A role for *AXIN2* in oncogenesis

by

Serina Marie Mazzoni

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Human Genetics)
in The University of Michigan
2014

Doctoral Committee:

Professor Eric R. Fearon, Chair
Professor John V. Moran
Professor Diane M. Robins
Associate Professor JoAnn Sekiguchi
Professor Stephen J. Weiss

“We choose to go to the moon in this decade and do the other things, not because they are easy, but because they are hard, because that goal will serve to organize and measure the best of our energies and skills, because that challenge is one that we are willing to accept…”

John F. Kennedy, 1962

Dedication

To all the caregivers- the doctors, nurses, families and friends- and the survivors.
In the lab, we can easily forget why we're here, but you are on the front lines,
and the home front, leading the battle against cancer every day.

And to my parents, who taught me to value hard work and education.

Acknowledgements

Thank you the members of my Dissertation Committee (Eric Fearon, John Moran, Didi Robins, JoAnn Sekiguchi, and Steve Weiss) for your time and advice; and thank you to Liz Petty, for giving me my start in grad school; the connections I made with you, Didi Robins, and Kathy Collins during my interview are what brought me to Michigan. Thank you also to Lori Isom and JoAnn Sekiguchi who helped me with my transition to the Fearon Lab. And finally, thank you to Eric for letting me continue my thesis project under your direction and special expertise.

I am grateful to all the members of the Fearon/Cho Lab, particularly Ying Feng, Naoya Sakamoto, Rong Wu, Yali Zhang, and most of all Maranne Greene for keeping the lab running smoothly and for teaching me all I know about working with mice. A special thank you to Albert Liu for always being available, to Xiao-wei Chen for your help with my TALEN project, and to Eileen Whiteman for your help and support, and for always being joyful. Thank you also to Beth Walczak, my fellow BSRB grad student and Wnt Signaling buff.

I should also thank the support staff who helped organize all the paperwork, scheduling, and other tasks that are necessary to complete research and a PhD: Karen Grahl, Mary Greene, Sue Kellogg and Michelle Melis. And I would be remiss if I didn't mention all the other experts who have helped me with my research projects: Tony Antonellis, Elizabeth Hughes, Bob Lyons, Judy Poore, Thom Saunders, and I'm sure there are many others whom I've forgotten to mention.

I would especially like to thank those people in my life who have been my sources of inspiration and support during grad school. I can't say enough about all my friends and our Friday night dinners where we can forget our sorrows over cookie bars and troubleshoot protein interactions. And the Petty Lab lunch crew: Jake Higgins and Hande Kocak for being my PhD comrades; Laura Nejedlik for being an inspiration as a mother, wife, and scientist; and Jenny Keller, who has taught me so much about science and has been a role model for both grad school and life.

Ultimately, I must thank my family: my mom and dad have been the greatest, constant source of support and encouragement all my life; and to Dan who has been with me from prelim through PhD defense with an endless supply of cheer and funny animal GIFs.

Table of Contents

List of Figures

List of Tables

List of Appendices

Glossary of Acronyms and Abbreviations

ACA	Adrenocortical adenoma
ACC	Adrenocortical carcinoma
AdCC	Adenoid Cystic carcinoma
APC	AdenomatouspPolyposis coli
BrdU	Bromodeoxyuridine (5-bromo-2'-deoxyuridine)
CC	Cervical carcinoma
cDNA	Complementary DNA
CKI	Casein kinase I
CRC	Colorectal cancer
Cre	Cre recombinase
CTNNB1	Catenin (cadherin-associated protein), beta 1
DIX	Disheveled-AXIN
DVL	Dishevelled
EM	Endometrial carcinoma
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FAM	Familial melanoma
FAP	Familial adenomatous polyposis
fl	Floxed
GC	Gastric carcinoma
gDNA	Genomic DNA
GSK3β	Glycogen synthase kinase 3 beta
HB	Hepatoblastoma
HCC	Hepatocellular carcinoma
hpf	Hours post-fertilization
HRP	Horse radish peroxidase
IP	Immunoprecipitation
JNK	c-Jun N-terminal kinase
LEF	Lymphoid enhancer factor
LOH	Loss of heterozygosity
LRP	Low density lipoprotein receptor-related protein
MB	Medulloblastoma
MLPA	Multiplex ligation-dependent probe amplification

MMR	Mismatch repair
MSI-H	Microsatellite instability-high
MSI-H	Microsatellite instable- high
MSS	Microsatellite stable
MYH	MutY homolog
NMD	Nonsense-mediated decay
OA	Okadaic acid
OEA	Ovarian endometrioid adenocarcinoma
PAR	Poly(ADP-ribose)
PBL	Peripheral blood lymphocyte
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PP2A	Protein phosphatase-2A
RGS	Regulator of G protein signaling
RNF	RING finger
SAPK	Stress-activated protein kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAM	Tamoxifen
TCF	T-cell Factor
TNKS1/2	Tankyrase 1/2
trAXIN2	Truncated AXIN2
USP	Ubiquitin-specific protease
UTR	Untranslated region
WB	Western blot

Chapter 1: Introduction

The Wnt/β-catenin pathway

The Wnt pathway is typically divided into two arms, the canonical and non-canonical Wnt pathways. Both pathways serve to transmit an extracellular signal, a Wnt ligand, to the nucleus via different mechanisms. In humans, there are 19 Wnts ligands, and the first Wnt, Wnt-1 (*int-1*) was discovered as the gene activated by the insertion of mouse mammary tumor virus elements in virally-induced mouse mammary tumors[1]. The Wnts are secreted glycoproteins that can activate or repress canonical or non-canonical signaling, depending on the Wnt ligand and the receptor/co-receptor combination that is bound[2; 3]. The canonical, or β-catenin dependent, Wnt pathway regulates cell fate during development and cellular homeostasis in adult tissues, and canonical Wnt pathway dysregulation is seen in many cancer types (reviewed in Anastas and Moon, 2013)[4]. The canonical Wnt pathway transmits extracellular Wnt signals to the nucleus via effects on β-catenin levels and localization[5]. In the remainder of my thesis, when I discuss the Wnt pathway, it will be in reference to the canonical, β-catenin dependent pathway.

β-catenin (encoded by the *CTNNB1* gene) was first identified as the *Drosophila* protein, armadillo, that played a key role in body patterning and in the assembly and function of adherens junctions[6-8]. A*rmadillo* mutants were found to have a similar phenotype to *wingless* (*Drosophila int-1* homolog[9]) mutants and armadillo protein accumulated in cells that expressed

wingless[7; 8]. This led to the conclusion that the wingless and armadillo proteins cooperate in the cell, and specifically, that armadillo protein stability is dependent on wingless; or in vertebrate terms, that β-catenin protein stability is dependent on a Wnt signal. In the absence of an activating Wnt ligand, a complex of proteins assembles in the cell to phosphorylate β-catenin at multiple residues in its amino-terminal domain. The phosphorylated β-catenin is then recognized by a ubiquitin ligation protein complex and subsequently targeted for degradation by the proteasome[10]. Amongst other proteins, APC[11-14], AXIN1[15; 16], and AXIN2[17; 18] provide a scaffold for the assembly of a β-catenin destruction complex, while GSK3β and CKI are responsible for phosphorylating β-catenin[19-22]. The phosphorylation of β-catenin occurs at four residues: S33, S37, T41, and S45, and mutation of these residues prevents phosphorylation, rendering β-catenin resistant to degradation and thus, constitutively activated[23; 24].

When an activating Wnt ligand is present, the destruction complex is inhibited[5] and the "free", signaling pool of β-catenin accumulates and translocates to the nucleus, where β-catenin binds to TCF/LEF transcription factors to modulate the expression of target genes (Figure 1.1)[25-27]. If and how the destruction complex disassembles and β-catenin becomes "free" is still debated[28]. However, the prevailing model is that upon Wnt ligand binding to the LRP receptor, the receptor is phosphorylated by GSK3β and CKI and destruction complex components, such as the AXIN proteins, are re-localized to the cell membrane where they bind to LRP and Dishevelled[29]. This re-localization of destruction complex proteins reduces the number of functional destruction complexes available, so newly synthesized β-catenin will not be bound, phosphorylated and targeted for degradation, but will instead be free to go to the nucleus to drive transcriptional programs[30].

The AXIN1/2 proteins and their functions

There are two AXIN proteins: AXIN1 and AXIN2. In the case of AXIN2, the mouse protein is also known as conductin and the rat protein is referred to as Axil. *Axin1* was initially identified as the locus responsible for a series of dominant "kinky" tail mouse phenotypes, all resulting from spontaneous retrotransposon insertions into an exon of *Axin1*[31]. When co-injected with Wnt pathway components into the ventral side of *Xenopus* embryos, *Axin1* mRNA inhibits ectopic axis formation, identifying the protein as a negative regulator of Wnt signaling[32]. The primary function of AXIN1 in the Wnt pathway is in the assembly of the β-catenin destruction complex, thus inhibiting the expression of Wnt- and β-catenin-dependent target genes. Aberrant activation of the Wnt pathway and dysregualtion of β-catenin protein levels and localization is widely presumed to be a main driver of colorectal cancer (CRC). Because AXIN1 is a negative regulator of β-catenin, AXIN1 has been classically thought of as a tumor suppressor protein. Experiments in CRC and hepatocellular carcinoma (HCC) cell lines showed that ectopic expression of Axin1 can cause growth suppression, reduce total β-catenin protein levels and inhibit a TCF-responsive luciferase reporter[33]. Additionally, a mouse model of loss of *Axin1* in the liver has an increased risk of liver cancer[34], further evidence of a role for AXIN1 in tumor inhibition.

AXIN2 was initially identified by yeast-two-hybrid interactions with β-catenin and GSK3β and named for its homology to AXIN1[17; 18]. AXIN2, like AXIN1, acts as a scaffold to help assemble the β-catenin destruction complex, and the two proteins how high similarity to one another in several domains (see Figure 1.2). Similar to AXIN1, AXIN2 negatively regulates β-catenin-

dependent Wnt signaling. Injection of the rat *Axil* cDNA in *Xenopus* inhibits the axis duplication caused by activated Wnt signaling, and dorsal *Axil* cDNA injection results in ventralized embryos[18]. The two AXIN proteins are considered functionally equivalent, as an *Axin2* cDNA inserted into the *Axin1* mouse locus rescues the *Axin1*-null lethality[35]. However, while the *Axin1*-null mouse is lethal in embryogenesis at e9.5[36; 37], mice carrying homozygous null mutations in *Axin2* are viable and fertile, with a mild skull abnormality, indicating that the two genes are not redundant *in vivo*[38]. This difference in phenotypes is likely due to the differential expression patterns of the two genes. The expression of *AXIN1* is ubiquitous in various tissues[32], while *AXIN2* shows a more restricted developmental and cell-type-specific expression pattern[18]. Uniquely, *AXIN2*, but not *AXIN1*, is a robust transcriptional target of β-catenin-dependent Wnt signaling[39-41], and *AXIN2* expression is elevated in cancers with activating Wnt pathway mutations. Because *AXIN2* is positively regulated by upstream Wnt- and β-catenin-dependent signals and because the AXIN proteins are the least abundant members of the destruction complex[42], changes in AXIN2 protein levels could be a key negative feedback mechanism for the regulation of Wnt/β-catenin signaling in cells.

The AXIN proteins contain multiple conserved functional domains. Starting at the N-terminus is the Tankyrase (TNKS) binding domain which contains two discrete sequences that regulate AXIN protein stability[43]. The AXIN proteins interact with TNKS, which modifies AXINs by the addition of a poly(ADP-ribose) molecule(ref). This PARsylation then directs the AXIN proteins for ubiquitination and degradation[44; 45]. The RGS domain is named for the Regulators of G-protein Signaling protein family. This domain interacts with a specific form of the G-alpha12 protein, but unlike other RGS-containing proteins, experiments with AXIN1 showed

that it does not regulate the GTP-ase activity of this G-protein[46]. The RGS domain of AXIN1/2 is most known for mediating binding to APC[17]. Near the center of the AXIN1 or AXIN2 protein is the β-catenin-binding domain[17; 18], and at the C-terminus is the DIX domain, a dimerization domain named for two proteins which share this motif - Dishevelled and AXIN[16; 17; 47-49].

The classical view of the AXIN proteins has been that they function as tumor suppressors via inhibition of Wnt signaling. Other *in vitro* and mouse studies suggest the contrary; that AXIN2, in particular, which is highly expressed in cancer cells with gain-of-function Wnt pathway mutations, could promote tumor phenotypes such as invasion or metastasis[50; 51]. However, in a study of CRCs, *AXIN2* expression had no correlations with survival or prognostic factors, so the clinical significance of *AXIN2* expression changes in cancer is unclear[52]. While the *Axin2*-null mouse has no reported cancer phenotype, studies with mammary stem cells have shown that *Axin2*-null cells have a sustained response to Wnt ligands and that *Axin2*-null mammary stem cells outcompete wild-type cells to repopulate the mammary fat pad[53]. Additionally, stabilization of AXIN1 and AXIN2 protein in breast cancer cell lines inhibited Wnt signaling, migration, and under low serum conditions, reduced colony formation[54]. Moreover, using a wound-healing model, it was shown that *Axin2*-null mice display increased Wnt signaling and cell proliferation, and decreased cell death when compared to heterozygous mice[55]. Taken together, these data suggest that loss of *AXIN2* expression or function, rather than increased *AXIN2* expression, would confer favorable traits to cancer cells.

Both AXIN1 and AXIN2 have been implicated in non-Wnt signaling pathways. AXIN1 has been reported to inhibit Ras/ERK signaling, although the effect appeared to be dependent on β-

catenin so it is not clear if AXIN plays a direct role in the modulation of ERK signaling[56]. A second study linking AXIN1 to Ras regulation found that both AXIN1 and APC can promote Ras degradation via the same E3-ligase that targets β-catenin[57]. AXIN1 has also been show to interact with MEKK1 and MEKK4 and to activate JNK when overexpressed[58; 59]. AXIN2 has also been implicated in other cellular functions including repression of the *MYC* gene via competition with β-catenin for TCF factors[60], regulation of the Wnt pathway in a cell-cycle dependent manner[61] and genomic instability[62].

The Wnt pathway and colorectal cancer

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the U.S. with a five-year survival rate of 64.7% (http://seer.cancer.gov/statfacts/html/colorect.html). Mutations that lead to Wnt pathway activation are found in approximately 90% of sporadic CRCs[63], and germline, inactivating mutations in the *APC* gene cause the familial colon cancer syndrome: familial adenomatous polyposis (FAP)[12]. Activation of the Wnt pathway is believed to be one of the important first steps in the genesis of CRC[64] and this key role is well-supported by mouse models harboring mutations in *Apc*. Loss of *APC* is believed to activate the Wnt pathway by inhibiting the assembly of the β-catenin destruction complex, thus increasing the levels of "free" β-catenin which activate a transcriptional program that drives cell proliferation and survival. In tumors, this activation of the Wnt pathway is frequently seen as shift in β-catenin localization from the membrane to the nucleus[65].

The most common mode of Wnt pathway activation in CRC is by complete loss of the APC protein by a combination of nonsense and frameshift mutations, loss of one *APC* copy, or

by transcriptional silencing[63]. Other Wnt pathway mutations have been reported in CRC, and 16 Wnt pathway-associated genes were identified as mutated in CRCs by the Cancer Genome Atlas study of 2012 (see Appendix A)[63]. Indeed, mutations in both *AXIN1* and *AXIN2* have been reported in association with CRC, although the functional significance of these mutations and their role in cancer is largely untested (to be discussed in Chapter 2).

AXIN stabilization as a cancer therapeutic

Because the Wnt pathway is activated in a wide variety of cancers, there has been broad interest in therapeutic approaches that target Wnt signaling. The Wnt pathway can be targeted at the level of Wnt production or Wnt response, and a number of small molecules exist that can activate or repress Wnt signaling[66; 67]. As negative regulators of the Wnt signaling pathway, the AXIN proteins are attractive targets, especially given the increased level of AXIN2 protein in most cancers with activated Wnt signaling (ref.). AXIN protein stability is regulated, in part, by poly-ADP-ribosylation (PARsylation). Two PARsylating enzymes, TNKS1 and TNKS2, PARsylate AXIN1 and AXIN2[43]. This modification primes the AXIN proteins for ubiquitination by the E3 ubiquitin ligase, RNF146, directing them to the ubiquitin/proteasome pathway for degradation[44; 45]. The ubiquitin-specific protease, USP34, counters RNF146 by removing ubiquitin molecules and stabilizing the AXIN proteins[68]. Regulation of this PARsylation-dependent turnover of the AXIN proteins has been targeted with small molecules that inhibit the TNKS1/2 enzymes[43; 69-71]. TNKS inhibition with several small molecules (XAV939, JW55 and G007-LK) has been shown to stabilize AXIN1 and AXIN2, and to inhibit Wnt signaling in CRC cells: XAV939 treatment of selected CRC cell lines resulted in decreased colony formation in cell

lines that are dependent on Wnt signaling due to inactivating mutations in *APC*, but not in cell lines lacking Wnt pathway defects[43]; experiments with JW55 saw similar growth inhibition only in CRC cell lines with *APC* and *CTNNB1* mutations, but not in a CRC cell line with intact Wnt signaling, or in the HeLa cell line; and G007-LK was shown to block cell-cycle progression, induce differentiation and inhibit colony formation in an *APC* mutant CRC cell line.

In vivo, G007-LK reduced the expression of Wnt target genes in the small intestine and inhibited xenograft growth of one out of three CRC cell lines tested[71]. JW55 was found to inhibit *Apc*-dependent tumorigenesis in a mouse model of CRC, implying that stabilization of the AXIN proteins could be a treatment option for cancers with *APC* mutations[69]. Presumably, the increased levels of the AXIN scaffold proteins can compensate for the loss of the APC scaffold and increase the number of functional β-catenin destruction complexes in the cell. Furthermore, the TNKS inhibitor JW74 was found to stabilize AXIN2 in osteosarcoma cell lines, resulting in differentiation and a decrease in proliferation[70]. Together, these data suggest that small molecules that stabilize the AXIN proteins could be useful therapeutics for CRC and other cancers with activated Wnt signaling. Whether or not AXIN stabilization has any effects on normal tissues and stem cells remains to be determined, although staining of normal mucosa in JW55 treated mice suggests that there is no effect on proliferation or the expression of intestinal stem cell markers[69].

Concluding remarks

The canonical Wnt pathway is an important regulator of differentiation and cell fate and aberrations in the Wnt pathway are seen in many cancers. Wnt signaling transmits cellular

responses via the effector molecule β-catenin. A β-catenin destruction complex composed of APC and the AXIN proteins restricts the levels of β-catenin that are free to go to the nucleus, thus regulating Wnt signaling. Germline mutations in *APC* lead to an inherited CRC syndrome, and somatic mutations in *APC* or other genes that activate Wnt signaling are seen in roughly 90% of sporadic CRCs. Modulation of the Wnt pathway has potential for the prevention or treatment of cancers, and the AXIN proteins have been proposed as specific targets for inhibition of the Wnt pathway, although their role in cancer is still debated. In my thesis work, I have addressed this question by testing the functional consequences of a CRC-associated germline *AXIN2* mutation on AXIN2 protein function, and by examining the effects of loss of *Axin2* in a mouse model of CRC.

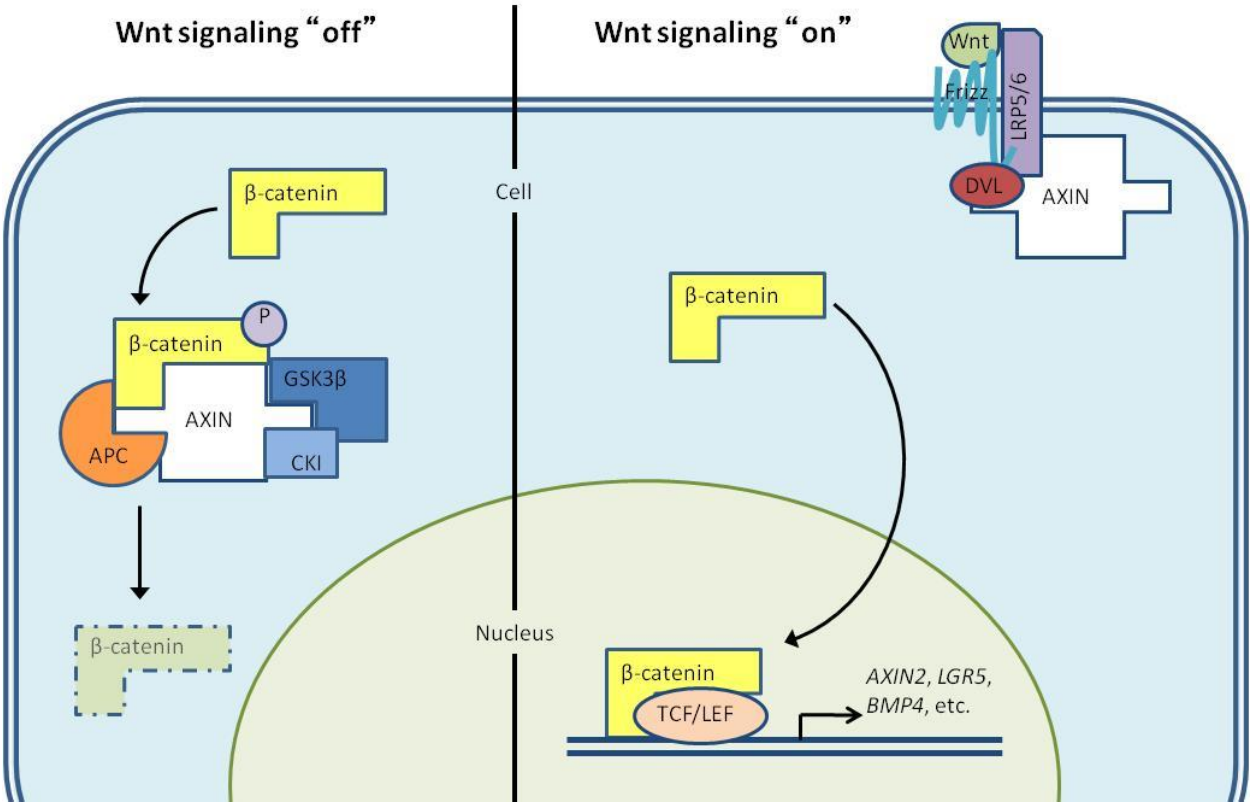


Figure 1.1- Schematic of Canonical Wnt/β-catenin signaling
In the absence of an activating Wnt ligand (left), β-catenin is bound by the β-catenin destruction complex, phosphorylated, and targeted for ubiquitin-mediated degradation. When an activating Wnt ligand is present (right), AXIN molecules are recruited to the plasma membrane and β-catenin is free to translocate to the nucleus where it interacts with TCF/LEF factors to activate specific target genes, including *AXIN2*.

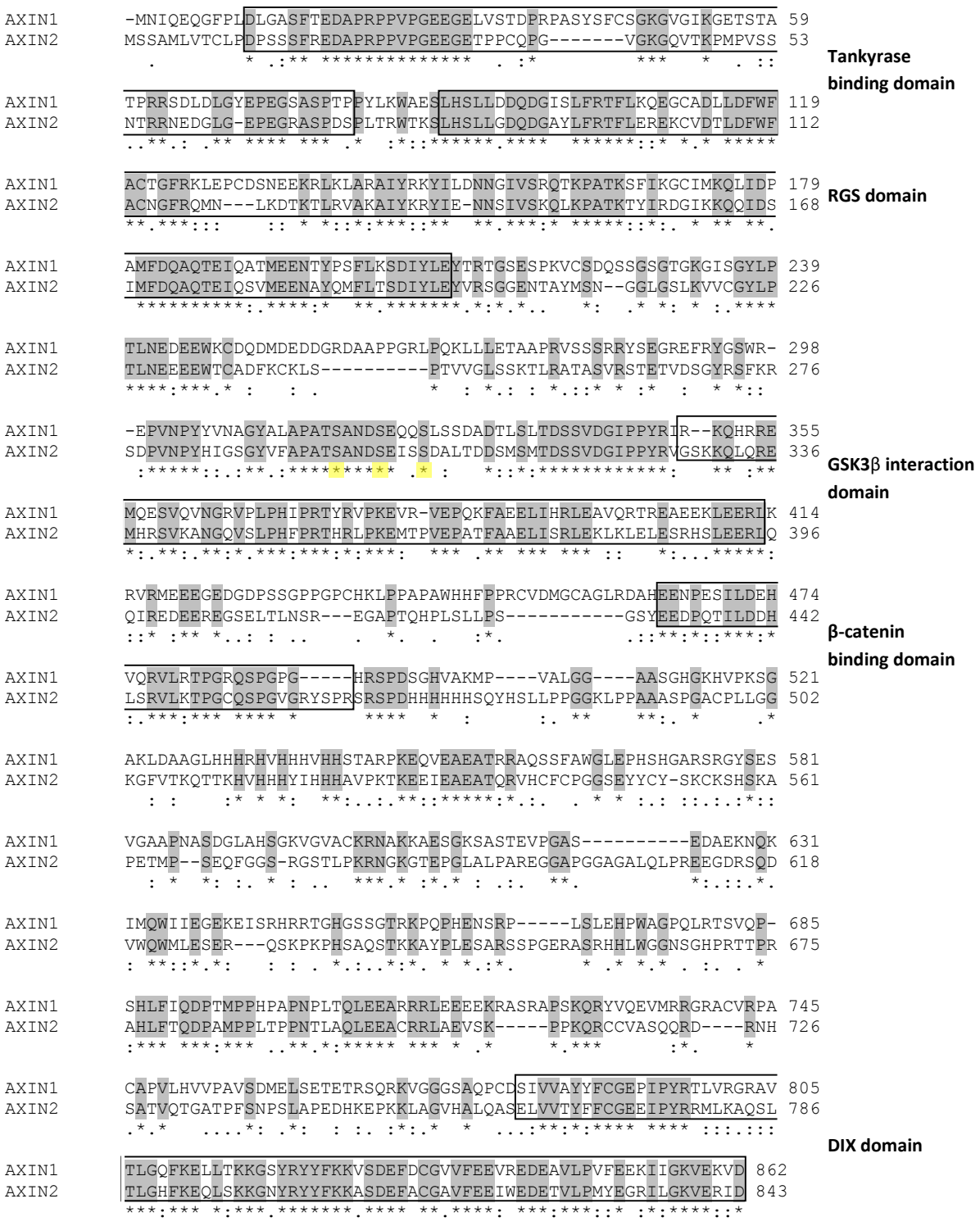


Figure 1.2- Conservation of important functional domains in the AXIN proteins
Functional domains of AXIN1/2 are boxed in with amino acid conservation measured by Gonnet[72] PAM 250 scores: >1.5 (+), 0.5 to 1.5 (:), 0.5 to -0.5 (.).

Chapter 2: *AXIN1* and *AXIN2* sequence alterations associated with cancer

Abstract

Mutations in the *APC* gene, which encodes a multi-functional protein with a well-defined role in the canonical Wnt pathway, underlie a rare, inherited form of CRC and the majority of sporadic CRCs. However, not all sporadic and familial CRCs can be explained by mutations in *APC* or other genes with well-established roles in CRC. The AXIN1 and AXIN2 proteins function in the canonical Wnt pathway, and *AXIN1/2* alterations have been proposed as key defects in some cancers. Here, we review the *AXIN1* and *AXIN2* sequence alterations reported in association with cancer, with the goal of vetting the evidence that some reported variants may have key functional roles in cancer development.

Introduction

Somatic mutations in genes of the canonical, or β-catenin-dependent, Wnt pathway are found in approximately 90% of CRCs[63]. These mutations contribute to CRC development by stabilizing the "free" signaling pool of β-catenin via disruption of the "β-catenin destruction complex", which includes the APC, AXIN, and GSK3β proteins. The most common destruction complex defects in CRCs are loss-of-function mutations in the *APC* tumor suppressor gene, although a subset of CRCs have *CTNNB1* mutations encoding a mutant β-catenin protein that is resistant to regulation by the destruction complex[63]. While *APC* is the most frequently mutated

Wnt pathway tumor suppressor gene, both germline and somatic mutations in the *AXIN1* or *AXIN2* genes have been identified in a subset of CRCs and in several other cancer types. Following the first reports of *AXIN1/2* mutations in cancer, other studies have been completed and *AXIN1/2* sequence polymorphisms in the general population have been better defined. Many of the previously reported "mutations" in *AXIN1/2* have now been included in dbSNP[73], suggesting that rather than somatic alterations, these sequence changes are likely germline polymorphisms. The following is a comprehensive update of the reported constitutional and somatic *AXIN1* and *AXIN2* sequence variants described thus far, in order to better understand which of the sequence variants are most likely to confer functional consequences for cancer.

