ELUCIDATING THE ROLE OF ROR2 WITHIN THE WNT PATHWAY AND ITS CONTRIBUTIONS TO RENAL CELL CARCINOMA TUMORIGENESIS

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum in Genetics and Molecular Biology.

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ABSTRACT

NEAL R RASMUSSEN: Elucidating the role of the receptor tyrosine kinase Ror2 within the Wnt pathway and its contributions to renal cell carcinoma tumorigenesis (Under the direction of W. Kimryn Rathmell, MD, PhD)

Ror2, an important mediator of Wnt signaling cascades, has been shown to be aberrantly expressed in RCC promoting cell migration, invasion, and tumor growth. In this work, our goal was to elucidate the role of Ror2 as a Wnt receptor within its contextual expression of RCC. Utilizing microarrays we examined gene expression in RCC tumors and found Ror2 was significantly correlated with expression of several Wnt signaling genes including the classical feedback target gene, Axin2. Subsequent analysis in RCC cells showed that Ror2 expression results in a poised state for canonical Wnt signaling through an increased signaling pool of β-catenin, leading to an enhancement of target genes following Wnt3a stimulation. In addition, we utilized siRNA and dickkopf to inhibit LRP6 in order to show that Ror2 stabilization of β-catenin was independent of LRP6 but required for the downstream response to Wnt3a. Finally, we also saw that the Ror2 kinase domain is required for stabilization of soluble β-catenin and enhancement of canonical signaling.

Due to Ror2's correlation with aggressive disease in several cancers we have aimed to examine its contributions to cell migration and tumor growth, and its potential as a prognostic biomarker for RCC. Utilizing RCC cells and human tumors we have shown that both MMP2 and SFRP2 exhibit a significant correlation with Ror2. We show that Ror2 expression results in increased cell migration that is dependent upon an intact Ror2 kinase domain. We also examined the effects of Ror2 overexpression in xenografts and found that Ror2 expression results in increased tumor growth and vascularity. To ascertain the

potential of Ror2 as a prognostic biomarker we first examined Ror2 expression in relation to the ccA and ccB molecular subtypes in RCC tumors, finding that Ror2 expression was significantly higher in ccB. Finally we assessed Ror2's potential as an independent prognostic biomarker for RCC; we show that high Ror2 expression correlates with increased tumor growth and significant reduction in overall survival. These results exhibit Ror2's potential as a prognostic biomarker and therapeutic target for RCC.

This dissertation is dedicated to my wife, Annie Atkin Rasmussen and my two wonderful sons, Joseph and John, who have given me so much and helped me to continually strive to reach my true potential.

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PREFACE

Author Contributions

Neal R Rasmussen – Was responsible for the design of the experiments and execution of all experiments not specifically noted below. Additionally was the primary author for each manuscript.

Tricia M Wright – Responsible for the design of the V5-HisA hRor2 constructs and development of the 786-0 hRor2 overexpression cell lines. She also initiated the xenografts studies utilizing the 786-0 hRor2 overexpression cell lines.

Rose Brannon – Provided ccA and ccB assignment along with all survival analyses of Ror2 in the ccRCC TCGA dataset.

Samira A Brooks – Was responsible for the RT-PCR utilizing the Human Wnt Signaling RT^2 Profiler PCR Array and our 786-0 cell panel.

Kathryn E Hacker – Responsible for SAM analysis of 95 human ccRCC tumors and helped with the analysis of the Human Wnt Signaling RT^2 Profiler PCR Array results.

Adam B Sendor – Helped with the *in-vitro* transwell migration assays.

Oishee Sen – Peformed all IHC staining for hRor2 overexpression xenografts.

Matthew P Walker & M Ben Major – Provided siLRP6, NFAT-Luciferase reporter, and Tap-hRor2 construct or use in *in-vitro* assays.

Jennifer Green & Geoffrey M Wahl – Provided eGFP and hRor2-eGFP constructs for use in *in-vitro* assays.

W. Kimryn Rathmell - Was responsible for helping in the design of experiments and editing of manuscripts.

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LIST OF ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
Ang1	Angiopoietin 1
Ang2	Angiopoietin 2
APC	adenomatous polyposis coli
aPKC	atypical protein kinase C
CAIX	carbonic anhydrase IX
CCND1	cyclin D1
ccRCC	clear cell renal cell carcinoma
CK1α	casein kinase 1α
CRD	cysteine-rich domain
DAAM1	Disheveled-Associated Activator Of Morphogenesis 1
DKK1	dickkopf-1
DVL	dishevelled
DKK	dickkopf
DVL	Dishevelled
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
Fzd	Frizzled

GIST	gastrointestinal stromal tumor
Glut1	glucose transporter 1
GFP	green fluorescent protein
GSK3-β	glycogen synthase kinase beta
HIF	hypoxia inducible factor
HSP90	heat shock protein 90
IGFR	insulin-like growth factor receptor
IRP1	iron-regulatory protein-1
JNK	c-Jun N-terminal kinase
LDA	lactate dehydrogenase-A
LEF	lymphoid enhancer-binding factor
LMS	leiomyosarcoma
LRP	low-density lipoprotein receptor-related protein
LRP5/6	lipoprotein receptor-related protein 5 or 6
MAPK	mitogen activation protein kinase
MuSK	muscle-specific kinase
MMP	matrix metalloprotease
MMP1	matrix metalloprotease 1
MMP2	matrix metalloprotease 2
MMP9	matrix metalloprotease 9
MMP13	matrix metalloprotease 13
mTOR	mechanistic target of rapamycin
mTORC1	mTOR complex 1

mTORC2	mTOR complex 2
NFAT	nuclear factor associated with T cells
NFκB	nuclear factor kappa B
PCP	planar cell polarity
PDGF	platelet-derived growth factor
PDGFR	platelet derived growth factor receptor
PDK1	phosphoinositide dependent protein kinase 1
PI3K	phosphatidylinositide 3-kinase
PIP3	phosphoinositol-3,4,5-triphosphate
PKA	phospho-glycerase kinase
PTEN	phosphatase and tensin homolog
pRS	pRetroSuper
pVHL	von Hippel-Lindau protein
PYGO1	pygopus 1
Raptor	regulatory-associated protein of mTOR
RCC	renal cell carcinoma
Rictor	Rapamycin-Insensitive companion of mTOR
ROCK1	Rho kinase 1
Ror2	receptor tyrosine kinase-like orphan receptor
RTK	receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SAM	Significance Analysis of Microarrays
SEM	standard error of mean

SFRP	secreted Frizzled-related protein
shRNA	short hairpin RNA
siRNA	small interfering RNA
TCF	T cell factor
Trk	tropomyosin receptor kinase
VHL	Von Hippel-Lindau
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau gene

Chapter One

INTRODUCTION

Renal cell carcinoma

Renal cell carcinoma (RCC) is a common epithelial tumor that continues to rise in prevalence in the face of recent advances in its treatment. It is the sixth most commonly diagnosed cancer in men and the eighth most common in women. Though the cause of the disparity of incidence rates between the genders is as yet undetermined, men are twice as likely to develop RCC as women. It is estimated there will be over 65,000 new cases and 13,000 deaths in the United States in 2013, nearly one-third of these new cases presenting with advanced RCC (1). For those patients with metastases upon diagnosis, the 5-year survival rate is bleak at less than 10% (2). Moreover, advanced RCC is quite difficult to treat because it is highly unresponsive to traditional single-agent or combination chemotherapeutic strategies. While cytokine therapies have consistently shown promise for a small subset of patients, the overall toxicity of current cytokine therapies interferes with their widespread use(3).

RCC consists of several distinct histological subtypes including chromophobe, papillary, and clear cell renal cell carcinoma (ccRCC). ccRCC accounts for 70-75% of RCC cases and derives its name from its histologically characteristic clear cytoplasm, setting it apart from the other subtypes (4). The clear cytoplasm is due to ccRCC's highly glycolytic

(This work is modified from Rasmussen and Rathmell, Current Clinical Pharmacology, 2011)

and vascular nature, which results in a buildup of glycogens and lipids within the cytoplasm. All subsequent work and discussion presented here will focus solely on ccRCC, being referred to as either ccRCC or RCC.

VHL/HIF signaling in clear cell renal cell carcinoma

ccRCC is molecularly characterized by inactivation of the von Hippel-Lindau tumor suppressor gene (*VHL*), leading to constitutive stabilization of the hypoxia-inducible factors (HIF-1α and HIF-2α) (5). This feature is seen both in sporadic ccRCC and in patients with *VHL* syndrome, as a result of either biallelic mutations or functional inactivation of the *VHL* protein (pVHL) in 75-85% of ccRCC cases (6-9). Under normoxia or normal oxygen conditions, pVHL serves as part of the E3 ubiquitin ligase complex that recognizes hydroxylated prolyl residues of HIFα subunits mark them for proteasomal degradation. However, under hypoxic conditions or when pVHL is inactivated, the HIFα subunits accumulate and heterodimerize with HIF-1β (ARNT), translocating into the nucleus and regulating transcription of target genes. The skewed VHL/HIF axis seen in ccRCC results in increased expression of a variety of hypoxia-inducible genes shared by both HIF-1α and HIF-2α including the pro-angiogenic factors platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), the extracellular matrix (ECM) remodeling protein matrix metalloprotease 2 (MMP2), and glucose transporter 1 (Glut1) (Figure 1.1). Although HIF-1α and HIF-2α are highly similar, with 48% overall amino acid identity, they exhibit unique expression profiles and induction of distinct target genes (10). HIF-1α-targeted induction of lactate dehydrogenase-A (LDA) and phospho-glycerase kinase (PKA) are representative of the increased metabolic activity observed in RCC. However, HIF-2α expression, can increase *in-vivo* tumor growth in RCC cells when acting alone, uniquely targets erythropoietin, Cyclin D1 (CCND1), and Oct-4, an important factor in stem cell regulation and de-differentiation (11,12).

In addition to its function as part of the E3 ubiquitin ligase complex regulating HIF, pVHL has been shown to regulate cell cycle, apoptosis, fibronectin binding, and extracellular matrix assembly, independent of HIF (13-18). The established hallmarks of cancer include the escape of programmed cell death and cell cycle checkpoints leading to sustained cell proliferation (19). As previously noted, traditional therapeutics have had limited efficacy in RCC because of its resistance to radiotherapy and cytotoxic treatments. These features of RCC may be partially attributed to increased anti-apoptotic signaling within the tumor. The contribution of loss of pVHL to these tumorigenic signaling networks is well illustrated by its regulation of several proteins known to be central to these cellular processes.

The tumor suppressor p53 serves as a key regulator of cell cycle and has been observed to be mutated in a significant portion of solid tumors. While many studies have noted the distinct lack of p53 mutations in RCC (6), pVHL has been shown to directly associate with p53, inhibiting Mdm-2 ubiquitination and the promotion of its transactivation. The loss of pVHL and proper regulation of p53 in RCC cells results in attenuated apoptosis or abnormal cell cycle arrest upon DNA damage (15). pVHL also serves as a negative regulator of the transcription factor nuclear factor κB (NFκB) through inhibitory phosphorylation of the NFκB agonist Card9 (20,21). Thus the loss of pVHL drives RCC tumorigenesis through the resulting increased anti-apoptotic signaling and improper cell cycle control. Finally, pVHL has been found to bind and inhibit atypical protein kinase C (aPKC) which is associated with cell proliferation, cellular survival, and in establishment and maintenance of cell-cell junctions (22,23).

Another hallmark of cancer is the ability of tumor cells to migrate and invade, allowing the cancer to metastasize throughout the body (19). Critical to cells' ability to do this is the loss of cell-cell adhesion, along with the remodeling of the surrounding ECM, which normally acts as a structural support for cells. pVHL has been shown to directly bind and play a crucial role in the regulation of fibronectin matrix assembly (17,18). pVHL also

requires the ECM to mediate cell signaling,as removal of the ECM substrate *in-vitro,* results in a reduction of epithelial differentiation and growth arrest at high cell density (16). Correlating with these changes in epithelial differentiation, pVHL loss in RCC cells results in an epithelial-mesenchymal transition (EMT) marked by decreased expression of epithelial markers that promote cellular adhesion (e.g. E-cadherin and γ-catenin) and accompanying increased expression of N-cadherin, vimentin, integrins, MMP2, and MMP9 characteristic of motile mesenchymal cells (21,22). Reintroduction of *VHL* expression or inhibition of NFκB in *VHL* null cells is also capable of reverting expression of these EMT markers in RCC cells (21). Taken together, these findings coalesce into a clearer understanding of how loss or inactivation of *VHL* drives many aspects of RCC tumor biology including resistance to traditional therapies, tumor initiation, and tumor progression.

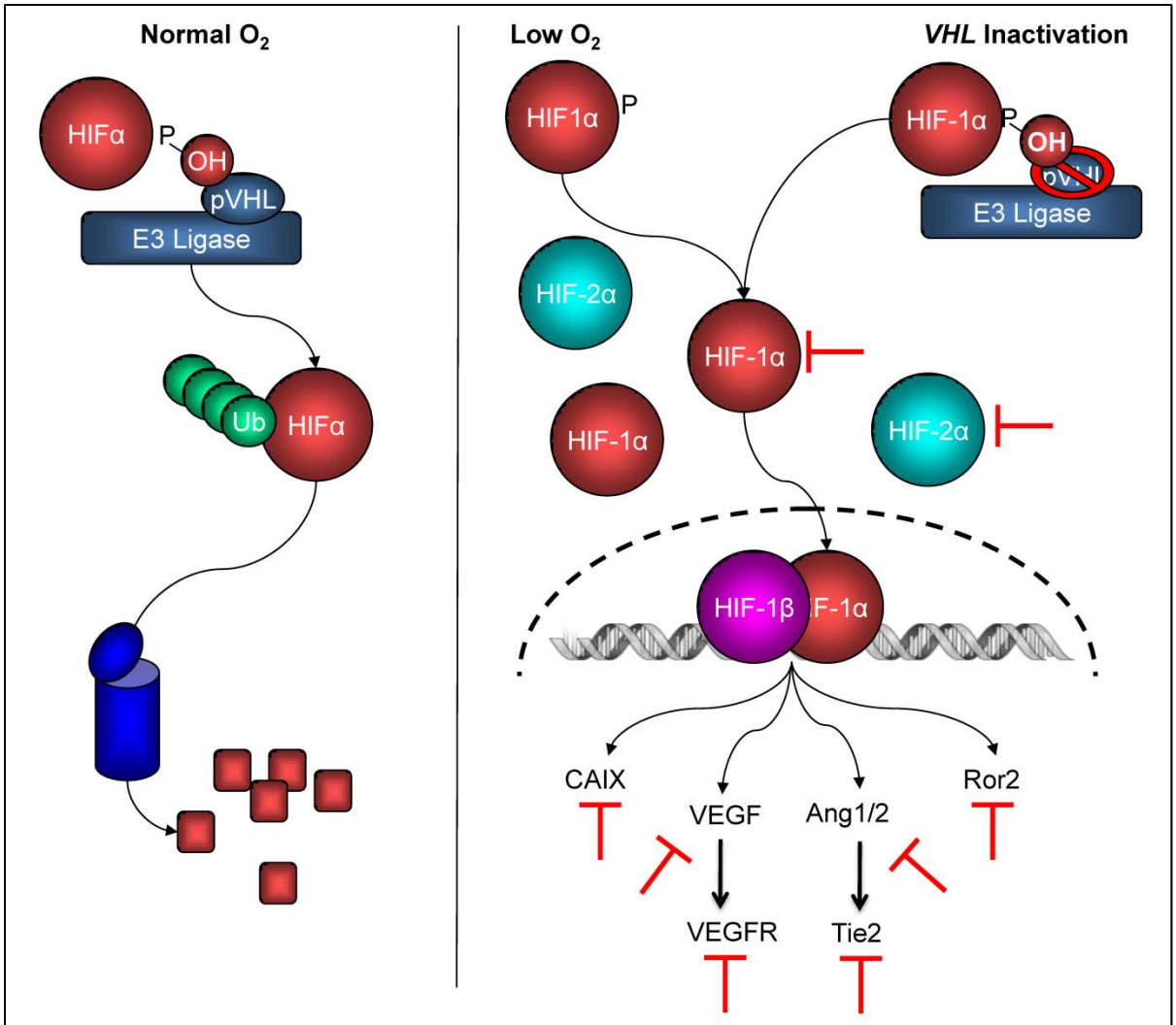

Figure 1.1: Overview of the VHL/HIF pathway and dependent targets in RCC
Under normoxic conditions the HIFα subunits are hyrodoxylated, allowing recognition and binding of E3 ligase complex via pVHL. The HIFα subunits are polyubiquitinated and subsequently degraded by the proteosome. However under hypoxic conditions or when pVHL is inactivated, HIF-1α and HIF-2α are stabilized and translocated to the nucleus, following binding with HIF-1β/ARNT. The heterodimerized HIF transcription factors promote transcription of various targets including VEGF, CAIX, Ang1/2, and Ror2. VEGF and the angiopoietins are ligands which activate cognate receptors VEGFR (family of VEGF receptors), and Tie2 (also a target of HIF activation) on endothelial cells. Red inhibitory arrows show potential targets for the VHL/HIF pathway for RCC.

RCC targeted therapeutic development

Better understanding of the molecular underpinnings of RCC has led to the advent of the use of targeted therapies such as the anti-angiogenic VEGF and PDGF receptor tyrosine kinase inhibitors sorafenib (Nexavar, Bayer/Onyx), pazopanib (Votrient, Glaxo Smith Kline) and sunitinib (Sutent, Pfizer), and the VEGF-neutralizing antibody bevacizumab

(Avastin, Genentech) (24-28). Each of these compounds has contributed significantly to improving the progression-free survival of patients with RCC, thus advancing the standard of care for the disease. Two mTOR inhibitors, temsirolimus (Torisel, Wyeth), and everolimus (Afinitor, Novartis) have also shown clinical efficacy in the care of advanced RCC, with temsirolimus extending overall survival for patients with extremely poor-risk disease (29,30).

