A is at an early stage. There is accumulating evidence regarding the substrates of Dyrk1A, for example, including caspase-9, ASF (Alternative splicing factor), Tau, HPV16E7 and p53 (104, 150-154). Substrates have shown variable responses, in some instances being destabilized in conjunction with GSK3β activity, while in others showing increased or unchanged levels. Until now, to our knowledge, there has been no prior reports indicating a functional relationship linking Dyrk1A with the Wnt/ p120/ Kaiso pathway. Likewise,

knowledge gaps exist concerning upstream signals relevant to Dyrk1A activity. Intriguingly, a previous report from our lab identified Frodo as an upstream binding-mate and modulator of p120-catenin, but not of β-catenin (70, 119). The depletion of Frodo decreases p120's levels, seemingly similar to the depletion effect of Dyrk1A. Thus, in the context of addressing p120 levels, and thereby the p120/ Kaiso signaling trajectory, Frodo and Dyrk1A might functionally interact. Based upon these interactions between Frodo, Dyrk1A and p120, one conceivable model is that Frodo acts as a scaffold to recruit Dyrk1A and p120, resulting in p120's increased level. Some of such accumulated 120 might then enter the nucleus to assist in recruiting Kaiso back to the cytoplasm, thereby de-repressing/ activating certain Wnt target genes such as Siamois and Wnt11 by (Figure 29A). Dyrk1A mainly localizes in the nucleus, while p120-catenin localizes largely to the cytoplasm. Thus, another potential model is that Frodo might promote p120's shuttling into the nucleus to be phosphorylated by Dyrk1A, with the resulting increased level of p120 re-localizing Kaiso from the nucleus to the cytoplasm (Figure 29B). Further study will increase our knowledge of the p120/Kaiso trajectory of the canonical Wnt signaling pathway.

Figure 29. Potential models of Dyrk1A/ p120/ Kaiso signaling in the context of the Wnt pathway. (A) The first model hypothesizes that Frodo acts as a scaffolding protein. In the presence of Wnt ligand, Frodo is activated by unknown mechanisms and recruits p120 and Dyrk1A to enable their close association and the phosphorylation of p120. Phosphorylated/ stabilized p120 enters the nucleus and assists in trans-locating the Kaiso repressor from the nucleus to the cytoplasm, resulting in gene activation. (B) Given that Dyrk1A mainly localizes to the nucleus, p120-catenin might interact with Dyrk1A in the nucleus, as a result of p120 entry in complex with Frodo. P120-catenin would then become phosphorylated by nuclear Dyrk1A, bind Kaiso and shuttle with Kaiso to the cytoplasm to result in gene target de-repression.


A
Wnt
Fz
Lrp
Dsh
destruction complex:
Axin/ APC
CK1α
GSK3β
β-TrCP
Frodo
p120
Dyrk1A
P
p120
Kaiso
TCF
Kaiso
Siamois
Wnt-11
others . . .
B
Wnt
Fz
Lrp
Dsh
destruction complex:
Axin/ APC
CK1α
GSK3β
β-TrCP
Frodo
p120
P
p120
Kaiso
Dyrk1A
P
p120
TCF
Kaiso
Siamois
Wnt-11
others . . .

Recently, in *Xenopus*, another member of the DYRK family of protein kinases, Hipk2 was found to promote Wnt signaling via phosphorylation and relief of TCF3 repression of ventral target gene (155). Hipk2 has also been implicated in phosphorylation of β-catenin (156). It is possible that canonical Wnt signals reside upstream of Dyrk1A within the (Frodo)/ p120/ Kaiso trajectory, just as we and others recently revealed is the case for p120, and likely for ARVCF- and δ-catenin (60, 61, 70, 119, 157, 158). Dyrk1A is a dual specificity protein kinase, which auto-phosphorylates on tyrosine residues, and phosphorylates other substrates on serine/threonine residues. The activation loop is characterized by a conserved YXY sequence whose tyrosine residues have been found phosphorylated in vivo (159). Upstream or regulatory signaling components such as phosphatases have not yet been identified. Given that Dyrk modulates certain downstream Wnt target genes through the p120/Kaiso trajectory, upstream Wnt components might be involved in Dyrk1A auto-phosphorylaton/activation . Future studies will be needed to define upstream components of the Dyrk/p120/Kaiso pathway, possibly including Wnt ligands.