Colorectal Cancer - Germline Mutations in *AXIN1/2*

Inactivating mutations in the *APC* gene are the most frequent mechanism for activating the Wnt pathway in CRCs, with about 80% of CRCs harboring somatic *APC* mutations[63]. About 0.5-1.0% of CRCs arise in individuals with FAP, who carry an inactivating germline *APC* mutation[74]. Colon and rectal adenomas and CRCs arise in those individuals with FAP, in part, due to somatic genetic or epigenetic inactivation of the remaining wild-type *APC* allele, leading to outgrowth of cells with no functional *APC* alleles and dysregulated β-catenin levels. However, germline *APC* mutations only account for a subset of the CRC cases with a hereditary component. *AXIN1* was analyzed as a candidate gene that might harbor CRC-associated germline mutations in a set of 124 CRC patients from the UK[75]. Several silent mutations were identified as well as seven heterozygous, missense mutations in various exons. Four of these mutations were found only in patients, while the other three were also found in controls, but at

lower frequencies. One of the patient-specific mutations has also been described in CRC cell lines[76] and is suggested to interfere with the interaction of AXIN1 and GSK3β[77]. The four patient-specific mutations have since been added to dbSNP.

There are only three reported germline mutations in *AXIN2* that have been associated with CRC risk (see Table 2.1 for more detailed information). The first is a premature stop codon in exon 7 that was found in a family with oligodontia (the absence of multiple adult teeth) and a strong history of CRC[78]. The CRC phenotype in this family was variable, and the age at CRC diagnosis for carriers ranged from 27 to 64 years. A second report of a family with oligodontia and a CRC risk found another premature stop codon, also in exon 7[79]. This family also had a variable cancer phenotype including one case of early-onset breast cancer. A third germline *AXIN2* mutation was found in a family with an apparently milder form of familial polyposis, known as attenuated FAP. Of note, this family had no oligodontia or ectodermal dysplasia phenotype[80]. The *AXIN2* mutation in this third family was a missense mutation in exon 5 with possible deleterious effects. However, the role for this *AXIN2* mutation in tumorigenesis seems quite uncertain, as the mutant allele was lost in adenomas and polyps from the proband and his sister. The three reported germline *AXIN2* mutations are heterozygous, suggesting that *AXIN2* mutation could perhaps predispose to cancer via a classical tumor suppressor model where the second allele is inactivated during tumor formation. However, loss of the wild-type *AXIN2* allele was not seen in the missense mutation family (third family), and the state of the wild-type *AXIN2* alleles has not been studied in cancer tissues from individuals carrying the two different *AXIN2* nonsense mutations. It is also possible that the nonsense alleles could produce truncated proteins with dominant or haplo-insufficient effects.

A 2005 paper aimed to counter some prior reports suggesting that constitutional *AXIN2* sequence variants play an important role in CRC development. Specifically, the paper reported that analysis of 82 probands from families with hereditary CRC revealed 20 *AXIN2* sequence variants in 19 individuals[81]. Silent and intronic mutations were ruled non-pathogenic and none of the identified missense mutations were enriched in patient populations when compared to controls. The authors thus concluded that germline *AXIN2* mutations are not associated with an increased CRC risk. While the findings in this 2005 paper do not support a role for germline *AXIN2* mutations in CRC risk, the collection of familial CRC cases studied was moderate and none of the probands had oligodontia. Another paper reporting on analysis of 31 patients with multiple adenomas or CRC found two notable *AXIN2* germline variants in a single patient, but neither variant was clearly pathogenic based on Polyphen analysis: T510T and N412S[82], and the N412S allele is now included in dbSNP.

Colorectal Cancer - Somatic Mutations in *AXIN1/2*

Shortly after the identification of a role for the AXIN1 protein in the β-catenin destruction complex, screening of *AXIN1* for mutations in selected patient samples and cell lines from various cancers was undertaken[76]. Two missense alterations were seen in the CRC cell lines studied. The first was S215L, found in the LS513 cell line, which also exhibited loss of heterozygosity (LOH) of the wild-type *AXIN1* allele. The second variant, L396M, was found in three cell lines: HCT-8, HCT-15, DLD-1, and the authors chose to follow up on this alteration because of the conservation of the affected amino acid and its location in the GSK3β binding domain. Expression of a fragment of the mouse *Axin1* gene with an analogous mutation indeed

showed reduced binding to GSK3β, suggesting this variant allele may have functional consequences on the ability of the AXIN1 protein to regulate β-catenin. Because these variants were identified in cell lines, with no matched normal DNA, it was unclear if the *AXIN1* variants are somatic mutations or germline polymorphisms. Both The S215L and L396M variants have since been listed in dbSNP, likely suggesting that these variants represent germline sequence variants, and not somatically acquired mutations.

A 2002 report comparing CRCs harboring mismatch repair pathway (MMR) defects (so-called microsatellite instability-high or MSI-H CRCs) versus CRCs with intact MMR (microsatellite-stable or MSS CRCs) suggested that *AXIN1* mutations may be important in MSI-H CRCs[83]. Out of 33 MSI-H CRC samples studied, eight somatic, missense mutations in *AXIN1* were found in seven tumors. No somatic mutations predicted to alter AXIN1 protein encoding sequences were found in the MSS samples. In the MSI-H CRCs with *AXIN1* somatic mutations no LOH analysis was done. But, one tumor did show two different *AXIN1* mutations, potentially representing selection for *AXIN1* bi-allelic defects, perhaps akin to the situation seen for some tumor suppressor genes. Another study of *AXIN1* mutations in 54 CRC samples reported five missense mutations and one nonsense mutation[84]. No matching normal tissue or blood samples were available to determine if these were somatic or germline changes. *AXIN2* was not assessed in either study.

A study by Liu et al. reported that 11 of 45 MSI-H CRCs tested had somatic mutations in *AXIN2*[85]. All of the mutations were frameshifts in exon 7 mononucleotide tracts that led to premature stop codons in the *AXIN2* open reading frame. In 60 CRCs with intact MMR, none contained an exon 7 *AXIN2* frameshift. Only two of the eleven MMR-defective CRCs with *AXIN2*

frameshifts showed loss of the wild-type allele. This result is not unexpected because LOH is infrequent throughout the genome of MMR-defective CRCs[86]. Assuming the remaining *AXIN2* allele was not silenced by epigenetic mechanisms in the other 9 of the 11 MMR-deficient CRCs with a frameshift mutation in one allele, the absence of demonstrable bi-allelic *AXIN2* defects suggests that if the *AXIN2* variants were key in promoting colorectal tumorigenesis, they might have done so via dosage-dependent effects and/or through some potential gain-of-function of the truncated AXIN2 proteins encoded. The authors did not rule out nonsense-mediated decay for *AXIN2* transcripts with premature stop codons. Of some interest, they did show that overexpression of a truncated AXIN2 protein, predicted to result from the frameshifts, promoted β-catenin accumulation in cells in culture, consistent with a possible gain-of-function role for the truncated AXIN2 protein.

In an analysis of 39 sporadic MSI-H CRCs, frameshifts at *c.1994* or *c.1995* in the exon seven polyG-tract of *AXIN2* were found in eight tumors[87]. The connection between *AXIN2* exon 7 frameshifts and MMR-deficient CRCs was further supported by a study of 310 CRCs[88]. Sequencing of exon 7 revealed heterozygous frameshift mutations in seven tumors, all of which were MSI-H. Four of the seven tumors also had *APC* mutations, perhaps raising some uncertainties about whether the *AXIN2* frameshift mutations were functionally significant in CRC development or just a potential marker of the MMR-defective phenotype. Finally, a survey of *AXIN2* sequence alterations in sporadic CRCs and some CRC cell lines identified several silent mutations, one 12-bp in frame deletion (*del2013-2024*), and potentially pathogenic frameshift mutation (*1993del1G*), which led to a premature stop at L688X[89]. This frameshift was seen in tumors with or without *APC* mutation, but only in MSI-H cancers, as reported previously[85]. The

12-bp deletion occurred in a MSS tumor and resulted in the in-frame loss of four amino acids, including two potential phosphorylation sites[89].

A study of a Kashmiri population looking for *AXIN* mutations in CRCs found two previously reported *AXIN2* single nucleotide polymorphisms and a novel missense variant A695S[90]. The three patients with this sequence variant were diagnosed at ages 50, 57, and 65 and all presented with well-differentiated tumors with LOH of the DCC region. While the authors reported using adjacent normal tissue in their analysis, this variant has since been included in dbSNP, and because it was found in 6% of the patients, it is likely a germline polymorphism.

In addition to somatic sequence changes, changes in *AXIN2* expression levels have been proposed as a potential contributing factor in CRC progression[91]. In a study of gene expression data *AXIN2* expression was significantly reduced in MSI-H CRCs compared to MSS cases. Of note, the *AXIN2* gene is directly regulated by the Wnt/β-catenin pathway, and elevated *AXIN2* expression is observed in most CRCs with *APC* mutations or other canonical Wnt pathway defects. Consistent with the gene expression data, the *AXIN2* promoter was found to be hypermethylated in 10 out of 27 MSI-H samples, but in none of the MSS samples. Additionally one of the nine MSI-H CRCs with *AXIN2* promoter hypermethylation had a frameshift leading to a premature stop codon (L688X). Ectopic expression of *AXIN2* in a MSI-H CRC cell line with low levels of endogenous protein resulted in inhibition of cell growth, suggesting that AXIN2 may act as a tumor suppressor in MSI-H CRCs. A second study also found *AXIN2* methylation and reduced expression in MSI-H CRCs[92]. The authors additionally reported that *AXIN2* expression was correlated with certain clinical and pathological features of Sessile Serrated Adenomas.

Hepatocellular Carcinoma (HCC) and Hepatoblastoma (HB)

Germline mutations in *APC* are associated with an increased risk of HB, suggesting that other components of the Wnt pathway may be important in HB tumorigenesis[93]. Sequencing of the *AXIN1* gene in 22 HBs revealed eight exon variants, seven of which were silent[94]. The eighth variant, T58M, was also found in peripheral blood, so it may be a potentially benign polymorphism. A second study of sporadic HB tumors and HB cell lines found two large, out of frame deletions (52-bp and 1624-bp) and one somatic, missense mutation (S762N) in *AXIN2*[95].

HCCs frequently display activated Wnt signaling, but often lack mutations in *CTNNB1* or *APC;* hence, the *AXIN1* gene was screened for mutations. Out of 100 primary HCCs studied, six *AXIN1* alterations were found in five tumors, including three deletions and three premature stop codons[33]. In three of the four *AXIN1*-mutant tumors that could be fully analyzed, LOH of the wild-type *AXIN1* allele was seen. Out of six HCC cell lines, two were found to contain deletions in both *AXIN1* alleles. The authors then investigated the *AXIN1* alterations in the HCC lines dysregulated Wnt signaling and/or cell proliferation. One of the two AXIN1-mutant HCC cell lines showed increased β-catenin reporter activity, and, in all six cell lines, overexpression of *AXIN1* was growth suppressive. The authors therefore concluded that *AXIN1* may act as a tumor suppressor in HCC.

Further analysis of HCC and HB has revealed some additional alterations in *AXIN1* and *AXIN2*[96]. While no *AXIN2* variants were seen in 27 HBs, two *AXIN1* missense variants were found (G650S and R841Q). Both of these sequence variants have since been added to dbSNP with minor allele frequencies of 0.0147 and 0.0101, respectively. Even though the authors

stated that they used matched normal samples to specifically identify somatic alterations, it is possible that these variants are just germline polymorphisms. In a study of 73 HCCs, seven somatic variants were found in *AXIN1*: five missense alterations, one 12-bp insertion, and a 1-bp deletion leading to a premature stop codon. LOH of the wild-type AXIN1 allele was seen in four of the five *AXIN1*-mutant HCCs tested, further supporting a role for *AXIN1* as a tumor suppressor in HCC. In this same collection of 73 HCCs, two *AXIN2* sequence changes were identified, both in exon 7: a missense mutation (R659W) and a 12-bp deletion (del2013-2024). The 12-bp deletion has since been identified in many other cancer types, and the missense allele has been included in dbSNP.

Gastric Carcinoma

Because of the highly mutable mononucleotide repeats present in *AXIN2*, one study looked for exon 7 *AXIN2* alterations in gastric cancers with MSI[97]. Nine of the 60 cancers had an *AXIN2* frameshift mutation, and all of the cancers with an *AXIN2* mutation were MSI-H.

Medulloblastoma (MB)

The incidence of MB is higher in individuals with *APC* mutations[98]. Because of the involvement of the Wnt pathway in MB, studies have looked for *AXIN1* and *AXIN2* mutations in MBs. Two reports identified variants in *AXIN1.* The first report of 86 MBs and 11 MB cell lines found one somatic missense alteration in exon 1 (P218S) and ten other single base pair changes, three that were previously identified silent polymorphisms, and four that resulted in amino acid changes[99]. The authors deemed these alterations to all be polymorphisms because

they were germline, rather than somatic. The study also found seven large deletions, three spanning exons 1-5, and four in the C-terminus. LOH studies in these MBs were inconclusive.

A second report looked in more detail at 39 primary MBs and concluded that the previously reported deletions were artifacts, but that an *AXIN1* SNP at nucleotide 16 of intron 4 is significantly over-represented in MBs and could be informative[100]. The authors also found several silent variants and two missense variants in exon 1, P218S and S226C.

Based on the work with *AXIN1,* a report looking for *AXIN2* mutations in MB biopsies and cell lines found two missense mutations in exons 7 and 8 (Q696R and S738F)[101]. A somatic, 2-bp insertion in exon 5 that led to a premature stop codon (V506X) was also found in the same MB as the somatic, exon 7 missense mutation. Western blot analysis of this biopsy showed an additional, lower molecular weight band suggesting that this allele produces a truncated protein. Cell-based reporter assays with a C-terminal deletion suggest that these truncated proteins up-regulate β-catenin signaling.

Ovarian Edometrioid Adenocarcinoma (OEA), Edometrial Carcinoma (EC), and Cervical Carcinoma (CC)

A study of 30 primary OEAs and one tumor-derived cell line looked for mutations in *AXIN1* and *AXIN2*[102]. The study primarily looked in previously identified mutation hotspots, and only in samples lacking *APC or CTNNB1* mutations. Sequence analysis found two potentially pathogenic variants in *AXIN1*: a nonsense mutation in exon 4 (K379X), a missense mutation in exon 5 (V517I), and three silent mutations including two previously published SNPs. A frameshift mutation was also found in exon 7 of *AXIN2* (leading to L668X), and similar to previous reports in CRC, the *AXIN2* mutation was found in a tumor with MSI[103].

A study of 35 endometrial cancers found three variants in *AXIN1*, two silent and one causing an A360V change in exon 1[104]. Three sequence alterations were also found in *AXIN2*, two silent changes and one missense variant in exon 7, S658C. The authors ruled out any causality for these six alleles as they were all present in corresponding normal tissue. Since publication, the A360V variant in *AXIN1* has become the reference allele.

To assess the importance of Wnt pathway defects in cervical cancer, 30 CC samples were tested for mutations in *CTNNB1* and *AXIN1*[105]*.* Six silent, germline polymorphisms and one missense variant were found in *AXIN1*. Additionally, the authors used the polymorphisms to look for deletions or loss of one allele, but neither was detected.

Adrenocortical (AC) Carcinoma

In a survey of 49 AC tumors and two AC carcinoma (ACC) cell lines, two SNPs and a 12-bp deletion were found in exon 7 of *AXIN2*[106]. The same 12-bp deletion was reported in CRC and melanoma, and interestingly, two of the four ACC samples containing the deletion also had a S45 mutation in *CTNNB1*, as previously reported[89; 107].

Adenoid Cystic Carcinoma (AdCC)

In a survey of 20 AdCC, five contained *AXIN1* mutations including two tumors that contained the same two *AXIN1* mutations, possibly representing bi-allelic targeting of *AXIN1*[108]. However, both mutations are in the far C-terminus of the protein, so it's unclear if they have any functional consequence. No analysis was done to determine if any of the mutations were germline, so the incidence of the same two mutations in two patients could represent

polymorphisms. These *AXIN1* mutations don't appear sufficient to activate Wnt signaling as immunostaining for β-catenin localization showed that only one of the five *AXIN1* mutant tumors had nuclear β-catenin, but this tumor also contained a *CTNNB1* mutation.

Melanoma

A 2008 report found a germline, heterozygous mutation in *AXIN2*. However, this was an in-frame, 12-bp deletion that probably didn't drive tumorigenesis as the tumor also had a somatic, hemizygous S45F *CTNNB1* mutation and was MMR deficient [107]. This deletion is the same as one previously reported in a sporadic CRC[89].

A follow-up study of 84 familial melanoma (FAM) families found six heterozygous *AXIN2* mutations[109]. Two of these were polymorphisms that showed similar allele frequencies in healthy controls. Two were intronic variants not detected in controls, but also not predicted to have any functional consequences. Of the remaining mutations, one was the 12-bp deletion previously identified in melanoma[107] and the other was a missense mutation previously seen in HCC (R659W)[96]. While these mutations weren't seen in controls, analysis of affected family members ruled out any association of these mutations with FAM.

Discussion

The potential tumor suppressor function of the AXIN1 and AXIN2 proteins has long been hypothesized, based on their role in the β-catenin destruction complex. However, the importance of the AXIN proteins, *in vivo*, for the inhibition of tumor development or progression is not well established, and the functional consequences of *AXIN1* or *AXIN2*

mutations in cancer remain largely uninvestigated. Some reported *AXIN1/2* mutations can be deemed non-contributory in cancer development, based on the lack of a clear link to familial cancer predisposition or because the variants are present at similar frequencies in controls. As noted in Table 2.1, many of the *AXIN1* mutations reported in cancers have since been added to the dbSNP database or identified in the 1000 Genomes Project. Further studies to compare allele frequencies in control and patient populations could shed light on whether these polymorphisms have any cancer significance. Other reported mutations need to be more thoroughly tested in clinical samples, human populations, and in cell culture and animal models to determine if they are functionally significant. Studies that address the loss of both alleles of *AXIN1* or *AXIN2* versus mutation of one allele in patient samples or animal models could help to clarify if AXINs function as classic "two-hit" tumor suppressor genes, or if the mutant alleles have a dominant effect in cancer development. A recent study found that complete loss of *Axin1* leads to a liver cancer predisposition in a mouse model[34]. Combined with *AXIN1* somatic inactivating mutations and LOH in HCCs, these data strongly support a role for *AXIN1* as a tumor suppressor in HCC.

In addition to the many *AXIN2* mutations reported in various cancer types, a few reports have linked *AXIN2* SNPs to the disease risk in breast, prostate, and lung cancer[110-115]. Further efforts may support the role of *AXIN2* variant alleles in cancer predisposition and perhaps might uncover some value for the variants in informing approaches for the prevention or early diagnosis of cancer. The vast majority of the *AXIN2* mutations in cancer have been found in exon 7 and many are frameshifts. Because the frameshift mutations are present in highly mutable mononucleotide tracts and because the mutations arise in MMR-defective CRCs, it is

difficult to determine whether these frameshifts, which lead to premature stop codons, play a role in cancer development or progression, or whether they are passenger mutations. The association with a major tooth phenotype and a very strong predisposition to colon and perhaps other cancers in some individuals carrying heterozygous, germline, truncating *AXIN2* mutations strongly suggests that these mutant alleles may be have major contributing roles in development and in cancer predisposition. Because the tooth phenotype is constitutional, it could inform on the function of these germline, truncating mutations. However, the role of the Wnt pathway in tooth development is complex and the tooth defect in these two families affects the development of a subset of secondary teeth that cannot be readily modeled in the mouse. Nonetheless, it is interesting to note that some FAP patients (carrying heterozygous *APC* germline mutations) will develop extra teeth and benign odontomas[116]. If we assume that the *APC* mutations lead to activation of Wnt signaling, then the reduction in teeth in *AXIN2* mutation carriers suggests that the *AXIN2* mutant alleles might not be simple loss of function alleles, or that perhaps AXIN2 plays an important role outside the β-catenin destruction complex. Further studies of the function of the wild-type and mutant AXIN2 proteins may yield important clues into AXIN2 function in development and cancer.

If the AXIN proteins are indeed important suppressors of cancer development or progression, then small molecules that stabilize these proteins could be a valuable treatment strategy. While some findings suggest that increased levels of AXIN proteins inhibit colon cancer cells that are dependent on β-catenin signaling, a recent mouse xenograft study suggests the opposite: that high levels of AXIN2 actually promote cancer cell invasion and perhaps metastasis [50]. Without clear evidence of the pathogenicity of the mutations identified to date or

strong evidence from animal models, the importance of AXIN1 and AXIN2 as tumor suppressor genes or oncogenes in cancer remains an unresolved issue of keen interest.

Table 2.1- Summary of *AXIN1* sequence alterations reported in cancer

	Sequence alteration identified*		Cancer type	Reference	Notes
Insertions/Deletions	Deletion of exons 1-2		HCC cell line: SNU475	33	Homozygous, no transcript detected
	Deletion of exon 4		HCC cell line: Alexander	33	Homozygous
	c.178_1597del		MB	99	
	c.266_1585del		MB	99	Germline
	c.355_1712del		MB	99	
	c.1938_2704del		MB	99	Deletion ends after stop codon
	c.2168_3098del		MB	99	
	c.2426_3101del		MB	99	
	c.2325_3106del	codons 346-350	MB	99	
	5-bp deletion	codon 386	HCC	33	
	13-bp deletion	codons 444-448	HCC	33	
	25-bp deletion†	codons 485-493	HCC	33	
	c.1076 del 1 bp	M418X	HCC	96	
	c.1714 ins 12 bp	Insert: QVHH	HCC	96	
Missense		T58M	HB	94	Germline
		L101P†	CRC	83	MSI CRC, heterozygous
		R103M	CRC	83	MSI CRC, heterozygous
		L106R	HCC	96	
		T122A	CRC	83	MSI CRC, heterozygous
		K203M‡	CRC	84	
		S215L‡	CRC cell line: LS513	76	
		P218S	MB	99, 100	Somatic (99)
		S226C	MB	100	
		P263T‡	CRC	75	Heterozygous
		N307K	CRC	84	
		P345L	HCC	96	
		R349H‡	CRC	75	Heterozygous
		R353H†	CRC	83	MSI CRC, heterozygous
		A360V	EM	104	Now the reference allele
		R382C‡	ESCC	117	Germline, seen in 2/81 patients
		H394N	CRC	84	
		R395C	CRC	83	MSI CRC, heterozygous
		L396M‡	CRC, CRC cell lines: HCT-8, HCT-15, DLD-1	75, 76	Heterozygous (75)
		E411D	CRC	83	MSI CRC, heterozygous
		M418I	CRC	83	MSI CRC, heterozygous
		G425S‡	HCC	96	
		G433E	AdCC	108	
		D495E‡	CRC	75	Heterozygous
		V517I‡	OEA	102	
		G583S	CRC	83	MSI CRC, heterozygous
		G650S‡	HB, HCC, CRC	96, 75	
		V517I‡	OEA	102	
		P661L	AdCC	108	
		A740T	CC	105	
		F824K	AdCC	108	
		S828G	AdCC	108	
		R841Q‡	HB, CRC	96, 75	Heterozygous (75)
		E842K	AdCC	108	
		P848L	CRC	84	
		E852G	CRC	84	
Non-sense		W247X	HCC	33	
		Y305X	CRC	84	
		E406X†	HCC	33	Seen in 2/100 cases
		K397X	OEA	102	

* Nucleotide and amino acid numbering modified to match modern sequences: NM_003502.3, NP_003493.1.

† These two mutations were found in same individual, references [33] and [83] respectively.

‡ These variants are now listed in dbSNP (http://www.ncbi.nlm.nih.gov/SNP).

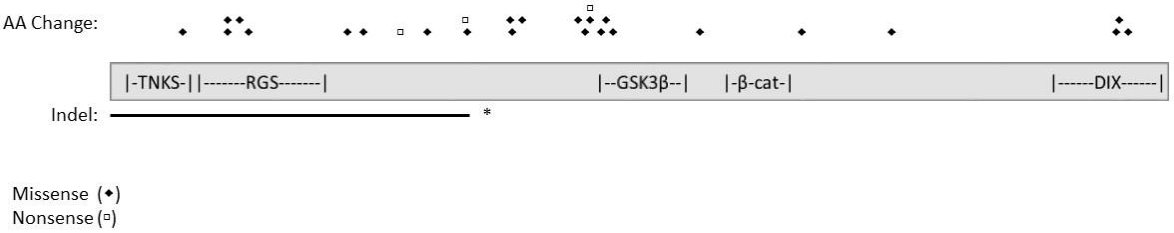
Table 2.2- Summary of *AXIN2* sequence alterations reported in cancer

	Sequence alteration identified*		Cancer type	Reference	Notes
Insertions/Deletions	52bp del	delE745-S762	HB cell line D272	[95]	
	del1624	delS217-E823	HB cell line HUH6	[95]	
	c.2010del12bp	delT672-R675	CRC	[89]	MSS CRC
	c.2013del12bp	delR671-P674	ACA, ACC, ACC cell line H295R, Melanoma cell line PR-Mel	[107, 106]	
	c.1209insAT	V506X	MB	[101]	
	c.1925delA	L688X	CRC	[88]	MSI
	c.1926insA	E706X†	CRC	[85]	MSI
	c.1993delG	L688X	CRC	[89]	MSI
	c.1994delG	L688X	CRC, GC, OEA	[85, 87, 88, 97, 102]	MSI CRC, GC, OEA
	c.1995insG	E706X†	CRC	[85, 87, 88]	MSI
	c.2007delC	L688X	CRC	[91]	MSS
	c.2011delC	L688X	CRC	[85]	MSI
	c.2023delC	L688X	CRC	[85, 88]	MSI
Missense		N412S‡	CRC	[81, 82]	Also seen in controls
		R463C	CRC	[80]	Germline, LOH wt allele
		A603P†,‡	CRC	[81]	Germline, also seen in controls
		S658C	EM	[104]	
		R659W‡	HCC, Melanoma	[96, 109]	
		A695S‡	CRC	[90]	
		Q696R	MB	[101]	
		S738F†,‡	CRC, MB	[81, 101]	Also seen in controls ([81])
		S762N‡	CRC, HB cell line D165	[95, 81]	Also seen in controls
Non-sense		R656X	CRC syndrome with oligodontia	[78]	Germline
		W663X	CRC syndrome with oligodontia	[79]	Germline

ACA/C-Adrenalcorical Adenoma/Carcinoma, AdCC-Adnoid Cystic Carcinoma, CC-Cervical Carcinoma, EM-Edometiral Carcinoma MB-Medulloblastoma, OEA- Ovarian Endometrioid Adenocarcinoma

* Nucleotide and amino acid numbering modified to match modern sequences: NM_004655.3, NP_004646.3.
† These two mutations were found in same individual, reference [81] and [85] respectively.
‡ These variants are now listed in dbSNP (http://www.ncbi.nlm.nih.gov/SNP).