Despite the progress seen with the introduction of these targeted therapies, there remains considerable room for improvement, considering that no available targeted therapies are currently capable of inducing remission, and *de novo* or acquired resistance to these targeted therapies is an on-going challenge facing clinicians. Further complicating matters, several of the currently approved therapies exhibit undesirable and dose-limiting side effects due to toxicity, most likely on account of both on- and off-target effects. Given the limitations of current targeted therapies there is a great need in the field for more targets for development of therapeutics for use as first-line agents or in combination with available therapies.

Because loss of *VHL* is one of the defining molecular characteristics of RCC, its interactions in the disease have been well characterized. As mentioned above, upon loss or inactivation of pVHL the HIF subunits accumulate and lead to the transcriptional induction of various target genes including VEGF and PDGF (Figure 1.1) (31). The molecular targeted therapies described above were designed to take advantage of this aspect of *VHL* deficiency, and are thought to primarily affect the VEGF and PDGF receptor tyrosine kinases expressed on tumor-supporting endothelial cells and pericytes. The efficacy of these drugs in inducing disease response and stabilization implicates this pathway as one important to the maintenance of renal tumors. Promising new targets exist at an array of points all along the *VHL*-HIF axis which could possibly address the problems of side effects and acquired resistance to VEGF-receptor targeted therapies.

Potential therapeutic targets in VHL/HIF signaling in RCC

The most immediate potential targets for therapeutics are the HIF transcription factors themselves, with HIF-2α expression being sufficient to induce *in-vivo* tumor growth (12). Various efforts have focused on targeting one or both HIF factors, with initial strategies directed toward the first of these factors to be characterized, HIF-1α. Transcription factors are notoriously difficult to inhibit compared to targets with enzymatic activity. Even so, strategies aimed at reducing the production or stability of these factors can be effective. HIF-1α is regulated by a variety of mechanisms in addition to the E3 ubiquitination provided via it's interaction with pVHL. These include the phosphatidylinositide 3-kinase (PI3K)/mTOR pathway, mitogen activation protein kinase (MAPK) signaling, and other autocrine circuitry (32). Agents intersecting all of these signaling pathways are presently in development for RCC and other tumor types and need to be evaluated in the context of ccRCC.

Several unique approaches have been undertaken to therapeutically reduce HIF-1α levels. EZN-2968, has highlighted the potential of antisense oligos, as it is able to effectively reduce both HIF-1α and target gene expression *in-vitro* and *in-vivo* (33) (Table 1.1). Oxygen-independent stablization of HIF-1α is also mediated by heat shock protein 90 (HSP90), providing an alternate target which has shown significant promise in reducing HIF-1 levels in the setting of *VHL* loss, and is currently being evaluated in clinical trials (34-36). YC-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole), originally identified as agent-targeting cyclic GMP, has been found to exhibit activity in repressing HIF-1α and to inhibit tumor growth (37). The small molecule inhibitor PX-478 (*S*-2-amino-3-(4'-*N*,*N*,-bis(2-chloroethyl)amino) phenyl propionic acid *N*-oxide dihydrochloride) has been shown to inhibit translation of HIF-1α and to exhibit antitumor activity (38,39). Other mechanisms also modulate HIF-1α production, but they are not as well understood. For example, one group has demonstrated a direct effect of treatment with the antihelmenthic agent albendazole on

tumor cell HIF-1α levels (40). These demonstrate some unique and upcoming inhibitors to HIF-1α; a more comprehensive review of all potential inhibitors can be found here (41).

Because several reports have suggested a primary role for HIF-2α in promoting ccRCC tumorigenesis, HIF-2α has begun to set itself apart as the more promising therapeutic target for pharmaceutical development. HIF-2α may in fact provide an *essential* function in ccRCC, and thus emerge as the key target. Recent studies inhibiting HIF-2α in *VHL*-null cells resulted in inhibition of cell growth (42,43). *In vitro* and *in vivo* experiments have further shown that stable expression of HIF-2α can drive tumor growth even in the presence of pVHL (12,44,45). Finally, examination of ccRCC tumors reveals that these tumors either express HIF-2α exclusively (H2 tumors) or HIF-2α along with HIF-1α (H1H2 tumors), leaving HIF-2α expression as the common denominator of *VHL*-mutated cancers (46). Thus, the evidence that HIF-2α is possibly a driving force in RCC tumorigenesis sets it apart as a promising target.

Less is understood about the regulation of HIF-2α, but efforts to target this factor are rapidly emerging from *in vitro* studies. One intriguing compound, emetine, is a protein synthesis inhibitor that has been shown to specifically inhibit HIF-2α protein stability in the setting of *VHL* loss, although the effect of sustained treatment with emetine in functional tumor assays remains unknown (47) (Table 1.1). An alternate class of inhibitors has shed light on the transcriptional regulation of HIF-2α. In a screen for small molecules to blunt HIF-2α transcription, the identified compounds enhance the binding of iron-regulatory protein-1 (IRP1) to an iron-responsive element in the HIF-2α 5'-untranslated region, effectively suppressing transcription of HIF-2α (48). This novel approach provides an effective strategy to limit HIF-2α accumulation, but remains to be studied in *in vitro* or *in vivo* assays of tumorigenesis.

These opportunities to effectively reduce the HIF transcriptional signal might be considered a strategy that would supplant VEGF or VEGF receptor inhibition, considering

the seeming linearity of these events. However, preliminary evidence suggests that removing the powerful stimulus of HIF transcriptional signals on a broad array of pro-angiogenic and other factors may enhance the activity of VEGF pathway-directed agents. This interesting pre-clinical observation heralds important considerations for ccRCC clinical trial design using vertical combination inhibitory strategies.

Table 1.1 Stage of development for new targeted therapies for RCC

Agent	Drug Class	Target	Developmental Stage	Study
HIF				
EZN-2968	Anti-sense oligo	HIF-1α	Phase1	(33)
PX-478	Small molecule	HIF-1α	In Vitro/In Vivo	(38)
albendazole	Small molecule	HIF-1α	In Vitro	(40)
YC-1	Small molecule	cGC/HIF-1α	In Vitro/In Vivo	(37)
17-AAG	Small molecule	HSP-90/HIF-1α	Phase II	(36)
emetine	Small molecule	HIF-2α	In Vitro	(47)
compound 76	Small molecule	HIF-2α	In Vitro	(48)
HIF-Targets				
cG250	AB	CAIX	Phase I/II	(49)
compound 7	Small molecule	Tie-2	In Vitro/In Vivo	(50)
AMG 386	AB	Ang-1/2	Phase I/II	(51)
PI3K/mTOR				
XL147	Small molecule	PI3K	Phase I/II	(52)
NVP-BKM120	Small molecule	PI3K	Phase I	(53)
XL765	Small molecule	PI3K/mTOR	Phase I/II	(54)
NVP-BEZ235	Small molecule	PI3K/mTOR	Phase I	(55)
GSK2334470	Small molecule	PDK1	In Vitro	(56,57)
AR-12	Small molecule	PDK1	Phase I	(56,57)
Palomid 529	Small molecule	mTORC1/2	In Vitro/In Vivo	(58)
OSI-027	Small molecule	mTORC1/2	In Vitro/In Vivo	(59)
PP-242	Small molecule	mTORC1/2	In Vitro/In Vivo	(60)
AZD-8055	Small molecule	mTORC1/2	In Vitro/In Vivo	(61)

HIF, hypoxia-inducible factor; cGC, cyclic GMP; HSP-90, heat shock protein-90; AB, antibody; CAIX, carbonic andhydrase; Ang, Angipoietin; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; PDK1, phosphoinositide dependent protein kinase 1, mTORC, mTOR-containing complexes

Developing targeted therapies for downstream HIF targets

In addition to targeting the HIF transcription family members themselves, HIF factors promote the transcription of over 100 immediate targets and innumerable secondary targets. In terms of broad classification, this spectrum of targets provides the opportunity to selectively inhibit angiogenesis with current or new putative targets, interfering with HIF-mediated cell growth and apoptotic resistance, impacting cell migration and extracellular matrix regulation, and finally identifying novel ways to harness altered cellular metabolism. A few selected targets downstream of HIF stabilization have already begun to present particularly intriguing possibilities for therapeutic development in RCC (Figure 1.1).

Carbonic anhydrase IX (CAIX) is one of many hypoxia-inducible target genes, but is unique in that it is found to be highly expressed in many ccRCC tumors (62). Due to these high expression levels, CAIX in RCC is now being pursued as a prognostic indicator, diagnostic tool, and prospective target for chemotherapeutics. CAIX serves a regulator for maintaining the pH in the cell and the microenvironment surrounding the cell (63,64). Strategies have emerged utilizing anti-CAIX antibody (also known as cG250) to identify tumors which are highly expressive of this moiety using molecular directed nuclear imaging (49). As a highly expressed cell surface marker in RCC, this antibody has also been investigated as a therapeutic modality to target agents more effectively to the tumor or as an agent with direct antitumor activity in the context of a large placebo-controlled adjuvant study (Table 1.1).

Anti-angiogenic therapies have become the standard of care for RCC due to its highly vascular nature and its resistance to traditional chemotherapeutic approaches, but resistance is common. Because this resistance appears to occur as a result of angiogenic escape mechanisms, expanding the repertoire of anti-angiogenic factors related to ccRCC is highly desirable. Additional angiogenic targets have been identified in RCC including the kinase Tie2 which serves as a receptor for the family of angiopoietin ligands, pro-angiogenic

targets of HIF transcriptional activity. Like other targeted angiogenic kinases, Tie2 expression is mostly restricted to endothelial cells, but has been shown to correlate with angiopotietin-2 expression in RCC tumors (65). Being an enzymatically active kinase, conventional small molecule inhibitor screens have been effective in producing lead compounds (50) (Table 1.1). These have been shown in preclinical models to be effective at reducing aberrant tumor vessel growth. Several Tie2 inhibitors are being seriously considered for clinical trials as stand-alone anti-angiogenics, or as therapeutic options to combat VEGF receptor inhibitor resistance. Similarly, the inhibition of angiopoietins themselves provides another tractable anti-angiogenic strategy. Angiopoietins 1 and 2 (Ang1, Ang2) are both targets and mediators of HIF expression (66). These molecules therefore present an interesting opportunity to dampen HIF expression while having a direct effect on tumor angiogenesis. One drug, a neutralizing peptibody, AMG 386 (Amgen) is currently being evaluated in clinical trials (51) (Table 1.1). These targets not only present possible new therapeutic options for the care of RCC patients but a unique opportunity to better understand the underlying biology of these tumors. Sequential or combinatorial therapies involving targeting Ang1, Ang2, and Tie2 also would serve to better understand the mechanisms of resistance in RCC, if it is through compensatory upregulation of HIF or simply engagement of untargeted angiogenic receptors among the endothelial cells. In addition, as Tie2 expression is limited solely to endothelial cells, as is thought to be the case for the angiogenic VEGF receptor, this presents an opportunity to examine the interplay between the tumor and the supporting microenvironment.

PI3K/AKT/mTOR signaling in clear cell renal cell carcinoma

VHL loss alone in mice has been unable to completely recapitulate the clinically observed pathology of ccRCC , therefore additional driver mutations and signaling cascades must play a role as well (67-69). The collaborative efforts found in the Cancer Genome Atlas (TCGA) have validated previous findings and identified genes in addition to *VHL* which

exhibit significantly high rates of mutation. Of note, two members of the mechanistic target rapamycin (mTOR) signaling cascade were included; mechanistic target of rapamycin (mTOR) and phosphatase and tensin homolog (PTEN) were among the eight most frequently mutated members (6).

The mTOR signaling cascade serves as a major hub for signals from the extracellular milieu, regulating a variety of intracellular processes including metabolism, survival, protein and lipid synthesis, and autophagy (70). Mutations and misregulation within the mTOR signaling pathway are implicated in a wide spectrum of cancers. Initiation of the mTOR signaling cascade occurs through multiple receptor tyrosine kinases (RTK) including epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR), and platelet-derived growth factor receptor (PDGFR) leading to activation of PI3K, which produces the secondary messenger phosphoinositol-3,4,5-triphosphate (PIP3) and is tightly regulated by the tumor suppressor PTEN. The resulting PIP3 recruits AKT (protein kinase B, PKB) to the membrane, where AKT is phosphorylated by phosphoinositide dependent protein kinase 1 (PDK1) to its fully activated form. The signaling cascade continues with activated AKT directly activating mTOR by phosphorylation and indirectly activating MTOR through inhibition of tuberous sclerosis 1 or 2 (TSC1/2), negative regulators of mTOR. Subsequent formation of either mTOR complex 1 (mTORC1) or mTOR complex 2 (mTORC2), dependent upon the inclusion of either regulatory-associated protein of mTOR (Raptor) or Rapamycin-Insensitive companion of mTOR (Rictor) respectively, regulates a wide variety of cellular effects including cell growth, survival and angiogenesis (71) (Figure 1.2).

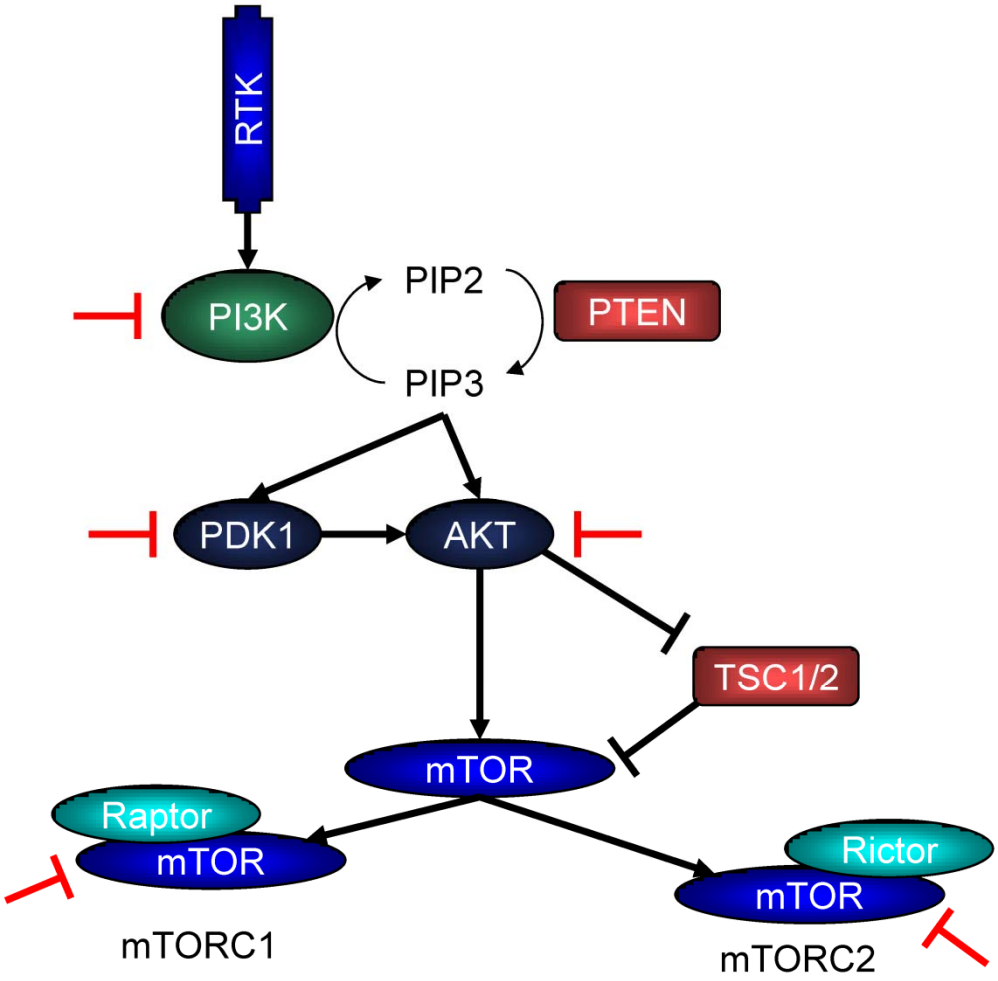

Figure 1.2: Overview of the PI3K/AKT/mTOR pathway and potential targets in RCC
PI3K is activated in response to various receptor tyrosine kinases leading to production of the secondary messenger PIP3, which is dephosphorylated by PTEN. PIP3 activates AKT following recruitment to the membrane through PDK1, providing several opportunities for therapeutic inhibition. mTOR activation is mediated directly by AKT and through indirect inhibition of TSC1/2, and is found in complexes mTORC1 and mTORC2 differentiated by the binding partners Raptor and Rictor. Potential targets within the PI3K/AKT/mTOR pathway for RCC are marked by red inhibitory marks.

Developing PI3K/AKT/mTOR pathway targeted therapeutics

The PI3K/AKT/mTOR pathway represents a prime target for the development of therapeutics since it is one of the most common aberrant pathways activated in cancer, regulating many known oncogenic pathways including apoptosis, proliferation, and cell

migration (72,73). Capitalizing on the known effects of the mTOR pathway, mTORC1 complex inhibitors have become an additional tool for treatment of advanced RCC (29,30) (Figure 1.2). Despite the initial successes of the mTOR inhibitors their efficacy has been limited, highlighting the need for additional therapeutic targets.

Because PI3K serves as a bottleneck in response to several RTKs and is the most proximal element, it has become another attractive target for RCC to be used alone or concert with current mTOR inhibitors. Two prospective PI3K inhibitors, LY294002 and wortmannin, have been shown to decrease AKT activation and significantly reduce cell growth *in vitro* through induction of cell apoptosis. Use of the small molecule inhibitor LY294002 *in vivo* was able to induce tumor regression (74). Although LY294002 and wortmannin exhibit limited selectivity and high toxicity, they demonstrate the possibilities associated with inhibiting PI3K in RCC. Ongoing studies using PI3K and dual PI3K/mTOR inhibitors modified for clinical use are ongoing (52-55) (Table 1.1).