Likewise as noted, it also remains to be determined whether Dyrk1A, through p120, regulates small GTPases such as RhoA and Rac, perhaps thereby regulating spine and synapse formation in the developing brain. Given that Dyrk1A dosage imbalance is correlated with Down Syndrome, study of Dyrk1A-mediated effects upon p120-catenin, and thereby upon nuclear Wnt-target genes and cytoplasmic small-GTPases, warrants further examination in brain development. Understanding

such functional interactions may assist in addressing Dyrk1A's contributions to Down syndrome, possibly for example, via effects upon p120-catenin and thus Wnt signaling in CNS development.

Chapter V

Significance and Future Directions

P120-catenin was originally identified as a preferred Src substrate (160). A number of studies have focused upon p120's phosphorylation within its amino-terminal regulatory domain (77). Since p120 becomes phosphorylated in mammalian cell lines and its phosphorylation is relevant to its binding to cadherin and thus cadherin stability, the identification of kinases that modulate p120-catenin's function or levels have been sought.

In this regard, my principal advancements were made in three areas. First, I reveal two different molecular mechanisms wherein p120 stability is regulated. These mechanisms are mediated through phosphorylation conferred by GSK3β, CK1α or Dyrk1A. Second, I provide evidence that additional members of the p120-catenin sub-family, ARVCF-catenin and δ-catenin, are further subjected to modulation by the destruction complex. Third, I found that p120 isoform-1 among multiple isoforms is more responsive to the Wnt pathway. Thus, in the future, p120 isoforms will require careful examination in both developmental and tumorigenesis contexts. To achieve a better understanding of the p120 / Kaiso pathway in the context of canonical Wnt signals, further studies are required as discussed here.

[Molecular mechanisms regulating p120-catenin]

The broad impact of Wnt signaling on other catenin family members

As mentioned earlier, other p120 subfamily members such as ARVCF-catenin and δ-catenin were found to associate with Axin, a scaffolding component of the destruction machinery. The protein levels of these additional p120 subfamily members were likewise responsive to the presence of destruction complex components. Based upon findings illustrated in Chapter III, it may be beneficial to identify conserved destruction motifs within further p120 subfamily members, which presumably become phosphorylated by GKS3β or other kinases to initiate degradation. Based upon alignment data and in silico analysis employing programs to predict phosphorylation sites, (NetPhospho and GPS2.1), I have found that ARVCF, δ-catenin and plakophilin have conserved sequences predicted to be phosphorylated by GSK3β and CK1α (Figure 30). One potential GSK phosphorylation region, that in turn contains a predicted β-TrCP binding site, was identified in the C-terminal end of human, mouse, *Xenopus*, *Zebrafish* and cow plakophilin-3. Interestingly, two serines in this region were mutated to alanines in *Xenopus* and *Zebrafish*, leaving open the possibility of differences in the plakophilin-3 Wnt response of mammals and amphibians. Another conserved potential GSK sites were found in the amino-terminal region of pkp-3, but without an integrated β-TrCP binding site. Each potential GSK3β, CK1α phosphorylation site in ARVCF- and δ-catenin is illustrated in Figure 30. Future study of the molecular mechanisms

regulating p120 subfamily members will contribute to our larger understanding of the canonical Wnt pathway, as will the identification of downstream gene targets of ARVCF-catenin, δ-catenin or the plakophilins (desmosomal catenins).

Figure 30. Cross-species sequence alignment of the region of p120-catenin subfamily members harboring conserved predicted GSK3β phosphorylation and β-TrCP binding sites. In plakophilin-3, the red box includes conserved potential GSK3β sites, and a DSEXXS motif consistent with the possibility of being a β-TrCP binding site.