A



B

```
Alteration:      59        101,103,106        122         203         215         263
                  M           P M  R           A           M           L           T
Human       …GIKGETSTATP…QDGISLFRTFLKQEGC…FWFACTGFRKL…YPSFLKSDIYLEYTRTGSESPKV…GRDAAPPGRLP…
Chimp       …GIKGETSTATP…QDGISLFRTFLKQEGC…FWFACTGFRKL…YPSFLKSDIYLEYTRTGSESPKV…GRDAAPPGRLP…
Mouse       …SIKSETSTATP…QDGISLFRTFLKQEGC…FWFACSGFRKL…YPSFLKSDIYLEYTRTGSESPKV…GRDPLPPSRLT…
Dog         …GVKGETSAATP…QDGINLFRTFLKQEDC…FWFACSGFRKL…YPSFLKSDIYLEYTRTGSESPKL…GRDPAPPGRLT…
Chicken     …AVRNETSTATP…QDGINLFRTFLKQEDC…FWFACSGFRKL…YPLFLKSDIYLEYTRTGGESPKI…SRDSAPSSRLT…
Zebrafish   …SLKNEASIATP…QDGIHLFRTFLKQEEC…FWFACSGFRKQ…YPLFLKSDIYLEYTRTGGESPKL…ESDPTPSNRLT…
                 -           - -  -           :           -           -           .

Alteration:    307          345,349,353       394,395,396      411     418     425         495
                K             L   H   H           NCM           D       I       S           E
Human       …NPYYVNAGYAL…VDGIPPYRIRKQHRREMQE…AEELIHRLEAVQR…EEKLEERLKRVRMEEEGEDGDPSSG…-HRSPDSGHVA…
Chimp       …NPYYVNAGYAL…VDGIPPYRIRKQHRREMQE…AEELIHRLEAVQR…EEKLEERLKRVRMEEEGEDGDPSSGP-HRSPDSGHVA…
Mouse       …NPYYVNSGYAL…VDGIPPYRIRKQHRREMQE…AEELIHRLEAVQR…EEKLEERLKRVRMEEEGEDGEMPSGP-HRSPDSGHVA…
Dog         …HPYYVSSGYAL…VDGIPPYRIRKQHRREMQE…AAELIHRLEAIQR…EEKLEERLKRVRMEEEGEDGDVPSGP-HRSPDNAHVP…
Chicken     …NPYYVNTGYAM…IDGIPPYRLRKQHRREMQE…AAELINRLEEVQK…EEKLEERLKRVRAEEEGEDADISSGPMPRSPESGHLG…
Zebrafish   …NPYYVNSGYAL…VDGVPPYRYRKPHRREIHE…AAELISRLEGVLR…QEKLEERLKRVRLEEEGDDADISTGPLSRSPDGLPAG…
                :             -   :   :           :-+           +       +       .           +

Alteration:      583         650          841     848 852
                  S           S            Q       L   G
Human       …YSESVGAAPNA…RHRRTGHGS-S…VFEEVREDEAVLPVFEEKIIGK
Chimp       …YSESVGAAPNA…RHRRTGHGS-S…----------------------
Mouse       …YSENAGTTLSA…RHRKAGHGS-S…VFEEVREDEAVLPVFEEKIIGK
Dog         …HSESVGAAPNA…RHRKAGHGS-S…VFEEVREDEAVLPVFEEKIIGK
Chicken     …YSENLGMAPVP…RHKKTNHGS-S…VFEEVREDDTILPIFEEKIIGK
Zebrafish   …YADGMSVGPNT…RYKRGPYGSIS…VFEEVREDDAILPIFEEKIIGK
                 .           .            :       -   -
```

Figure 2.1- Location and amino acid conservation of *AXIN1* sequence alterations reported in association with GI cancers

(A) Diagram of the location of germline and somatic *AXIN1* variants implicated in GI cancers. The locations of important functional domains in the AXIN1 protein are indicated. Missense and nonsense mutations are shown above the diagram, insertions and deletions are shown below. (B) Amino acid conservation of the reported missense variants. Conservation designated by Gonnet [15] PAM 250 scores: >1.5 (+), 0.5 to 1.5 (:), 0.5 to -0.5 (.), <0.5 (-).

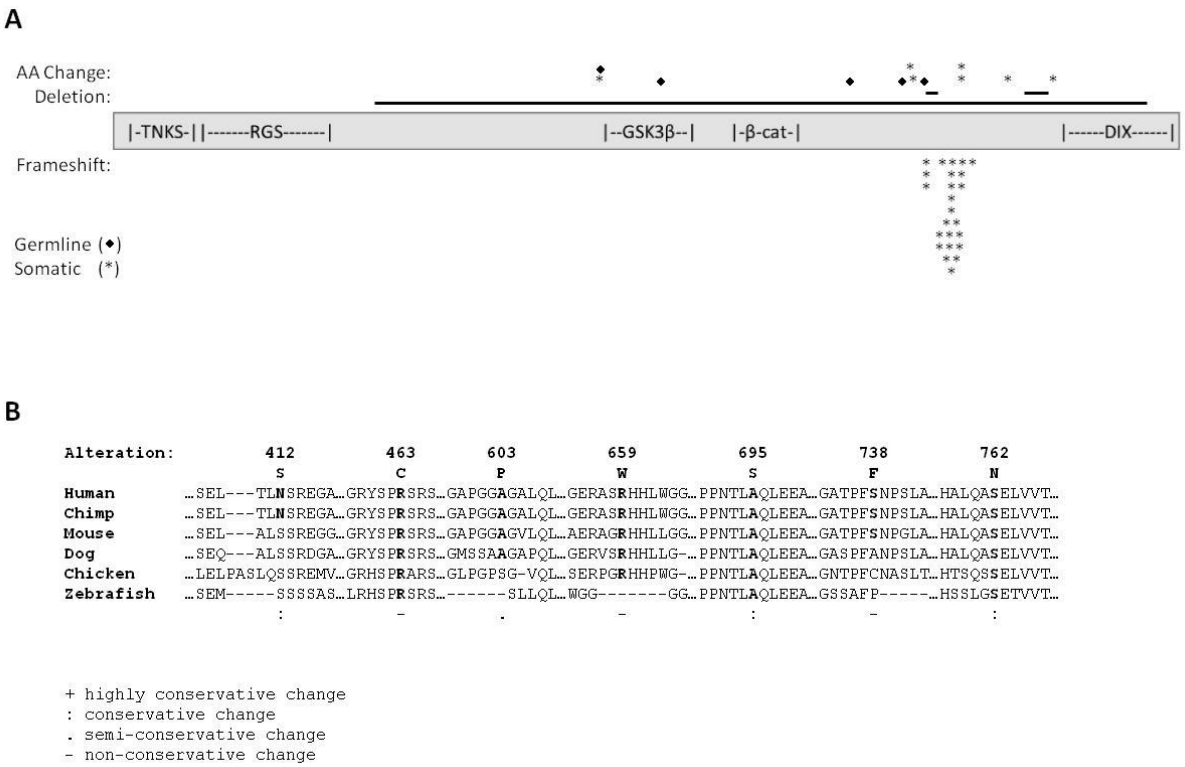


Figure 2.2- Location and amino acid conservation of *AXIN2* sequence alterations reported in association with GI cancers

(A) Diagram of the location of germline and somatic *AXIN2* variants implicated in GI cancers. The locations of important functional domains in the AXIN2 protein are indicated. Missense and nonsense variants and deletions are shown above the diagram, frameshifts are shown below. Note the density of frameshifts in the exon 7 mononucleotide repeat regions. (B) Amino acid conservation of the missense variants. Conservation designated by Gonnet [15] PAM 250 scores: >1.5 (+), 0.5 to 1.5 (:), 0.5 to -0.5 (.), <0.5 (-).

Chapter 3: An *AXIN2* mutation associated with an autosomal dominant ectodermal dysplasia and neoplastic syndrome[1]

Abstract

We describe a family with a novel, inherited *AXIN2* mutation (*c.1989G>A*) segregating in an autosomal dominant pattern with oligodontia and variable other findings including a mild ectodermal dyplasia phenotype with sparse hair and eyebrows, colon polyposis, and early onset colorectal and breast cancers. This novel mutation introduces a stop codon at amino acid 663, predicted to encode a truncated protein that is missing the last three exons, including the DIX (Disheveled and AXIN interacting) domain. This nonsense mutation is predicted to alter the inhibitory action of AXIN2 on WNT signaling. Previous authors have described an unrelated family with autosomal dominant oligodontia and a variable colorectal phenotype segregating with a nonsense mutation of *AXIN2*, as well as a frameshift *AXIN2* mutation in an unrelated individual with oligodontia. Our report provides additional evidence supporting an autosomal dominant *AXIN2*-related ectodermal dysplasia and neoplastic syndrome.

[1] Originally published as *AXIN2*-associated autosomal dominant ectodermal dysplasia and neoplastic syndrome in American Journal of Medical Genetics Part A. (2011 Apr;155A(4), 898-902) with authors listed as Marvin ML, Mazzoni SM, Herron CM, Edwards S, Gruber SB, and Petty EM.
My contribution to this paper included writing the "Truncation Analysis" section, generating Figure 3.2, and assisting with the "Discussion" section and proof-reading of the manuscript. I cloned the *AXIN2* and *trAXIN2* cDNA expression constructs and expressed them in 293T cells to see if the truncated protein was stable. CM Herron performed the PCR to amplify the patient DNA.

Introduction

Several inherited CRC syndromes have been described that result from germline mutations in tumor suppressor or mismatch repair genes. These conditions include FAP resulting from germline *APC* mutations, *MYH* associated polyposis resulting from biallelic germline *MYH* mutations, and Lynch syndrome, also known as hereditary nonpolyposis colorectal cancer syndrome, resulting from mutations in one of four mismatch repair genes, *MSH2, MLH1, MSH6, or PMS2*[118; 119] . These syndromes often include extracolonic manifestations. However, not all familial colon cancer cases can be attributed to these or other known, CRC-associated genes, suggesting that other uncharacterized cancer syndromes and genes remain to be described.

Because mutations in several genes of the canonical WNT pathway (i.e. *APC* and *CTNNB1* which encodes β-catenin) have been implicated in colorectal tumorigenesis, other genes in this pathway may play a role in these uncharacterized families, including *AXIN2*. Mutations in genes of the WNT signaling pathway that prevent the degradation of β-catenin lead to tumorigenesis by inappropriately activating WNT signaling. Within the WNT pathway, AXIN2 acts as a negative regulator by contributing to the assembly of the β-catenin degradation complex[120]. Somatic *AXIN2* mutations have been described in a variety of human cancers, including colorectal cancers[76; 111].[85; 121] *AXIN2* has also been independently implicated in tooth agenesis and oral clefts[122-124].

Of particular interest is the description of a large, four-generation Finnish kindred in which both oligodontia and a variable colorectal phenotype segregated with a nonsense

mutation in *AXIN2*[78]. In this family, the oligodontia phenotype (defined as congenital absence of six or more permanent teeth, third molars excluded) was entirely penetrant in mutation carriers. No other ectodermal findings such as those involving the nails, hair, or skin were described. The colorectal phenotype in this family was variable. Six of the seven family members with oligodontia had colorectal neoplasms, ranging from polyposis to colorectal malignancy with no polyps. Lammi et al. also screened oligodontia patients for *AXIN2* mutations and identified a *de novo* germline mutation in a 13-year-old boy with oligodontia. Of note, the germline mutation in this patient was a 1-bp insertion in exon 7 that was identical to a frameshift mutation described in a colorectal cancer tissue[85].

We describe an unrelated family with an inherited *AXIN2* mutation segregating in an autosomal dominant pattern with oligodontia and variable other findings including colonic polyposis, gastric polyps, a mild ectodermal dyplasia phenotype with sparse hair and eyebrows, and early onset colorectal and breast cancers. This report provides additional clinical description of and support for an autosomal dominant AXIN2-related ectodermal dysplasia and neoplastic syndrome.

Case Report

A 35-year-old woman presented to Medical Genetics for evaluation of a possible ectodermal dysplasia syndrome due to a personal history of oligodontia, including the congenital absence of greater than 10 secondary teeth, and a family history of oligodontia (Figures 3.1a and 3.1b). She reported normal body hair, normal nails, and normal sweating. Her past medical history was noted for intermittent non-bloody diarrhea, gastric reflux, peptic ulcer

disease, iron deficiency anemia, polycystic ovary disease, decreased fertility, fibromyalgia, insulin resistance, fundic gland polyps, Sjogren's disease, anxiety, and depression.

Previous evaluations included a normal colonoscopy and upper endoscopy exams revealing multiple fundic gland polyps at ages 33 and 35, normal capsule endoscopy at age 34, and normal rheumatology and celiac sprue evaluations. Physical exam was notable for hypognathia, malar hypoplasia, broad nasal bridge, very sparse eyebrows, fine scalp hair, and slightly upslanting palpebral fissures. Oropharyngeal exam was noted for a high arched palate, several missing teeth with increased spacing and conical shaping of several teeth. She was otherwise noted to have normal body hair distribution, normal nails, and normal skin.

Her family history was notable for oligodontia, absent eyebrows, sparse hair, colon polyps, early onset colon cancer, and early onset breast cancer (Figure 3.1b and Table 3.1). The proband's mother (II, 2) had oligodontia with absence of most of her secondary teeth. She began colonoscopic screening at age 60 and was found to have greater than 100 adenomatous polyps requiring a sigmoid colectomy at age 60 and a right hemicolectomy at age 62. She was evaluated in our clinic and was also noted to have soft skin with particularly scant body hair, minimal axillary hair, short eyelashes, and very sparse eyebrows, especially laterally.

The proband had two maternal aunts, including one (II, 1) with oligodontia, breast cancer diagnosed at age 44, metachronous colon cancers diagnosed at ages 50 and 59, and 5 adenomatous polyps of the transverse and ascending colon at age 69. The other maternal aunt (II, 3) had a reported history of colon polyps, oligodontia, absent eyebrows, and sparse hair. Confirmation of her history was not available. The index patient's sister (III, 2) had no dental or other ectodermal findings and had not undergone endoscopic screening. One cousin (III, 1) also

had no dental or ectodermal findings and had normal colon screening at age 41. The proband's maternal grandmother (I, 2) died at age 97 with a reported history of oligodontia, absent eyebrows, and sparse hair, and no known history of colon polyps or cancers.

Methods and Results

Genetic Testing

After genetic counseling and informed consent, the proband was enrolled in the University of Michigan Cancer Genetics Registry and a blood sample was obtained. Bidirectional direct sequencing of exon 7 of *AXIN2* and flanking intronic regions revealed the *1989G>A* gene alteration (W663X) (Figure 3. 2). This is a novel mutation that introduces a stop codon at amino acid 663.

Following the identification of the AXIN2 mutation in the proband, her sister (III, 2), mother (II, 2), one maternal aunt (II, 1), and one maternal cousin (III,1) also enrolled in the University of Michigan Cancer Genetics Registry and underwent genetic testing for the *c.1989G>A* alteration. The proband's mother (II, 2) and maternal aunt (II, 1) tested positive and her sister (III, 2) and cousin (III,1) tested negative (Table 3.1 and Figure 3.1b). The proband's mother (II, 2) also underwent clinical genetic testing of the *APC* and *MYH* genes with normal results. *APC* testing included sequence analysis of exons 1-14 and the 5' end of exon 15 and protein truncation testing for mutations in exon 15. Gene dosage analysis (MLPA) was also performed to test for the presence of large deletions, duplications, and other genomic rearrangements. *MYH* testing included a PCR-based analysis (restriction enzyme digest) for the presence of the Y165C and G382D mutations.

Truncation Analysis

The *AXIN2* DNA sequence was cloned from DLD1 cDNA, PCR amplified using primers specific to the *AXIN2* sequence, and inserted into the pCR2.1-TOPO vector (Invitrogen). The 1989G>A mutation was introduced by site-directed mutagenesis using the QuickChangeII site-directed mutagenesis kit (Stratagene). *In vitro* transcription and translation (TNT, Promega) was performed following manufacturer's instructions. Biotinylated proteins were visualized following se paration by SDS-PAGE with streptavidin-conjugated HRP. *In vitro* transcription and translation of the mutated construct produced an approximately 80kDa protein representing a truncated AXIN2 product, missing the last three exons. Further, 293T cells were transfected with *AXIN2* or *1989G>A AXIN2* expression constructs and protein expression analyzed by Western blot using an AXIN2 antibody (Cell Signaling, 76G6).

Discussion

Our findings provide further evidence of an autosomal dominant multisystem ectodermal and neoplastic phenotype associated with a germline *AXIN2* mutation. The novel mutation found in our family introduces a stop codon at amino acid 663. The truncated protein predicted to be encoded by this mutation is missing the last three exons, including the DIX domain. The DIX domain is required for AXIN2 dimerization, and dimerization is believed to be essential for AXIN2 to act as a scaffold for the assembly of protein complexes, in particular, the β-catenin destruction complex[49]. Formation of this complex is important for the inhibition and

negative feedback of WNT signaling, therefore, we predict that loss of the DIX domain will have functional consequences for the inhibitory action of AXIN2 on WNT signaling.

Similar to the findings of Lammi et al., the oligodontia phenotype in our family is highly penetrant. In addition, members of our family had a mild ectodermal dysplasia phenotype characterized by absent or sparse eyebrows and body hair. Furthermore, three of the four *AXIN2* mutation carriers in our family had colorectal neoplasia, including one with a modest number of adenomas who had two primary colon cancers in her 50's (II,1); one with polyposis reminiscent of FAP (II,2); and one with an unspecified number of colon polyps (II,3). The youngest mutation carrier in our family, the proband (III,3), had normal colonoscopic findings in her mid 30's, but was noted to have multiple fundic gland polyps. Given that upper gastrointestinal screening has not been performed in other mutation carriers and that the proband's fundic gland polyps could be related to proton-pump inhibitor use, further exploration is needed to determine if fundic gland polyps are related to *AXIN2* mutations[125].

The diagnosis of early onset breast cancer in one member of this family (II, 1) is intriguing given that others have hypothesized that genetic variation influencing the expression of *APC* and *AXIN2* contributes to breast cancer risk[113; 114]. Supporting evidence includes the finding that *AXIN2* maps to a chromosomal region at band 17q24 that has demonstrated allelic imbalance in breast cancer samples, and an increased rate of breast cancer in mouse models with up-regulated WNT signaling,[126]. Additionally, AXIN2 is involved in epithelial to mesenchymal transitions in mammary epithelial cells, which is thought to be a critical in the development of invasive and metastatic breast cancer[51]. It is unclear if the observed

development of early onset breast cancer in one member of this kindred is related to the germline *AXIN2* mutation identified. Further studies are needed to substantiate a causal role.

AXIN2 mutations have also been associated with the development of oral clefts, such as cleft lip and cleft palate. A recent analysis of 75 families found that there was a significantly higher incidence of cancer in families with cleft lip and palate compared to controls. In fact, the rates of breast and colon cancer in these families were significantly higher[127]. Additional studies to examine the correlation of *AXIN2* mutations with cleft lip/cleft palate associated with an increased risk of breast or colon cancer are needed to determine if a unique AXIN2-related syndrome of oral clefs associated with an increased risk for cancer exists.

This report describes a second family with an *AXIN2* mutation co-segregating in an autosomal dominant pattern with oligodontia and early onset cancers. In addition, this study extends the phenotypic spectrum associated with germline heterozygous *AXIN2* mutations to include other ectodermal dyplasia features and raises the possibility of an association with increased breast cancer risk. Further studies are needed to determine the role of *AXIN2* mutations in inherited cancer predisposition and better characterize the phenotype associated with this newly recognized autosomal dominant AXIN2-associated ectodermal dysplasia neoplasia syndrome. Similar to other inherited cancer predisposition syndromes, a careful family and personal medical history is critical to developing a complete differential. The co-occurrence of colorectal neoplasia, including polyposis and colorectal cancer, with oligodontia, other ectodermal dysplasia findings such as sparse hair and eyebrows, or oral clefts should prompt an evaluation for *AXIN2* mutations.

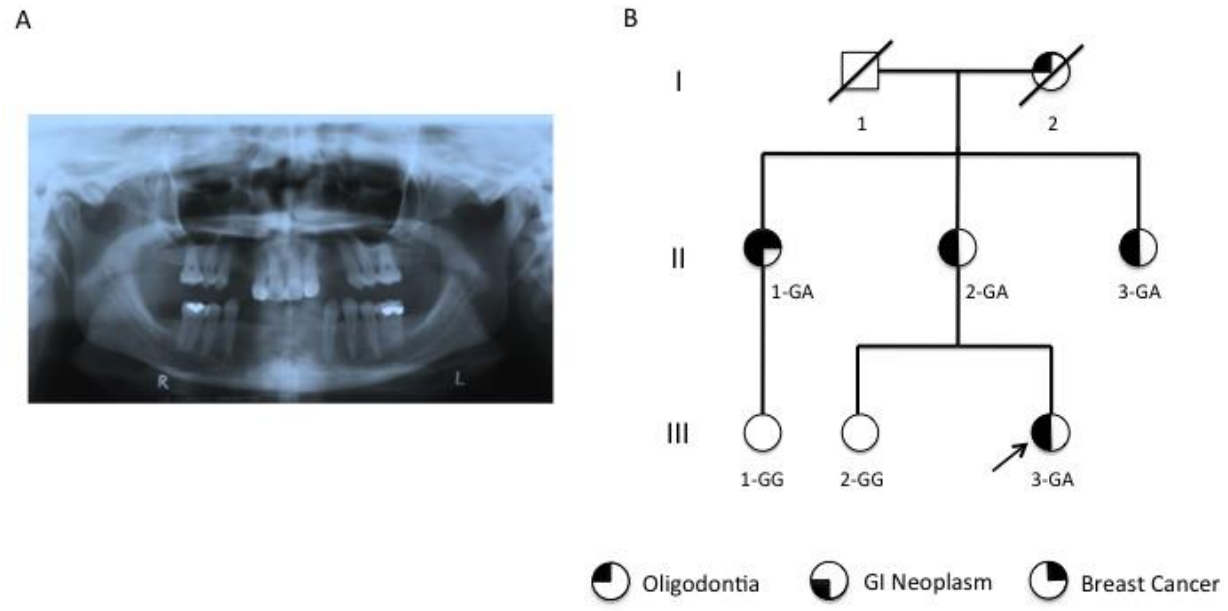


Figure 3.1- Oligodontia phenotype and pedigree
(A) Panoramic radiograph demonstrating the proband's congenital absence of teeth 2, 7, 10, 15, 18, 23, 24, 25, 26, 27, and 31. (B) Autosomal dominant inheritance of oligodontia and variable colorectal phenotype associated with *c.1989G>A*.

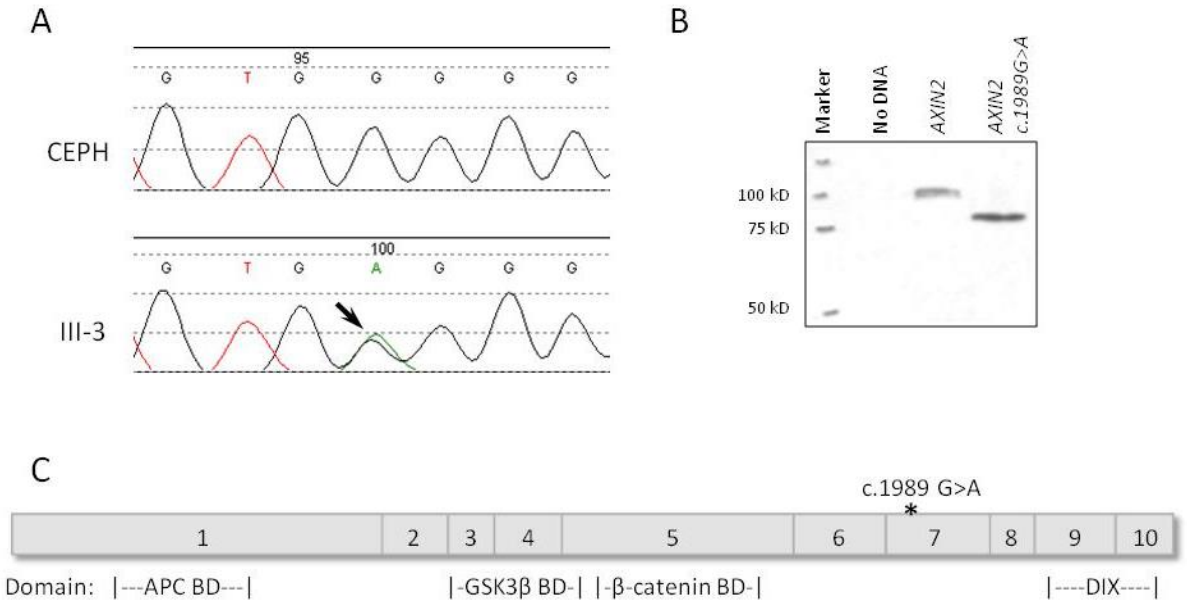


Figure 3.2- *c.1989G>A* mutation produces a truncated protein *in vitro*
(A) Sequencing of the proband's DNA (III, 3) revealed a heterozygous mutation, *c.1989G>A*, which encodes a premature stop codon. (B) 293T cells were transfected with wild-type *AXIN2* or *AXIN2 c.1989G>A* cDNA constructs. Western blot of cell lysates revealed that *AXIN2 c.1989G>A* produces an approximately 80kDa protein representing a truncated AXIN2 product. (C) Schematic of the AXIN2 protein and important binding domains shows the site of the identified mutation.

Chapter 4: A truncated AXIN2 protein- functional consequences of deletion of the DIX domain

Abstract

Two heterozygous, germline mutations in the *AXIN2* gene have been identified in families with oligodontia and a CRC predisposition. To determine how these nonsense alleles might contribute to a cancer predisposition, I used the *1989G>A* mutation as a model for the germline and somatic mutations seen in a subset of many cancers. Analysis of lymphocytes from an individual carrying the *1989G>A* mutation suggested that the mutant transcripts escape nonsense-mediated decay and could then encode a truncated protein. Cell culture experiments using a cDNA encoding the truncated protein (trAXIN2) demonstrated that the truncated protein is more abundant, possibly due to increased protein stability. Unlike the wild-type AXIN2 protein, the regulation by GSK3β that inhibits AXIN2 turnover does not seem to be a main source for the stabilization of the trAXIN2 protein. In zebrafish embryo injections, the *trAXIN2* mRNA produced a developmental phenotype, suggesting that this protein is not a simple loss of function protein. Additionally, while the trAXIN2 protein may be hypomorphic in Wnt-target gene inhibition assays, it retains binding with destruction complex components and inhibits colony formation in a Wnt-dependent CRC cell line. These data support the conclusion that the germline mutations in AXIN2 could encode a truncated protein with reduced function that might be present in cells at a higher concentration than the wild-type protein.

Introduction

In 2011 we reported a germline *AXIN2* mutation associated with oligodontia and a colorectal cancer predisposition[79]. This mutation introduces a premature stop codon and because of the location of the mutation, this allele would be predicted to undergo nonsense-mediated decay (NMD). NMD would support a classical tumor suppressor model where one allele is non-functional and the second is lost during tumor development. In addition to two other reports of germline *AXIN2* alleles associated with CRC predisposition, somatic *AXIN2* mutations have been identified in a subset of several cancer types (reviewed in Chapter 2). The majority of these somatic mutations are in exon 7, near the *1989G>A* mutation, and lead to premature stop codons. It is possible that these mutations are simple passenger mutations, but they could also escape NMD and have functional significance as has been seen with truncating mutations in *APC*[128]. We have been unable to directly assess AXIN2 protein expression in patient samples to determine if these truncating mutations create stable proteins, but since the *Axin2*$^{lacZ/+}$ mouse lacks a haplo-insufficient phenotype[38], the functional consequence of these truncating mutations merited further exploration. The *1989G>A* germline allele was used as a model truncating mutation to ask how the function of the truncated protein it encodes, trAXIN2, differs from the wild-type protein, and whether these truncating mutations impact cancer development.

Materials and Methods

Expression constructs and cell culture

The *AXIN2* sequence was cloned using cDNA isolated from the DLD-1 cell line. The *trAXIN2* sequence was made from *AXIN2* by site-directed mutagenesis and the sequences were cloned in to the following expression constructs: ppGS-CMV-Cite-Neo for retroviral expression; pCMV-3Tag for FLAG-tagged expression; and ppCS2+MT for myc-tagged expression. The *AXIN1* cDNA was cloned into pCDNA3.1 with a FLAG- and Myc-tag, and the *β-catenin* S33Y expression construct has been previously published[129].

All cells were cultured under sterile conditions at 37°C, 5% CO_2. The 293T, DLD-1 (ATCC-CCL-221), RKO (ATCC CCL-2577) and SW480 (ATCC CCL-228) cell lines were cultured in DMEM (GIBCO) with the addition of 10% FBS and 1% penicillin/streptomycin. IEC-6 cells (ATTC CRL-1592) were grown in DMEM (Gibco) with the addition of 10% FBS (Fisher), 1% penicillin/streptomycin (Gibco), and 0.1 Unit/ml recombinant human insulin (Gibco).