Similar to PI3K, PDK1, a key mediator of AKT activation, is poised to respond to targeted inhibition by blockade of AKT signaling. Both highly specific inhibitors such as AR-12 (Arno) and inhibitors with dual function on PDK1/PI3K or PDK1/AKT are in development (56,57) (Table 1.1). These targets present another possibly robust way to render the AKT pathway completely inhibited, mitigating the confounding issues of inhibition of each of the AKT family members.

Upon phosphorylation AKT is known to interact with a large set of substrates, impacting many key cellular processes such as cell cycle progression and apoptosis, both of which execute a vital function in oncogenesis (75). In addition, AKT is constitutively activated in RCC cell lines (74). Recent work has suggested mTORC2 provides feedback to AKT, leading to its compensatory activation (76,77). AKT targeting via direct or indirect methods to impair its activation should prove to be effective therapy in RCC, given the high

expression of activated AKT, and as coordinate therapy with current mTOR inhibitors curbing possible refractory responses.

mTOR performs a vital role in regulating critical cellular processes including cell growth, proliferation, transcription, and protein synthesis (78). In addition to the aforementioned effects of mTOR activation, mTOR plays an important part in regulating HIF-1α expression. Targeting mTOR is already an FDA-approved strategy for the treatment of RCC but both rapalogs temsirolimus and everolimus suffer from the drawbacks of seeming to be solely selective for inhibiting mTORC1 and, as previously mentioned, can lead to upstream activation of AKT through mTORC2 (76,77). This highlights the need for targeted agents for mTORC2 or dual inhibitors of both mTORC1 and mTORC2. Inhibitors in these categories are also being developed for clinical use (58-61) (Table 1.1).

Overcoming resistance in RCC

A continuing impediment to the treatment of RCC in the clinic is resistance to established targeted therapies. The driving forces behind initial or developed resistance in RCC are not yet fully understood. However, RCC xenografts submitted to a daily regimen of the VEGF receptor inhibitor sorafenib showed a reduction in supporting tumor vasculature that was reestablished prior to the onset of resistance (79). These findings suggest a potential mechanism of resistance where, through either compensatory activity of the VEGF or alternative angiogenic pathways, the supporting vasculature to the tumor is reestablished (80). Several of the targets discussed above lend themselves as potential tools in overcoming and better understanding these mechanisms of resistance in RCC.

As previously discussed, RCC is characterized by loss or inactivation of *VHL*, leading to constitutive stabilization of HIFα. A plausible mechanism for this observed resistance is through upregulation of HIF transcription factors and targeted pro-angiogenic genes VEGF and PDGF. If a compensatory increase in HIF levels is truly driving the development of resistance in RCC, targeting HIF either directly or indirectly through inhibition of the

PI3K/AKT/mTOR pathway may lead to renewed potentiation of VEGF-receptor targeted therapies. This approach was examined in colon cancer xenografts using the VEGF receptor inhibitor, sunitinib, with and without disruption of HIF-1α and HIF-2α, showing an additive effect on angiogenesis and tumor cell proliferation (81). Additionally, the use of mTOR inhibitors temsirolimus and everolimus has been shown to lead to a compensatory activation of AKT which could spur a resurgence of vasculature through an upregulation of HIF (76,77). Vertical combination approaches targeting PI3K, AKT, and PDK1 or use of inhibitors that more fully target both mTORC complexes pose an intriguing possibility that calls for further research. Another avenue for escape from treatment with the established anti-angiogenic therapies lies in additional angiogenic pathways independent of VEGF. One of these VEGF independent pathways, receptor Tie2 and ligands Ang1/2, has been discussed previously here as a new target for RCC. Inhibition of the kinase Tie2 and blocking of the angiopoietins or other adjunctive angiogenic signals offers a plausible route to more fully restrict the reestablishment of the supporting tumor vasculature. Further research using these strategies either in sequential or combination with available targeted therapies is needed to establish their efficacy in reducing resistance in RCC.

Receptor tyrosine kinase, Ror2

An exciting, novel RCC therapeutic target recently identified is the developmentally regulated receptor tyrosine kinase-like orphan receptor 2 (Ror2) which has been shown to be expressed in a *VHL*- and HIF-dependent manner in RCC (82,83). Ror2 is a member of the Ror family of receptor tyrosine kinases (RTK) which also includes Ror1. Ror1 and Ror2 are both characterized by a conserved series of domains, including an Immunoglobin-like domain, a Frizzled-like cysteine-rich domain (CRD) allowing Ror RTK to serve as Wnt receptors, and a kringle domain in their extracellular portion. The intracellular portion of Ror

RTK includes a tyrosine kinase domain, which shows a high degree of amino acid identity with both muscle-specific kinase (MuSK) and the tropomyosin receptor kinase (Trk), kinases, which served as the basis of the original identification of *hRor1* and *hRor2* genes in humans by PCR screen (84). Both the mouse and human orthologs of Ror RTK also include a series of domains following the tyrosine kinase domain, consisting of a proline-rich domain flanked on both sides by serine/threonine-rich domains. These serine/threonine-rich domains have been shown to be necessary for activity and mediating protein interactions (85-89). This general structure of domains is well conserved with a few deviations across species within the Ror othologs identified in *A. californica* (Apror) (90), *C. elegans* (CAM-1) , *D. melanogaster* (dRor) (91), *X. laevis* (*xRor2*) (92), into the split of two genes in *M. musculus* (*mRor1, mRor2*) (93), and humans (*hRor1, hRor2*) (84) (Figure 1.3). Although Ror1 and Ror2 share this overall conserved structure of domains they diverge in that overall they share only 58% amino acid identity. However, there is a 92% amino acid identity between *mRor2* and *hRor2*, allowing studies in both to be reasonably used to inform one another.

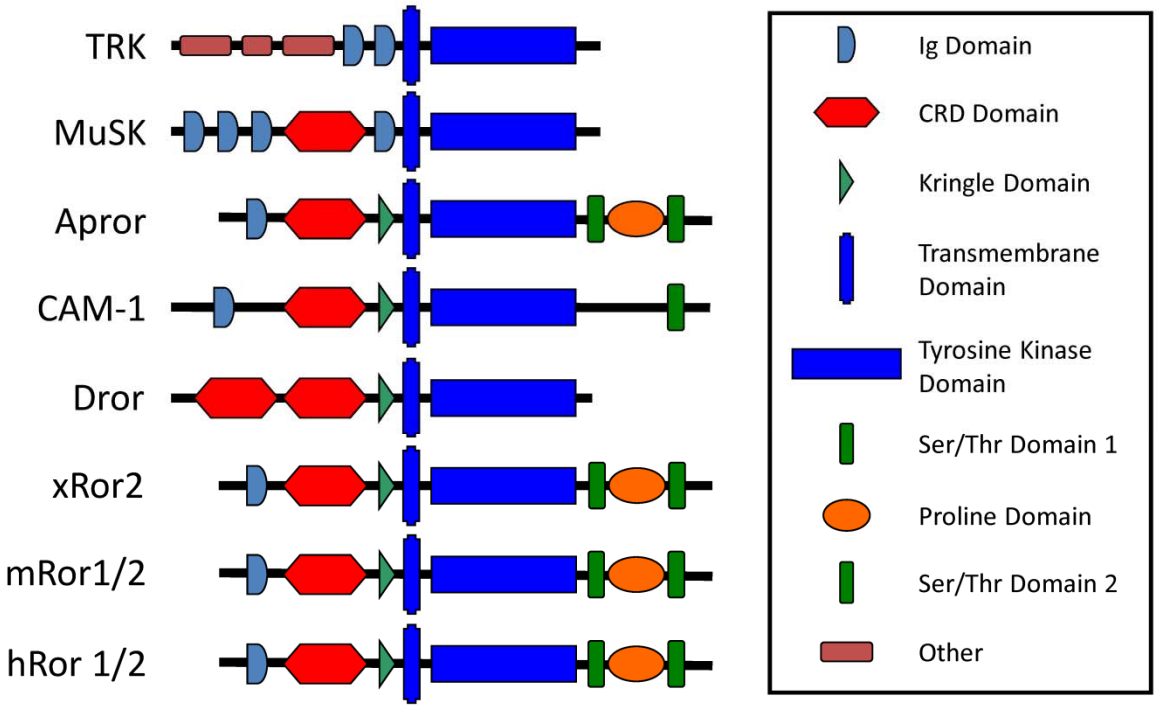

Figure 1.3: Structural representations of Ror orthologs.
A schematic representation of the structural domains found within the various orthologs Ror2. Despite some variations across species Ror2 is most notably characterized by its extracellular cysteine-rich domain which has been shown to bind to various Wnt ligands, sometimes flanked by immunoglobin-like and Kringle domains. The intracellular portion consists of tyrosine kinase domain often followed by a series of serine/theonine and proline rich-domains.

Ror2 a developmental receptor tyrosine kinase

The role of Ror2 as a developmental kinase was first evident in CAM-1 mutants, which exhibit improper cell migration, defects in asymmetric cell divisions, and axon outgrowth in *C. elegans* (94-96). These mutational analyses also highlight the function of Ror2 as a Wnt receptor, since overexpression of EGL-20 (a Wnt) phenocopies mutant CAM-1 migration defects (97). Several independent knockout studies of mouse *mRor1* and *mRor2* have further elucidated their expression patterns and roles in development. Ror2 expression is largely restricted to embryogenesis, with expression seen in the heart, brain, nervous system, lungs, midgut, developing limb buds, and kidney (83,93,98-105) although some limited expression of Ror2 has been observed in the adult within the cycling uterus, colon, and small intestine (106,107).

Knockout of Ror2 results in perinatal lethality, most likely due to respiratory defects. Loss of Ror2 also results in widespread skeletal abnormalities, ventricular septal defects in the heart, and decreased branching of sympathetic neuron axons (93,98-102). Loss of *mRor1,* however, exhibits none of the developmental defects seen in *mRor2*, though *mRor1*/*mRor2*-null mice experience an increased severity of these developmental defects, suggesting some level of redundancy or interaction within Ror RTKs (100-102). The skeletal defects are the result of loss of Ror2 in proliferating chrondocytes, leading to reduced endochondral ossification (98,99). Many of the developmental defects observed in the *mRor2* knockout are phenocopied in Wnt5a-null mice (108). In addition the spatiotemporal expression of Wnt5a and Ror2 during development overlaps in many tissues, including the facial primordia, limb mesenchyme, neural crest-derived tissues, and the genital tubercle (100,101,108). Together, these observations suggest that Ror2 may function as a receptor for Wnt5a during development.

Due to the widespread skeletal defects observed in *mRor2*-null mice, it is unsurprising that mutations in *hRor2* in humans have been linked to two skeletal

developmental disorders: recessive Robinow syndrome and autosomal dominant Brachydactyly type B (109-112). Robinow syndrome is characterized by skeletal dysplasia, limb foreshortening, brachydactyly, craniofacial malformations, and genital hypoplasia. These features are strikingly similar to the phenotypes observed in Ror2 knockout mice, suggesting that Robinow syndrome is indeed due to loss of Ror2. However, Brachydactyly type B effects are limited to hypoplasia of the distal phalanges and nails. The differences in Robinow syndrome and Brachydactyly type B phenotypes is reflected by the mutational profile of Ror2 for each. Robinow Syndrome is characterized by missense, nonsense, and frameshift mutations within the CRD, Kringle, and tyrosine kinase domains, resulting in loss of Ror2 function. However, the Ror2 mutations resulting in Brachydactyly type B are due to truncation of the protein within or after the kinase domain (112).

Ror2 in Cancer

In addition to its contribution during development, expression of Ror2 has been observed in an increasing array of cancers. Ror2 expression was first identified in SH-SY5Y cells derived from a neuroblastoma (84). The aberrant expression of Ror2 in primary human RCC tumors was initially shown in 2009, where its expression in RCC cells was found to be *VHL*- and HIF-dependent (82,83). Expression of Ror2 in RCC cells was shown to promote cell migration, anchorage-independent growth and *in-vivo* growth in xenografts (83). Since that time Ror2 expression has been observed in several other cancers including osteosarcoma, melanoma, prostate cancer, gastric cancer, gastrointestinal stromal tumor (GIST), leiomyosarcoma (LMS), and squamous cell carcinoma of the head and neck (113-120) (Table 1.2).

Table 1.2: Summary of the role of Ror2 in various cancers

Tumor promotion

Cancer	Reference	Role of Ror2
Osteosarcoma	(114)	Cell proliferation Cell invasion Wnt5b/Ror2 signaling
	(113)	Cell migration Cell invasion Formation of invadapodia Wnt5a/Ror2 Signaling MMP13 expression
	(121)	Correlates with disease severity
Melanoma	(122)	Correlates with metastasis Cell migration Cell invasion Pulmonary metastasis formation Wnt5a/Ror2 signaling
	(89)	Higher expression in metastatic cells
GIST and LMS	(116)	Correlates with decreased survival Cell invasion Xenograft tumor growth
Renal cell carcinoma	(83)	Cell migration Anchorage independent growth Xenograft tumor growth MMP2 expression
Prostate	(118)	Cell migration Cell invasion Ror2/Wnt5a signaling MMP1 expression
Gastric Cancer	(119)	Ror2 mutated in activation domain
	(120)	Cell invasion
Head & Neck	(117)	Correlates with disease malignancy Cell motility Cell polarity

Tumor suppression

Cancer	Reference	Role of Ror2
Head & Neck	(123)	Decreased Ror2 expression
Colorectal	(124)	Ror2 methylation Decreased Ror2 expression Cell viability Colony formation Xenograft tumor growth Wnt5a/Ror2 signaling
Hepatocellular	(125)	Decreased Ror2 expression Correlates with shorter overall survival

Since changes in Ror2 expression result in drastic defects in skeletal development, it is fairly unsurprising to find the reexpression of Ror2 in osteosarcoma, a malignant adolescent bone cancer. *In-vitro* analysis shows aberrant expression of Ror2 in osteosarcoma increases cell proliferation, migration, invasion, and formation of invadapodia (113,114). These invasive and migratory characteristics are mediated by Ror2-dependent expression of the extracellular matrix remodeling protease, matrix metalloprotease 13 MMP13 (113,114,126). Surprisingly, either Wnt5a or Wnt5b in connection with Ror2 were able to mediate these aggressive features in osteosarcoma cells. An analysis of primary osteosarcoma tumors through IHC for Wnt5a and ROR2 revealed a positive correlation between their high degree of expression in over 70% of samples. In addition, both Wnt5a and Ror2 correlated with Enneking surgical stage and tumor metastasis. These findings further confirm the role of Ror2 in tumor progression in osteosarcoma and its potential as prognostic biomarker (121).

Previous works elucidating the process of tumor metastasis in melanoma have shown Wnt5a to be overexpressed in metastatic melanoma tissues and correlated with decreased survival in melanoma patients (127,128). In addition to Wnt5a's role in promoting tumor progression and metastasis in melanoma, it also serves in regulating Ror2 expression in melanoma cells. Utilizing *in-vitro* and *in-vivo* approaches, suppression of Wnt5a/Ror2 signaling resulted in decreased cell invasion, migration, and metastasis (122). Reflective of the shared role Wnt5a and Ror2 have exhibited in melanoma, Ror2 is similarly correlated with metastasis (89,122).

Soft-tissue sarcomas are heterogeneous malignant tumors that currently have limited therapeutic options and prognostic biomarkers. Edris et al. undertook an analysis using a library of sarcomas to uncover novel RTK targets or biomarkers (116). Ror2 expression was detected in both LMS and GIST utilizing both gene expression and IHC as validation.

Knockdown of Ror2 reduced cell invasion in both GIST and LMS cells. Similar to our previous finding in RCC cells (83), suppression of Ror2 in LMS significantly reduced *in-vivo* tumor growth. Finally, Edris et al. showed that Ror2 can serve as an independent prognostic factor for both LMS and GIST, with Ror2 expression predicting poorer clinical outcome (116).

For most prostate cancer patients the rate of response to treatment is high if the disease is caught in its early stages. However for the 20-30% of patients that experience recurrence the picture is much more grim highlighting the need for identification of novel biomarkers to predict recurrence and identify new therapeutic targets. Yamamoto et al. showed that Wnt5a expression correlates with high Gleason scores, which are linked to increased risk of recurrence. Suppression of Ror2 resulted in decreased Wnt5a-dependent cell migration and invasion in prostate cancer-derived cells. These invasive characteristics are partially mediated by MMP1 in a Wnt5a/Ror2 dependent signaling manner (118).

An analysis undertaken to identify additional RTK targets expressed in gastric cancer screened all RTKs for mutations within the kinase domain in a series of gastric cancer cell lines and 52 microdissected primary gastric cancer tumors. Ror2 was identified among other RTKs to be expressed in poorly-differentiated invasive gastric cancers and to be a frequent target of non-synonymous mutagenesis. The identified D644N mutation is found within the kinase domain of Ror2, likely altering activity of the RTK Ror2. The indication that Ror2 is a potential driving factor in the development and/or progression of RCC is strengthened by the combined weight of these studies with similar suggestive correlations between increased invasive malignancy and Ror2, marking it as a promising therapeutic target. These studies also emphasize the potential of Ror2 as a prognostic and/or diagnostic biomarker.

Conflicting reports on whether Ror2 is more highly expressed in squamous cell carcinoma have been published. Kobayashi states that Ror2 is more highly expressed in comparison to normal tissue and is linked to cell polarity, motility, and increased malignancy

(117), but Liu et al. states that Ror2 expression is downregulated in comparison to adjacent corresponding normal tissues (123). Seeing that both studies rely upon very small sample sizes, any firm conclusions cannot be made at this time and further studies are required to clarify this discrepancy.