A
890
900
910
920
930
human pkp3
mouse pkp3 isoform2
danio rerio pkp3
xenopus pkp3
cow pkp3
β-TrCP binding site
B
280
290
300
310
320
human d-catenin
rat
mouse d-catenin
xenopus
C
360
370
380
390
400
xenopus ARVCF
mouse ARVCF
human ARVCF
rat ARVCF

What selective signaling entities act upon particular p120-catenin subfamily members?

During my graduate study, I found that upstream Wnt components modulate the p120/Kaiso pathway by inhibiting the destruction complex in a manner similar to that used in regulating β-catenin. In our group's earlier report, we showed that Frodo stabilizes p120, thereby assisting in the transmission of Wnt/ p120/ Kaiso signals to downstream target genes upon Wnt stimulation. My findings here suggest that Frodo acts upon p120-catenin, but not (or less so) upon β-catenin. For example, immuno-precipitations from *Xenopus* embryo extracts revealed the existence of a Frodo:p120 complex, while a Frodo:β-catenin complex was not resolved. Furthermore, Frodo depletion only diminished p120's levels, whereas β-catenin levels remained steady, suggesting a selective role of Frodo downstream of canonical Wnt signals. It is thus conceivable that there exist other currently unknown proteins that confer a form of selectivity for facilitating canonical Wnt signals to particular p120-catenin family members. If more such molecules are identified, another layer of complexity of canonical Wnt signaling will be revealed. For example, in my hands, Dapper, which is structurally similar to Frodo, appears to have a protective impact upon δ-catenin as well as p120-catenin (Figure 18). This suggests that Dapper might modulate catenins in response to Wnt signals as does Frodo. Since Dishevelled is a focal point in the transmission of both canonical- and non-canonical-Wnt signals, the context dependent modulation of β-catenin versus

p120 subfamily members may further involve “choices” made at the level of Dishevelled.

As mentioned earlier, a number of Wnt ligands and Frizzled receptors exist (19 & 13 in mammals, respectively). Different combination of Wnt:Frizzled complexes have distinct roles in development. For example, xWnt5a is often characterized as a non-canonical Wnt ligand. However, in the presence of hFz5, Wnt5a can induce an ectopic Spemann organizer and axis duplication in Xenopus embryos, well established properties reflecting canonical activity (161). Upon the association of Wnt ligand with the Frizzled receptor, Dishevelled becomes localized to the plasma membrane, associating with Frizzled. Given that Frodo binds Dishevelled and apparently assists in transmitting canonical Wnt signals to the p120/ Kaiso trajectory, I envisage that different Wnt:Frizzled combinations may recruit distinct modulators (Frodo, Dapper or others), with these modulators selecting one or another catenin family member in a context dependent manner. Over-expression or knock-down of Wnt ligand or Frizzled receptor components could be employed to test if we can identify differential effects upon the level of β-catenin, p120-catenin or other p120 subfamily members. Once endogenous gene targets of the differing catenins become known, they may also serve as reporters for the activity of differing Wnt ligand:receptor combinations, to again address the hypothetical differential effects upon distinct p120 subfamily members.