For transfection-based studies, cells were transfected in 6-well plates at 60-80% confluence using 2µg of total DNA per well with the TransIT-LT1 transfection reagent (Mirus).

Mutant allele identification

After obtaining informed consent, peripheral blood lymphocytes (PBLs) were isolated from whole blood using Ficoll-Hyaque separation. The buffy coat layer was removed and plated for adherence depletion of monocytes. PBLs were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS (Fisher), 1% penicillin/streptomycin (Gibco), 1% HEPES (Gibco), 0.5% β-mercaptoethanol (Gibco), and L-Glutamine (Gibco). PBLs were cultured for 48 hours and then treated with 5µM BIO (Cayman Chemical #13123) or DMSO for 18 hours.

Total RNA was isolated using Trizol and cDNA was generated with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Genomic DNA was isolated from PBLs by boiling the cells in 50mM NaOH followed by neutralization with 1M Tris, pH 8.0.

PCR to specifically amplify gDNA or cDNA was done using Taq polymerase (Promega). Bulk PCR products were gel extracted and sequenced. PCR products from gDNA and cDNA were TOPO cloned using the pCR2.1 TOPO cloning vector (Invitrogen). Single TOPO clones were then isolated and sequenced to confirm the *1989GA* genotype and the presence of the *AXIN2* 3'UTR.

Table 4.1- *AXIN2* PCR primers

Primer	**Primer location**	**Primer sequence**
AXIN2 gDNA forward	*AXIN2* intron 5	CCGACTTGCTGAATTGTCTG
AXIN2 gDNA reverse	*AXIN2* intron 6	TGGATGAGTAAGCTGCTGCTT
AXIN2 cDNA forward	*AXIN2* exon 6	GGGAGGAAGGAGACAGGTCGC
AXIN2 cDNA reverse	*AXIN2* 3' UTR	CAAAGCCAGACCCCAGGG

Quantitative PCR (qPCR)

For Wnt target gene analysis, IEC-6 cells were transduced with retrovirus and then grown under G418 selection to create stable lines. To test inhibition of β-catenin target genes, 100,000 stable IEC-6 cells were plated in a 6cm dish. 24 hours after plating, the cells were treated with 200ng/ml recombinant Wnt3a (ProSci) to induce target gene expression, or with PBS as a control. After 16 hours of induction, RNA was collected by Trizol extraction, cDNA synthesized, and target genes analyzed by qPCR using the primers shown below. Target gene induction was measured six independent times.

For quantification of *AXIN2* or *trAXIN2* transcripts, 293T cells were transfected in 6-well dishes with 2µg of FLAG-tagged expression constructs. 24 hours after transfection, RNA was isolated for analysis by qPCR. Ectopic transcripts were quantified by detection of the FLAG sequence.

Table 4.2- qPCR primers for Wnt target gene and *AXIN2/trAXIN2* transcript quantification

Gene	Primer sequence
U6	F- GTGCTCGCTTCGGCAGCACATAT R- AAAAATATGGAACGCTTCACGAA
Axin2 (rat)	F- CTCTAACGCTAGGCGGAATG R- CCAGAAGTCCAGGGTATCCA
Lgr5	F- GCTGCCAAATTGTTGGTTTT R- CAGGCTAGAAAGGGGAGCTT
Irs1	F- CCAGAAGCAACCAGAGGA R- CCATGAGTTAAAAAGGAGGAT
Nkd1	F- AGGACGACTTCCCCCTAGAA R- TGCAGCAAGCTGGTAATGTC
β-actin	F- GCCTTCCTTCTTGGGTATGG R- GCCTGGGTACATGGTGGT
FLAG	F- AAGGACGATGATGACAAGGACTACA R- TCCGGGAGGCAAGTCACCAA

Protein interaction studies

Cells were transfected with plasmids using TransIT-LT1 (Mirus) or Fugene-HD (Promega). Total protein was extracted using RIPA buffer followed by immunoprecipitation (IP) with the indicated antibody and protein A/G agarose with 200µg of total protein. Proteins were eluted from agarose by boiling in Laemmli buffer and analyzed by SDS-PAGE and Western blot. β-catenin IPs were performed following a five-hour treatment with 10µM of the proteasome inhibitor, MG132 (Cayman Chemical).

The following antibodies were used for Western blotting: α-AXIN2 (Cell Signaling, 76G6), α-FLAG (Sigma, M2), α-myc (Sigma, C3956), total β-catenin (BD-Transduction Laboratories), phospho-33, 37, 41 β-catenin (Cell Signaling, 9561) and β-actin (Sigma, AC-15); and for IP: α-FLAG (Sigma, F7425) and α-myc (as above).

For protein stabilization studies, 293T cells were plated in a 6-well dish and transfected at 70-90% confluence with 2μg of FLAG-tagged proteins. Six hours after transfection, each well was split to four wells of a six-well plate. 24 hours post-transfection, the cells were treated with DMSO, 100nM OA (Cell Signaling, #5934), or 1μM BIO. Cells were collected for Western blotting after 6 hours of inhibitor treatment. XAV939 (Cayman Chemical) treatment was performed using the indicated concentrations and cells were collected for Western blotting after 24 hours of inhibitor treatment. Proteasome inhibition was tested in DLD-1 cells that stably express a FLAG-tagged AXIN2 or trAXIN2 protein. Cells were treated with 10μM MG132 for five hours and then total protein lysates were analyzed by Western blot.

Colony formation assays

Cells were transfected with vector, *AXIN2*, or *trAXIN2* pCMV3Tag expression constructs. 24 hours post-transfection, cell were counted, plated in triplicate in 24-well dishes, and grown under selection in 250μg/ml G418. RKO cells were plated at a density of 5,000 cells per well. SW480 cells were plated at a density of 1,000 cells per well. After 21 days, colonies were fixed in 4% PFA, stained with crystal violet, and counted. Colony formation was performed in triplicate and repeated in three independent transfections. Colony numbers were analyzed using a t-test to compare *AXIN2* or *trAXIN2* transfections to vector-only colony number.

Zebrafish embryo injections

Zebrafish were bred and maintained in standard conditions under the direction of Dr. Anthony Antonellis. The *AXIN2* and *trAXIN2* cDNAs were cloned into pCSDest using Gateway technology (Invitrogen). Messenger RNA was generated using the mMESSAGE mMACHINE kit (Abmion). The yolks of one- to two-cell stage embryos were injected with mRNA (~500ng/µl). The embryos were monitored up until 48 hours post-fertilization (hpf). The embryos injected with *trAXIN2* mRNA were only analyzed at 26 or 27-hpf because of the severity of their phenotype.

Image quantification

Relative protein abundance in Western blots was estimated using ImageJ software (nih.gov) by comparison to a β-actin loading control band.

Results

My first step was to address if the *1989G>A* allele was a null allele that produced no AXIN2 protein. It is possible that this premature stop codon triggers NMD, but there are exceptions to the canonical NMD formula. Alternatively, the mutation introduces an AG, which could serve a cryptic splice site that could alter splicing and create a new protein product. While the sequence change does not predict the introduction of a consensus splice site, I further ruled out this possibility by using Spliceport[130] (http://spliceport.cbcb.umd.edu/) to analyze the genomic DNA sequence of the two alleles from intron 5 to exon 8 (the mutation is in exon 7).

The Spliceport analysis found no changes in the predicted splice sites by introduction of the *1989A* allele (See Figure 4.1).

To address NMD of the *1989G>A* transcripts, we examined lymphocytes from the Marvin et al. proband[79]. Peripheral blood lymphocytes (PBLs) were isolated from the whole blood and cultured to obtain RNA. NMD is triggered by the pioneering round of translation. To enhance transcription and translation of AXIN2, all the PBLs were cultured with BIO, a GSK3β inhibitor that activates the expression of Wnt target genes, including *AXIN2*[131] (Appendix B). Total RNA and genomic DNA were then isolated from cells and the region immediately surrounding the *1989G>A* allele was PCR amplified. These bulk PCR products were sequenced and TOPO cloned to detect the presence of wild-type and mutant alleles. The *1989G>A* allele was confirmed in gDNA from the proband, but was absent in gDNA from the DLD-1 cell line (Figure 4.2b). Sequencing of the bulk PCR product from cDNA identified the *1989G>A* allele in roughly equivalent levels to the wild-type allele (Figure4.2c-d). This was true in RNA from PBLs that were cultured with or without PHA and IL-2 to enhance lymphocyte proliferation. To confirm the presence of the mutant allele, individual TOPO clones were sequenced to genotype the *1989* allele and to verify that the 3'UTR of the transcript was present (further ruling out plasmid contamination). The ratio of TOPO clones were as follows: *4G:4A* in gDNA, *9G:3A* in cDNA from PBLs, and *6G:4A* in cDNA from PBLs treated with BIO. These results suggest that many of the *1989G>A* transcripts escape NMD and would be free to produce truncated AXIN2 proteins. Additionally, these results argue against an alternative transcript that uses the *G>A* alteration as a novel splice site.

One of the primary functions of AXIN2 is the inhibition of Wnt/β-catenin target genes. To test the effect of the trAXIN2 protein on regulation of this function, IEC-6 cells stably expressing AXIN2 or trAXIN2 were treated with Wnt3a to activate β-catenin target gene expression. RNA was then collected and analyzed by qPCR. As expected, expression of AXIN2 reduced Wnt target gene expression, but trAXIN2 failed to inhibit Wnt target genes, similar to the vector control (Figure 4.3).

Because trAXIN2 lacks the DIX domain, I hypothesized that the failure to inhibit target genes could be due to an inability to dimerize, and therefore the formation of inefficient destruction complexes[48; 49; 132]. To test this hypothesis, differentially tagged *AXIN2* constructs were co-transfected and the interaction between AXIN2 molecules assessed by immunoprecipitation (IP). trAXIN2 interacted with both AXIN2 and trAXIN2 in ratios similar to their expression levels (Figure 4.4b). Similar results were seen when AXIN2 was used for IP. Because trAXIN2 does not contain the DIX domain, it is most likely that the trAXIN2 interactions are not direct, but are facilitated by a protein that contains multiple AXIN binding sites, such as APC[17]. Interactions with AXIN1 were also tested because AXIN1 acts in the β-catenin destruction complex and also contains a DIX domain that is used for dimerization with AXIN2 and other AXIN1 molecules. Interestingly, the AXIN1 interaction with wild-type AXIN2 pulled down a greater amount of AXIN2 protein, despite lower AXIN2 protein expression, suggesting that the AXIN1:AXIN2 interaction is a direct interaction due to heterodimerization, while the AXIN1:trAXIN2 interaction is indirect (Figure 4.4c). Finally, both AXIN2 and trAXIN2 were shown to interact with β-catenin, proving that the inhibition of target gene repression is not the result of trAXIN2 failing to interact with β-catenin (Figure 4.4c). Overall, these data suggest that

trAXIN2 is still capable of assembling in β-catenin destruction complexes, although they may be less efficient than wild-type AXIN2 destruction complexes, based on the Wnt-target gene inhibition results.

The trAXIN2 protein was found to be more abundant than wild-type AXIN2, regardless of tag, vector backbone or the antibody used to detect it. To rule out a discrepancy in RNA levels, cells were transfected with either FLAG-tagged *AXIN2* or *trAXIN2*. Total protein and RNA were isolated from the same cell pellets and analyzed by Western blotting and qPCR. No significant difference was seen in the abundance of transcripts, while trAXIN2 protein was consistently more abundant (Figure 4.5a-b). I next looked at AXIN2 stability by inhibiting the proteasome. Inhibition of the proteasome stabilized AXIN2, but the protein did not achieve equivalent levels with the trAXIN2 protein (Figure 4.5c).

The stability of AXIN1 is regulated by GSK3β phosphorylation and PP2A dephosphorylation[133; 134]. The predicted GSK3β phosphorylation sites are conserved between AXIN1 and AXIN2 (see Figure 1.2), so presumably, AXIN2 is subject to this same phosphorylation-mediated stability whereupon phosphorylated AXIN2 is more stable and dephosphorylated AXIN2 is less stable (Figure 4.6a). Additionally, the PP2A interaction domain has been mapped to the C-terminus of AXIN1[135], so the interaction between PP2A and trAXIN2 might be disrupted, which could account for the increased stability of the trAXIN2 protein. To test the effects of PP2A and GSK3β activity on the AXIN2 and trAXIN2 proteins I used the GSK3β inhibitor, BIO, and the PP2A inhibitor, okadaic acid (OA)[136]. As expected, treatment with BIO reduced the levels of AXIN2, while OA increased AXIN2 levels (Figure 4.6b). However, while the trAXIN2 protein was stabilized by OA, protein levels were unchanged upon BIO treatment,

suggesting that the trAXIN2 protein retains the interaction with PP2A, but does not rely on GSK3β phosphorylation to maintain protein stability. Another mode of AXIN2 protein regulation is via PARsylation which targets AXIN2 for degradation[43]. Treatment of cells expressing AXIN2 and trAXIN2 with a small molecule that inhibits TNKS-mediated PARsylation, XAV939, showed that the AXIN2 protein is more sensitive to PARsylation-mediated stabilization than trAXIN2 (Figure4.6c).

It has previously been shown that stabilization of AXIN2 inhibits colon cancer cell lines with *APC* mutations[43; 69]. I tested whether trAXIN2 retains this ability to inhibit the growth or survival of colon cancer cell lines in the colony formation assay. I chose the SW480 cell line, which has activated Wnt signaling due to *APC* mutation[137], and the RKO cell line with intact Wnt signaling[138]. Like the wild-type protein, trAXIN2 only inhibits colony formation in a cell line that is dependent on activated β-catenin signaling (Figure 4.7). In some replicates, the trAXIN2 protein even seemed better at inhibiting SW480 colony formation, although this is likely due to the much higher levels of trAXIN2 protein expression in these cells compared to AXIN2 protein levels (Appendix C).

To assess the function of this truncated protein *in vivo*, I turned to the zebrafish model. The first studies of β-catenin established an important role for the Wnt pathway in development, and overexpression of rat Axin2 in *Xenopus* has been shown to alter the dorsal/ventral axis and oppose Wnt-induced axis duplication[18]. Previous studies have also found that the amount of Axin-2 protein must be carefully titrated to achieve normal development in the zebrafish (Figure 4.8a)[139]. To test if the trAXIN2 protein displays a loss of function *in vivo*, one to two-cell stage zebrafish embryos were injected with mRNA encoding either *AXIN2* or

trAXIN2. First, I confirmed that overexpression of human *AXIN2* creates a phenotype similar to overexpression of the zebrafish *Axin-2*, suggesting that the human protein maintains its functions in zebrafish cells (Figure 4.8b). Next, I asked if the *trAXIN2* mRNA resulted in the same phenotype, or a less severe one. I actually found that injection with *trAXIN2* mRNA resulted in a more severe developmental phenotype (Figure 4.8c), which could be due to higher protein levels as was seen in cell culture-based assays. I was unable to measure the relative protein levels in the developing embryos, and the severe phenotype made it difficult to address a potential mechanism for the difference in phenotype. However, the trAXIN2 zebrafish phenotype supports the conclusion that the trAXIN2 protein retains some protein function, or potentially acquires new functions.

Discussion

A germline *AXIN2* mutation was found to segregate with an ectodermal dysplasia and polyposis phenotype. This mutation is in the same exon as the majority of somatic *AXIN2* mutations reported in cancers[85], and may alter *AXIN2* mRNA or protein stability. We studied the consequences of this mutation on protein function using the trAXIN2 protein, which lacks the DIX domain. This trAXIN2 protein can serve as a model for the truncated AXIN2 proteins that potentially result from somatic exon 7 frameshift mutations.

The *1989G>A AXIN2* mutation segregates in an autosomal dominant manner[79]. Whether this mutation influences cancer development via a gain-of-function or loss-of-function is not understood. If the *1989G>A* allele mRNA undergoes nonsense-mediated decay, then it would point to a loss-of-function mechanism of disease. This model is consistent with the current

belief that AXIN proteins act as tumor suppressors. However, transcripts from both alleles were detected in PBLs from a heterozygous individual. Cell culture-based studies suggest that the trAXIN2 protein is hypomorphic in Wnt-target gene regulation and more stable than the wild-type protein. Gross overexpression studies make it difficult to estimate the functional consequences of this truncated protein, *in vivo*, where the expression levels are likely much lower. It is conceivable that trAXIN2 could produce less functional destruction complexes, and because it is more abundant than wild-type AXIN2, could be sequestering destruction complex components away from more efficient AXIN1 or AXIN2 complexes, thereby slowing down the turnover of β-catenin, or potentiating the activation of β-catenin by a Wnt ligand.

This truncated protein was found to have a reduced function in the inhibition of Wnt/β-catenin target genes, but not because it can't complex with β-catenin or other AXIN1/2 molecules. *In vitro* colony formation studies suggest that the trAXIN2 protein retains the functions of the wild-type AXIN2 protein, although *in vivo* studies using a zebrafish model suggested a stronger developmental defect. This apparent disconnect between the reduced function and maintained or enhanced/gain of function behavior could be explained by the increased stability of the trAXIN2 protein. The inhibition of Wnt target genes was performed in cell lines that had been retrovirally transduced to express moderate, and more equivalent, levels of the AXIN2 and trAXIN2 proteins. The reduced function in inhibition of Wnt target genes suggests that the trAXIN2 protein could be hypomorphic. The zebrafish and colony formation assays were performed with gross, transient over-expression; therefore, the higher trAXIN2 protein levels in these studies could compensate for a reduced functionality. Additionally, while the inhibition of colony formation was only significant in a CRC cell line with

mutant *APC*, it's possible that AXIN2 and trAXIN2 exert their inhibitory effects via a non-β-catenin dependent mechanism. The trAXIN2 protein could indeed have a reduced function in the inhibition of Wnt-target genes, but still retain functions in non-canonical pathways such as the ERK[56] or JNK pathways[58], or by regulation of GSK3β[51].

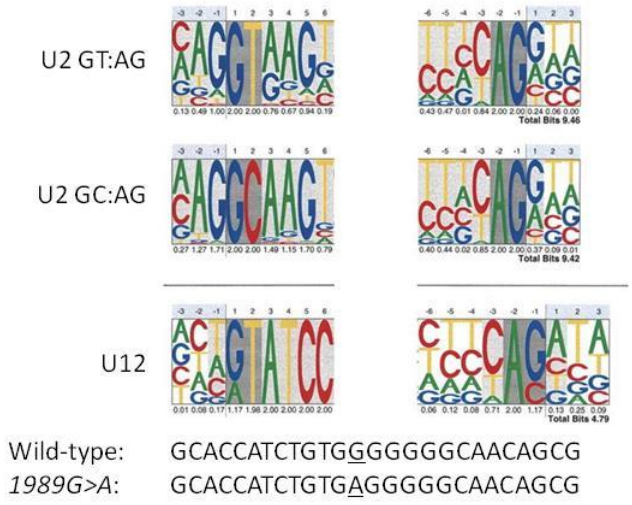
The observed difference in the level of AXIN2 and trAXIN2 proteins suggest that there is a functional difference in the two proteins. This could be due to a difference in the regulation of protein stability, as suggested by the differential stabilization by GSK3β, but it is also possible that higher levels of the wild-type protein simply aren't tolerated by the cell because they inhibit growth or survival. Preliminary flow cytometry data in 293T cells using fluorescently-tagged *AXIN2* and *trAXIN2* constructs saw no clear sub-G1 peak to indicate the induction of apoptosis (Appendix D), although further studies at different time-points will be needed to clarify if cells expressing high levels of AXIN2 are indeed lost to apoptosis or a cell cycle arrest. There are likely multiple cellular pathways involved, but the exact mechanism(s) for why the truncated protein accumulates to such levels remains unclear.

It is important to also consider the possibility that the trAXIN2 protein has a gain-of-function effect in cells. Some publications have suggested that in certain contexts, AXIN2 can promote tumor invasion and progression[50; 51]. One proposed mode of AXIN2-mediated tumor invasion is via the kinase GSK3β[51]. Overexpression of AXIN2 has been shown to alter the nuclear to cytoplasmic ratio of GSK3β, influencing the expression of genes involved in the epithelial to mesenchymal transition[51]. A more stable trAXIN2 protein could recreate the situation of AXIN2 overexpression and exert broader effects via GSK3β. Another argument against this human allele being a simple loss of function or null allele is that the *Axin2* knockout mouse has no

reported cancer phenotype[38]. To further address the function of this allele, a knock-in mouse model of the *1989G>A* allele has been created and ongoing studies will address mRNA stability and protein function *in vivo*.

As negative regulators of Wnt signaling, AXINs promote the assembly of complexes that target β-catenin for degradation. The AXINs are considered to be tumor suppressors because of this ability to inhibit β-catenin, although the clinical evidence to support their role in cancer is limited. Interestingly, *AXIN2* is a β-catenin/TCF target gene and is actually up-regulated in many cancers with activating Wnt pathway mutations[39; 40]; therefore, it is important to understand what role wild-type or truncated AXIN2 protein products may play in cancer development or progression.

A



B

Wild-type Spliceport Report		
Site	**Position**	**Score**
Acceptor	220*	0.312315
Donor	455*	0.72764
Acceptor	770*	0.36914
Acceptor	823	0.318951
Acceptor	906	0.459046
Donor	1004*	1.47053
Acceptor	1602*	1.43483

Mutant Spliceport Report		
Site	**Position**	**Score**
Acceptor	220*	0.312315
Donor	455*	0.72764
Acceptor	770*	0.36914
Acceptor	823	0.225624
Acceptor	906	0.253692
Donor	1004*	1.47053
Acceptor	1602*	1.47053

*Actual exon 7 splice sites

Figure 4.1- The *1989G>A* allele does not introduce a cryptic splice site
(A) Consensus splice acceptor and donor sequences are shown with the *AXIN2* genomic DNA sequence for the *G* and *A* alleles. (B) Spliceport prediction for the *AXIN2 1989G* or *1989A* genomic DNA shows no change in the predicted splice sites.

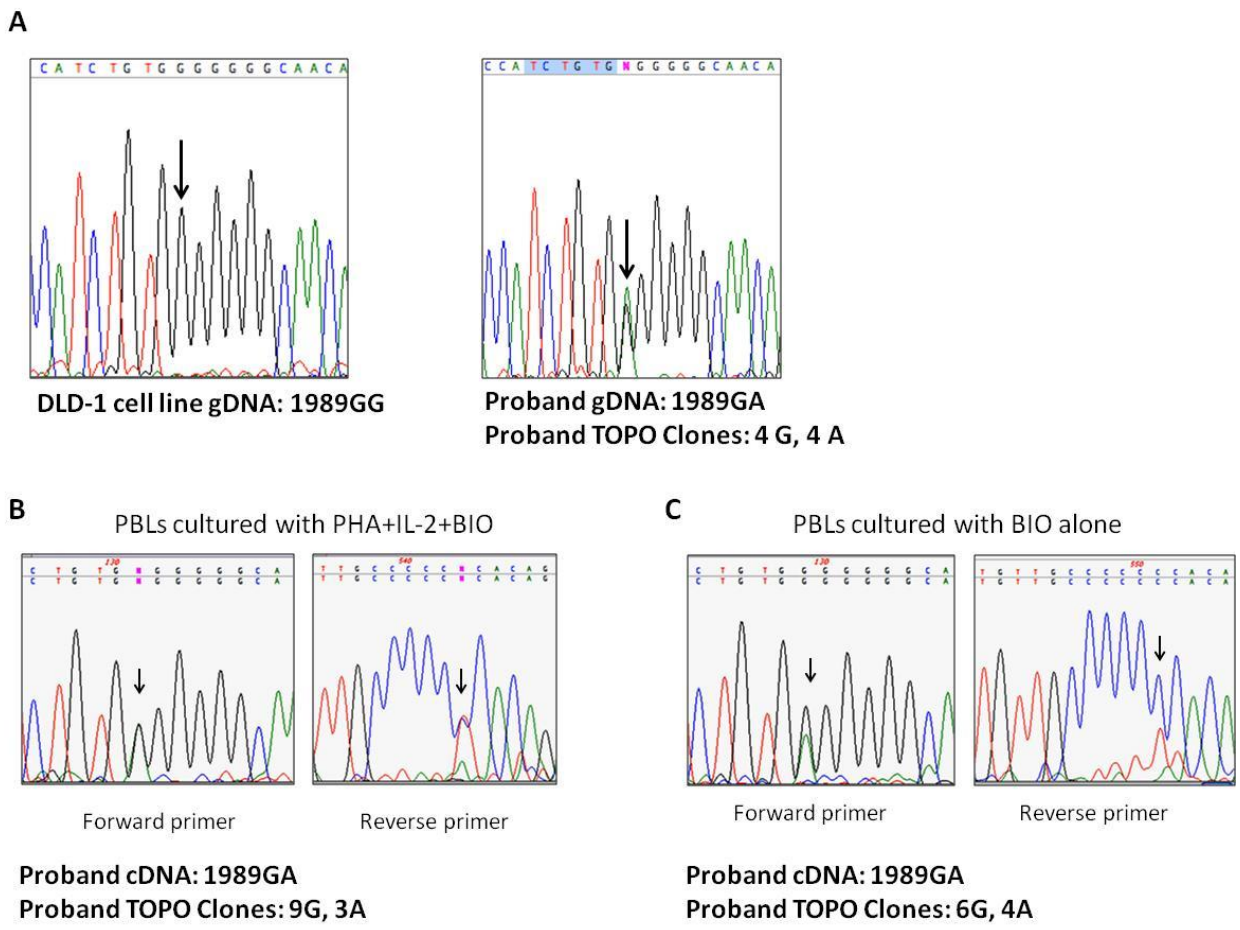


Figure 4.2- Transcripts from the *1989G>A* allele escape NMD
(A) Genomic DNA was PCR amplified to identify the 1989 allele(s) present in cells. Chromatograms confirming the absence of the *A* allele in a wild-type cell line, and of the *GA* genotype in the proband's cells. (B) cDNA isolated from a heterozygous carrier (the proband) was amplified by PCR to determine which transcripts are expressed in PBLs. Sequencing results of bulk PCR products identifies transcripts from both the *G* and *A* alleles. Individual TOPO clones were cloned from bulk PCR products and also sequenced, the results of which are shown below the chromatograms.

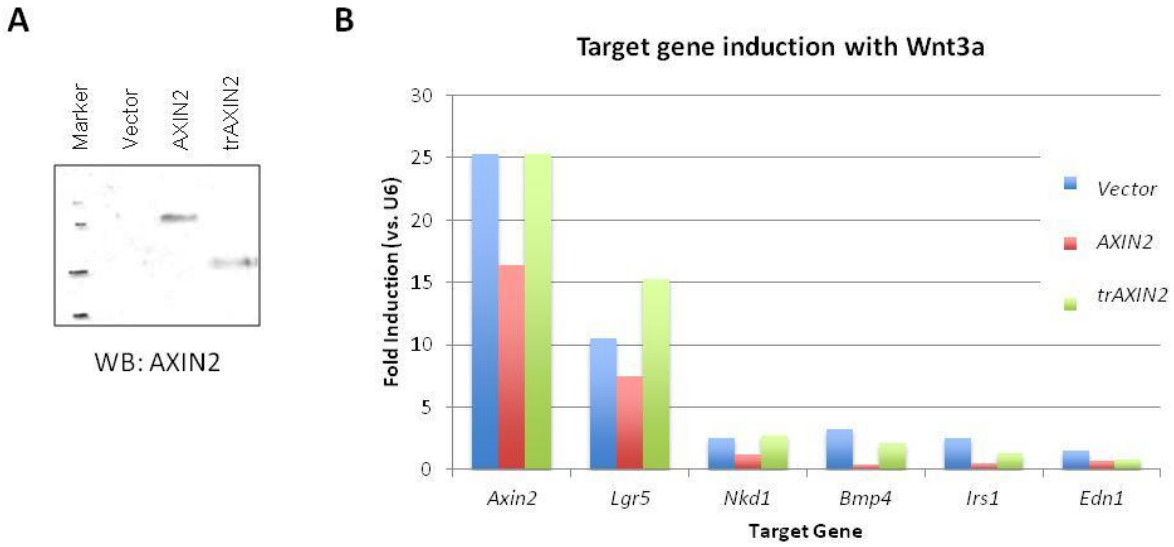


Figure 4.3- Wnt target gene inhibition
(A) IEC-6 cells were transduced with either vector, *AXIN2* or *trAXIN2* expression constructs to create cell lines that stably express the full-length and truncated AXIN2 proteins. (B) Stable transductants were treated with 200ng/ml Wnt3a to induce target gene expression. After 16 hours, RNA was collected and target genes measured by qPCR. Panel B is a representative experiment.