This ambiguity is further reflected in Ror2's purported tumor-suppressive capacity in both colorectal cancer and hepatocellular carcinoma. Epigenetic silencing of Ror2 through hypermethylation was observed in both colorectal cancer-derived cell lines and in primary tumors, with rescue of Ror2 inhibiting *in-vitro* and *in-vivo* tumor growth (129). Similarly reduced Ror2 expression levels were seen in 63% of hepatocellular patients in comparison to adjacent paired normal tissue samples and correlated with a significantly reduced overall survival rate (125). Though these results are seemingly inconsistent with previous findings regarding Ror2's tumor-promoting capacity, it is important to note that reintroduction of Ror2 in colorectal cancer cells resulted in a decrease in β-catenin-dependent signaling, constitutive activation of which serves as the driving force in colorectal cancer (129). These results suggest Ror2's identity as a tumor promoter or suppressor is dependent upon the context of expression and the state of Wnt/β-catenin-dependent signaling within the tumor. A precedent for this scenario may be observed in several of the Wnt antagonists including dickkopf -1 (DKK1) and secreted Frizzled-related proteins 1-3 (SFRP1-3) which have shown the ability to serve in both roles of tumor promoter and suppressor in various cancer contexts (130,131).

Wnt Signaling

Wnt signaling is a highly conserved network effecting a diverse set of processes within adult tissue homeostasis and embryonic development, including migration, cell/tissue polarity, cell proliferation, cytoskeletal arrangement, stem cell renewal, and differentiation

(132,133). Considering that Wnt signaling contributes to such a varied array of cellular functions it is unsurprising that disruptions in the activity and/or regulation of Wnt signaling have been linked to various developmental defects including skeletal diseases (134), polycystic kidney disease (135-137), and many forms of cancer (134,138). The large range of processes and signaling networks regulated by Wnt signaling can be partially attributed to the multitude of ligand/receptor combinations possible between the 19 members of the Wnt family, 10 Frizzled (Fzd) receptors, and additional Wnt receptors found in mice and humans. In addition the tight regulation of the spatiotemporal expression of these Wnt ligands and receptors during development allows for crosstalk between the Wnt signaling pathways.

Following the original discovery of the Wnt-1 (int-1) ligand, members were originally divided into two classes: canonical (β-catenin-dependent) and non-canonical (β-catenin-independent), according to their ability to transform C57mg mammary epithelial cells and activate β-catenin-mediated transcription (139-141). This classification placed Wnt1, 3, 3a, and 7a as canonical Wnts, with Wnt2, 4, 5a, 5b, 6, 7b and 11 as non-canonical Wnts. However, this classification of Wnt ligands may be artifactual or incomplete since crossover between the β-catenin-dependent and independent Wnt signaling has been well documented as a result of receptor context. The most extensively used ‘non-canonical’ ligand, Wnt5a, has been shown to be able to either stimulate or repress β-catenin dependent signaling both *in vitro* and *in vivo* depending upon the context of the spatiotemporal expression of various Wnt receptors (142-144). Thus future examinations of Wnt signaling cascades need to be viewed through cellular and receptor context.

β-catenin-dependent Wnt signaling

β-catenin-dependent Wnt signaling is the most well-characterized cascade and is triggered by Wnt ligand engaging Fzd and low density lipoprotein receptor-related protein 5 or 6 (LRP5/6) heterodimers. This signal is transduced through activation of dishevelled (DVL) which inhibits phosphorylation of β-catenin within the destruction complex consisting

of casein kinase 1α (CK1α), glycogen synthase kinase-β (GSK3-β), adenomatous polyposis coli (APC), and Axin. The resulting accumulating stabilized B-catenin is translocated into the nucleus, binding to transcription factors including T cell factor (TCF) and lymphoid enhancer-binding factor (LEF), regulating expression of target genes such as the negative feedback regulator Axin2. However, under conditions where no Wnt ligand is bound, β-catenin is sequentially phosphorylated by CK1α and GSK3-β within the destruction complex. β-catenin is then polyubiquitinated following recruitment of the β-TrCP-containing E3 ubiquitin ligase, marking it for proteasomal degradation (134,145) (Figure 1.4A).

β-catenin-independent Wnt signaling

β-catenin-independent Wnt signaling does not utilize the coreceptor LRP5/6 or β-catenin and is mediated solely through Fzd or other non-canonical Wnt receptors. The first of the β-catenin-independent pathways is referred to as the planar cell polarity pathway (PCP). Wnt/PCP cascade is initiated through Fzd receptor engagement of the Wnt ligand resulting in activation of DVL, then Disheveled-Associated Activator of Morphogenesis 1 (DAAM1) and/or the GTPases Rac1 and RhoA. The signal is further transduced with the activation of the kinases c-Jun N-terminal kinase (JNK) and Rho kinase 1 (ROCK1), resulting in changes in cell polarity, movement, and inhibition of β-catenin-dependent signaling (Figure 1.4B).

The second of the β-catenin-independent pathways is the Wnt/Ca^{2+} pathway which, akin to the Wnt/PCP pathway, is mediated solely through the Fzd receptor. Upon engagement with the Wnt ligand the Fzd receptor leads to the activation of DVL. The cascade continues through activation of phospholipase C (PLC) resulting in a release of intracellular Ca^{2+} and activation of calcineurin, calcium-calmodulin kinase II (CamKII) and protein kinase C (PKC). Activated calcineurin rapidly dephosphorylates NFAT resulting in its translocation into the nucleus to mediate transcription of target genes (Figure 1.4C).

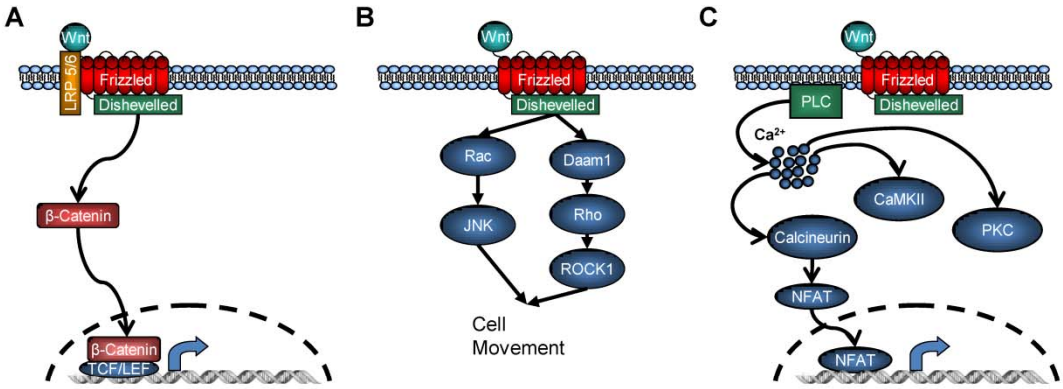

Figure 1.4: Overview of Wnt signaling pathways.
A schematic representation of the various Wnt signal transduction cascades. **A)** The canonical Wnt pathway cascade is initiated following engagement of Wnt ligand with the Frizzled and LRP5/6 receptor complex induces the stabilization of β-catenin via Dishevelled and inhibition of the destruction complex consiting of Axin, APC, glycogen synthase kinase 3-β and casein kinase 1. β-catenin translocates into the nucleus where it complexes with members of the LEF/TCF family of transcription factors to mediate transcription of various target genes. **B)** The non-canonical planar cell polarity (PCP) signaling pathway is likewise characterized by Wnt ligand engaging the Frizzled receptor, but this occurs independent of LPR5/6. Dishevelled transduces this signal through DAAM1, and/or, through activation of the small GTPases Rho and Rac eventually resulting cytoskeletal changes. **C)** The Wnt-Ca^{2+} signal cascade is also solely mediated following Wnt binding to the Frizzled receptor leading to activation of Dishevelled. The signal continues through Phospholipase C resulting in a release of intracellular Ca^{2+} and activation of Calcineurin, Calcium-calmodulin kinase II (CamKII) and protein kinase C (PKC). Activated Calcineurin dephosphorylates NFAT resulting in its translocation into the nucleus to mediate transcription of target genes.

Wnt antagonists

An additional level of regulation of these Wnt signaling pathways is through several families of secreted proteins referred to as Wnt antagonists. These factors have been classified into two broad groups based upon their mechanisms of action. The first class of secreted antagonist factors comprised of the SFRPs, Wnt inhibitory factor 1 (WIF-1), and Cerberus bind directly to Wnt ligands allowing for inhibition of any the Wnt signaling cascades. The SFRP family is comprised of 5 members, SFRP1-5, which were originally identified in mammals for their regulatory contribution in embryonic development (146,147). All members of the SFRP family consist of two domains, a CRD domain, allowing for binding of Wnt and Fzd receptors or Tolloid metalloproteases; and a Netrin domain, which has also been shown to participate in binding of Wnts (148-152). Although WIF-1 and Ceberus both lack a CRD domain they have been shown to be able to bind Wnts and serve as antagonists (153,154). The second class of Wnt antagonists consists of the DKK family, which bind the

Wnt coreceptors LRP5/6. The DKK family is comprised of four members, DKK1-4, which are characterized by two CRD domains (155,156). DKKs mediate their inhibition of β-catenin-dependent signaling by binding LRP5/6 and initiate either the internalization or disruption of its heterodimer with Fzd (157-159). DKK1-2's inhibition of LRP5/6 requires the concomitant binding of the transmembrane protein Kremen.

As with the other members, many of these Wnt signaling members are able to serve in multiple capacities dependent upon the context of their expression. Although the SFRP family has been classified as Wnt antagonists, further research has shown their part to be much more complicated as they are capable of inhibiting and enhancing Wnt signaling in a context-dependent manner (160). DKK2 is further set apart from its family members because it is also capable of acting as LRP agonist (161).

Wnt signaling in renal development and RCC

As previously described, Wnt signaling plays an extensive role in development, but of particular note for this work, previous studies have highlighted the function of Wnt signaling in nephrogenesis. The loss of either Wnt9b or Wnt4 results in hypoplasia within the developing kidney, due to their regulation of the epithelial differentiation of metanephric mesenchymal progenitor cells (162,163). Wnt11 expression is restricted to the tip of the ureteric bud, with its loss causing a mild form of hypoplasia within the kidney (164). Expression of both SFRP1 and SFRP2 is present during nephrogenesis in tightly-regulated spatiotemporal patterns within the developing mesenchyme, which complement or overlap the expression of aforementioned Wnt4 (165-167). SFRP1 binds Wnt4, inhibiting β-catenin-dependent signaling, resulting in decreased tubular differentiation and bud branching. SFRP2 competes with SFRP1 for the binding of Wnt4, allowing a dynamic balance to achieve proper differentiation and tubule formation within the kidney (165,166). In addition to these secreted factors, several Wnt receptors including Ror2 and Fzd are also expressed in the developing mesenchyme of the kidney.

Beside its function in development of the kidney, Wnt signaling also plays a role in pathology of RCC. Mutations and misregulation of the β-catenin-dependent signaling cascade resulting in tumorigenesis or tumor progression have been observed in multiple cancers, the most notable being colorectal carcinoma (138,168). However, mutations in the typical players (e.g. APC, Axin, and β-catenin) found in other cancers are absent from RCC. Several independent groups have shown epigenetic silencing of Wnt antagonists SFRP1-5, DKK1-3, and WIF1 in RCC, providing one possible avenue in which Wnt/β-catenin signaling may be constitutively activated in RCC (169-176). In seeming contrast to these findings, high expression of SFRP1 & SFRP3 in RCC metastases suggests that low SFRP expression may be beneficial for tumorigenesis but that high expression may help spur the transition to a more aggressive cancer (177,178). Overexpression of SFRP2 and SFRP3 in RCC cells resulted in decreased apoptosis, increased cell invasion, and *in-vivo* tumor growth (178,179). Surprisingly, SFRP2 expression in RCC cells also resulted in increased β-catenin mediated transcription, measured by TopFlash luciferase reporter (179). These findings as a whole lend considerable weight to the importance of Wnt signaling in the initiation and progression of RCC.

Significance

Although in recent years progress has been made in the care and treatment of RCC patients, the need for new targeted therapies for RCC remains. Owing to increased understanding of the underlying molecular biology of RCC, many new targets are being discovered or rediscovered. The identification of the RTK Ror2 as a novel target for RCC provides a needed window for the development of new therapeutics. However, a lack of knowledge of what role Ror2 plays as a receptor in Wnt signaling and its function in tumor progression of RCC greatly inhibits this goal. This thesis begins to provide the framework of

Ror2 signaling within the various Wnt signaling cascades in the context of RCC, providing the tools for further characterization in future studies. Furthermore this thesis expands our knowledge of the effects of Ror2 expression in RCC on cell migration, angiogenesis, and the potential of Ror2 as a prognostic biomarker for RCC.

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Chapter Two

ROR2 EXPRESSION CREATES A POISED STATE OF WNT SIGNALING IN RENAL CANCER

Introduction

Cancer cells usurp many normal signaling processes in the course of transformation or acquisition of transformed cell traits. The Wnt signaling pathway is a growth and differentiation control pathway central to embryogenesis and cancer (1). The canonical Wnt pathway is characterized by secreted Wnt factors, Wnt3a being the most widely studied, engaging the receptor complex consisting of low-density lipoprotein receptor-related protein (LRP) and a member of the Frizzled (Fzd) family of receptors, halting the basal state of ubiquitination and degradation of β-catenin within the destruction complex. As β-catenin becomes stabilized it results in the saturation of the destruction complex leading to an increase of cytosolic β-catenin and localization to the nucleus (2). Nuclear β-catenin drives the transcription of genes involved in promoting cell proliferation, maintenance of a primitive state, or induction of differentiation dependent upon cellular context. Wnts can initiate other signaling processes, known as non-canonical Wnt signaling pathways, and promote migration, establishment of polarity, or programming of morphogenesis (3). In tumors, these pathways provide signals for cancer cells to proliferate, acquire stem cell-like properties, and secure invasive characteristics as a result of mutations or differential expression of key members of these pathways.

(This work is modified from Rasmussen et al Journal of Biological Chemistry, 2013)

One of the recently described non-canonical Wnt receptors, the receptor tyrosine kinase-like orphan receptor 2 (Ror2) has been implicated in a diverse set of cancers (4-9), including renal cell carcinoma (RCC), where aberrant expression occurs as a result of the constitutive deregulation of the hypoxia response pathway resulting from loss of the Von Hippel-Lindau (*VHL*) gene (4,10). Ror2 is best known for its role in bone morphogenesis, as mutation or loss of this protein in early embryogenesis results in limb foreshortening in humans and mouse models (11-13). The expression of Ror2 in mice is restricted to early gestation and mesenchymal stem cell niches (14,15). Ror2 is emerging as an intriguing mediator of various context-specific Wnt signals in developmental processes as well as cancer (16-18). Ror2 has been shown to engage both canonical and non-canonical Wnts, and it has been shown to transmit signals related to convergent extension movements, planar cell polarity, and proliferation (19,20). Ror2 engagement of Wnt5a has been shown to inhibit canonical signaling, however, recent work utilizing primary cell lines showed this effect to be Ror2-independent (18,21). The ability of Ror2 to convey situation-appropriate signals may contribute to cell-type or situation specific effects, but likely involves availability of cofactors, binding partners, or downstream substrates to affect the signal and functional outcome.

Although we have previously shown that Ror2 expression is associated with cellular migration and invasion phenotypes in renal carcinoma cells, growth of RCC is also facilitated by β-catenin transcription-mediated canonical signals (22). The mediators of this signal are thought to be dependent upon the lost expression of the Von Hippel-Lindau tumor suppressor (23,24). We therefore decided to explore the signaling pathways related to Ror2 expression in renal carcinoma and renal-derived cellular systems. Here, we demonstrate that human RCC tumors and cell lines show concordance of Ror2 expression with a large number of established canonical Wnt pathway targets, suggesting a role of Ror2 in mediating β-catenin-dependent signaling. Expression of Ror2 increased the stabilization of

β-catenin as well as the transcriptional activity of canonical Wnt target genes, independently of exogenously added Wnt3a, and led to the enhancement of the canonical signal when exogenous Wnt3a was supplied. Inhibition of the canonical coreceptor LRP6 reduced the responsiveness to Wnt3a; however, it did not ablate the Wnt3a-independent increase in β-catenin. Mutations in the kinase domain of Ror2 abrogated the stabilization of β-catenin and Axin2 transcription observed with expression of wild-type protein. These findings demonstrate a novel LRP-independent function of Ror2 to maintain an increased signaling pool of stable β-catenin in renal cells, while priming cells for additional responsiveness to Wnt3a activation of the β-catenin transcriptional pathway.

Results

Expression of Ror2 in primary human RCC tumors correlates with expression of Wnt signaling component gene

We previously established that Ror2 is expressed in RCC and contributes to three-dimensional growth, migration, and *in vivo* tumor growth (4). However, the role of Ror2 as a Wnt receptor in RCC remains largely undetermined. Ror2 has been shown to engage both canonical and non-canonical Wnt ligands (16-18). We first sought to elucidate the dominant pattern of interaction of Ror2 with signaling networks through examination of gene expression patterns from 95 human clear cell RCC tumors. An analysis of transcript diversity was performed using Agilent microarray data using significance of microarrays (SAM) (25). This evaluation yielded 723 genes (Online S. Table 1) that exhibited a significant correlation or anti-correlation with Ror2 (False Discovery Rate (FDR) <0.043%). A heat map of these genes following an unsupervised clustering exhibited two cluster patterns correlating with the level of Ror2 transcript in these tumors (Figure 2.1A). Using DAVID (26,27), a variety of significantly enriched gene ontologies (Online S. Table 2) were identified from the genes

exhibiting a positive correlation with Ror2. Similar to previous results (4), Ror2 expression correlated with many genes playing a role in extracellular matrix (GO:0031012, *P*-value= $3.16x10^{-25}$) (Figure 2.1B) and skeletal system development (GO:0001501, *P*-value= $6.11x10^{-08}$) (Figure 2.1C). In addition, enrichment for genes in the Wnt receptor canonical signaling pathway (GO:0016055, *P*-value=$2.6x10^{-4}$) was also observed (Figure 2.1D). These results suggest that the role of Ror2 as a Wnt receptor in RCC tumors may not be limited to non-canonical Wnt pathways.