With regards to the second part of my graduate work focused upon Dyrk1A, recent reports by others show that the kinases Hipk1 and Hipk2 contribute to Wnt signaling in certain embryonic context, either positively or negatively affecting β-catenin levels (156, 162, 163). For example, in early ventral tissue of *Xenopus*, it was shown that Hipk2 is recruited by a β-catenin-mediated mechanism to the TCF complex, leading to TCF phosphorylation and dissociation from target gene promoters (155). Interestingly, although I did not observe p120 levels altered upon Hipk2 expression in mammalian cells, Hipk2 was also identified in yeast two-hybrid screening as a binding partner of the p120 modulator Frodo (personal communication from Dr. Sergei Y. Sokol). Dyrk family members include Dyrk1, 2, 3, and Hipk1, 2. Interestingly, our group previously reported that Frodo depletion diminished p120 levels, as I have observed upon Dyrk1A depletion. Thus, it is conceivable that Dyrk1A might act upon p120's level through phosphorylation of Frodo or an association with Frodo. Upon study of Frodo's primary sequence (GPS2.1 software), I resolved multiple potential Dyrk1A phosphorylation sites. Thus, I envisage that Dyrk1A either phosphorylates p120-catenin or Frodo, and that phosphorylation recruits Frodo to the Dyrk1A / p120 complex, stabilizing p120-catenin. Intriguingly, one of the predicted Dyrk phosphorylation sites in Frodo is conserved in Dapper, suggesting this could be a critical site for both proteins. Another possibility is that Frodo serves as a scaffold to recruit Dyrk1A to p120-catenin. Being similar in structure to Frodo, Dapper is another candidate that may participate in the Dyrk/ p120 pathway. Since Frodo associates with Dishevelled, acts upon the p120/ Kaiso pathway and modulates small GTPases, it is germane to

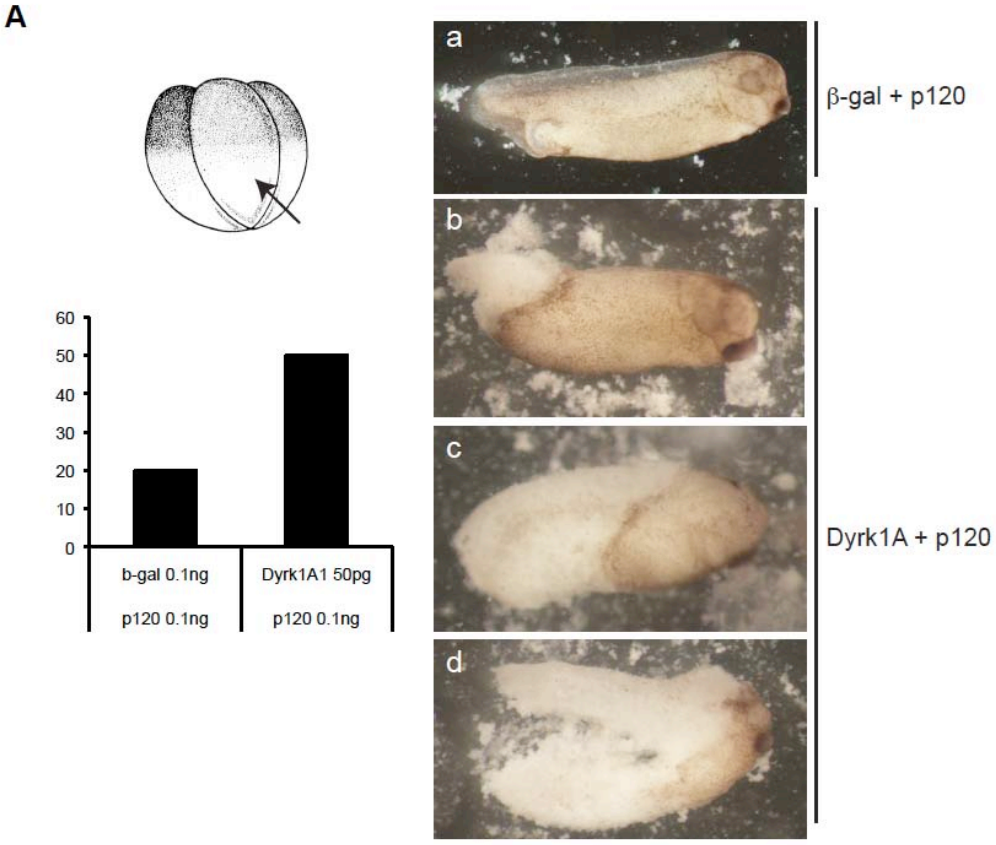
examine the potential role of Dyrk1A in these same processes. These data may provide the research community with new insights on the molecular mechanisms of Dyrk1A and its role in development or pathologies including Down syndrome.

Does Dyrk1A modulate Cadherin or small GTPases through p120?