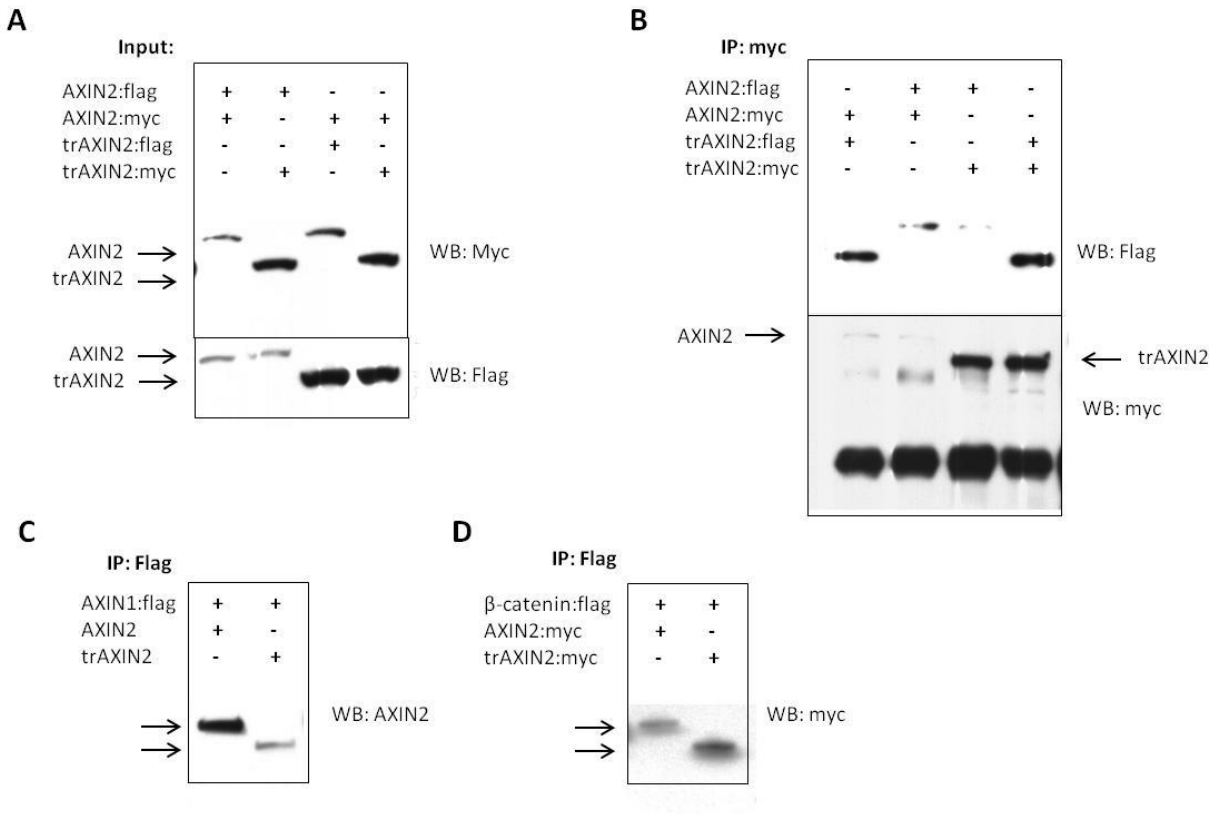
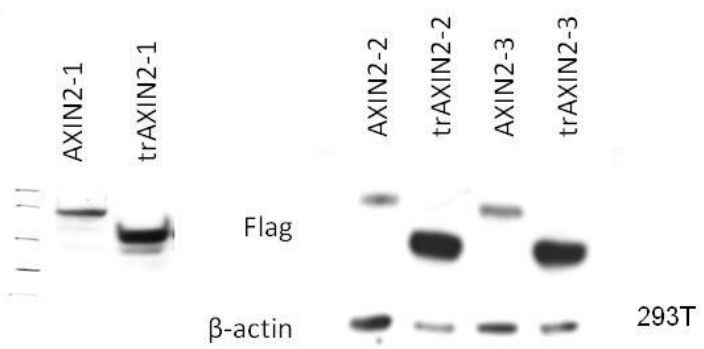


Figure 4.4- The trAXIN2 protein retains destruction complex associations
293T cells were transfected with various expression constructs to test protein interactions. (A) Input (20μg) for AXIN2, trAXIN2 homodimerization analysis. (B-C) Immunoprecipitation experiments show that trAXIN2 interacts with trAXIN2, AXIN2, AXIN1 and β-catenin.

A



B

Replicate	*AXIN2:trAXIN2* transcript ratio	AXIN2:trAXIN2 protein ratio
1	1.0 : 0.74	
2	1.0 : 1.37	1.0 : 23.4
3	1.0 : 1.33	1.0 : 6.34

C

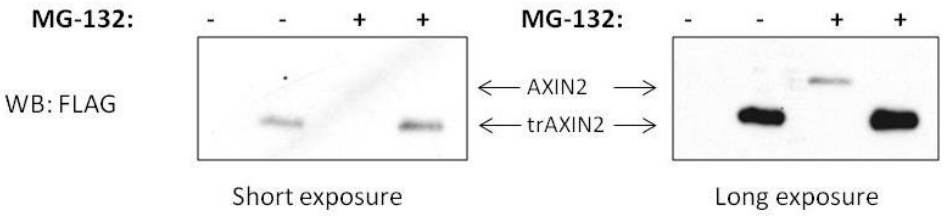


Figure 4.5- trAXIN2 protein and transcript levels do not correlate
(A) Western blot of 293T cells transfected with *AXIN2* or *trAXIN2* expression constructs. Relative protein level was quantified using ImgaeJ. (B) Quantification of *AXIN2* transcripts by qPCR shows that trAXIN2 protein levels are not solely a reflection of transcript levels. (C) Proteasome inhibition of DLD-1 cells expressing AXIN2 or trAXIN2 with MG132 stabilizes AXIN2, but not to the level of trAXIN2 protein.

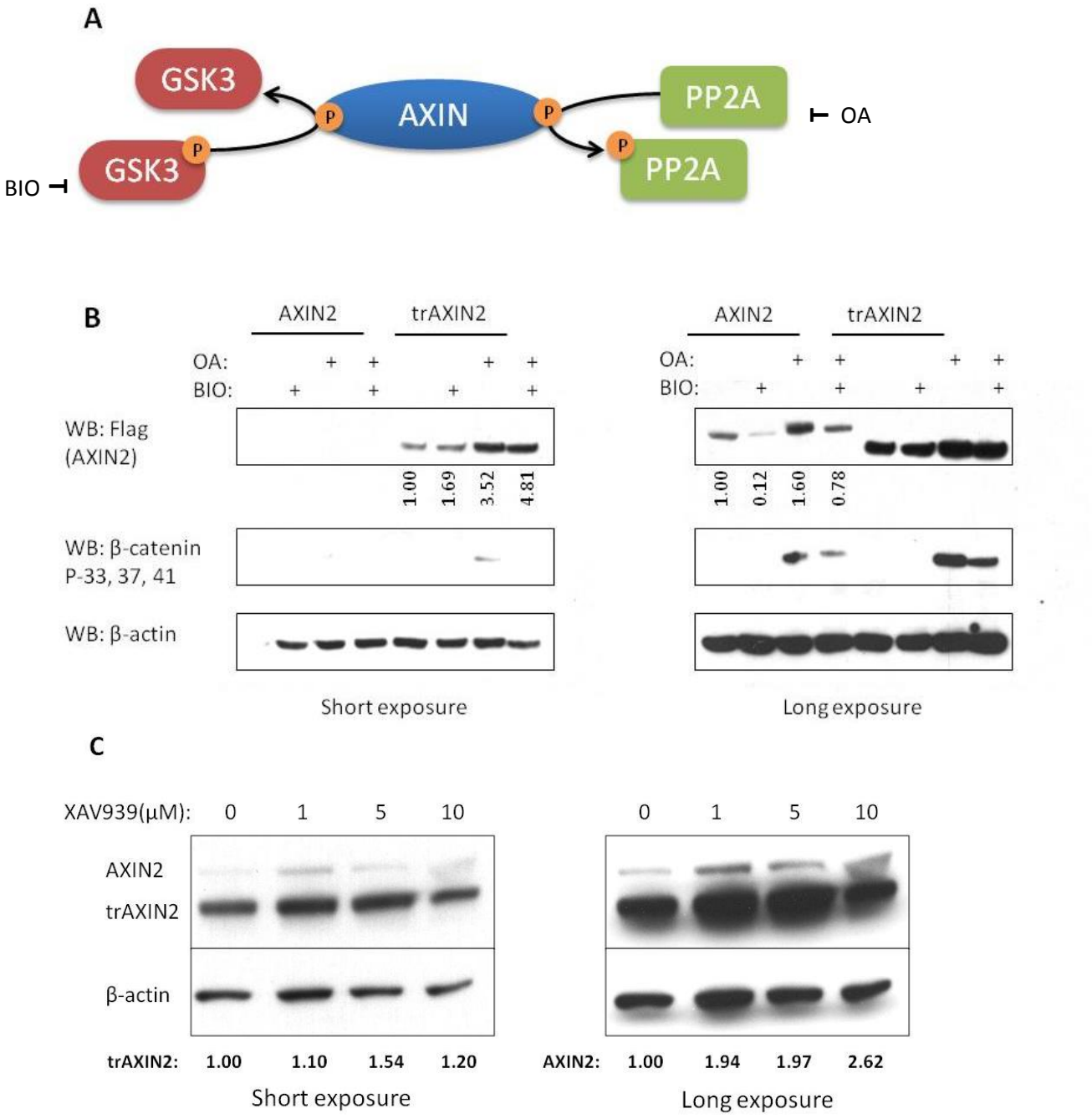


Figure 4.6- trAXIN2 stability is not GSK3-β dependent and does not respond to XAV treatment
(A) Diagram of the regulation of AXIN phosphorylation by GSKβ and PP2A. (B) Treatment of 293T cells expressing full-length or truncated AXIN2 proteins with 1μM BIO and 100nM Okadaic Acid (OA) shows a differential stabilization by GSK3β-inhibtion. Quantification of AXIN2 protein levels is shown in relation to the β-actin loading control. A Western blot of phospho-β-catenin is included as a control for OA activity. (C) Treatment with the TNKS inhibitor, XAV939, stabilizes AXIN2 to a greater extent than trAXIN2 protein.

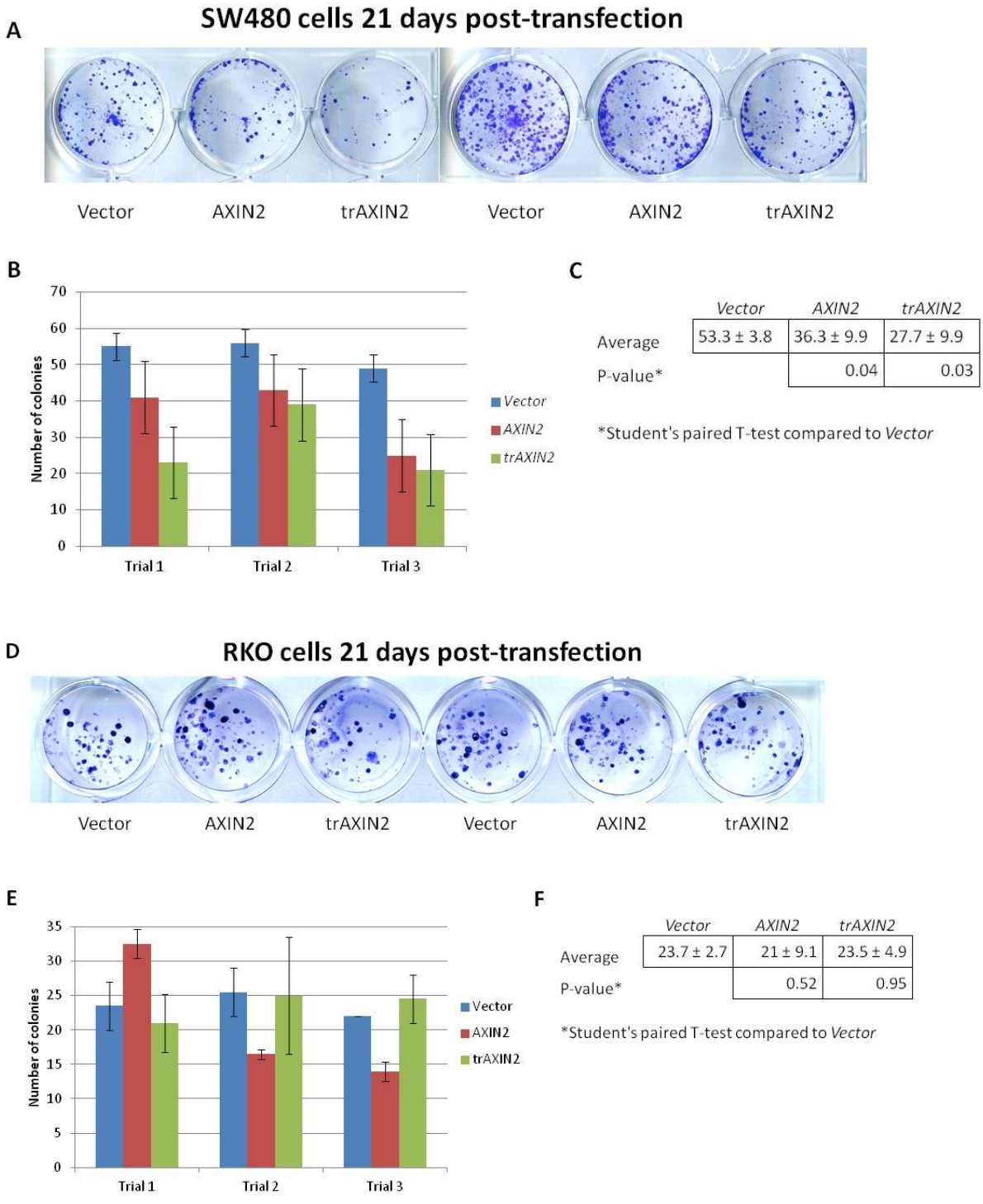


Figure 4.7- AXIN2 and trAXIN2 inhibit colony formation in Wnt-dependent colon cancer cells
Drug-resistant colony formation assays in SW480 (A-C), and RKO (D-F). Similar to AXIN2, trAXIN2 expression inhibits colony formation in SW480 cells, which have aberrantly activated Wnt/β-catenin signaling, but not in RKO cells, which have intact Wnt/β-catenin signaling.

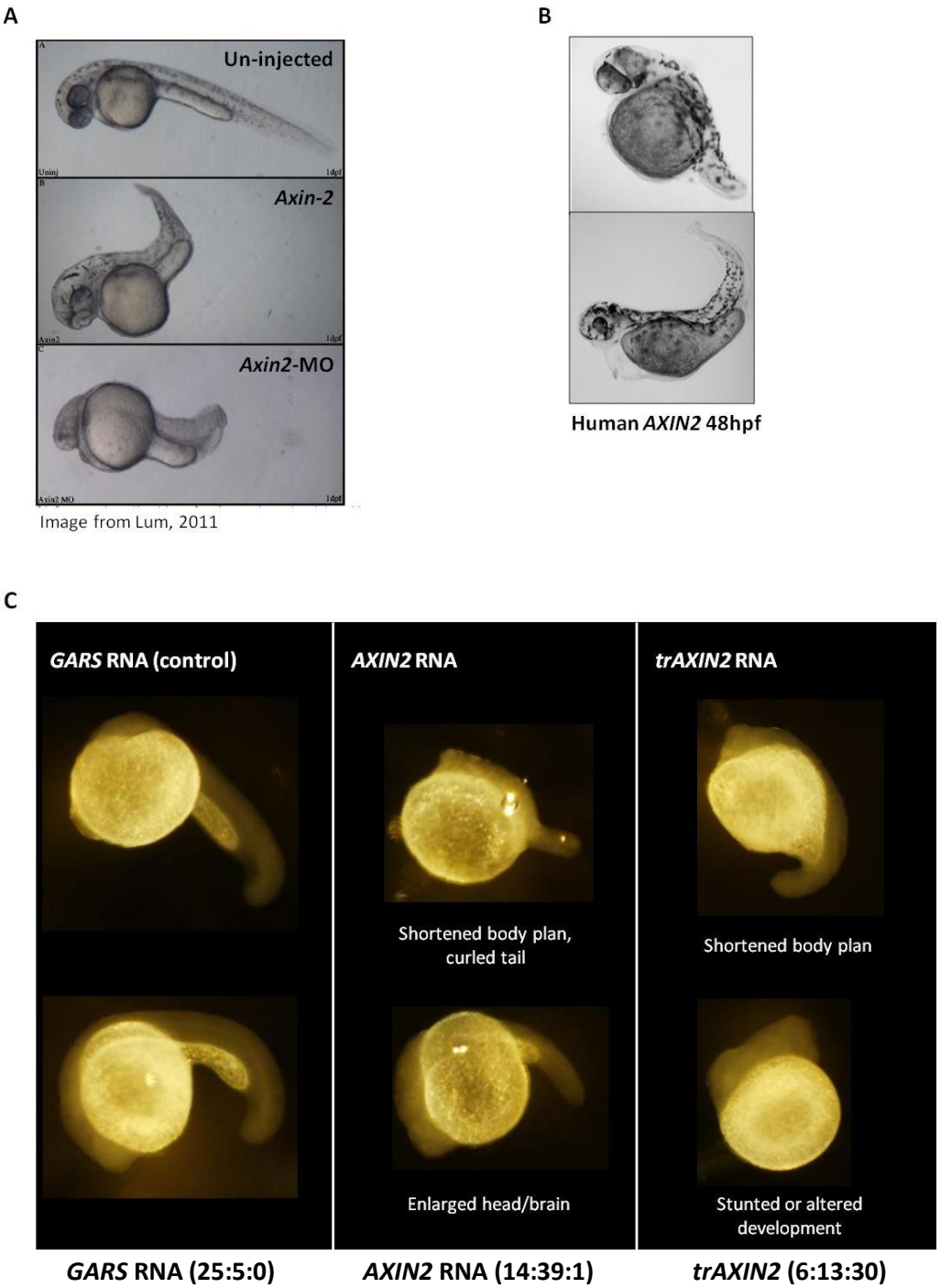


Figure 4.8- *AXIN2* and *trAXIN2* produce a developmental phenotype in zebrafish
(A) Previously published work showed that over-expression and knockdown of zebrafish *Axin-2* causes a developmental phenotype. (B) Ectopic expression of human *AXIN2* results in a developmental phenotype similar to zebrafish *Axin-2*. (C) Developmental phenotypes of zebrafish embryos 27hpf show that *trAXIN2* produces a more severe developmental phenotype. Quantification of phenotypes shown below (wild-type: altered body plan: severely stunted).

Chapter 5: The role of *Axin2* in a mouse model of colon cancer

Abstract

In order to further explore the significance of the *1989G>A* truncating mutation in disease, it is necessary to more properly define the role of wild-type AXIN2 under normal physiological conditions. If the AXIN2 protein indeed acts as a tumor suppressor, this could help inform predictions of loss-of-function vs. gain-of-function mechanisms. Ongoing studies with a mouse model of the *Axin2*-null allele aim to determine how loss of *AXIN2* might alter normal colon epithelial development or contribute to cancer progression. Additionally, although RNA studies in the proband's PBLs suggests otherwise, if the *1989G>A* allele is a null allele, this mouse model would be an appropriate model for the constitutional loss of one allele of Axin2. Studies of these *Axin2* mice found that mice with one or no functional *Axin2* alleles have normal teeth. Analysis of colon tissues in a preliminary cohort found no change in proliferation across genotypes, but in liver tissue, some Wnt target genes were elevated with loss of *Axin2*. Using a mouse model of colon cancer, I am also testing whether loss of *Axin2* enhances the tumorigenic phenotype, a result that could support AXIN2 as a tumor suppressor in CRC. While the tumor-based studies are still ongoing, the first cohorts of $Apc^{fl/fl}$ mice suggest that *Axin2* genotype has no effect on survival, but may have more subtle influences on proliferation or the distribution of adenomas in the colon.

Introduction

Mutations in the *APC* tumor suppressor gene are found in approximately 80% of human CRCs and germline mutations in *APC* underlie the hereditary CRC syndrome FAP[11; 12; 63; 137; 140]. The Fearon lab previously developed a mouse model of colorectal cancer using a floxed (fl) *Apc* allele[141] and a Tamoxifen-inducible Cdx2-Cre (Cdx2P-CreER^{T2})[142]. This model restricts the expression of Cre recombinase to caudal embryonic tissues and to the epithelium of the distal ileum, cecum, colon, and rectum in the adult mouse, with the strongest induction of Cre activity occurring in the cecum and proximal colon (Appendix D). Cdx2P-CreER^{T2}*, Apc$^{fl/fl}$* mice rapidly develop adenomas following intraperitoneal (IP) injection with Tamoxifen (TAM) and become moribund in 20-30 days.

Interestingly, *AXIN2* itself is a β-catenin/TCF target gene and is up-regulated in cancers with activating Wnt pathway mutations[39; 40]. In the context of the germline mutations associated with oligodontia/CRC predisposition, it is important to better understand what role the wild-type AXIN2 protein may play in cancer development or progression in order to further explore the functional significance of germline and somatic truncating mutations. AXIN2 is hypothesized to be a tumor suppressor in CRC and studies with a small molecule that stabilizes both AXIN1 and AXIN2 found that increased AXIN protein levels reduced the number and size of tumors in an *Apc*-dependent model of CRC[69]. However, some reports suggest that high levels of AXIN2 protein may actually promote invasive and metastatic phenotypes in cancer cells as seen in tissue culture and mouse xenograft experiments[50; 51]. To test the hypothesis that AXIN2 acts as a tumor suppressor in CRC, mice carrying an *Axin2*-null allele, *Axin2^{LacZ}*, were crossed to

the Cdx2P-CreER^{T2} mouse model of *Apc*-dependent colon cancer to ask whether loss of *Axin2* modifies the *Apc* tumor phenotype.

Materials and Methods

Mouse Models and Tamoxifen Treatment

An *Axin2^{LacZ}* mouse was obtained from The Jackson Laboratory on a C57BL/6 background (stock number: 009120) and bred to C57BL/6 mice to generate a colony of *Axin2^{LacZ}* mice. These mice were then bred to our floxed *Apc* tumor model to create *Apc$^{fl/fl}$,* Cdx2P-CreER^{T2} mice with varying *Axin2* genotypes. Mice were aged to at least three months and then injected with 150µl of TAM (Sigma-Aldrich) on two consecutive days. Injections were delivered IP with 20mg/ml TAM dissolved in corn oil. The mice were then monitored daily and euthanized at 15 days post TAM treatment or when they appeared moribund. One hour prior to euthanization, the mice received an IP injection of 100µl of 10mg/ml BrdU. All animal care and experimental procedures were performed in compliance with the guidelines set by the University of Michigan's University Committee on Use and Care of Animals.

Analysis of teeth

The teeth of six *Apc $^{fl/fl}$*, *Axin2$^{LacZ/LacZ}$* mice; two *Apc $^{fl/fl}$, Axin2$^{LacZ/+}$* mice; two *Apc $^{fl/fl}$, Axin2$^{+/+}$* mice; and two *Axin2$^{LacZ/LacZ}$* mice were examined by eye for enamel coloration, incisor shape and size, and the number of upper and lower molars.

Immunohistochemistry

Tissues were fixed overnight in 10% buffered formalin, processed, embedded in paraffin, and 5μM sections cut. Slides were stained with H&E and BrdU (BrdU antibody from BD Pharmigen). Tissues were stained for BrdU using the Vector Labs M.O.M. (Mouse on mouse) and DAB kits. Briefly, the BrdU staining protocol is as follows. Slides were baked at 58˚C and then rehydrated in xylenes and a gradient of ethanol. Antigen retrieval was done Citra Plus buffer (BioGenex) and slides were incubated in 2N HCl at 37˚C for 30 minutes and then endogenous peroxidase activity was quenched by incubation in 80%MeOH/ 3%H_2O_2 for 20 minutes. BrdU antibody was used at a concentration of 1:400 following the M.O.M. and DAB published protocols with the modification of an overnight incubation at 4˚C with the primary antibody. The number of BrdU positive cells were counted using ImageJ software (nih.gov).

RNA isolation

RNA was isolated by homogenizing tissues in Trizol followed by RNA isolation. RT-PCR using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize cDNA and gene expression was analyzed by qPCR. The primers used for qPCR are shown in the table below.

Table 5.1- qPCR primers for Wnt target genes

Gene	Sequence
β-actin	F- GCCTTCCTTCTTGGGTATGG R- GCCTGGGTACATGGTGGT
Axin2	F- GAGGATGCTGAAGGCTCAAA R- GCAGGCAAATTCGTCACTC
Lgr5	F- GGAATAAAGACGACGGCAAC R- GGATCAGCCAGCTACCAAA
Irs1	F- GACGCTCCAGTGAGGATTT R- AGGTCCTGGTTGTGAATTGTG
Nkd1	F- CCGAGCAGACACCAAACC

	R- TCCTCTCAATGTTCTCATCCA

Results

I began by analyzing the *Axin2* mice under normal conditions. Mice carrying the *Axin2*^{LacZ} allele were aged to 6 months and then the colon and intestine were collected from each mouse for tissue analysis, as well as a sample of the liver, a tissue with high expression of Axin2 protein, for RNA analysis. I found that without exogenous stimulation, the expression of some Wnt target genes is elevated in the liver tissue of mice with loss of *Axin2* (*Axin2*$^{LacZ/\ LacZ}$ genotype) (Figure 5.1). This finding sets the stage for tissues that could be primed for Wnt activation, or that could display a potentiated response to Wnt stimulation as previously seen in mouse mammary stem cells[53].

Wnt signaling is activated at the base of colon crypts[143] and Wnt signaling is essential for normal colon epithelial cell proliferation[144]. As a negative regulator and feedback repressor of Wnt signaling, Axin2 could be playing an important role in the maintenance of Wnt signaling in the colon, and loss of Axin2 protein could alter the distribution of Wnt signaling, skew the differentiation pattern of colon epithelial cells, or change the number of proliferating cells[145]. To assess the impact of loss of *Axin2* on proliferation, I analyzed the number of BrdU positive cells in the proximal and distal colon of these mice (Figure 5.2, Table 5.2). From this first small cohort, it is not clear that a change in the levels of Axin2 protein influences proliferation in the colon. Analysis of more mice is ongoing. Finally, because the family carrying the *1989G>A* allele has oligodontia, I examined the teeth of the *Axin2*$^{LacZ/LacZ}$ mice and *Apc* $^{fl/fl}$, *Axin2*$^{LacZ/LacZ}$ mice but found no abnormalites in the shape of their incisors or the number of molars (Figure 5.3).

To assess the consequence of loss of *Axin2* on the *Apc* tumor phenotype, three mice of each *Axin2* genotype (wild-type, heterozygous, or homozygous for the *LacZ* allele) were injected with two consecutive doses of TAM and monitored for 15 days at which time the mice were euthanized and their tissues collected for analysis. One $Axin2^{LacZ/LacZ}$ mouse was euthanized on day 14 at the request of the University Laboratory Animal Medicine team (ULAM). His paired littermates were also euthanized at this time point. While two of the three $Axin2^{LacZ/LacZ}$ mice appeared ill and were flagged by ULAM for rapid weight loss within the first eight days post-TAM injection, the other genotypes appeared moderately healthy at the 15-day euthanization time point. Colon tissues from these mice were analyzed for β-catenin localization and proliferation via BrdU staining. However, while the colon tissues displayed hyperplasia and increased crypt height, there were few adenomatous areas and little nuclear β-catenin staining.