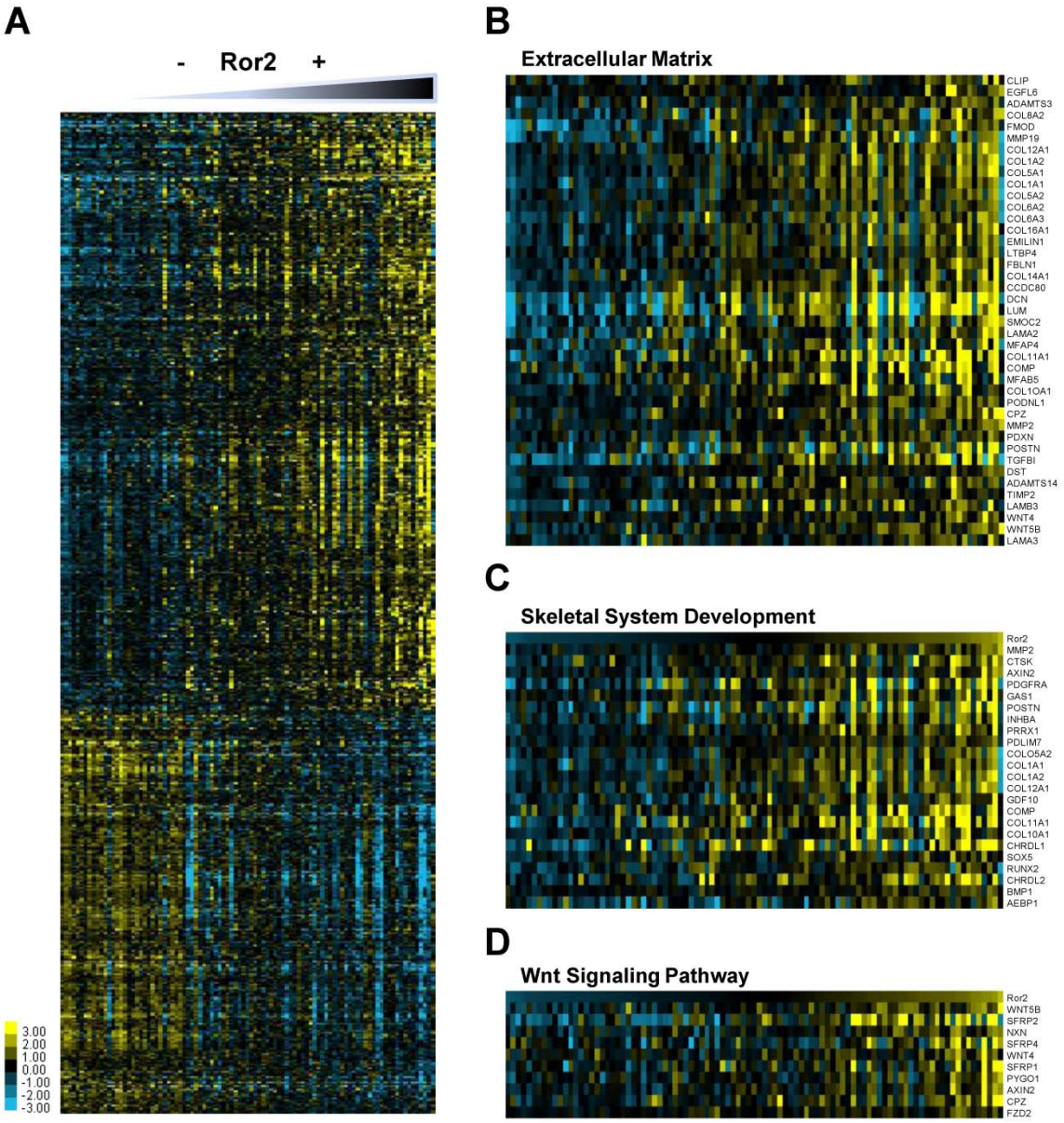

Figure 2.1: Expression of Ror2 in primary human RCC tumors correlates with expression of Wnt signaling component genes.

A) Heat map of 721 probes exhibiting significant (false discovery rate (FDR) < 0.043) correlation and anti-correlation with Ror2 expression as determined by SAM analysis in a panel of 95 primary human RCC tumors.

Magnified heat maps for the following significantly enriched gene ontologies identified using DAVID from genes positively correlating with Ror2 expression **B)** Extracellular Matrix **C)** Skeletal Development and **D)** Wnt Signaling Pathway.

Expression of Ror2 in RCC cells results in changes of canonical Wnt regulators and target genes

Building on our findings in our human RCC tumors, we examined the effects of Ror2 expression on gene expression using the Human Wnt Signaling RT^2 Profiler PCR Array (SABiosciences, Frederick, MD, USA). Three cell lines were used to determine alterations in gene expression under basal conditions: the RCC-derived cell line 786-0, which exhibits endogenous Ror2 expression; a derived shRNA Ror2 knockdown cell line, 786-0 shRor2; and an overexpression cell line, 786-0 TAP-hRor2 (Figure 2.2A). We observed a large number of canonical genes displaying concordant expression with Ror2 (Figure 2.2B), suggesting a potential association between Ror2 expression and canonical β-catenin transcriptional activity independent of Wnt3a stimulation with exogenous ligand. This concordant expression pattern with Ror2 was independently verified for targets Fzd1 and Jun using independent samples and primer pairs (Figure 2.2C-D).

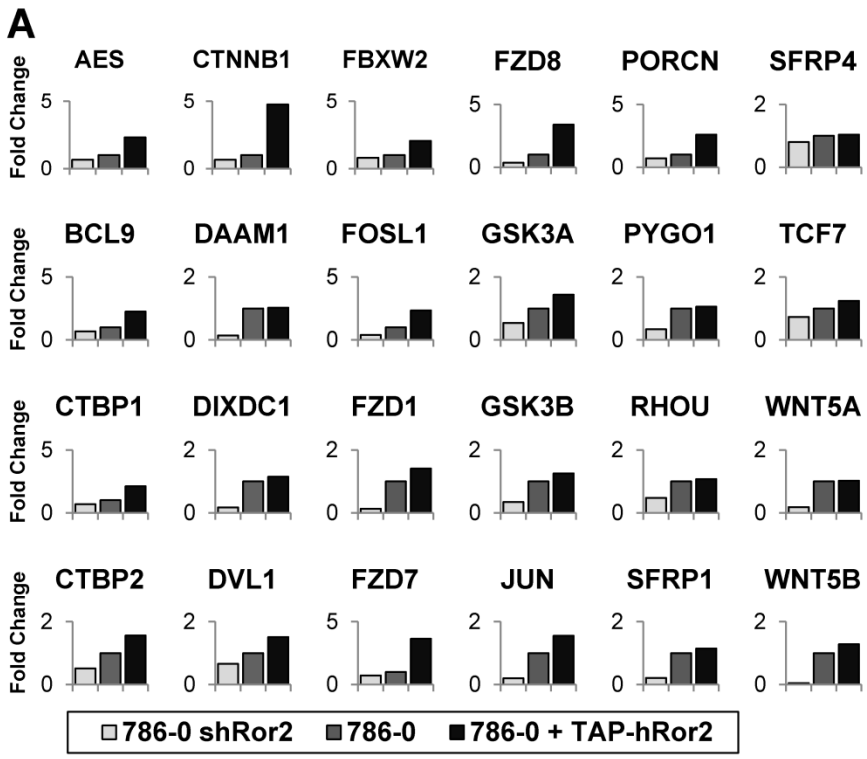

Figure 2.2: Expression of Ror2 in RCC cells results in changes of canonical Wnt regulators and target genes.
A) RNA expression was paneled across various Wnt component and target genes using the Human Wnt Signaling RT^2 Profiler PCR Array with cDNA from 786-0 cells which endogenously express Ror2, 786-0 shRor2 were Ror2 is stably suppressed, and the Ror2 overexpression cell line 786-0 + TAP-hRor2. Transcript values for each sample were normalized to the geometric mean of a 5 internal housekeeping genes. Changes in the level of expression correlating with Ror2 expression are displayed for the indicated 24 genes with fold change calculated relative to 786-0.
B) Representative quantitative RT-PCR for shows Ror2 expression levels for all 786-0 cell lines relative to 786-0.
Quantitative RT-PCR shows a decrease in 786-0 shRNA Ror2 knockdown relative 786-0 cells for **C)** FZD1 and **D)** JUN. For all quantitative RT-PCR assays, transcript values were normalized to B-Actin RNA internal standard. Error bars represent SEM across triplicates in triplicate experiments. *P*-values calculated using an Anova one-way analysis (*<.05, **<.001).

Suppression of Ror2 results in decreased β-catenin mediated transcription in RCC cells

786-0 cells which endogenously express Ror2 were used to more fully determine if the aberrant expression of Ror2 in RCC cells contributes to β-catenin-dependent signaling. To directly examine canonical Wnt target gene activation in these cells, we first examined the mRNA expression of a classic canonical Wnt target gene, Axin2, using quantitative RT-

PCR. Using two independent shRNAs targeting unique domains of Ror2, we observed a significant suppression of Ror2 (Figure 2.3A) and of Axin2 transcription following treatment with Wnt3a (Figure 2.3B) in comparison to the control empty vector (786-0 RC3), which also exhibited an increase in Ror2 expression with Wnt3a stimulation. These results were corroborated using the TOPFlash luciferase-reporter, in which 786-0 pRS (pRetroSuper) control showed a significant induction of signal in response to Wnt3a, but no induction was observed with shRor2 knockdown cell lines (Figure 2.3C).

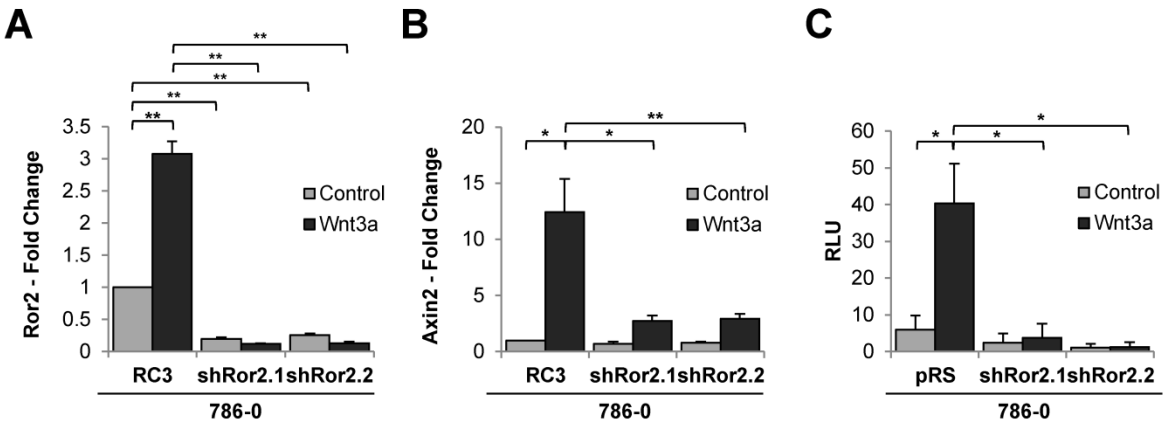

Figure 2.3: Suppression of Ror2 results in decreased β-catenin mediated transcription in RCC cells.
Quantitative RT-PCR in 786-0 cells for **A)** Ror2 shows a significant increase in control RC3 cells with Wnt3a (100 ng/ml) stimulation, while levels are significantly reduced in both shRNA Ror2 knockdown cell lines. **B)** Likewise, a significant attenuation in Axin2 transcription in response to Wnt3a (100 ng/ml) is seen in both shRNA Ror2 knockdowns using quantitative RT-PCR. For all quantitative RT-PCR assays, transcript values were normalized to 18S RNA internal standard with fold change calculated in reference to unstimulated 786-0 RC3.
C) A significant reduction of β-catenin mediated transcriptional response following Wnt3a (100ng/ml) stimulation with the TOPFlash luciferase reporter in 786-0 Ror2 suppressed cells in comparison to control 786-0 pRS cells. Relative luciferase units (RLU) were calculated by normalizing TOPFlash luciferase to internal renilla luciferase activity.
Error bars represent SEM across triplicates in duplicate experiments. *P*-values calculated using an Anova one-way analysis (*<.05, **<.001).

Overexpression of Ror2 enhances β-catenin mediated transcription in renal and RCC cells

Prior studies expressing Ror2 in HEK293 cells have focused on concomitant treatment of Wnt3a and Wnt5a, but have not elaborated on how Ror2 expression may also

potentiate Wnt3a canonical signaling, nor examined the potential for differential signaling (18,28). To further examine Ror2 contributions as a mediator of canonical β-catenin signaling in RCC, we utilized 786-0 and HEK293T cells expressing a tetracycline-dependent fusion protein, Ror2-GFP, or GFP control. Robust expression of Ror2 mRNA was observed in 786-0 Ror2-overexpressing cells, relative to control GFP cells following the addition of doxycycline, and remained unaltered by treatment with Wnt3a (Figure 2.4A). Upon expression of Ror2 there was a significant increase in basal Axin2 mRNA as well as a heightened response to Wnt3a treatment (Figure 2.4B). This enhancement of β-catenin mediated transcription in the absence of Wnt3a with expression of Ror2, and further potentiation was also observed in 786-0 cells transfected with a TOPFlash luciferase-reporter (Figure 2.4D).

To validate these findings in an additional renal-derived cell background, we used HEK293T cells expressing either GFP or Ror2. Upon the induction of Ror2 in HEK293T cells, we again observed significant enhancement of Axin2 transcription and a further increase following stimulation with Wnt3a (Figure 2.4F). Likewise, β-catenin-mediated transcription measured using the TOPFlash luciferase reporter showed an enhanced response to Wnt3a stimulation, although no significant change was detected under basal conditions (Figure 2.4H). Inhibition of GSK3-β using LiCl resulted in an additive effect of Axin2 transcription with Ror2 expression, suggesting Ror2 effects are mediated through β-catenin (Figure 2.4C&G).

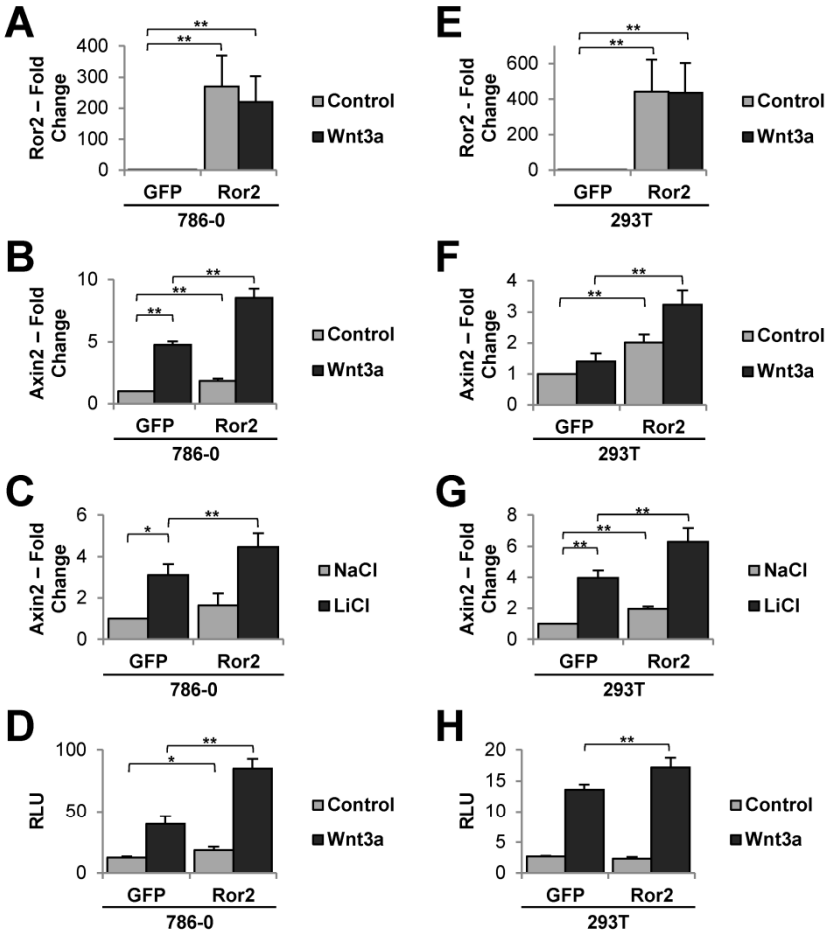

Figure 2.4: Overexpression of Ror2 enhances β-catenin mediated transcription in renal and RCC cells.
Quantitative RT-PCR for Ror2 in **A)** 786-0 and **E)** HEK293Tcells shows a strong induction of Ror2 following treatment with Doxycycline (500 ng/ml) and **B&F)** concordant significant increase in basal expression of the canonical Wnt target gene Axin2 that is further heightened upon the addition of Wnt3a (100 ng/ml) relative to the control cells. Quantitative RT-PCR in **C)** 786-0 and **G)** 293T cells for Axin2 again shows an increase in basal levels with Ror2 expression and a significant increase in response to treatment with LiCl (10mM). For all quantitative RT-PCR assays, transcript values were normalized to β-actin RNA internal standard with fold change calculated in reference to unstimulated GFP expressing cells.
D) The expression of Ror2 in 786-0 transfected with a TOPFlash-luciferase reporter exhibited a significant increase in activity basally with Ror2 expression leading to a significant increase in response to Wnt3a (100ng/ml) stimulation. **H)** HEK293T cells transfected with TOPFlash-luciferase reporter displayed a significantly enhanced response to Wnt3a (100 ng/ml) stimulation.
Error bars represent SEM across triplicates in duplicate experiments. *P*-values calculated using an Anova one-way analysis (*<.05, **<.001).

Ror2 expression results in an increased pool of stable β-catenin independent of exogenous Wnt stimulation

Both TCF-luciferase reporters and target gene expression of Axin2 demonstrated that Ror2 expression is associated with heightened β-catenin target gene transcription in response to Wnt3a stimulation. In addition, expression of Ror2 resulted in enhanced transcriptional activity in the absence of exogenous ligand. Therefore, we sought to determine if these findings also correlated with the availability of β-catenin for transcriptional activation.