Intriguingly, I found that ventral expression of p120-catenin with Dyrk1A results in skin fragility in *Xenopus* embryos (Figure 25). Embryos at stage 26 displayed impaired skin integrity potentially due to an effect upon cadherin-mediated adhesion and/ or upon small GTPases such as RhoA and Rac. Microinjection of either p120 or Dyrk1A alone did not confer these phenotypes. Based upon p120's positive role in the stabilization of cadherin and its modulation of small GTPases, I envisage that Dyrk1A acts through p120 to regulate either cadherin stability and/ or small GTPases. Dissociation/ association tests using cells derived from *Xenopus* embryo animal caps will be employed to test if Dyrk1A has an impact upon p120's regulation of small GTPases and cadherin. In mammalian cells, exogenous p120 is known to induce branching phenotypes via the modulation of small GTPases. Interestingly, Dryk1A expression in 293T cells results in a branching morphology similar to that seen following expression of p120-catenin. Thus, Dyrk1A depletion or over-expression will be employed to test Dyrk1A's involvement in modulating small GTPases through the elevation of p120 levels. This study may additionally provide some information regarding how the Dyrk/ p120 pathway conceivably contributes to Down syndrome pathology.

Figure 31. Skin fragility results from co-injection of Dyrk1A with p120

(A) Myc-p120-catenin was co-injected with either β-gal or Dyrk1A into the ventral-vegetal region of 4-cell stage embryos. Skin defects were observed at stage 26, when the images were taken.


A
a
b
c
d
β-gal + p120
Dyrk1A + p120
60
50
40
30
20
10
0
b-gal 0.1ng
p120 0.1ng
Dyrk1A1 50pg
p120 0.1ng

[P120-catenin in cancer and development]

P120-catenin isoforms in development and cancer

P120-catenin has multiple isoforms and its expression and localization varies depending on tissues and cell types. The ablation of p120-catenin in mouse intestine, skin, salivary gland and enamel resulted in severe developmental defects (51, 164-167). Interestingly, p120 isoform-1 is present during rat brain development, with its expression decreasing in adult brain. P120 isoform-1 is rich in the cortex, cerebellum, the olfactory bulb and the hippocampus (107). The spatial-temporal expression of p120-catenin isoforms in most tissues is not yet defined. Thus, the expression pattern of p120-catenin isoforms having distinct functions need to be evaluated. Furthermore, to investigate the roles of distinct p120 isoforms during development, rescue of p120 knock-down in Xenopus embryos would seem a reasonable approach.

P120 misexpression is associated with multiple cancer types and poor prognosis of several cancer. P120 variables include its total level of expression, intracellular localization and the relative ratios of its expressed isoforms. For example, p120 isoform-1 over-expression is evident in several lung cancers and breast cancer, correlating with poor prognosis and lymph node metastasis, poor differentiation, histological type and high TNM stage (92, 168). In stark contrast to metastatic lymph nodes wherein p120 isoform-1's level is high, in normal lung, p120

isoform-1 expression is very low. Additionally, cytoplasmic Kaiso co-localization with p120 was discovered in metastatic lung cancer lesions, fitting with p120's known capacity to displace Kaiso from gene promoters and sequester it in the cytoplasm (hence relieving Kaiso-mediated gene repression, resulting in the transcription of multiple downstream genes, most of which are unknown) (91). Although it is unclear which p120 isoform(s) is primarily responsible for Kaiso relocalization in cancer, I hypothesize that p120-catenin, especially isoform-1 is a contributing modulator of Wnt signaling (and possibly also small GTPases) in cancer metastasis. One recent study in breast cancer showed that a long p120 isoform (presumably isoform-1) was present in the nucleus of invasive ductal and lobular carcinomas. Together with studies in lung cancer, I envisage that the canonical Wnt pathway may relay signals through p120 isoform-1 that are relevant to cancer metastasis. Although considerable work has been committed to the role of p120-catenin in cancer, there remain mixed ideas of how the varying isoforms regulate tumor progression (87, 169-172). P120-catenin's role in cancer has primarily been studied with regard to E-cadherin stability, and to a lesser extent, small GTPase activity. P120-catenin was known to stabilize cadherin, inhibit Rho and activate Rac. Our recently published work together with that of others points to a new possibility, wherein the relationship between the Wnt pathway and p120 nuclear functions needs future evaluation.