The $Axin2^{LacZ/LacZ}$ mice are frequently flagged for their small size and altered body stature, so the study was repeated with a second cohort, using both *Cre*-positive and *Cre*-negative mice to assess whether the weight loss seen in the $Axin2^{LacZ}$ homozygous mice was specific to their genotype, and if it would impair survival for tumor studies. These mice were injected with the same two-dose TAM scheme and then weighed daily and monitored until they became moribund. In this small cohort, TAM injection caused a uniform loss in weight regardless of *Axin2* or *Cre* genotype, and all mice recovered from the TAM-induced weight loss until becoming moribund (Figure 5.4). Unlike the first cohort of mice, we saw adenomas and nuclear β-catenin staining in all Cre-positive mice. This second cohort survived longer than expected, although a new batch of Tamoxifen was used, so there could be lot to lot variation in

the potency of the drug. All the subsequent studies of these mice used this same, second batch of Tamoxifen to maintain consistency.

A final, third cohort with two mice of each *Axin2* genotype was injected with the same two-dose scheme and monitored until moribund. In examining these mice along with the second cohort, there was no significant change in survival (Table 5.3), although there did appear to be a possible difference in the gross physiology of the mice based on *Axin2* genotype. The usual phenotype seen in $Apc^{fl/fl}$, Cdx2P-CreER^{T2} mice is hyperproliferation of the cecum and proximal colon with polyposis present in the proximal to upper middle colon with few distal alterations. Loss of *Axin2* appeared to potentially reduce hyperproliferation and polyposis in the proximal colon, and to shift the adenoma phenotype more distally (Figure 5.5). There also appeared to be an increase in the number of lymphoid cells seen in the $Axin2^{LacZ/LacZ}$ mice, although the cohort size is small and further histological analysis of these mice is ongoing.

Discussion

The truncating germline *AXIN2* mutations reported in CRC predisposition syndromes could be null or loss of function alleles, in which case, the $Axin2^{LacZ/+}$ mouse could model the developmental and tumorigenic effects of these alleles. Additionally, the $Axin2^{LacZ/LacZ}$ mouse could model the loss of the second *AXIN2* allele during cancer development, if *AXIN2* adheres to a traditional two-hit tumor suppressor model. The two families with germline, truncating *AXIN2* mutations have oligodontia, a phenotype that was not seen in the $Axin2^{LacZ}$ mice, but this does not prove that the oligodontia is unrelated to an *AXIN2* mutation, or that the *AXIN2* mutation is not a loss-of-function. Missense mutations in *AXIN2* have been linked to oligodontia[123; 146; 147]

and assessment of the oligodontia phenotype in mice is complicated by the differences in mouse and human dentition. Humans have two stages of dentition, and the defect in the *1989G>A* individuals is in the second dentition (permanent teeth). Mice, on the other hand, have only one stage of dentition and a pair of continuously growing incisors, neither of which is an accurate recapitulation of human secondary dentition.

The colon epithelium of *Axin2*$^{LacZ/+}$ and *Axin2*$^{LacZ/LacZ}$ does not appear grossly different than in wild-type mice and no difference in proliferation was seen as evidenced by BrdU staining. Some Wnt target genes were found to be elevated in the liver, a tissue that expresses AXIN2 protein under normal conditions, suggesting that there are potentially differences in Wnt activity in colon tissue as well. AXIN2 is one of many feedback repressors of Wnt signaling, and if Wnt signaling is elevated at baseline, then it could "prime" the cells, making them more susceptible to a pro-oncogenic hit. Further histological analysis of the cell lineages present in the colon of these mice will ask if there are any developmental defects due to this difference in Wnt regulation, as an activation of Wnt activity has been shown to alter differentiation, survival, migration, and proliferation in the intestine[148]. The analysis of tumor studies with mice on the *Apc*$^{fl/fl}$ background is still underway, but the data thus far suggests that there is no difference in survival although there may be a shift in the hyperproliferation and adenomatous phenotype toward the distal end of the colon, which matches the gradient of *Axin2* expression in the mouse intestine and colon. It is possible that the impact of a change in Axin2 protein level cannot be observed in this mouse model because the loss of both *Apc* alleles creates such a severe distortion of Wnt signaling, or because the defects in *Axin2* are redundant to *Apc* and would only impact cancer progression in the absence of other Wnt pathway abrogation. To

address this issue, I am also testing the *Axin2* mice in a heterozygous *Apc* background and in a *Kras* hyperplasia model (see Chapter 6). Two families have been reported to have a colorectal cancer syndrome including oligodontia that is associated with a germline truncating mutation in *AXIN2*. The mechanism by which this mutation predisposes to colon cancer remains unclear. Mice lacking one or two copies of *Axin2* display no tooth phenotype, but loss of *Axin2* may enhance baseline Wnt signaling and alter the distribution of the hyperplasia/adenoma phenotype seen in $Apc^{fl/fl}$ mice. Further studies with a knock-in mouse model of one of the human mutations, and looking at the role of *Axin2* in other models of CRC could clarify the role *AXIN2* plays in cancer predisposition.

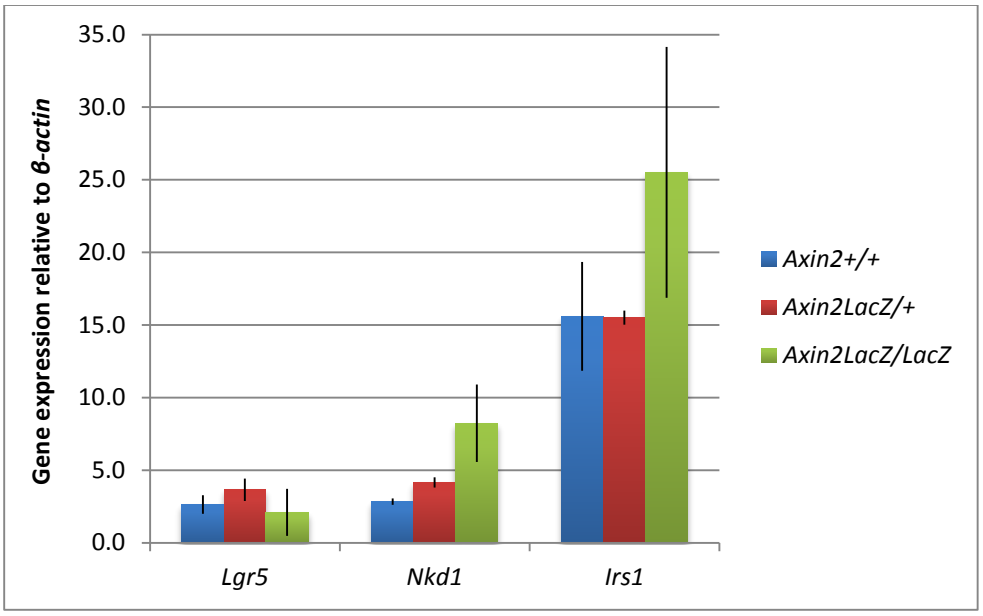
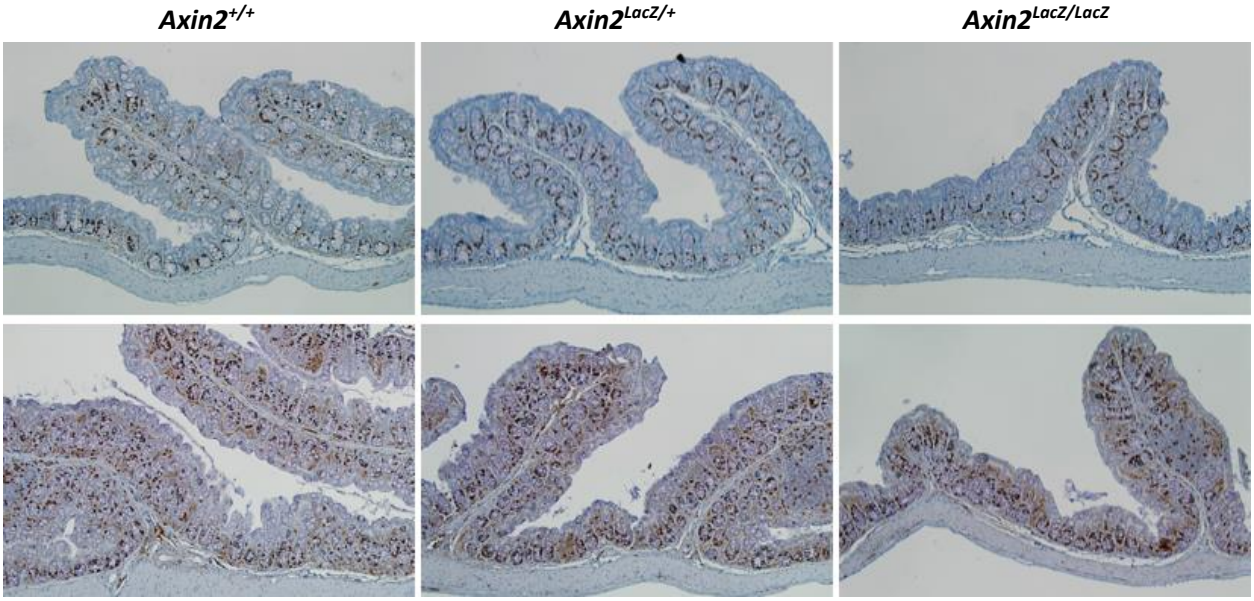


Figure 5.1- Expression of some Wnt target genes is elevated in *Axin2*^{LacZ} mouse liver tissue
Wnt/β-catenin target gene expression in liver tissue was measured by qPCR. Data are shown as the average of two mice per genotype ± standard deviation.

Proximal Colon



Distal Colon

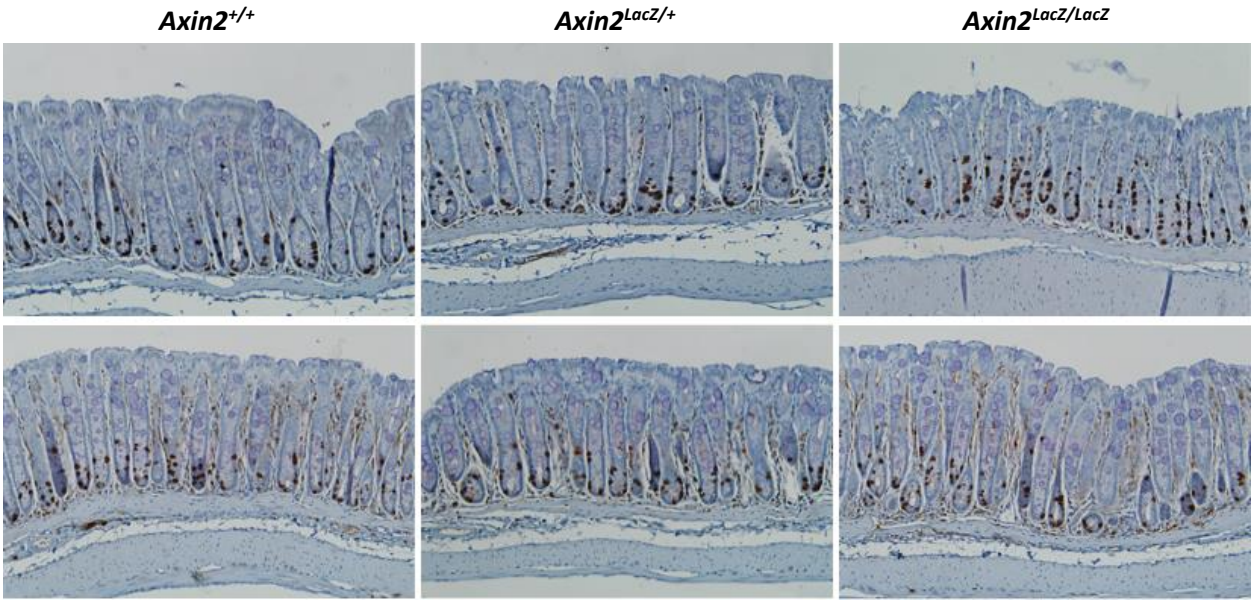


Figure 5.2- BrdU staining to measure proliferation in the colon of *Axin2*^{LacZ} mice
Tissue sections of the proximal and distal colons of six month old mice were stained for BrdU positive cells. A quantification of the staining is shown in Table 5.2.

Table 5.2- Quantification of BrdU-positive cells in the colon of *Axin2*^{LacZ} mice

	Genotype	Number of crypts counted	Total number of BrdU+ cells	BrdU+ cells per crypt
Proximal colon	*Axin2* $^{+/+}$	42	424	10.1
	Axin2 $^{+/+}$	23	160	7.0
	Axin2 $^{LacZ/+}$	33	189	5.7
	Axin2 $^{LacZ/+}$	25	208	8.3
	Axin2 $^{LacZ/LacZ}$	34	295	8.7
	Axin2 $^{LacZ/LacZ}$	44	278	6.3
Distal colon	*Axin2* $^{+/+}$	19	109	5.7
	Axin2 $^{+/+}$	47	255	5.4
	Axin2 $^{LacZ/+}$	44	330	7.5
	Axin2 $^{LacZ/+}$	59	360	6.1
	Axin2 $^{LacZ/LacZ}$	37	179	4.8
	Axin2 $^{LacZ/LacZ}$	50	338	6.8

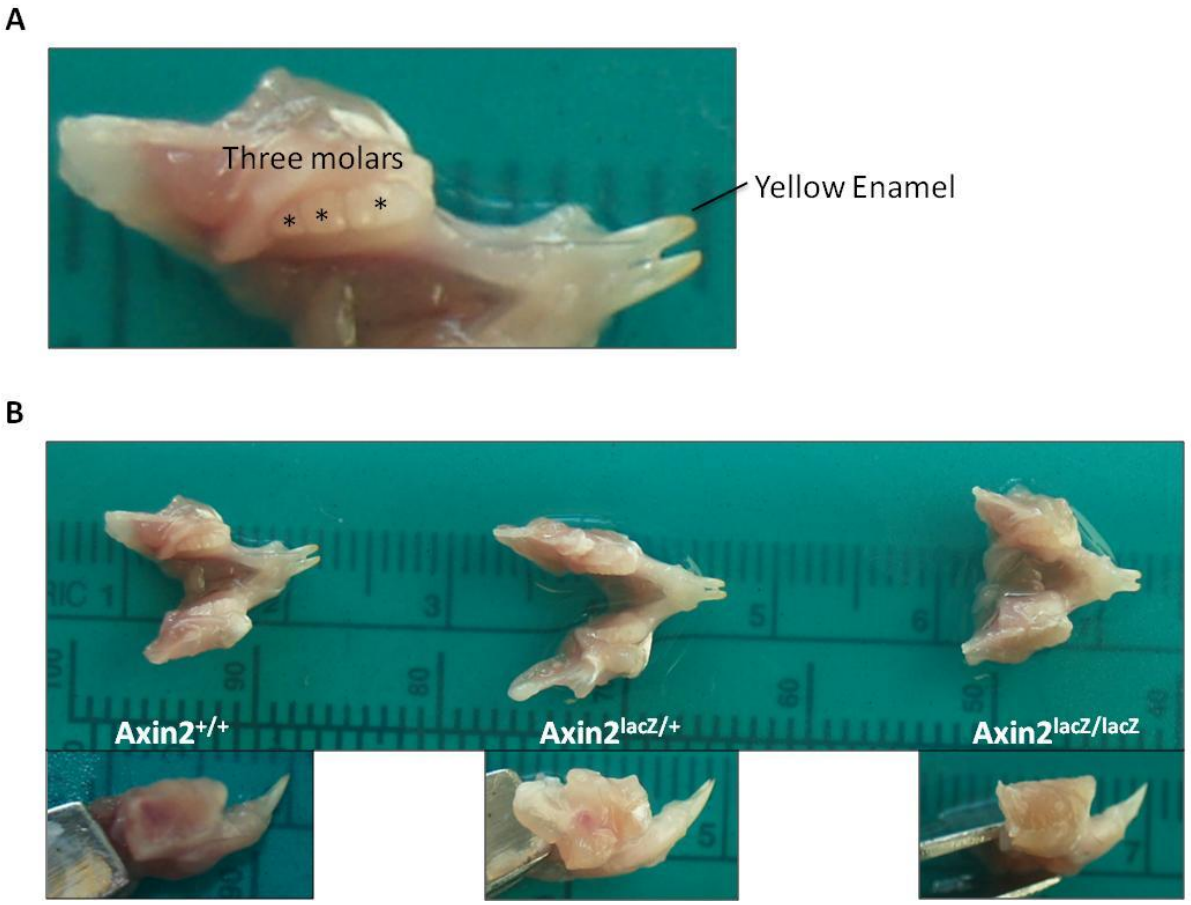


Figure 5.3- Analysis of the tooth phenotype in *Axin2^{LacZ}* mice
(A) A close-up image identifying the three lower molars and proper yellow shade indicating enamel on the incisors. (B) Sample side-by-side comparison of *Apc$^{fl/fl}$* mice shows no significant difference in the size or shape of the incisors.

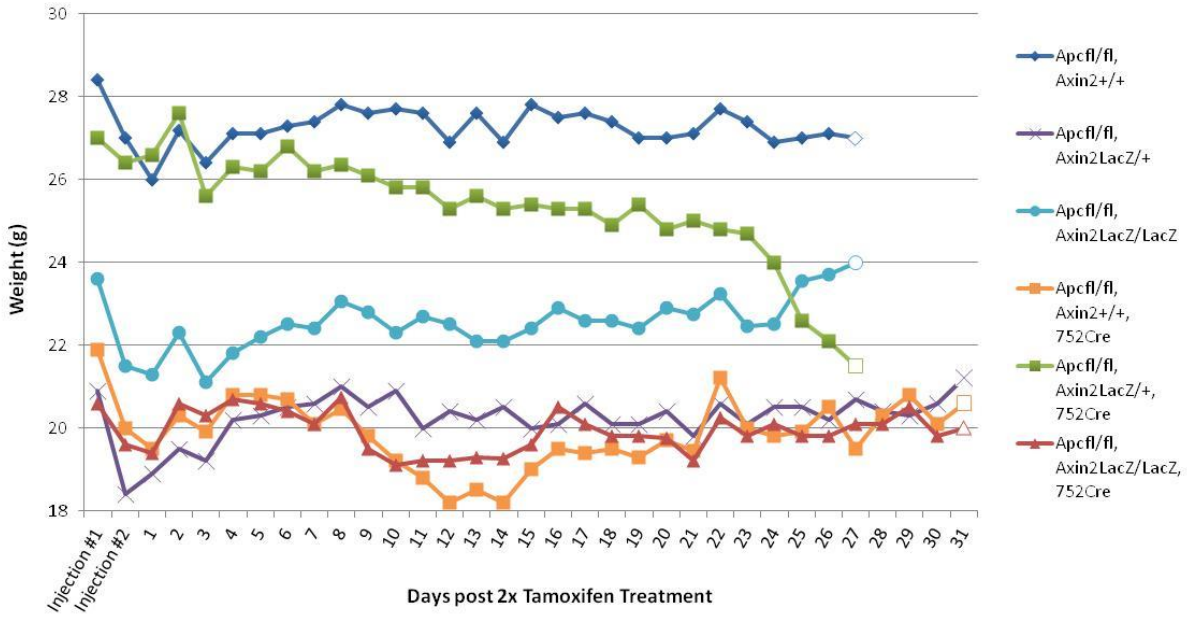


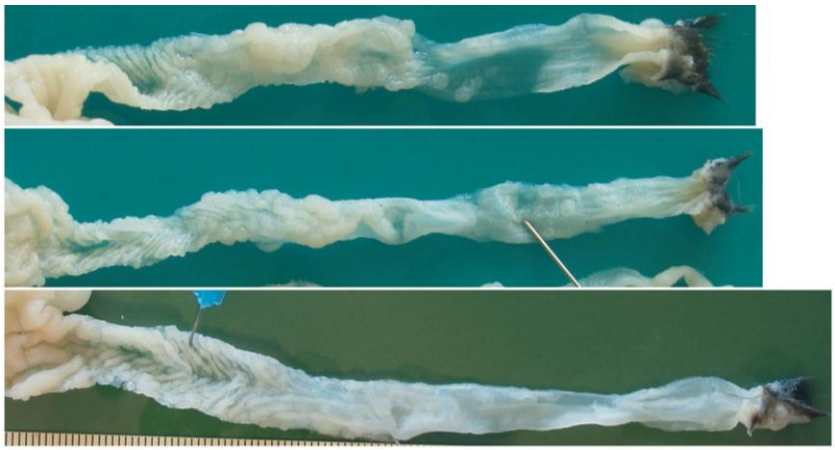
Figure 5.4- Analysis of weight loss in *Apc$^{fl/fl}$, Axin2^{LacZ}* mice
Following two doses of TAM, mice were monitored and weighed daily until becoming moribund. Cre-negative mice were euthanized with their Cre-positive littermates. Of note, the Cre-positive *Axin2$^{LacZ/+}$* mouse was male, while the *Axin2$^{+/+}$* and *Axin2$^{LacZ/LacZ}$* mice were females.

Table 5.3- Survival statistics of *Apc*$^{fl/fl}$ mice

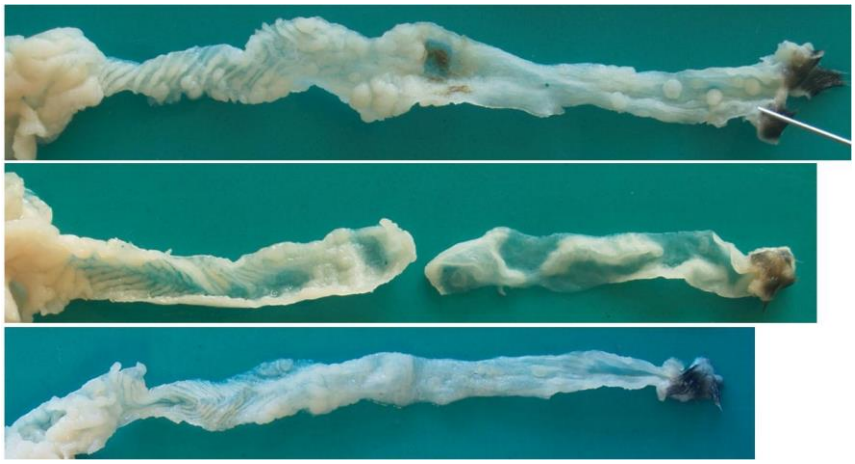
Ear tag	Sex	*Axin2*	Age @ inj.	Days post-inj.	
SM-34	F	*+/+*	149	31	
SM-55	M	*+/+*	201	28	
SM-112	F	*+/+*	118	32	30 ± 2 days
SM-35	M	*lacZ/+*	149	27	
SM-57	F	*lacZ/+*	201	29	
SM-111	F	*lacZ/+*	118	47	34 ± 11 days
SM-40	F	*lacZ/lacZ*	149	31	
SM-53	M	*lacZ/lacZ*	215	29	
SM-90	M	*lacZ/lacZ*	153	22*	27 ± 5 days

*This mouse was euthanized at 22 days at the request of ULAM because of anemia
Mean survival ± standard deviation.

Apc$^{fl/fl}$, Axin2$^{+/+}$



Apc$^{fl/fl}$, Axin2$^{LacZ/+}$



Apc$^{fl/fl}$, Axin2$^{LacZ/LacZ}$

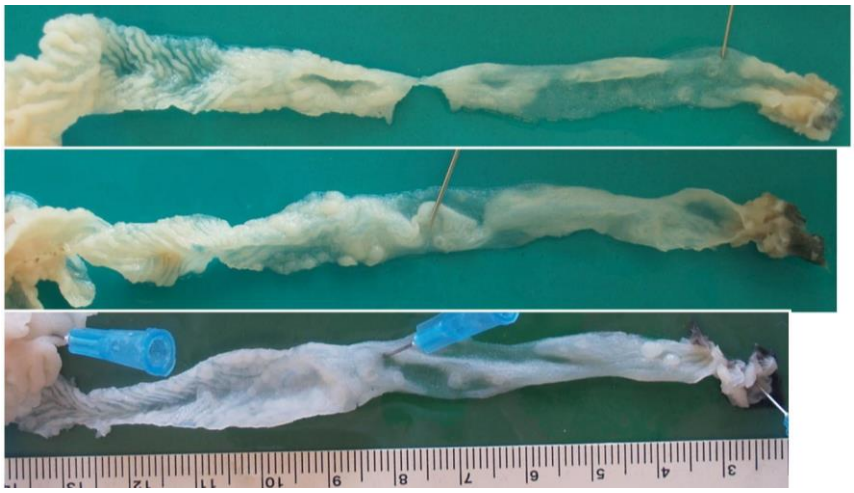


Figure 5.5- Gross colon phenotype of *Apc$^{fl/fl}$* mice post-TAM treatment
After two doses of TAM, mice were followed until moribund. Tissues were collected, fixed, and photographed. Colon images from three cohorts of mice show the thinner proximal colon tissue and perhaps greater distal involvement in the *Axin2$^{LacZ/+}$* and *Axin2$^{LacZ/LacZ}$* mice.

Chapter 6: Conclusions

Summary of findings

The Wnt pathway has essential roles in development, differentiation, and maintenance of cellular homeostasis[4]. In the canonical Wnt pathway, activating Wnt ligands stabilize β-catenin, freeing it to translocate to the nucleus where it activates expression of β-catenin/TCF target genes. Somatic mutations that constitutively stabilize β-catenin are found in many cancers, including about 90% of colorectal cancers. Many issues regarding Wnt signaling in cancer remain unresolved, and genetic and cell-based studies may offer important insights. In my thesis work, I have sought to clarify the role that AXIN2, one of the proteins involved in regulation of β-catenin/Wnt-signaling, potentially plays in cancer.

Analysis of PBLs from an individual heterozygous for the *1989G>A* allele shows that transcripts from both the *A* and *G* allele are present in roughly equal amounts, suggesting that this transcript escapes NMD and can encode a truncated protein. Testing of this truncated protein *in vitro* found that the trAXIN2 protein can still interact with destruction complex proteins, including β-catenin and other AXIN1/2 molecules. Additionally, the trAXIN2 protein retains function in the inhibition of colony formation in a Wnt-dependent CRC cell line. However, the trAXIN2 protein in stable cell lines, has reduced function in the inhibition of Wnt target genes, suggesting that this allele could be hypomorphic. The trAXIN2 protein was also found to be more abundant, presumably more stable, than wild-type protein and this increased

protein level could account for the equivalent colony inhibition and zebrafish developmental phenotype, even if the allele is hypomorphic. Finally, the trAXIN2 protein stability was found to be regulated by PP2A, but inhibition of TNKS1/2 and GSK3β had minimal effect on protein levels. This difference in the regulation of protein levels points to a functional difference between the trAXIN2 and AXIN2 proteins, but the exact mechanism needs further investigation.

Overall, this data supports the conclusion that the *1989G>A* allele is neither a null allele nor a complete loss of function. Rather, this allele appears to be hypormorphic in the inhibition of Wnt/β-catenin signaling. It is also possible that other non-canonical protein interactions have been disrupted, creating loss-of-function phenotypes; or the trAXIN2 protein could acquire novel protein interactions or traits, creating a gain-of-function phenotype. The increased protein stability could be due to either a gain or loss of some protein interaction, but with more trAXIN2 protein in the cell hypomorphic actions could be masked, or gain-of-function actions amplified. Additionally, with more trAXIN2 in a cell, β-catenin destructions complex components may be tied up with a more abundant, but less functional version of AXIN2, perhaps slowing the turnover of β-catenin and delaying negative feedback repression.

Significance

Individuals carrying the *1989G>A* allele have a predisposition to colon cancer. *AXIN1* and *AXIN2* mutations have been reported in a subset of many cancers, but clear functional evidence to support the role of these two proteins as tumor suppressors in CRC is lacking. In addition to creating a better understanding of the truncating germline and somatic mutations reported in association with CRC, it is important to clarify the role of AXIN2 in cancer. The role of AXIN2 in

promoting or suppressing cancer is debated; therefore, a better understanding of the role of the protein, and mutant forms of the protein, in normal and cancer contexts will be key for determining if AXIN proteins are an appropriate therapeutic target.