To examine the pool of cytosolic β-catenin available for signaling, protein lysates were incubated with concanavalin-A sepharose beads. Immunoblot of 786-0 cell lysates revealed an increase of free β-catenin levels (indicated with a *, as compared to total levels) upon expression of Ror2, with a further increase following stimulation with Wnt3a CM (Figure 2.5A). These results were corroborated in HEK293T cells, demonstrating increased stabilized β-catenin upon Ror2 expression that is enhanced with the addition of Wnt3a CM in comparison to GFP expressing cells (Figure 2.5B). Quantification of stabilized β-catenin relative to total β-catenin is shown below the corresponding bands in each immunoblot.

To understand the mechanism Ror2 could be using to initiate this poised signaling state, we examined upstream pathway components. Dishevelled proteins (DVL2 and DVL3) play an important role in canonical Wnt signaling and DVL protein activation via phosphorylation has been previously reported as a mediator of Ror2 signaling (21,29-31). Expression of Ror2 in 786-0 and HEK293T cells resulted in increased levels of phosphorylated DVL2 and DVL3, detected as a shift from the faster migrating band to a slower migrating, phosphorylated form, indicative of activation (Figure 2.5A&B). Quantitation of pDVL signal relative to total DVL demonstrates increased activation with Ror2 expression and in response to Wnt3a.

We also examined the receptor LRP6 previously established to be required for Wnt ligand-induced β-catenin signaling in mice and *Xenopus* (32,33). However, while we observed expression of LRP6 and its phosphorylation in response to Wnt3a stimulation, no effect was observed related to Ror2 expression (Figure 2.5A-C). These data suggest that Ror2 expression mediates an LRP6-independent signal resulting in DVL2/3 activation and subsequent stabilization of β-catenin. However, Wnt-ligand enhanced signal proceeds via LRP6 phosphorylation.

When we examined suppression of Ror2, 786-0 cells demonstrated no effect on LRP activation in response to Wnt3a, consistent with the overexpression studies prior (Figure 2.5C). However, Ror2 shRNA abrogated the stabilized β-catenin both basally and upon Wnt3a CM stimulation (Figure 2.5C). The extent of suppression of Ror2 protein differs between the two shRNAs, targeting different sequences in Ror2, with the more potent hairpin, shRor2.2, eliciting the most significant reduction in β-catenin stabilization with or without Wnt3a. This is demonstrated visually as well as quantitatively.

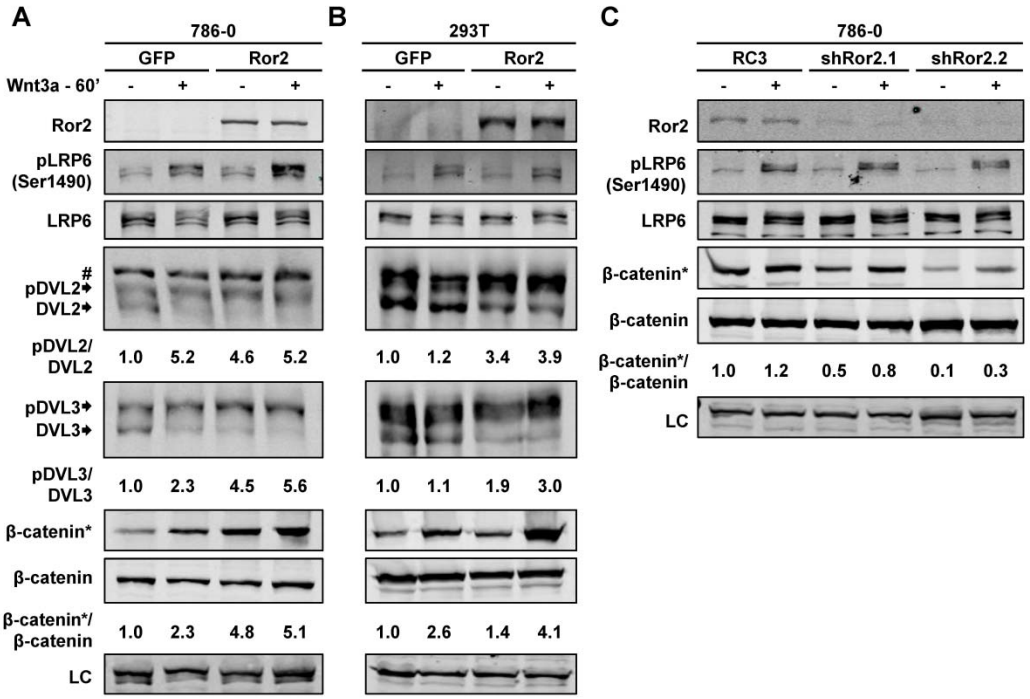

Figure 2.5: Ror2 expression results in an increased pool of stable β-catenin independent of exogenous Wnt stimulation.
Immunoblot for the indicated antibodies of whole cell lysates or concanavalin-A treated lysates (*) following stimulation with either control or Wnt3a CM (dil 1:2) for 1hour. **A)** 786-0 and **B)** HEK293T cells show increased levels of phosphorylated DVL2, DVL3, and stable β-catenin with Ror2 expression and an additional increase of stable β-catenin following treatment with Wnt3a CM. **C)** 786-0 shRor2 knockdown cell lines exhibited attenuated levels of stable β-catenin basally and in response to Wnt3a CM stimulation in comparison to 786-0 RC3 control cells. Changes in pDVL2, pDVL3, and stable β-catenin relative to unphosphorylated DVL or total β-catenin are shown with densitometric quantification below the corresponding bands. (LC = Loading Control).

Ror2 dependent stabilization of β-catenin is independent of LRP6

To further determine what contribution the coreceptor LRP6 has on Ror2 effects in β-catenin-dependent signaling, we utilized siRNA targeting to deplete cells of LRP6. Suppression of the coreceptor LRP6 resulted in the loss of β-catenin stabilization following treatment with Wnt3a CM in GFP control cells. Yet, the increased basal β-catenin concordant with Ror2 expression remained unaltered (Figure 2.6A). We did observe repression of the stimulated signal at the level of Axin2 transcription with siLRP6 in both GFP and Ror2 expressing cells, although in HEK293T cells, the basal increased level of Axin2 transcript was lost on LRP6 suppression (Figure 2.6C).

Dickkopf-1 (DKK1) is an established Wnt antagonist that binds to LRP5/6 leading to its internalization from the cell surface. Pretreatment of HEK293T cells with recombinant human DKK1 prior to the addition of Wnt3a CM resulted in decreased stabilization of β-catenin in GFP control cells. However, this addition of DKK1 preceding Wnt3a CM had no bearing on the increased β-catenin pool associated with Ror2 expression (Figure 2.6B). Quantitative RT-PCR for Axin2 in DKK1-treated cells likewise showed maintenance of higher basal levels of transcription than control cells, but with a loss of response to Wnt3a stimulation (Figure 2.6D). Together these data suggest that LRP6 cofactor is important primarily to transduce the Wnt3a ligand-driven enhanced signal. However, the reduction of basal target gene transcript by LRP6 siRNA indicates that LRP6 may retain a minor role in mediating this signal.

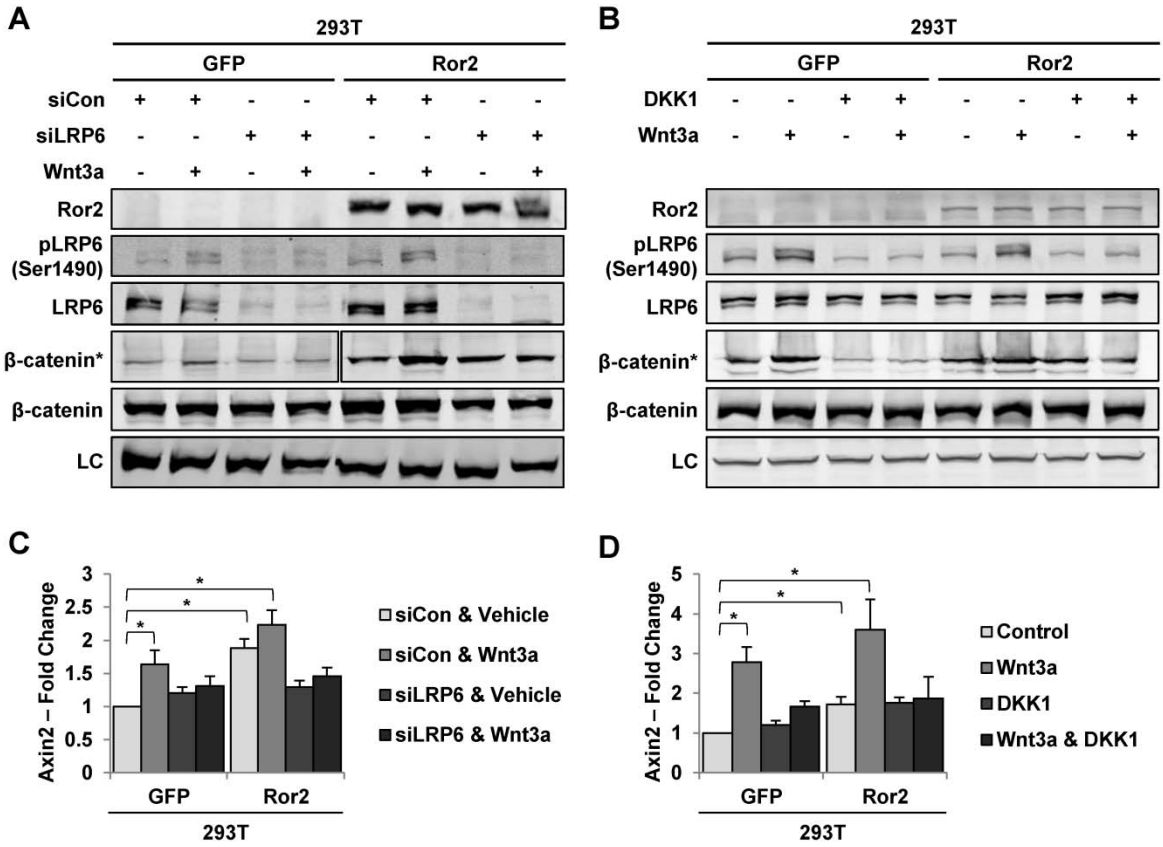

Figure 2.6: Ror2 dependent stabilization of β-catenin is independent of LRP6.
A) Immunoblot for the indicated antibodies of either whole cell lysates or concanavalin-A beads treated lysates (*) from 293T cells, 48 hours post-transfection with siCon (non-specific control) or siLRP6 and stimulated with either control or Wnt3a CM (dil 1:2) for 1hour shows increased levels of β-catenin is maintained with Ror2 expression. **B)** Immunoblot for the indicated antibodies of whole cell lysates or concanavalin-A treated lysates (*) following stimulation with either Wnt3a CM (dil 1:2) or DKK1 (400ng/ml) for 1 hour followed by Wnt3a CM stimulation showed increased levels of β-catenin concurrent with Ror2 expression. (LC = Loading Control).
Quantitative RT-PCR for Axin2 in HEK293T cells **C)** transfected with siCon or siLRP6 and stimulated 48 hours post transfection with Wnt3a (100ng/ml) show that LRP6 is needed for mediating Wnt3a/β-catenin dependent transcription. **D)** Axin2 expression is shown in 293T cells stimulated with either Wnt3a (100ng/ml) or DKK1 (400ng/ml) for 1 hour followed by Wnt3a stimulation, with high DKK1 inhibiting response to Wnt3a stimulation in both GFP and Ror2 expressing cells. For all quantitative RT-PCR assays, transcript values were normalized to B-Actin RNA internal standard with fold change calculated in reference to unstimulated GFP expressing cells.
Error bars represent SEM across triplicates in duplicate experiments. *P*-values calculated using an Anova one-way analysis (*<.05, *<.001).

Intact Ror2 kinase domain is required for poised Wnt signaling state

To determine if Ror2 is directly contributing to these changes in canonical Wnt signaling, site-directed mutagenesis was used to disrupt the putative Ror2 kinase domain. Ror2-DM contains mutations in two key residues of the catalytic site lysine 507 within the

putative ATP binding pocket and aspartate 633 of the DFG (found as DLG in Ror2) motif. Despite being expressed at similar levels in 786-0 cells to wild type Ror2, this mutant version failed to effectively stabilize β-catenin (Figure 2.7A). A similar pattern of increased stable β-catenin levels is seen in HEK293T cells with overexpression of wild-type Ror2, but not with expression of the DM mutant (Figure 2.7B). This effect is also shown quantitatively below the corresponding immunoblots (Figure 2.7A&B). Quantitative RT-PCR for Axin2 also shows a significant decrease with the expression of mutant Ror2 (Ror2-DM) in comparison to wild-type Ror2 in both 786-0 (Figure 2.7C) and HEK293T cells (Figure 2.7D). Thus, an intact Ror2 kinase domain is necessary for achieving the increased pool of stabilized β-catenin and for canonical transcript activity independent of Wnt3a stimulation.

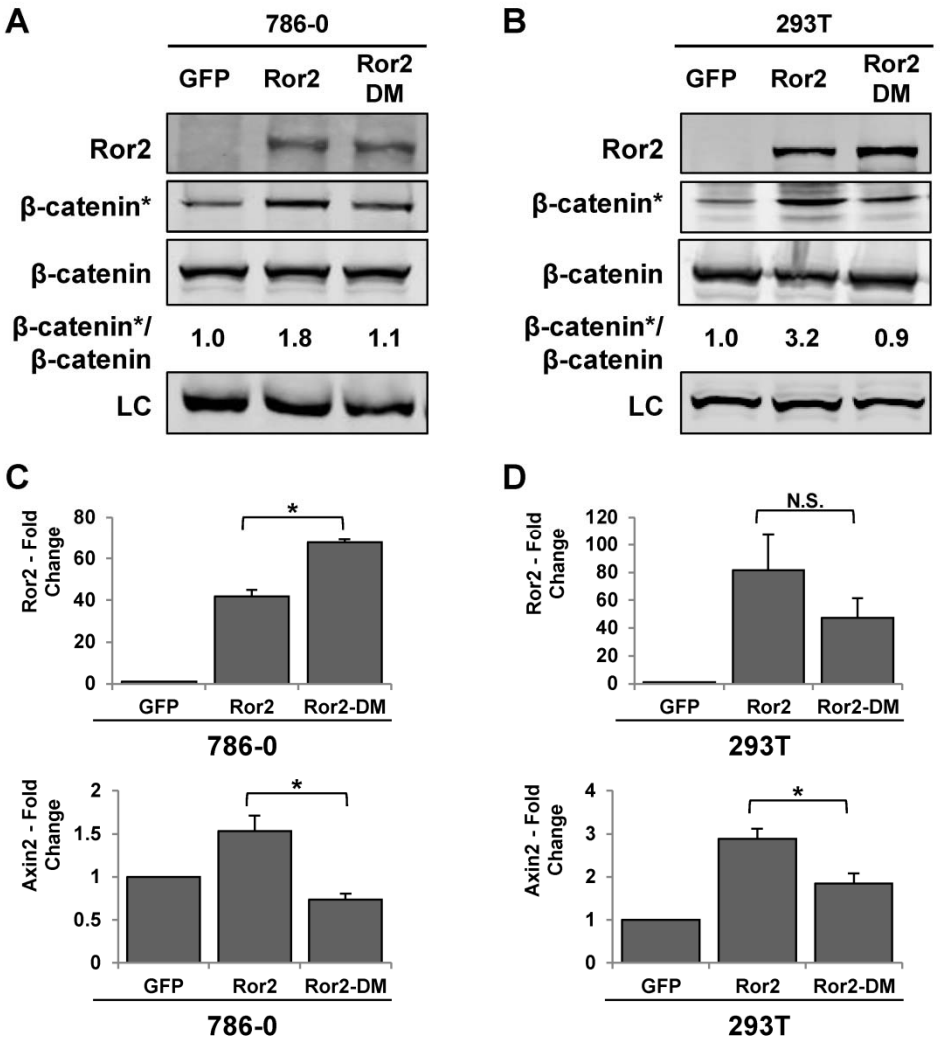

Figure 2.7: Intact Ror2 kinase domain is required for poised Wnt signaling state

Immunoblot of whole cell lysates or concanavalin-A treated lysates (*) from **A)** 786-0 or **B)** HEK293T cells expressing GFP, Ror2, or the double mutant Ror2-DM, 48 hour post induction with Doxycycline (500ng/ml) show increased levels of stable β-catenin with wild-type Ror2 compared with Ror2-DM. Densitometry quantification of stabilized β-catenin relative to total β-catenin is provided below the corresponding bands. (LC = Loading Control).

Quantitative RT-PCR for Ror2 in **C)** 786-0 or **D)** HEK293T cells shows expression of Ror2 and Ror2-DM cells 24 hours post induction, but a significant decrease of Axin2 expression in Ror2-DM expressing in both cell lines. For all quantitative RT-PCR assays, transcript values were normalized to β-actin RNA internal standard with fold change calculated in reference to unstimulated GFP expressing cells.

Error bars represent SEM across triplicates of a representative duplicated experiment. *P*-values calculated using an Anova one-way analysis (*<.05, **<.001).