Future study includes generating an antibody against active (unphosphorylated destruction box) p120 isoform-1, to detect the presence of stabilized p120 isoform-1 in cancer tissues, especially in lung and breast cancer tissues given that p120

isoform-1 is highly expressed in this pathologic context. Using this antibody, the expression levels and intracellular localization of active p120 isoform-1 will be assessed, together with that of additional Wnt pathway components using immunohistochemistry. To begin to address the role of p120 isoform-1 in cancer metastasis where it is present at elevated levels, cancer cell lines will be selectively depleted with siRNA for p120 isoform-1. Then, assays relevant to aggressive cell characteristics will be undertaken, such as measures of cell motility and invasion. These experiments will provide valuable information on p120 isoform-1 from the perspective of Wnt signaling and gene target activation.

Dyrk/ p120/ Kaiso in neural development

In rat brain development, p120-catenin is distributed at the plasma membrane (cell:cell junctions) and within the cytoplasm, suggesting a possible role of p120 in neural development. P120 is co-localized with a subset of synapses in cultured hippocampus neurons (107). In mouse forebrain, the depletion of p120-catenin reduced spine and synapse densities (52). Additionally, ectopically expressed p120 resulted in a branching phenotype in mammalian cell lines, roughly resembling neurite outgrowths (173). Interestingly, my preliminary evidence indicates that Dyrk1A promotes p120's branching phenotype (data not shown). Given that the branching phenotype was employed as an assay to uncover p120's relationship to small-GTPases in prior studies by others, this assay can also be applied to study of p120's roles in neuronal development. Appearing consistent with such p120

involvement, the p120 binding partner Kaiso, a POZ / zinc finger transcriptional repressor, is expressed strongly in brain, eye, spinal cord, and brancial arches during Xenopus embryonic development. It is thus conceivable that the Dyrk1A/p120/Kaiso pathway might participate neuronal development and differentiation. Because Dyrk1A is likely to be associated with Down syndrome, the study of this pathway in neuronal function and differentiation may provide valuable information on our larger understanding of Down syndrome and p120-catenin in brain development.

Understanding the molecular mechanisms of Dyrk1A is at early stage. There are several Dyrk1A substrates identified such as p53 and tau, and from my work, p120-catenin. However, there are no prior findings relating to upstream regulators of Dyrk1A. As alluded to above, Dyrk1A appears to participate in modulation of the Wnt/ p120-catenin pathway in development, and is thus perhaps itself regulated by more upstream events in Wnt signaling, such as those immediately following Wnt-ligand: receptor interactions. Future study will include testing upstream candidates that may impinge on Dyrk1A/ p120 pathway activity.

Vita

Ji Yeon Hong was born in Seoul, Korea on June 20, 1978, the Daughter of Young Kyu Hong and Soon Hae Lee. After completing her work at Yeouido Women's High School in Seoul, she entered Ewha Woman's University, Seoul, Korea in 1997. She received the degree of Bachelor of Science with a major in Chemistry and another degree of Bachelor of Science with a major in Biological Science. She then earned the degree of Master of Science in the Department of Dentistry, School of Medicine, Seoul National University in 2004, where she studied on Biomedical Science and Biomaterials. For the next one year, she worked as a research technician at National Institute of Agricultural Biotechnology, Suwon, Korea. In September of 2005 she left Korea to enter the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences to pursue a Doctor of Philosophy degree in Biomedical Science. Ji Yeon currently lives in Houston, Texas with her son Andrew Y. Jeong and her mother, Soon Hae Lee. Ji Yeon Hong's husband, Sung Hoon Jeong, lives in Seoul, Korea.

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