Ongoing work

Axin2 *modification of the* Apc *tumor phenotype*

While I am completing analysis of the inducible *Apc* homozygous mice, I have also been ageing mice that have a constitutional *Apc* defect[141]. Similar to the Cdx2P-CreER^{T2}mice, these Cdx2P-NLS Cre mice express a Cre recombinase specifically in the caudal portion of the body, but rather than being inducible, the NLS Cre transgene is constitutive. Cdx2P-NLS Cre*, Apc$^{fl/+}$* mice develop disease more distally than other mouse models of CRC and they survive for approximately 200 days of age with a sex-specific difference in survival[141]. Because the tumorigenic effects of Axin2 may act via the Wnt signaling pathway, and because the Apc$^{fl/fl}$ tumor phenotype develops so rapidly, the heterozygous state might allow us to observe more subtle changes caused by changes in Axin2 protein levels.

The findings of a recent study of Sessile Serrated Adenomas suggested that a decrease in *AXIN2* expression correlates with smaller, more distal adenomas[92]. While Sessile Serrated Adenomas are more associated with *BRAF* and *KRAS* mutations, it will be interesting to see if we find similar changes in phenotype as we continue to analyze our *Apc*-dependent CRC model.

Knock-in analysis and rescue

In order to answer, *in vivo*, what is the significance of the *1989G>A* allele, I have generated a knock-in mouse of the human allele. Because I have been unable to prove if the *1989G>A* allele is a loss of function allele or if it has some altered function, studies in the mouse will be highly valuable. These mice will be examined for an ectodermal phenotype (alterations in teeth or hair) and for a colon phenotype to determine if the *1989G>A* allele affects colon epithelial development or changes the steady state of Wnt signaling or proliferation. Crossing these mice to the *Apc* heterozygous background or a *Kras* background to sensitize them will help determine if the colon epithelium in these mice is predisposed to developing adenomas. Finally, these mice can be crossed to the $Axin2^{LacZ}$ mice to test if one or two copies of the *1989G>A* allele is sufficient to rescue the *Axin2*-null phenotype, perhaps answering whether this allele is hypomorphic.

Future directions

AXIN2 protein interactions required for Wnt signal transduction

I have shown that trAXIN2 still interacts with other AXIN molecules and β-catenin, but to further understand the impact of the trAXIN2 protein, I would test if the protein retains interactions known to be important for the transduction of a Wnt signal. A recent study on the conformation of AXIN proteins suggests that the protein auto-regulates its function via an interaction between the N- and C-termini of the protein that renders the protein in a "closed" conformation[149]. A Wnt-responsive luciferase screen identified a small molecule that potentiated stimulation with a Wnt ligand. It was found that this small molecule binds to the AXIN1 DIX domain and prevents the self-interaction between the N- and C-termini of the

protein. Without this interaction, the protein is always in the "open" conformation, with increased affinity for LRP5/6 binding. This means that "open" AXIN1 localizes to the membrane, rather than to β-catenin destruction complexes, increasing the levels of nuclear β-catenin and Wnt target gene expression[149]. It has been previously found that the AXIN/LRP interaction is necessary for Wnt signal transduction[29; 150], and concordantly, the authors saw a stabilization of β-catenin and an increase in Wnt target genes, although only in response to a Wnt ligand, not at steady state[149]. The authors went on to show that an AXIN1 protein lacking the N-terminal domain has a higher affinity for LRP5/6 binding[149]. The authors propose that the transition of AXIN1 from "closed" to "open" is kinetically difficult, but that this small molecule, or loss of the interacting regions of the AXIN1 protein create a kinetically favorable "open" configuration that has a higher affinity for LRP binding and can thus activate or potentiate Wnt signaling[149]. The authors only tested a ΔN-AXIN1 protein, not a ΔC-AXIN1 protein, but it is possible that ΔC-AXIN1 would similarly reside in the "open" conformation and potentiate Wnt signaling.

While most of the studies of Wnt signal transduction has been completed with AXIN1, it is predictable that these functions could extend to the AXIN2 or trAXIN2 protein, so I would propose to test the interaction of AXIN2 and trAXIN2 with LRP5/6 and DVL, another protein important for the Wnt membrane signalsome[77]. These interactions could provide useful insights because the DVL protein interacts via the DIX domain[77; 132], which is missing in trAXIN2, and the LRP-interaction domain has been mapped to C-terminal half of the AXIN1 protein[151], so it is possible that trAXIN2 does not interact with LRP or DVL, which could have functional consequences for Wnt signal transduction.

AXIN2 as an oncogene: the* Axin2 Canp *allele

I have begun to ask how the dose of *Axin2* alters the tumor phenotype in a mouse model of colon cancer, but we are only looking at loss of *Axin2*, never gain. I propose to study the *Axin2*^{canp} allele to model the impact of increased dosage Axin2 on tumor development. While homozygotes for the *Axin2*^{canp} allele are lethal, heterozygotes are viable and rescue the *Axin2*-null phenotype[152]. Analysis of this allele in a CRC or other mouse models of cancer could help clarify if there is a role for AXIN2 in promoting cancer progression, invasion, or metastasis. Studies with this allele would be additionally helpful because the *canp* allele is stabilized by a disruption of the interaction with TNKS1/2[152], so this allele is a genetic model that could support or discourage pharmacological intervention with Tankyrase inhibitors.

AXIN2 and non-canonical signaling

Studies of AXIN2 in the context of *APC* mutation might hide Wnt pathway effects or complicate analysis because of the already activated Wnt/β-catenin signaling. The AXIN proteins have been previously reported to be involved in a multiple non-canonical signaling pathways including the Ras/ERK[56; 57] and SAPK/JNK pathways[58], so it is valuable to examine if trAXIN2 or loss of *AXIN2* alters these interactions. To address this, I will test the effect of AXIN2 and trAXIN2 on Ras stability and signaling in cell lines and in our knock-in mouse model. Furthermore, I propose to use a *Kras* model of intestinal hyperplasia[153] to investigate whether loss or gain of Axin2 protein (or mutation via *1989G>A*) promotes the progression of *Kras*-mediated hyperplasia to the adenoma stage, perhaps by a cooperative Wnt pathway activation, or via regulation of Kras.

Work to address the functional significance of Axin1 protein domains in the mouse model found that both the RGS domain and the six C-terminal amino acids (C6) of Axin1 are necessary for normal mouse development[154]. Mice homozygous for mutations that deleted the RGS or C6 domain of the Axin1 protein had an embryonic lethal phenotype similar to the *Axin1*-null phenotype. The C6 domain has been implicated in the activation of JNK signaling[155], and it is known that the C6 domain is the site of a SUMO modification that stabilizes AXIN1[156]. Interestingly, the *ΔC6* homozygous ES cells had no detectable Axin1 protein, although the mRNA levels were equivalent to wild-type. Furthermore, the embryonic lethality was partially rescued by loss of one allele of *Ctnnb1*, suggesting that the lethal phenotype is, in part, due to over-activation of Wnt signaling[154]. The C6 domain of Axin1 is conserved in human AXIN1 and AXIN2, so I propose to ask whether AXIN2 similarly activates the JNK signaling pathway, and if so, whether the trAXIN2 protein, which lacks the C6 domain, has any effect on JNK signaling. I would predict that the trAXIN2 protein does not affect JNK signaling, as C-terminal deletions in AXIN1 had reduced function in JNK signaling assays[58].

Finally, to address the importance of AXIN2 function outside the canonical Wnt pathway, I would cross the *Axin2*$^{LacZ/LacZ}$ mouse to a mouse with only one allele of β-catenin to determine if this rescues the *Axin2*-null phenotype. The importance of the AXIN proteins in non-canonical signaling and whether these pathways are activated via effects on β-catenin protein levels is unclear. Analysis of this cross could begin to answer these questions, and to determine whether β-catenin-dependent, or non-canonical roles are the key to AXIN2's functional significance in development and cancer.

In summary, the AXIN proteins play an established role in the inhibition of β-catenin dependent Wnt signaling and individuals with germline, truncating mutations in *AXIN2* have a predisposition to CRC. Additionally, the AXIN proteins have been proposed as therapeutic targets in cancer. Many questions remain about AXIN1 and AXIN2 under normal physiological conditions, and in cancer development, but further molecular experiments and mouse models can clarify the function and value of these proteins.

Appendices

Appendix A- Table of CRC mutations identified in Wnt pathway components

(Adapted from the TCGA dataset on human colon and rectal cancer mutations[63])

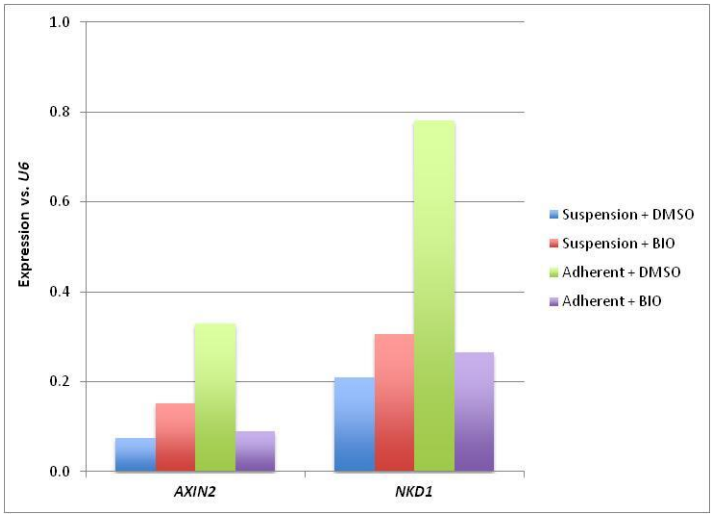
Gene	Frequency in non-HM CRCs	Frequency in HM CRCs
DKK1-4	4%	33%
LRP5	<1%	10%
FZD10	19%	13%
FAM123B	7%	37%
AXIN2	4%	23%
APC	81%	53%
CTNNB1	5%	7%
TCF7L2	12%	30%
SOX9	4%	7%
FBXW7	10%	43%
ARID1A	5%	37%
Total	**92%**	**97%**

non-HM, non-hypermutated
HM, hypermutated

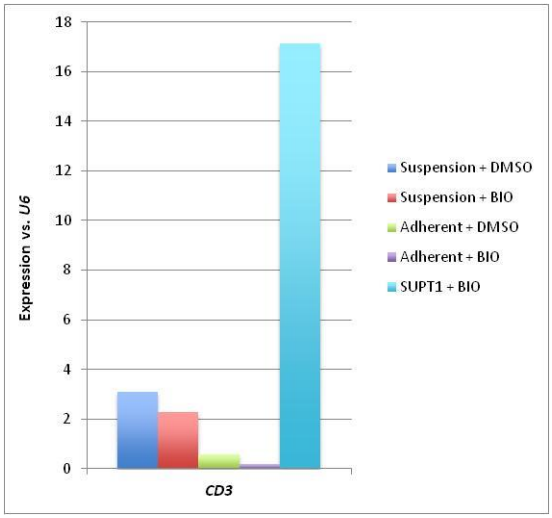
Appendix B- BIO induction of *AXIN2* expression in PBLs and the T-ALL cell line, SUPT-1

Target gene expression in cell populations treated with DMSO or BIO. (A) PBLs (Suspension) and PBMCs (Adherent) were treated with BIO to induce Wnt target genes, namely *AXIN2*. (B) The T-ALL cell line SUP-T1 was used to as a positive control for *CD3* expression to confirm that the isolated cells were indeed lymphocytes.

A

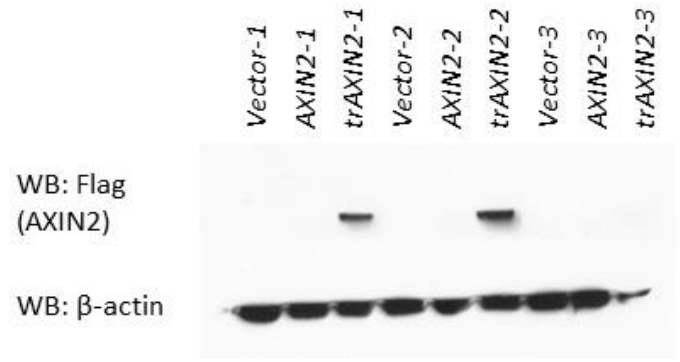


B



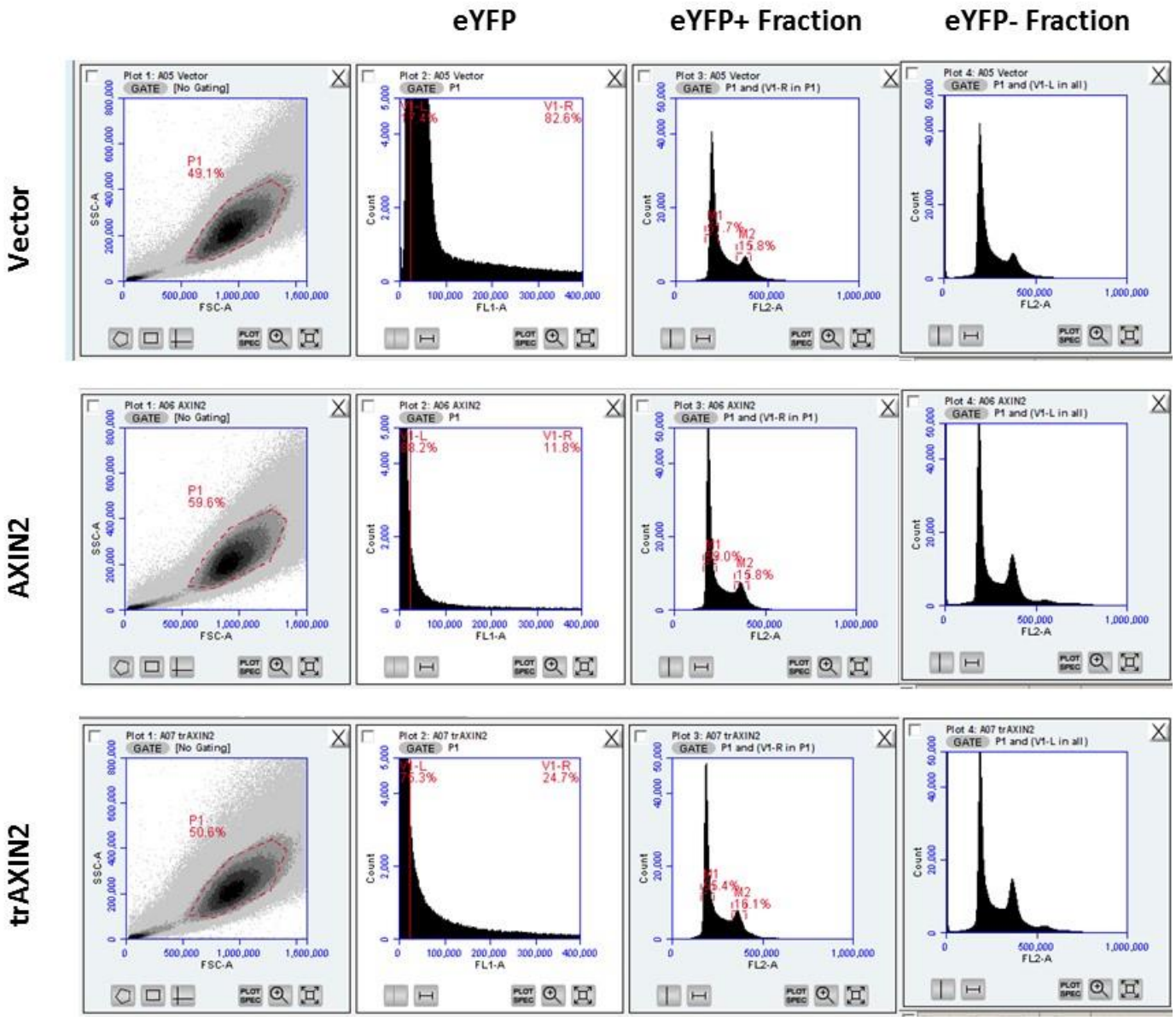
Appendix C- Western blot of AXIN2, trAXIN2 expression in SW-480 cells

SW-480 cells were transfected with expression vectors as indicated, counted and plated for drug-resistant colony formation. The remaining cells were grown under selection for 14 days and then lysed for Western blotting. Note the strong expression of trAXIN2.



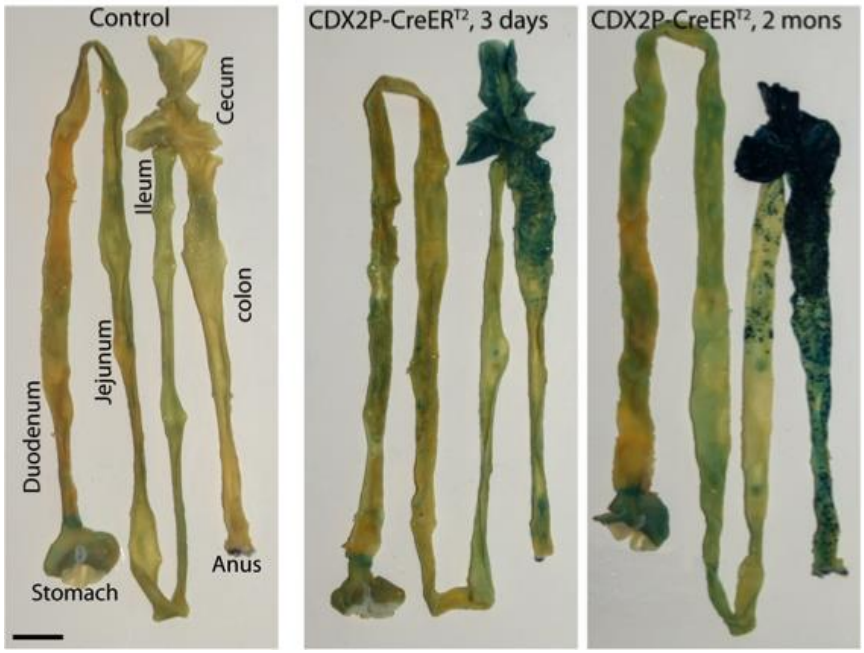
Appendix D- Flow cytometric analysis of cells expressing AXIN2 or trAXIN2 show no significant inhibition of proliferation or cell survival.

293T cells were transfected with eYFP-tagged *AXIN2*, *trAXIN2* or vector constructs. Sixteen hours post-transfection, cells were split 1:2 and cultured for 24 hours prior to fixation with PFA followed by propidium iodide staining. Cells were sorted by total DNA content and fluorescence to determine cell cycle distribution.



Appendix E- Cdx2P-CreER^{T2} activity is restricted to the colon and distal ileum

Mice carrying a floxed *LacZ* allele were treated with TAM to induce Cdx2P-CreER^{T2} expression. Staining for β-galactosidase activity shows that Cre expression is restricted to the colon, cecum, and distal ileum. (Image courtesy of Ying Feng)



References

1. Nusse, R., and Varmus, H.E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell 31, 99-109.
2. Liu, G., Bafico, A., and Aaronson, S.A. (2005). The mechanism of endogenous receptor activation functionally distinguishes prototype canonical and noncanonical Wnts. Molecular and cellular biology 25, 3475-3482.
3. Mikels, A.J., and Nusse, R. (2006). Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. PLoS biology 4, e115.
4. Anastas, J.N., and Moon, R.T. (2013). WNT signalling pathways as therapeutic targets in cancer. Nature reviews Cancer 13, 11-26.
5. Papkoff, J., Rubinfeld, B., Schryver, B., and Polakis, P. (1996). Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. Molecular and cellular biology 16, 2128-2134.
6. Peifer, M., Orsulic, S., Sweeton, D., and Wieschaus, E. (1993). A role for the Drosophila segment polarity gene armadillo in cell adhesion and cytoskeletal integrity during oogenesis. Development 118, 1191-1207.
7. Peifer, M., Rauskolb, C., Williams, M., Riggleman, B., and Wieschaus, E. (1991). The segment polarity gene armadillo interacts with the wingless signaling pathway in both embryonic and adult pattern formation. Development 111, 1029-1043.
8. Riggleman, B., Schedl, P., and Wieschaus, E. (1990). Spatial expression of the Drosophila segment polarity gene armadillo is posttranscriptionally regulated by wingless. Cell 63, 549-560.
9. Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D., and Nusse, R. (1987). The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. Cell 50, 649-657.
10. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. The EMBO journal 16, 3797-3804.
11. Kinzler, K.W., Nilbert, M.C., Su, L.K., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hedge, P., McKechnie, D., et al. (1991). Identification of FAP locus genes from chromosome 5q21. Science 253, 661-665.
12. Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., et al. (1991). Identification and characterization of the familial adenomatous polyposis coli gene. Cell 66, 589-600.
13. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. (1995). Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proceedings of the National Academy of Sciences of the United States of America 92, 3046-3050.
14. Xing, Y., Clements, W.K., Kimelman, D., and Xu, W. (2003). Crystal structure of a beta-catenin/axin complex suggests a mechanism for the beta-catenin destruction complex. Genes & development 17, 2753-2764.

15. Hart, M.J., de los Santos, R., Albert, I.N., Rubinfeld, B., and Polakis, P. (1998). Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta. Current biology : CB 8, 573-581.
16. Kishida, S., Yamamoto, H., Ikeda, S., Kishida, M., Sakamoto, I., Koyama, S., and Kikuchi, A. (1998). Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. The Journal of biological chemistry 273, 10823-10826.
17. Behrens, J., Jerchow, B.A., Wurtele, M., Grimm, J., Asbrand, C., Wirtz, R., Kuhl, M., Wedlich, D., and Birchmeier, W. (1998). Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta. Science 280, 596-599.
18. Yamamoto, H., Kishida, S., Uochi, T., Ikeda, S., Koyama, S., Asashima, M., and Kikuchi, A. (1998). Axil, a member of the Axin family, interacts with both glycogen synthase kinase 3beta and beta-catenin and inhibits axis formation of Xenopus embryos. Molecular and cellular biology 18, 2867-2875.
19. Peifer, M., Pai, L.M., and Casey, M. (1994). Phosphorylation of the Drosophila adherens junction protein Armadillo: roles for wingless signal and zeste-white 3 kinase. Developmental biology 166, 543-556.
20. Peifer, M., Sweeton, D., Casey, M., and Wieschaus, E. (1994). wingless signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. Development 120, 369-380.
21. Cook, D., Fry, M.J., Hughes, K., Sumathipala, R., Woodgett, J.R., and Dale, T.C. (1996). Wingless inactivates glycogen synthase kinase-3 via an intracellular signalling pathway which involves a protein kinase C. The EMBO journal 15, 4526-4536.
22. Amit, S., Hatzubai, A., Birman, Y., Andersen, J.S., Ben-Shushan, E., Mann, M., Ben-Neriah, Y., and Alkalay, I. (2002). Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes & development 16, 1066-1076.
23. Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science 275, 1787-1790.
24. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science 275, 1790-1792.
25. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. Cell 86, 391-399.
26. Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B.G., and Kemler, R. (1996). Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. Mechanisms of development 59, 3-10.
27. Behrens, J., von Kries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 382, 638-642.
28. Li, V.S., Ng, S.S., Boersema, P.J., Low, T.Y., Karthaus, W.R., Gerlach, J.P., Mohammed, S., Heck, A.J., Maurice, M.M., Mahmoudi, T., et al. (2012). Wnt signaling through inhibition of beta-catenin degradation in an intact Axin1 complex. Cell 149, 1245-1256.
29. Mao, J., Wang, J., Liu, B., Pan, W., Farr, G.H., 3rd, Flynn, C., Yuan, H., Takada, S., Kimelman, D., Li, L., et al. (2001). Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. Molecular cell 7, 801-809.
30. Hannoush, R.N. (2008). Kinetics of Wnt-driven beta-catenin stabilization revealed by quantitative and temporal imaging. PloS one 3, e3498.

31. Vasicek, T.J., Zeng, L., Guan, X.J., Zhang, T., Costantini, F., and Tilghman, S.M. (1997). Two dominant mutations in the mouse fused gene are the result of transposon insertions. Genetics 147, 777-786.
32. Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T.J., Perry, W.L., 3rd, Lee, J.J., Tilghman, S.M., Gumbiner, B.M., and Costantini, F. (1997). The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell 90, 181-192.
33. Satoh, S., Daigo, Y., Furukawa, Y., Kato, T., Miwa, N., Nishiwaki, T., Kawasoe, T., Ishiguro, H., Fujita, M., Tokino, T., et al. (2000). AXIN1 mutations in hepatocellular carcinomas, and growth suppression in cancer cells by virus-mediated transfer of AXIN1. Nature genetics 24, 245-250.
34. Feng, G.J., Cotta, W., Wei, X.Q., Poetz, O., Evans, R., Jarde, T., Reed, K., Meniel, V., Williams, G.T., Clarke, A.R., et al. (2012). Conditional disruption of Axin1 leads to development of liver tumors in mice. Gastroenterology 143, 1650-1659.
35. Chia, I.V., and Costantini, F. (2005). Mouse axin and axin2/conductin proteins are functionally equivalent in vivo. Mol Cell Biol 25, 4371-4376.
36. Gluecksohn-Schoenheimer, S. (1949). The effects of a lethal mutation responsible for duplications and twinning in mouse embryos. The Journal of experimental zoology 110, 47-76.
37. Perry, W.L., 3rd, Vasicek, T.J., Lee, J.J., Rossi, J.M., Zeng, L., Zhang, T., Tilghman, S.M., and Costantini, F. (1995). Phenotypic and molecular analysis of a transgenic insertional allele of the mouse Fused locus. Genetics 141, 321-332.
38. Yu, H.M., Jerchow, B., Sheu, T.J., Liu, B., Costantini, F., Puzas, J.E., Birchmeier, W., and Hsu, W. (2005). The role of Axin2 in calvarial morphogenesis and craniosynostosis. Development 132, 1995-2005.
39. Leung, J.Y., Kolligs, F.T., Wu, R., Zhai, Y., Kuick, R., Hanash, S., Cho, K.R., and Fearon, E.R. (2002). Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling. The Journal of biological chemistry 277, 21657-21665.
40. Jho, E.H., Zhang, T., Domon, C., Joo, C.K., Freund, J.N., and Costantini, F. (2002). Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Molecular and cellular biology 22, 1172-1183.
41. Aulehla, A., Wehrle, C., Brand-Saberi, B., Kemler, R., Gossler, A., Kanzler, B., and Herrmann, B.G. (2003). Wnt3a plays a major role in the segmentation clock controlling somitogenesis. Developmental cell 4, 395-406.
42. Lee, E., Salic, A., Kruger, R., Heinrich, R., and Kirschner, M.W. (2003). The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. PLoS biology 1, E10.
43. Huang, S.M., Mishina, Y.M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G.A., Charlat, O., Wiellette, E., Zhang, Y., Wiessner, S., et al. (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature 461, 614-620.
44. Callow, M.G., Tran, H., Phu, L., Lau, T., Lee, J., Sandoval, W.N., Liu, P.S., Bheddah, S., Tao, J., Lill, J.R., et al. (2011). Ubiquitin ligase RNF146 regulates tankyrase and Axin to promote Wnt signaling. PloS one 6, e22595.
45. Zhang, Y., Liu, S., Mickanin, C., Feng, Y., Charlat, O., Michaud, G.A., Schirle, M., Shi, X., Hild, M., Bauer, A., et al. (2011). RNF146 is a poly(ADP-ribose)-directed E3 ligase that regulates axin degradation and Wnt signalling. Nature cell biology 13, 623-629.
46. Stemmle, L.N., Fields, T.A., and Casey, P.J. (2006). The regulator of G protein signaling domain of axin selectively interacts with Galpha12 but not Galpha13. Molecular pharmacology 70, 1461-1468.
47. Ikeda, S., Kishida, S., Yamamoto, H., Murai, H., Koyama, S., and Kikuchi, A. (1998). Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. The EMBO journal 17, 1371-1384.