Wnt/PCP and Wnt/Ca^{2+} Signaling is independent of Ror2 in RCC cells

Ror2 has previously been shown to bind the non-canonical Wnt5a ligand effecting and serving as a receptor for non-canonical Wnt signaling pathways; however, these same effects have been shown to be Ror2-independent (20,21,29,30,34,35). Because cell context seems to influence the role of Ror2 in Wnt signaling, we sought to elucidate if Ror2 also serves as a receptor for non-canonical Wnt pathways in RCC cells. First, we looked at changes in Wnt/planar cell polarity (PCP) signaling using changes in activity of the GTPases RhoA and Rac1 as a functional readout. Although the addition of Wnt5a did result in an increase of GTP-bound/active RhoA there was no significant change in either of the stable shRor2 knockdowns (Figure 2.8A). Rac1 levels remain unchanged with addition of Wnt5a or suppression of Ror2 (Figure 2.8B). Likewise we observed no changes with Wnt5a stimulation or Ror2 knockdown in the downstream target of Rac1, c-Jun N-terminal kinase (JNK) (Figure 2.8C). To determine if Ror2 serves as receptor for Wnt/Ca^{2+} pathway, 786-0 cells were transfected with a nuclear factor associated with T cells (NFAT)-driven luciferase reporter which exhibited increases with the addition of Wnt5a that were unaltered with Ror2 expression. Although these results are not comprehensive they suggest that Wnt/PCP and Wnt/ Ca^{2+} signaling are independent of Ror2 in RCC cells.

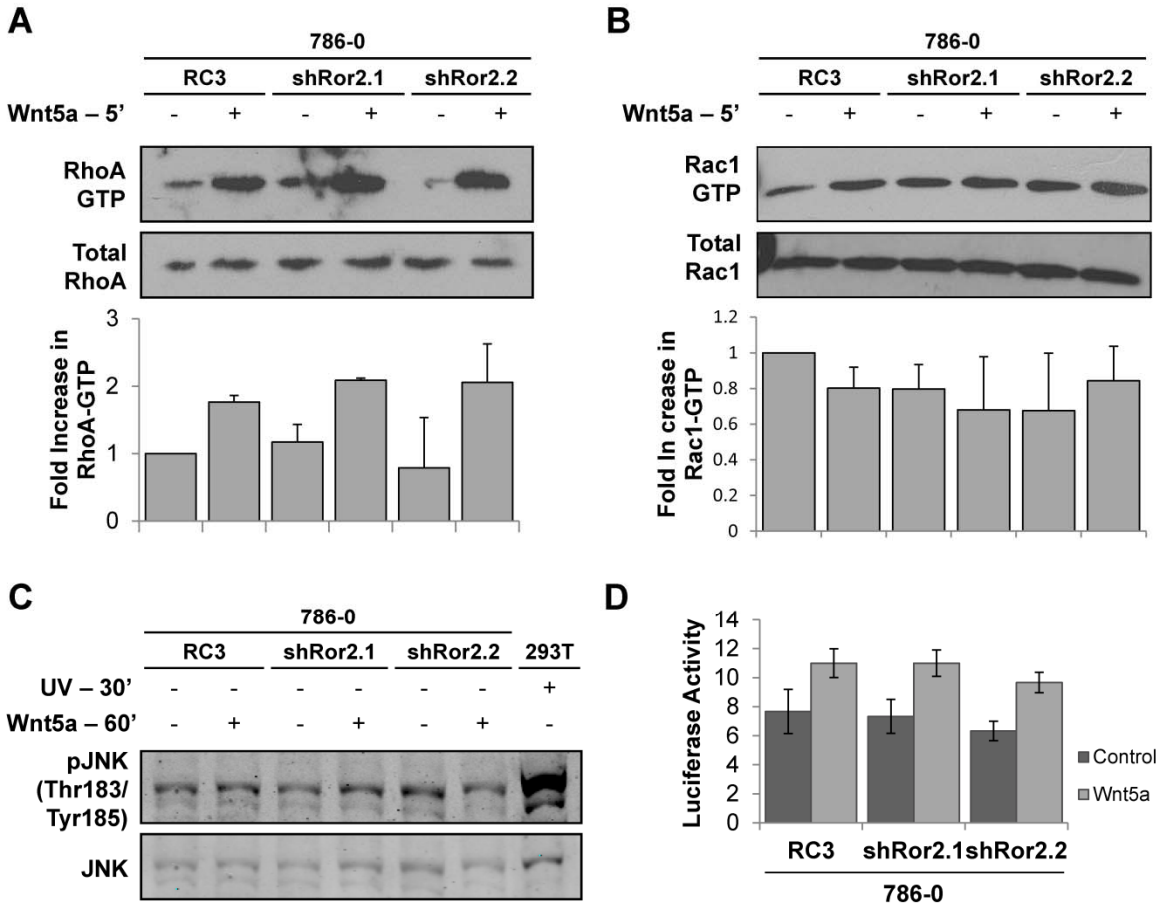

Figure 2.8: Wnt/PCP and Wnt/Ca^{2+} Signaling is independent of Ror2 in RCC cells
786-0 RC3 and independent shRor2 knockdown cell lines were incubated with Wnt5a (100ng/ml) for 5 minutes and immunobloted for **A)** active GTP bound RhoA and total RhoA **B)** active GTP bound Rac1 and total Rac1 with no significant differences observed. Densitometry for duplicate experiments with error bars representing SD is seen below each corresponding immunoblot.
C) Immunoblot for active and total levels of JNK following 60 minutes of Wnt5a (100ng/ml) stimulation show no differential activation in 786-0 RC3, shRor2.1, and shRor2.2. Although a strong induction is seen in the positive control HEK293T cells in response to UV irradiation.
D) Luciferase activity for pGreenFire NFAT-responsive luciferase reporter shows no significant difference in the luciferase activity between the control 786-0 RC3 and shRNA knockdowns shRor2.1, and shRor2.2 following treatment with Wnt5a (100ng/ml) with error bars representing SEM.

Discussion

Renal cell carcinoma has historically been a cancer that challenges conventional paradigms. Canonical Wnt signaling disregulation is associated with tumorigenesis and progression of RCC, transduced by a previously unknown set of mediating factors (22-24,36). Furthermore, increased expression of various Wnt ligands including Wnt3a have been observed in RCC tumors (36). We sought to identify what role Ror2 played as a Wnt

signaling mediator in RCC, as it is capable of engaging a variety of canonical and non-canonical Wnt ligands (17). In kidney tumors, we show that canonical β-catenin target genes, including Axin2, positively correlate with Ror2 expression. A translational validation of these results is seen in the multitude of target and canonical component genes showing correlated expression with Ror2 in RCC cells and primary human tumors, including pygopus 1 (PYGO1), secreted Frizzled-related protein 1 (SFRP1), and secreted Frizzled-related protein 4 (SFRP4).

Our data with both kidney-derived cells and kidney cancer cell lines show that Ror2 expression results in a state of basally enhanced canonical transcriptional signaling due to DVL activation and an increased pool of stabilized soluble β-catenin. Stimulation of Ror2-expressing cells with Wnt3a further enhances the β-catenin pool and transcriptional response (Figure 2.9).

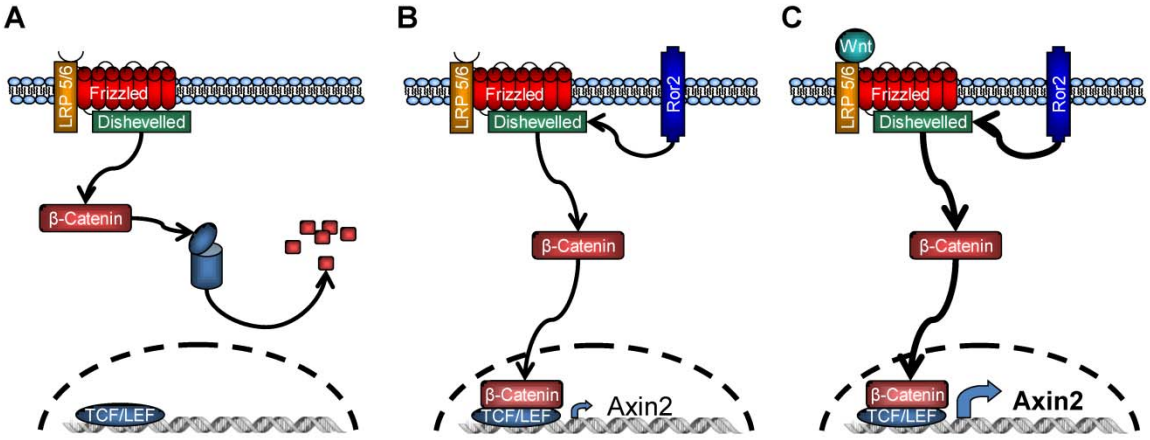

Figure 2.9: Ror2/Wnt3a signaling in RCC cells.
Schematic overview of Ror2/Wnt3a signaling in RCC cells. **A)** In canonical Wnt signaling in the absence of either Wnt ligand or Ror2 expression, β-catenin is degraded by the proteasome. **B)** However, with the expression of Ror2, DVL becomes phosphorylated, leading to stabilization of β-catenin and its translocation into the nucleus driving transcription of target genes (i.e. Axin2). **C)** In cells expressing Ror2 and stimulated with Wnt3a, a further enhancement of β-catenin stabilization and amplified transcription of target genes is observed.

The canonical signal cascade requires the canonical coreceptor LRP6 for pathway activation in response to Wnt3a, as we observed consistent inhibition with siLRP6 or DKK1 of β-catenin stabilization as well as Axin2 transcription with the concurrent addition of

Wnt3a. Inhibition or knockdown of LRP6, however, showed little effect on the increased pool of stabilized β-catenin associated with Ror2 expression in the absence of Wnt3a. This result would suggest that the Ror2-dependent tonic signal to stabilize β-catenin and enhance transcriptional signals is independent of LRP6. However, siRNA targeting of LRP6, but not inhibition with DKK1, inhibited the enhanced basal levels of Axin2 expression seen with Ror2, allowing the possibility that Ror2 also involves LRP6 in maintaining this tonic state of canonical Wnt signaling. Recent work has demonstrated that β-catenin remains bound in the destruction complex and that Wnt stimulation inhibits β-catenin degradation, leading to saturation of the complex. As a result, β-catenin accumulates in the cytosolic pool, and then is transported into the nucleus and binds to various target genes (2). These data suggest that Ror2 can play a role in regulating this available and active pool of β-catenin. The increase in measurable soluble β-catenin appears to occur independently of LRP6 co-factor recruitment; however, LRP6 may contribute to the transcriptional activation of target genes, perhaps via a nuclear influx of β-catenin that is not reflected in the soluble pool in the absence of LRP6 or an alternate mechanism.

Ror2 has been shown to mediate activation of various homologs of DVL, key signaling members in multiple Wnt pathways (21,29,30). Our data shows that Ror2 expression results in an increase in activated (phosphorylated) DVL2/3 in both 786-0 and HEK293T cells. These results, combined with the previous findings in the field, suggest DVL molecules as possible substrates for the kinase activity of Ror2. However, the potential for intermediate substrates or activators of DVL family members cannot be excluded based on these findings.

These findings highlight several relevant points to Wnt signaling and tumor biology. First, this model demonstrates that Ror2 activity may be multidimensional. We observed substantial changes in canonical signaling with Ror2 expression in RCC and renal-derived cell lines in contrast to previous studies reporting Ror2 expression having no effect on β-

catenin accumulation in response to Wnt3a (30). In addition, we did not observe any changes in either the Wnt/PCP or Wnt/Ca2+ signaling pathways dependent on Ror2 expression with addition of the non-canonical ligand Wnt5a. These expanding cell line specific roles of Ror2 can likely be attributed, at least in part, to available coreceptors as well as access to Wnt ligands. LRP5/6 is well established as part of the heterodimer responding to Wnt ligand binding. Although Ror2 expression results in Wnt3a-independent β-catenin-dependent stabilization and signaling, the dependence on the expression of LRP6 for Wnt induction exhibits the necessity of additional co-receptors for amplifying this signaling cascade from the poised state. Fzd2, which was previously reported to cooperate with Ror2 enhancement of canonical Wnt signaling (16), correlated with Ror2 expression in our set of human RCC tumors. Fzd2 represents a candidate cofactor in this regard, highlighting the future need for a comprehensive understanding of Ror2 interactions with possible coreceptors within the greater cellular context of cancer signaling.

Based on these observations, it is essential to examine cell signaling effects with an emphasis towards differential states of activation. Signals emanating from Wnt receptors such as Ror2 may produce partial or incomplete activation of the β-catenin transcriptional response repertoire, a feature we have labeled as a poised state of signaling. Engagement of the canonical Wnt3a ligand, expressed in RCC tumors, produces a robust enhancement that is dependent on the availability of the coreceptor LRP6, and exceeds the Wnt3a induced signal in the absence of Ror2. Together, this model system sheds light on the specific activity of Ror2 signaling in renal cancer and on the complex nature of this signaling pathway which may be critical for dissecting these events in understanding cancer progression. Furthermore, this model highlights the potential for a tumor modulator to effect moderate pathway activation in a way that primes the pathway for a more intense response when a ligand or other source of stimulation becomes available.

Ultimately, it is increasingly apparent that Ror2 expression in cancers advances signals that provide a variety of advantages for tumor progression. Ror2 serving as a mediator of β-catenin dependent signaling in RCC cells and tumors provides a highly promising therapeutic target for RCC.

Materials and Methods

Cell Culture

786-0 and derivative cell lines, 786-0 RC3 (kindly provided by Dr. Kaelin) (37), 786-0 pRS, stable monoclonal knockdown 786-0 shRor2.1 and shRor2.2 (4) cells , 786-0 Tap-hRor2 cells and HEK293T cells were grown in DMEM with 10% FBS, nonessential amino acids, L-glutamine, and penicillin/streptomycin. L cells and Wnt3a producing L cells were grown in DMEM with 10% FBS. Control and Wnt3a-conditioned media (CM) was collected at 24 and 48 hours after cells reached confluency. 786-0 cells were transfected with TAP-hRor2 and selected with 1μg/mL puromycin to generate polyclonal stable lines. HEK293T and 786-0 were transduced with lentiviruses carrying the eGFP control, TRE(tight)-eGFP-pEF1a-rtTA-IRES-Puro or hRor2-eGFP, TRE(tight)-hRor2eGFP-pEF1a-rtTA-IRES-Puro, with stable lines generated following selection with 1μg/mL puromycin.

Plasmids

The TAP-hRor2 was generated with hRor2 cloned into the pIRESpuro-GLUE backbone (38). The control TRE(tight)-eGFP-pEF1a-rtTA-IRES-Puro and TRE-tight-hRor2-eGFP-eF1a-rtTA-IRES-Puro vectors were generated with eGFP alone or the fusion hRor2-eGFP, being cloned into a PHAGE6 lentiviral backbone (39). Expression of eGFP or hRor2-eGFP was visually confirmed 48hr after induction with doxycycline (500ng/mL). Site directed mutagenesis using QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies,

LaJolla CA) was used to introduce the amino acid substitutions K507M and D633A for hRor2-eGFP-DM.

siRNA

Stealth siRNA for LRP6 (5 -ACGCAGCAUUGAGCGUGCCAACAAA - 3 and 5 - GAUCCCAUGGUUGGGUACAUGU AUU - 3) were pooled and Stealth negative control siRNA (Stealth RNAi Negative Control Low GC Duplex) were introduced into cells using Lipofectamine RNAiMAX (Invitrogen, Carlsbad CA) according to the manfacturer's instructions and allowed to incubate for 48 hours.

Quantitative RT-PCR and Human Wnt Signaling RT2 Profiler PCR Array

Total RNA was extracted from cells using Qiagen RNeasy Mini Kit (Valencia, CA, USA). cDNA was made from 500ng of total RNA using Random Primers (Invitrogen, Carlsbad CA) and Superscript II RT-PCR reagents (Invitrogen, Carlsbad CA) and analyzed using the ABI 7900HT Fast Real-Time PCR System with the following proprietary FAM labeled primers: Ror2, Axin2, Fzd1, Jun, 18S, and β-actin (Applied Biosystems, Foster City CA). Wnt-related gene expression was examined with the Human Wnt Signaling RT^2 Profiler PCR Array (SABiosciences, Frederick, MD, USA) using cDNA from 786-0 +TAP-hRor2, 786-0, and 786-0 shRor2.2 cells with the ABI 7500 Real-Time PCR System. Changes in gene expression were calculated following normalization to an internal pool of 5 housekeeping genes. Note Axin2 was not available in the RT^2 Profiler PCR Array.

Immunoblotting

Cells were lysed in 10mM Tris, pH 7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM Na4P2O7, 2mM Na3VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS , 0.5% deoxycholate, 1mM PMSF and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis IN) and quantitated using Bradford reagent to measure absorbance at 595nm. Lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes (GE,

Pittsburg, PA) and evaluated using the following antibodies: Ror2 (AF2064, R&D Systems, Minneapolis, MN), DVL2 (3216), DVL3 (3218), JNK (9258), pJNK (9255), LRP6 (3395) pLRP6 (2568, Cell Signaling Technology, Danvers, MA), Ku80 used as loading control (ab1273, AbCam, Cambridge, MA), β-catenin (C2206, Sigma-Aldrich, St. Louis, MO). For all immunoblots, following incubation with corresponding secondary antibodies conjugated to IR dye 680 or 800, membranes were scanned using the Odyssey IR imager (LI-COR Biosciences, Lincoln, NE) with densitometric analysis performed using Odyssey v3.0.21.

Concanavalin-A Separation of Cytosolic β-catenin

Cells were incubated for 1 hour with control conditioned media, Wnt3a conditioned media (1:2 dil), or DKK1 (400ng/ml, R&D Systems). 50μg of protein lysates from each sample as determined by Bradford assay were rotated with 10μl of prepared concanavalin-A Sepharose 4b beads (GE, Pittsburg, PA) overnight at 4˚C. Supernatants were removed following a brief centrifugation and analyzed by SDS-PAGE.

Affinity-Precipitation of cellular GTP-RhoA/Rac1

Active RhoA pulldown experiments were done as described previously (40). Cells were lysed in 300μl 50mM Tris, pH 7.4, 10mM MgCl2, 500mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deozycholate, 1mM PMSF, and 10μg/ml each of aprotinin and leupeptin. Lysates were cleared by centrifugation and supernatants were rotated with 20-30μg GST-RBD conjugated to glutathione–Sepharose beads (GE, Pittsburg, PA). Beads were washed with 50mM Tris, pH 7.4, 10mM MgCl2, 150mM NaCl, 1% Triton X-100, 1mM PMSF, and 10μg/ml each of aprotinin and leupeptin. For active Rac1 pulldown assays, cells were lysed in 300μl 50mM Tris, pH 7.4, 10mM MgCl2, 150mM NaCl, 1% Triton X-100, 1mM PMSF, and 10μg/ml each of aprotinin and leupeptin and rotated with 30-50μg GST-PBD conjugated to glutathione–Sepharose beads. Active and total levels of RhoA and Rac1 were analyzed by

SDS-PAGE. Densitometry for each scanned immunoblot was performed using ImageJ and averaged across duplicate experiments.