48. Fagotto, F., Jho, E., Zeng, L., Kurth, T., Joos, T., Kaufmann, C., and Costantini, F. (1999). Domains of axin involved in protein-protein interactions, Wnt pathway inhibition, and intracellular localization. The Journal of cell biology 145, 741-756.
49. Sakanaka, C., and Williams, L.T. (1999). Functional domains of axin. Importance of the C terminus as an oligomerization domain. The Journal of biological chemistry 274, 14090-14093.
50. Wu, Z.Q., Brabletz, T., Fearon, E., Willis, A.L., Hu, C.Y., Li, X.Y., and Weiss, S.J. (2012). Canonical Wnt suppressor, Axin2, promotes colon carcinoma oncogenic activity. Proceedings of the National Academy of Sciences of the United States of America 109, 11312-11317.
51. Yook, J.I., Li, X.Y., Ota, I., Hu, C., Kim, H.S., Kim, N.H., Cha, S.Y., Ryu, J.K., Choi, Y.J., Kim, J., et al. (2006). A Wnt-Axin2-GSK3beta cascade regulates Snail1 activity in breast cancer cells. Nature cell biology 8, 1398-1406.
52. Schaal, U., Grenz, S., Merkel, S., Rau, T.T., Hadjihannas, M.V., Kremmer, E., Chudasama, P., Croner, R.S., Behrens, J., Sturzl, M., et al. (2013). Expression and localization of axin 2 in colorectal carcinoma and its clinical implication. International journal of colorectal disease 28, 1469-1478.
53. Zeng, Y.A., and Nusse, R. (2010). Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. Cell stem cell 6, 568-577.
54. Bao, R., Christova, T., Song, S., Angers, S., Yan, X., and Attisano, L. (2012). Inhibition of tankyrases induces Axin stabilization and blocks Wnt signalling in breast cancer cells. PloS one 7, e48670.
55. Whyte, J.L., Smith, A.A., Liu, B., Manzano, W.R., Evans, N.D., Dhamdhere, G.R., Fang, M.Y., Chang, H.Y., Oro, A.E., and Helms, J.A. (2013). Augmenting endogenous Wnt signaling improves skin wound healing. PloS one 8, e76883.
56. Jeon, S.H., Yoon, J.Y., Park, Y.N., Jeong, W.J., Kim, S., Jho, E.H., Surh, Y.J., and Choi, K.Y. (2007). Axin inhibits extracellular signal-regulated kinase pathway by Ras degradation via beta-catenin. The Journal of biological chemistry 282, 14482-14492.
57. Kim, S.E., Yoon, J.Y., Jeong, W.J., Jeon, S.H., Park, Y., Yoon, J.B., Park, Y.N., Kim, H., and Choi, K.Y. (2009). H-Ras is degraded by Wnt/beta-catenin signaling via beta-TrCP-mediated polyubiquitylation. Journal of cell science 122, 842-848.
58. Zhang, Y., Neo, S.Y., Wang, X., Han, J., and Lin, S.C. (1999). Axin forms a complex with MEKK1 and activates c-Jun NH(2)-terminal kinase/stress-activated protein kinase through domains distinct from Wnt signaling. The Journal of biological chemistry 274, 35247-35254.
59. Luo, W., Ng, W.W., Jin, L.H., Ye, Z., Han, J., and Lin, S.C. (2003). Axin utilizes distinct regions for competitive MEKK1 and MEKK4 binding and JNK activation. The Journal of biological chemistry 278, 37451-37458.
60. Rennoll, S.A., Konsavage, W.M., Jr., and Yochum, G.S. (2014). Nuclear AXIN2 represses MYC gene expression. Biochemical and biophysical research communications 443, 217-222.
61. Hadjihannas, M.V., Bernkopf, D.B., Bruckner, M., and Behrens, J. (2012). Cell cycle control of Wnt/beta-catenin signalling by conductin/axin2 through CDC20. EMBO reports 13, 347-354.
62. Hadjihannas, M.V., Bruckner, M., and Behrens, J. (2010). Conductin/axin2 and Wnt signalling regulates centrosome cohesion. EMBO reports 11, 317-324.
63. Cancer Genome Atlas, N. (2012). Comprehensive molecular characterization of human colon and rectal cancer. Nature 487, 330-337.
64. Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B., and Kinzler, K.W. (1992). APC mutations occur early during colorectal tumorigenesis. Nature 359, 235-237.
65. Inomata, M., Ochiai, A., Akimoto, S., Kitano, S., and Hirohashi, S. (1996). Alteration of beta-catenin expression in colonic epithelial cells of familial adenomatous polyposis patients. Cancer research 56, 2213-2217.
66. Polakis, P. (2012). Drugging Wnt signalling in cancer. The EMBO journal 31, 2737-2746.

67. Voronkov, A., and Krauss, S. (2013). Wnt/beta-catenin signaling and small molecule inhibitors. Current pharmaceutical design 19, 634-664.
68. Lui, T.T., Lacroix, C., Ahmed, S.M., Goldenberg, S.J., Leach, C.A., Daulat, A.M., and Angers, S. (2011). The ubiquitin-specific protease USP34 regulates axin stability and Wnt/beta-catenin signaling. Molecular and cellular biology 31, 2053-2065.
69. Waaler, J., Machon, O., Tumova, L., Dinh, H., Korinek, V., Wilson, S.R., Paulsen, J.E., Pedersen, N.M., Eide, T.J., Machonova, O., et al. (2012). A novel tankyrase inhibitor decreases canonical Wnt signaling in colon carcinoma cells and reduces tumor growth in conditional APC mutant mice. Cancer research 72, 2822-2832.
70. Stratford, E.W., Daffinrud, J., Munthe, E., Castro, R., Waaler, J., Krauss, S., and Myklebost, O. (2014). The tankyrase-specific inhibitor JW74 affects cell cycle progression and induces apoptosis and differentiation in osteosarcoma cell lines. Cancer medicine 3, 36-46.
71. Lau, T., Chan, E., Callow, M., Waaler, J., Boggs, J., Blake, R.A., Magnuson, S., Sambrone, A., Schutten, M., Firestein, R., et al. (2013). A novel tankyrase small-molecule inhibitor suppresses APC mutation-driven colorectal tumor growth. Cancer research 73, 3132-3144.
72. Gonnet, G.H., Cohen, M.A., and Benner, S.A. (1992). Exhaustive matching of the entire protein sequence database. Science 256, 1443-1445.
73. Sherry, S.T., Ward, M.H., Kholodov, M., Baker, J., Phan, L., Smigielski, E.M., and Sirotkin, K. (2001). dbSNP: the NCBI database of genetic variation. Nucleic acids research 29, 308-311.
74. Lipton, L., and Tomlinson, I. (2006). The genetics of FAP and FAP-like syndromes. Familial cancer 5, 221-226.
75. Fearnhead, N.S., Wilding, J.L., Winney, B., Tonks, S., Bartlett, S., Bicknell, D.C., Tomlinson, I.P., Mortensen, N.J., and Bodmer, W.F. (2004). Multiple rare variants in different genes account for multifactorial inherited susceptibility to colorectal adenomas. Proceedings of the National Academy of Sciences of the United States of America 101, 15992-15997.
76. Webster, M.T., Rozycka, M., Sara, E., Davis, E., Smalley, M., Young, N., Dale, T.C., and Wooster, R. (2000). Sequence variants of the axin gene in breast, colon, and other cancers: an analysis of mutations that interfere with GSK3 binding. Genes, chromosomes & cancer 28, 443-453.
77. Smalley, M.J., Sara, E., Paterson, H., Naylor, S., Cook, D., Jayatilake, H., Fryer, L.G., Hutchinson, L., Fry, M.J., and Dale, T.C. (1999). Interaction of axin and Dvl-2 proteins regulates Dvl-2-stimulated TCF-dependent transcription. The EMBO journal 18, 2823-2835.
78. Lammi, L., Arte, S., Somer, M., Jarvinen, H., Lahermo, P., Thesleff, I., Pirinen, S., and Nieminen, P. (2004). Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer. Am J Hum Genet 74, 1043-1050.
79. Marvin, M.L., Mazzoni, S.M., Herron, C.M., Edwards, S., Gruber, S.B., and Petty, E.M. (2011). AXIN2-associated autosomal dominant ectodermal dysplasia and neoplastic syndrome. Am J Med Genet A 155A, 898-902.
80. Rivera, B., Perea, J., Sanchez, E., Villapun, M., Sanchez-Tome, E., Mercadillo, F., Robledo, M., Benitez, J., and Urioste, M. (2013). A novel AXIN2 germline variant associated with attenuated FAP without signs of oligondontia or ectodermal dysplasia. Eur J Hum Genet.
81. Peterlongo, P., Howe, L.R., Radice, P., Sala, P., Hong, Y.J., Hong, S.I., Mitra, N., Offit, K., and Ellis, N.A. (2005). Germline mutations of AXIN2 are not associated with nonsyndromic colorectal cancer. Human mutation 25, 498-500.
82. Lejeune, S., Guillemot, F., Triboulet, J.P., Cattan, S., Mouton, C., Group, P., Porchet, N., Manouvrier, S., and Buisine, M.P. (2006). Low frequency of AXIN2 mutations and high frequency of MUTYH mutations in patients with multiple polyposis. Human mutation 27, 1064.

83. Shimizu, Y., Ikeda, S., Fujimori, M., Kodama, S., Nakahara, M., Okajima, M., and Asahara, T. (2002). Frequent alterations in the Wnt signaling pathway in colorectal cancer with microsatellite instability. Genes, chromosomes & cancer 33, 73-81.
84. Jin, L.H., Shao, Q.J., Luo, W., Ye, Z.Y., Li, Q., and Lin, S.C. (2003). Detection of point mutations of the Axin1 gene in colorectal cancers. International journal of cancer Journal international du cancer 107, 696-699.
85. Liu, W., Dong, X., Mai, M., Seelan, R.S., Taniguchi, K., Krishnadath, K.K., Halling, K.C., Cunningham, J.M., Boardman, L.A., Qian, C., et al. (2000). Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling. Nature genetics 26, 146-147.
86. Matsuzaki, K., Deng, G., Tanaka, H., Kakar, S., Miura, S., and Kim, Y.S. (2005). The relationship between global methylation level, loss of heterozygosity, and microsatellite instability in sporadic colorectal cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 11, 8564-8569.
87. Domingo, E., Espin, E., Armengol, M., Oliveira, C., Pinto, M., Duval, A., Brennetot, C., Seruca, R., Hamelin, R., Yamamoto, H., et al. (2004). Activated BRAF targets proximal colon tumors with mismatch repair deficiency and MLH1 inactivation. Genes, chromosomes & cancer 39, 138-142.
88. Thorstensen, L., Lind, G.E., Lovig, T., Diep, C.B., Meling, G.I., Rognum, T.O., and Lothe, R.A. (2005). Genetic and epigenetic changes of components affecting the WNT pathway in colorectal carcinomas stratified by microsatellite instability. Neoplasia (New York, NY) 7, 99-108.
89. Suraweera, N., Robinson, J., Volikos, E., Guenther, T., Talbot, I., Tomlinson, I., and Silver, A. (2006). Mutations within Wnt pathway genes in sporadic colorectal cancers and cell lines. International journal of cancer Journal international du cancer 119, 1837-1842.
90. Khan, N.P., Pandith, A.A., Hussain, M.U., Yousuf, A., Khan, M.S., Wani, K.A., and Mudassar, S. (2011). Novelty of Axin 2 and lack of Axin 1 gene mutation in colorectal cancer: a study in Kashmiri population. Molecular and cellular biochemistry 355, 149-155.
91. Koinuma, K., Yamashita, Y., Liu, W., Hatanaka, H., Kurashina, K., Wada, T., Takada, S., Kaneda, R., Choi, Y.L., Fujiwara, S.I., et al. (2006). Epigenetic silencing of AXIN2 in colorectal carcinoma with microsatellite instability. Oncogene 25, 139-146.
92. Muto, Y., Maeda, T., Suzuki, K., Kato, T., Watanabe, F., Kamiyama, H., Saito, M., Koizumi, K., Miyaki, Y., Konishi, F., et al. (2014). DNA methylation alterations of AXIN2 in serrated adenomas and colon carcinomas with microsatellite instability. BMC cancer 14, 466.
93. Kingston, J.E., Draper, G.J., and Mann, J.R. (1982). Hepatoblastoma and polyposis coli. Lancet 1, 457.
94. Miao, J., Kusafuka, T., Udatsu, Y., and Okada, A. (2003). Sequence variants of the Axin gene in hepatoblastoma. Hepatology research : the official journal of the Japan Society of Hepatology 25, 174-179.
95. Koch, A., Weber, N., Waha, A., Hartmann, W., Denkhaus, D., Behrens, J., Birchmeier, W., von Schweinitz, D., and Pietsch, T. (2004). Mutations and elevated transcriptional activity of conductin (AXIN2) in hepatoblastomas. J Pathol 204, 546-554.
96. Taniguchi, K., Roberts, L.R., Aderca, I.N., Dong, X., Qian, C., Murphy, L.M., Nagorney, D.M., Burgart, L.J., Roche, P.C., Smith, D.I., et al. (2002). Mutational spectrum of beta-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas. Oncogene 21, 4863-4871.
97. Kim, M.S., Kim, S.S., Ahn, C.H., Yoo, N.J., and Lee, S.H. (2009). Frameshift mutations of Wnt pathway genes AXIN2 and TCF7L2 in gastric carcinomas with high microsatellite instability. Hum Pathol 40, 58-64.
98. Hamilton, S.R., Liu, B., Parsons, R.E., Papadopoulos, N., Jen, J., Powell, S.M., Krush, A.J., Berk, T., Cohen, Z., Tetu, B., et al. (1995). The molecular basis of Turcot's syndrome. The New England journal of medicine 332, 839-847.

99. Dahmen, R.P., Koch, A., Denkhaus, D., Tonn, J.C., Sorensen, N., Berthold, F., Behrens, J., Birchmeier, W., Wiestler, O.D., and Pietsch, T. (2001). Deletions of AXIN1, a component of the WNT/wingless pathway, in sporadic medulloblastomas. Cancer research 61, 7039-7043.
100. Baeza, N., Masuoka, J., Kleihues, P., and Ohgaki, H. (2003). AXIN1 mutations but not deletions in cerebellar medulloblastomas. Oncogene 22, 632-636.
101. Koch, A., Hrychyk, A., Hartmann, W., Waha, A., Mikeska, T., Schuller, U., Sorensen, N., Berthold, F., Goodyer, C.G., Wiestler, O.D., et al. (2007). Mutations of the Wnt antagonist AXIN2 (Conductin) result in TCF-dependent transcription in medulloblastomas. International journal of cancer Journal international du cancer 121, 284-291.
102. Wu, R., Zhai, Y., Fearon, E.R., and Cho, K.R. (2001). Diverse mechanisms of beta-catenin deregulation in ovarian endometrioid adenocarcinomas. Cancer research 61, 8247-8255.
103. Wu, R., Connolly, D.C., Ren, X., Fearon, E.R., and Cho, K.R. (1999). Somatic mutations of the PPP2R1B candidate tumor suppressor gene at chromosome 11q23 are infrequent in ovarian carcinomas. Neoplasia (New York, NY) 1, 311-314.
104. Moreno-Bueno, G., Hardisson, D., Sanchez, C., Sarrio, D., Cassia, R., Garcia-Rostan, G., Prat, J., Guo, M., Herman, J.G., Matias-Guiu, X., et al. (2002). Abnormalities of the APC/beta-catenin pathway in endometrial cancer. Oncogene 21, 7981-7990.
105. Su, T.H., Chang, J.G., Yeh, K.T., Lin, T.H., Lee, T.P., Chen, J.C., and Lin, C.C. (2003). Mutation analysis of CTNNB1 (beta-catenin) and AXIN1, the components of Wnt pathway, in cervical carcinomas. Oncology reports 10, 1195-1200.
106. Chapman, A., Durand, J., Ouadi, L., and Bourdeau, I. (2011). Identification of genetic alterations of AXIN2 gene in adrenocortical tumors. J Clin Endocrinol Metab 96, E1477-1481.
107. Castiglia, D., Bernardini, S., Alvino, E., Pagani, E., De Luca, N., Falcinelli, S., Pacchiarotti, A., Bonmassar, E., Zambruno, G., and D'Atri, S. (2008). Concomitant activation of Wnt pathway and loss of mismatch repair function in human melanoma. Genes, chromosomes & cancer 47, 614-624.
108. Daa, T., Kashima, K., Kaku, N., Suzuki, M., and Yokoyama, S. (2004). Mutations in components of the Wnt signaling pathway in adenoid cystic carcinoma. Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc 17, 1475-1482.
109. Pedace, L., Castiglia, D., De Simone, P., Castori, M., De Luca, N., Amantea, A., Binni, F., Majore, S., Cozzolino, A.M., De Bernardo, C., et al. (2011). AXIN2 germline mutations are rare in familial melanoma. Genes, chromosomes & cancer 50, 370-373.
110. Ma, C., Liu, C., Huang, P., Kaku, H., Chen, J., Guo, K., Ueki, H., Sakai, A., Nasu, Y., Kumon, H., et al. (2014). Significant association between the Axin2 rs2240308 single nucleotide polymorphism and the incidence of prostate cancer. Oncology letters 8, 789-794.
111. Pinarbasi, E., Gunes, E.G., Pinarbasi, H., Donmez, G., and Silig, Y. (2011). AXIN2 polymorphism and its association with prostate cancer in a Turkish population. Medical oncology 28, 1373-1378.
112. Kanzaki, H., Ouchida, M., Hanafusa, H., Yano, M., Suzuki, H., Aoe, M., Imai, K., Shimizu, N., Nakachi, K., and Shimizu, K. (2006). Single nucleotide polymorphism of the AXIN2 gene is preferentially associated with human lung cancer risk in a Japanese population. International journal of molecular medicine 18, 279-284.
113. Olson, J.E., Wang, X., Pankratz, V.S., Fredericksen, Z.S., Vachon, C.M., Vierkant, R.A., Cerhan, J.R., and Couch, F.J. (2011). Centrosome-related genes, genetic variation, and risk of breast cancer. Breast cancer research and treatment 125, 221-228.
114. Wang, X., Goode, E.L., Fredericksen, Z.S., Vierkant, R.A., Pankratz, V.S., Liu-Mares, W., Rider, D.N., Vachon, C.M., Cerhan, J.R., Olson, J.E., et al. (2008). Association of genetic variation in genes implicated in the beta-catenin destruction complex with risk of breast cancer. Cancer Epidemiol Biomarkers Prev 17, 2101-2108.

115. Stewart, D.J., Chang, D.W., Ye, Y., Spitz, M., Lu, C., Shu, X., Wampfler, J.A., Marks, R.S., Garces, Y.I., Yang, P., et al. (2014). Wnt signaling pathway pharmacogenetics in non-small cell lung cancer. The pharmacogenomics journal.
116. Wang, X.P., and Fan, J. (2011). Molecular genetics of supernumerary tooth formation. Genesis 49, 261-277.
117. Nakajima, M., Fukuchi, M., Miyazaki, T., Masuda, N., Kato, H., and Kuwano, H. (2003). Reduced expression of Axin correlates with tumour progression of oesophageal squamous cell carcinoma. British journal of cancer 88, 1734-1739.
118. Lindor, N.M. (2009). Hereditary colorectal cancer: MYH-associated polyposis and other newly identified disorders. Best practice & research 23, 75-87.
119. Lynch, P.M. (2008). Standards of care in diagnosis and testing for hereditary colon cancer. Familial cancer 7, 65-72.
120. Polakis, P. (2007). The many ways of Wnt in cancer. Current opinion in genetics & development 17, 45-51.
121. Salahshor, S., and Woodgett, J.R. (2005). The links between axin and carcinogenesis. J Clin Pathol 58, 225-236.
122. Mostowska, A., Biedziak, B., and Jagodzinski, P.P. (2006). Axis inhibition protein 2 (AXIN2) polymorphisms may be a risk factor for selective tooth agenesis. Journal of human genetics 51, 262-266.
123. Callahan, N., Modesto, A., Meira, R., Seymen, F., Patir, A., and Vieira, A.R. (2009). Axis inhibition protein 2 (AXIN2) polymorphisms and tooth agenesis. Archives of oral biology 54, 45-49.
124. Letra, A., Menezes, R., Granjeiro, J.M., and Vieira, A.R. (2009). AXIN2 and CDH1 polymorphisms, tooth agenesis, and oral clefts. Birth Defects Res A Clin Mol Teratol 85, 169-173.
125. Choudhry, U., Boyce, H.W., Jr., and Coppola, D. (1998). Proton pump inhibitor-associated gastric polyps: a retrospective analysis of their frequency, and endoscopic, histologic, and ultrastructural characteristics. American journal of clinical pathology 110, 615-621.
126. Zhang, J., Li, Y., Liu, Q., Lu, W., and Bu, G. Wnt signaling activation and mammary gland hyperplasia in MMTV-LRP6 transgenic mice: implication for breast cancer tumorigenesis. Oncogene 29, 539-549.
127. Menezes, R., Marazita, M.L., Goldstein McHenry, T., Cooper, M.E., Bardi, K., Brandon, C., Letra, A., Martin, R.A., and Vieira, A.R. (2009). AXIS inhibition protein 2, orofacial clefts and a family history of cancer. Journal of the American Dental Association (1939) 140, 80-84.
128. Smith, K.J., Johnson, K.A., Bryan, T.M., Hill, D.E., Markowitz, S., Willson, J.K., Paraskeva, C., Petersen, G.M., Hamilton, S.R., Vogelstein, B., et al. (1993). The APC gene product in normal and tumor cells. Proceedings of the National Academy of Sciences of the United States of America 90, 2846-2850.
129. Kolligs, F.T., Hu, G., Dang, C.V., and Fearon, E.R. (1999). Neoplastic transformation of RK3E by mutant beta-catenin requires deregulation of Tcf/Lef transcription but not activation of c-myc expression. Molecular and cellular biology 19, 5696-5706.
130. Dogan, R.I., Getoor, L., Wilbur, W.J., and Mount, S.M. (2007). SplicePort--an interactive splice-site analysis tool. Nucleic acids research 35, W285-291.
131. Polychronopoulos, P., Magiatis, P., Skaltsounis, A.L., Myrianthopoulos, V., Mikros, E., Tarricone, A., Musacchio, A., Roe, S.M., Pearl, L., Leost, M., et al. (2004). Structural basis for the synthesis of indirubins as potent and selective inhibitors of glycogen synthase kinase-3 and cyclin-dependent kinases. Journal of medicinal chemistry 47, 935-946.
132. Kishida, S., Yamamoto, H., Hino, S., Ikeda, S., Kishida, M., and Kikuchi, A. (1999). DIX domains of Dvl and axin are necessary for protein interactions and their ability to regulate beta-catenin stability. Molecular and cellular biology 19, 4414-4422.

133. Yamamoto, H., Kishida, S., Kishida, M., Ikeda, S., Takada, S., and Kikuchi, A. (1999). Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability. The Journal of biological chemistry 274, 10681-10684.
134. Strovel, E.T., Wu, D., and Sussman, D.J. (2000). Protein phosphatase 2Calpha dephosphorylates axin and activates LEF-1-dependent transcription. The Journal of biological chemistry 275, 2399-2403.
135. Hsu, W., Zeng, L., and Costantini, F. (1999). Identification of a domain of Axin that binds to the serine/threonine protein phosphatase 2A and a self-binding domain. The Journal of biological chemistry 274, 3439-3445.
136. Cohen, P., Klumpp, S., and Schelling, D.L. (1989). An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. FEBS letters 250, 596-600.
137. Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., and Hedge, P. (1991). Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science 253, 665-669.
138. da Costa, L.T., He, T.C., Yu, J., Sparks, A.B., Morin, P.J., Polyak, K., Laken, S., Vogelstein, B., and Kinzler, K.W. (1999). CDX2 is mutated in a colorectal cancer with normal APC/beta-catenin signaling. Oncogene 18, 5010-5014.
139. Lum, W. (2011). INVESTIGATION OF AXIN2 IN ZEBRAFISH (DANIO RERIO) DEVELOPMENT AND ITS ROLE IN CANONICAL WNT SIGNALING. Master of Science, The University of Guelph, Guelph, Ontario.
140. Joslyn, G., Carlson, M., Thliveris, A., Albertsen, H., Gelbert, L., Samowitz, W., Groden, J., Stevens, J., Spirio, L., Robertson, M., et al. (1991). Identification of deletion mutations and three new genes at the familial polyposis locus. Cell 66, 601-613.
141. Hinoi, T., Akyol, A., Theisen, B.K., Ferguson, D.O., Greenson, J.K., Williams, B.O., Cho, K.R., and Fearon, E.R. (2007). Mouse model of colonic adenoma-carcinoma progression based on somatic Apc inactivation. Cancer research 67, 9721-9730.
142. Feng, Y., Sentani, K., Wiese, A., Sands, E., Green, M., Bommer, G.T., Cho, K.R., and Fearon, E.R. (2013). Sox9 induction, ectopic Paneth cells, and mitotic spindle axis defects in mouse colon adenomatous epithelium arising from conditional biallelic Apc inactivation. The American journal of pathology 183, 493-503.
143. Kosinski, C., Li, V.S., Chan, A.S., Zhang, J., Ho, C., Tsui, W.Y., Chan, T.L., Mifflin, R.C., Powell, D.W., Yuen, S.T., et al. (2007). Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. Proceedings of the National Academy of Sciences of the United States of America 104, 15418-15423.
144. Kuhnert, F., Davis, C.R., Wang, H.T., Chu, P., Lee, M., Yuan, J., Nusse, R., and Kuo, C.J. (2004). Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. Proceedings of the National Academy of Sciences of the United States of America 101, 266-271.
145. van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P., et al. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 111, 241-250.
146. Bergendal, B., Klar, J., Stecksen-Blicks, C., Norderyd, J., and Dahl, N. (2011). Isolated oligodontia associated with mutations in EDARADD, AXIN2, MSX1, and PAX9 genes. Am J Med Genet A 155A, 1616-1622.
147. Wong, S., Liu, H., Bai, B., Chang, H., Zhao, H., Wang, Y., Han, D., and Feng, H. (2014). Novel missense mutations in the AXIN2 gene associated with non-syndromic oligodontia. Archives of oral biology 59, 349-353.

148. Sansom, O.J., Reed, K.R., Hayes, A.J., Ireland, H., Brinkmann, H., Newton, I.P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I.S., et al. (2004). Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes & development 18, 1385-1390.
149. Wang, S., Yin, J., Chen, D., Nie, F., Song, X., Fei, C., Miao, H., Jing, C., Ma, W., Wang, L., et al. (2013). Small-molecule modulation of Wnt signaling via modulating the Axin-LRP5/6 interaction. Nature chemical biology 9, 579-585.
150. Tamai, K., Zeng, X., Liu, C., Zhang, X., Harada, Y., Chang, Z., and He, X. (2004). A mechanism for Wnt coreceptor activation. Molecular cell 13, 149-156.
151. Tolwinski, N.S., Wehrli, M., Rives, A., Erdeniz, N., DiNardo, S., and Wieschaus, E. (2003). Wg/Wnt signal can be transmitted through arrow/LRP5,6 and Axin independently of Zw3/Gsk3beta activity. Developmental cell 4, 407-418.
152. Qian, L., Mahaffey, J.P., Alcorn, H.L., and Anderson, K.V. (2011). Tissue-specific roles of Axin2 in the inhibition and activation of Wnt signaling in the mouse embryo. Proceedings of the National Academy of Sciences of the United States of America 108, 8692-8697.
153. Feng, Y., Bommer, G.T., Zhao, J., Green, M., Sands, E., Zhai, Y., Brown, K., Burberry, A., Cho, K.R., and Fearon, E.R. (2011). Mutant KRAS promotes hyperplasia and alters differentiation in the colon epithelium but does not expand the presumptive stem cell pool. Gastroenterology 141, 1003-1013 e1001-1010.
154. Chia, I.V., Kim, M.J., Itoh, K., Sokol, S.Y., and Costantini, F. (2009). Both the RGS domain and the six C-terminal amino acids of mouse Axin are required for normal embryogenesis. Genetics 181, 1359-1368.
155. Rui, H.L., Fan, E., Zhou, H.M., Xu, Z., Zhang, Y., and Lin, S.C. (2002). SUMO-1 modification of the C-terminal KVEKVD of Axin is required for JNK activation but has no effect on Wnt signaling. The Journal of biological chemistry 277, 42981-42986.
156. Kim, M.J., Chia, I.V., and Costantini, F. (2008). SUMOylation target sites at the C terminus protect Axin from ubiquitination and confer protein stability. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 22, 3785-3794.