Luciferase Assays

To determine the activity of the canonical Wnt signaling in the RCC cells, 786-0 and HEK293T cells were transfected with TOPFlash and constitutive pHRG-renilla luciferase reporters. 24 hours post transfection, cells were seeded in triplicate with media being replaced after 8 hours with serum-free media and incubated for 20hr. Post serum starvation, cells were treated with control PBS or recombinant human Wnt3a (100ng/ml, R&D systems) and incubated for 24hr. Luciferase activity was measured from lysed samples using a luminometer and the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Relative luciferase units were calculated by the ratio of TOPFlash luciferase activity to internal renilla luciferase activity for each sample.

NFAT transcriptional induced activity was determined following transfection with the pGreenfire-NFAT GFP-Luciferase Reporter (System Biosciences, Mountain View, CA). Cells were treated with PBS or recombinant human Wnt5a (100ng/ml) and incubated for 24hr. Luciferase activity was measured from lysed samples using a luminometer and the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Microarray Analysis

Gene expression for 95 human RCC tumors was downloaded and prepared as described previously (41). Collection and analysis of these tumors was approved by the institutional biomedical ethics review committee. Significance of Analysis of Microarrays (SAM) (http://www-stat.stanford.edu/~tibs/SAM/) was used to determine genes that significantly correlated with Ror2 expression among the 95 human RCC tumors with a False Discovery Rate (FDR) <0.043% and analyzed using DAVID (http://david.abcc.ncifcrf.gov/) to identify significantly enriched gene ontologies.

Statistical Analysis

One-way ANOVA analysis was used to generate *p*-values in the comparison of each experimental condition with the control. A *p*-value of <0.05 was considered significant and <0.001 being highly significant. All error bars shown are the calculated standard deviation (SD) or standard error of the mean (SEM) across duplicate or triplicate experiments.

Supplementary material for this article can be found at the JBC Web site, http://www.jbc.org

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Chapter Three

ROR2 IS A NOVEL PROGNOSTIC BIOMARKER FOR RENAL CELL CARCINOMA

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer in adults, accounting for ~3% of all malignancies. Its incidence and mortality rate continue to climb steadily ~2–3% per decade (1). RCC is notoriously difficult to treat as it is radioinsensitive and highly unresponsive to traditional chemotherapeutic approaches. Approximately one-third of new patients present with metastatic RCC, for which the 5-year survival rate remains only 5-10% (1,2). As such there is an urgent necessity to identify novel therapeutic targets that contribute to RCC tumor progression and can also serve as prognostic biomarkers in RCC.

Receptor tyrosine kinase-like orphan receptor 2 (Ror2) is a recently identified tumor promoter. Ror2 was initially found to be expressed in RCC and later was found to be overexpressed in a variety of cancers including osteosarcoma, metastatic melanoma, gastric cancer, prostate cancer, gastrointestinal stromal tumor (GIST), leiomyosarcoma, and squamous cell carcinoma of the head and neck (3-10). The aberrant expression of Ror2 in these cancers has been shown to promote cell proliferation, migration, invasion, and metastasis, mirroring some of its roles in early development (3,4,6,8,11-13). Prior studies have effectively presented Ror2's potential as a prognostic biomarker, with high Ror2 expression correlating with Enneking surgical stage and tumor metastasis in osteosarcoma

(14), metastatic melanoma (12,15), and poorer clinical outcome in GIST and leiomyosarcoma (6). Because earlier work has shown that Ror2 expression is associated with cellular migration, invasion, and *in*-vivo tumor growth in RCC, we sought to expand our understanding of Ror2's tumor promoting role and its potential as a prognostic biomarker in RCC (16). As Ror2 signaling has been proven to be highly dependent on cellular context, we utilized both RCC cell lines and primary human RCC tumors to uncover additional genes correlating with Ror2 expression.

In accordance with previous research, expression of matrix metalloprotease 2 (MMP2) is associated with Ror2 expression in RCC cells (13). Ror2 regulation of MMP expression has been noted, affecting multiple members (MMP1, MMP2, and MMP13) across several cell types, with correlating changes in migration and invasion (3,8,11,13,17). The MMP family consists of zinc-dependent enzymes capable of degrading the extracellular matrix (ECM), including basement membrane components, resulting in increased cell motility. Tumor cells have exploited these features of MMPs to promote migration, invasion and metastasis. We examined MMP2 expression in renal cells, which increases with the addition of exogenous Wnt3a. This response is attenuated or enhanced with the suppression or overexpression of Ror2, respectively. Taking note of MMP2's role in mediating migration as extracellular matrix remodeling protease, we further explored the effects of Ror2 expression in migration utilizing a Boyden chamber assay. We observed that the introduction of mutations in key areas for activity within the kinase domain of Ror2 abrogates the increase in MMP2 transcription and cell migration observed with expression of wild-type Ror2.

In addition to the correlation of MMP2 with Ror2 expression in RCC cells and primary tumors we identified secreted Frizzled related protein 2 (SFRP2), a known regulator of Wnt signaling. We observed that Ror2 suppression and overexpression in RCC cells resulted in a correlating reduction or increase of protein levels of SFRP2. Prior work has determined

that expression of SFRP2 in RCC cells promotes cell proliferation, *in-vivo* tumor growth, and reduces UV-induced apoptosis (18). Although the role of SFRP2 in Wnt signaling is well established, more recent studies have unexpectedly shown SFRP2 to be a potent angiogenic factor which is expressed in RCC endothelial cells (19). Upon examination of the effects of Ror2 and SFRP2 expression *in*-vivo we observed an increase in both tumor growth and vascularity.

Like many cancers, RCC consists of an extremely heterogeneous group of tumors represented by the wide variability in metastasis, recurrence and survival in individual patients. For this reason, multiple prognostic nomograms have been developed using clinical and pathological factors to assist in stratifying patients into different categories of risk of recurrence or death. Prognostic nomograms currently in use for RCC fall into one of three categories: preoperative, post-operative and metastatic; a clear majority have been developed for use in the post-operative sphere (20). The preoperative nomogram put forth by Raj et al for non-metastatic RCC patients is used to predict likelihood of recurrence after resection using the variables of tumor size, evidence of lymphadenopathy and imaging to determine necrosis (21,22). Along with histology the American Joint Committee on Cancer's Tumor Node Metastasis (TNM) algorithm forms the core of post-operative prognostic nomograms with varying extensions to include necrosis in the Mayo Clinic's Stage, Size, Grade, and Necrosis (SSIGN) algorithm and performance status in UCLA's Integrated Scoring System (UISS) (20,23,24). The Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic nomogram from Motzer et al. incorporates a number of additional features for use in metastatic RCC: blood measurements of hemoglobin, serum calcium, and lactate dehydrogenase, as well as clinical evaluation of performance status and nephrectomy status allowing for stratification into low, intermediate, and high risk groups (25). These nomograms provide a framework for clinicians to offer assessments of recurrence and survival for patients.

Since the advent of microarrays, considerable work has been done using gene expression analysis to better understand the underlying heterogeneity of RCC and identify new biomarkers for use in current nomograms or as independent predictors of survival. A recent study using a set of 120 probes was able distinguish two distinct subtypes, ccA and ccB, across multiple gene expression datasets of primary ccRCC tumors, with substantially divergent gene expression patterns and survival curves (26). ccA is characterized by genes associated with classical ccRCC such as hypoxia, angiogenesis, fatty acid metabolism, and organic acid metabolism, whereas ccB tumors expression patterns detail a more aggressive panel of genes regulating cell adhesion, extracellular matrix remodeling, cell cycle progression, and epithelial-mesenchymal transition (EMT) (26). The more aggressive nature of ccB tumors is also reflected in a significantly shorter overall and disease-specific survival time in comparison to ccA tumors (26).

As we saw that Ror2 expression correlated with ccB tumors, we examined the potential of Ror2 as an independent prognostic biomarker and found that high Ror2 expression results in a significant increase in tumor growth and a significant decrease in overall survival in RCC. Together, these findings show that Ror2 is a novel prognostic biomarker and potential therapeutic target in RCC.

Results

Determination of Ror2 correlated genes in RCC cells and tumors

Prior research identified matrix metalloprotease 2 (MMP2) expression to be Ror2 dependent in RCC and renal tubular epithelial cells following injury (13,27). We sought to build on these results in finding additional genes whose expression in RCC correlated with Ror2. We utilized Agilent gene expression microarrays to assess mRNA transcripts from 786-0 cells infected with an shRNA scramble retrovirus serving as a control and two

independent shRor2 knockdown cell lines (786-0 shRor2) in duplicate. To identify the genes most significantly correlated with Ror2 (FDR <0.05), significance of microarrays (SAM) (28) was utilized to examine gene expression patterns following normalization to an internal human reference standard, with relative fold change being calculated in reference to control 786-0 scramble cells which retain endogenous expression of Ror2. The resulting genes were clustered in an unsupervised hierarchal fashion and visualized in a heat map. In addition to MMP2, over 1100 probes showed a significant reduction in expression correlating with suppression of Ror2.

An intriguing find among these genes was the Wnt signaling regulator, secreted frizzled-related protein 2 (SFRP2) which exhibited a strong correlation with Ror2 expression across independent probes (Figure 3.1A). SFRP2 is a member of a family of secreted factors capable of binding Wnt ligands, that serve primarily as antagonists of Wnt signaling; however, SFRP2 expression in RCC cells has been shown to lead to increased stabilized β-catenin and downstream transcription through a yet undetermined mechanism. SFRP2 expression in RCC has also been shown to promote cell proliferation, tumor growth, and prevent UV-mediated apoptosis. As these results mirror our previous results, with Ror2 expression resulting in increased β-catenin signaling independent of Wnt, we used quantitative RT-PCR to test SFRP2 expression in 786-0 cells to ensure that these results were not artifactual (29). SFRP2 showed a significant reduction along with MMP2 in both shRor2 knockdown cell lines in comparison to control 786-0 RC3 cells (Figure 3.1B).

Having determined that SFRP2 mRNA expression correlated with Ror2 expression in RCC cells, we sought to evaluate this relationship in primary tumors. Since SFRP2 has been proposed to be either a tumor suppressor or promoter in varying cancers we needed to determine its expression in RCC tumors. Also, because SFRP2 is a secreted factor its interplay with tumor microenvironment is unaccounted for *in-vitro*, necessitating we examine Ror2 and SFRP2 correlation in primary RCC tumors, more fully taking into account *in-vivo*

signaling conditions. Utilizing a panel of 95 primary human RCC tumors, relative gene expression was determined using Agilent gene expression microarrays. A heat map of the unsupervised clustering of the averaged expression across probes for Ror2, SFRP2, and MMP2 exhibited two discrete clusters. Both SFRP2 and MMP2 clustered quite distinctly with Ror2 expression, demonstrating that their correlating expression is not an artifact of our *in-vitro* system (Figure 3.1C).

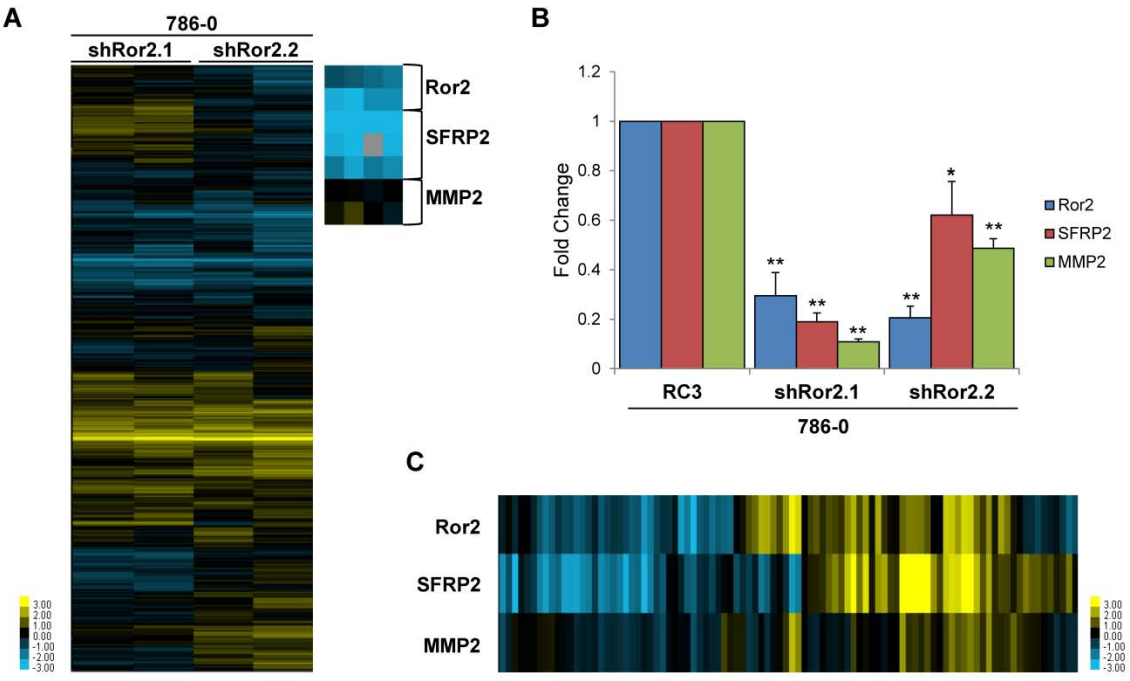

Figure 3.1: Determination of Ror2 correlated genes in RCC cells and tumors
A) Heatmap of the most significant Ror2 associated expressed probes (false discovery rate (FDR) <0.05) in two independent RCC cell lines suppressing Ror2 (786-0 shRor2) relative to 786-0 scramble cell line as determined by SAM. Magnified heatmaps showing a positive correlation of expression of both SFRP2 and MMP2 with Ror2.
B) Quantitative RT-PCR shows a significant reduction in both MMP2, and SFRP2 in both shRor2 knockdown cells relative to control 786-0 RC3 cells. For all quantitative RT-PCR assays, transcript values were normalized to B-Actin RNA internal standard. Error bars represent SEM across triplicates in triplicate experiments. *P*-values calculated using an Anova one-way analysis (*<.05, **<.001).
C) Heatmap of the averaged expression for Ror2, SFRP2, and MMP2 in 95 primary human RCC tumors.

Ror2 dependent MMP2 expression is enhanced by Wnt3a stimulation

Although previous work has shown MMP2 expression to be Ror2 dependent in RCC cells, the effects of Wnt stimulation on this expression have not yet been established (13).

Ror2 expression in RCC cells results in a potentiation of β-catenin-dependent signaling; accordingly, we utilized 786-0 cells which exhibit endogenous Ror2 expression to assess the role of Wnt3a activation of MMP2 in RCC cells (29). Quantitative RT-PCR for MMP2 showed a significant response to Wnt3a stimulation in control 786-0 RC3 cells, this response being attenuated in both shRor2 knockdowns (Figure 3.2A). Paralleling previous findings with Axin2, overexpression of Ror2 in HEK293T cells resulted in an increase in basal levels of MMP2 that were further enhanced with the addition of Wnt3a (29) (Figure 3.2B).

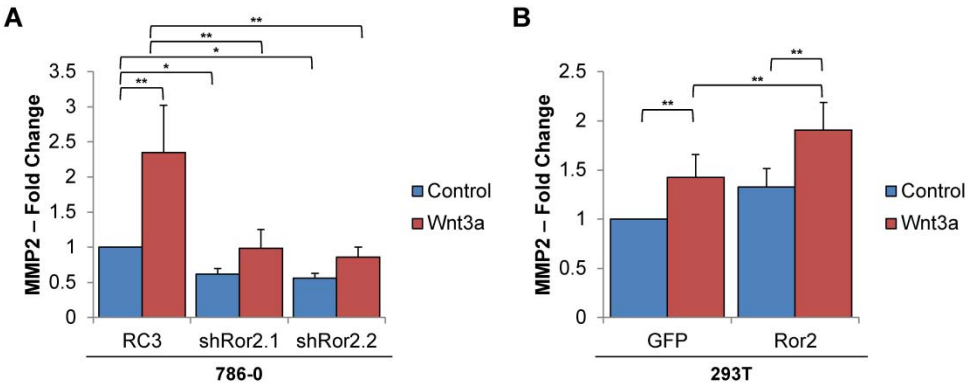

Figure 3.2: Ror2 dependent MMP2 expression is enhanced by Wnt3a stimulation
Quantitative RT-PCR in 786-0 cells for **A)** MMP2 shows a significant increase in control RC3 cells with Wnt3a (100 ng/ml) stimulation, while levels are significantly reduced in both shRNA Ror2 knockdown cell lines and exhibited an attenuated response to Wnt3a. **B)** Likewise, a concordant significant increase in the basal expression of MMP2 is seen with overexpression of Ror2 in 293T cells that is further heightened upon the addition of Wnt3a (100 ng/ml) relative to the control cells. For all quantitative RT-PCR assays, transcript values were normalized to B-Actin RNA internal standard. Error bars represent SEM across triplicates in duplicate experiments. *P*-values calculated using an Anova one-way analysis (*<.05, **<.001).

Migration in RCC cells is enhanced by Ror2 expression

Expression of Ror2 has been shown to mediate expression of several MMPs and cell migratory/invasive phenotypes in a multitude of cancers including RCC (3,8,11,13,17). To further clarify the contribution of Ror2 to cell migration in RCC we utilized a Boyden chamber assay, allowing us to observe single cell motility as cells migrated across the membrane in response to extracellular signaling cues. Both 786-0 shRor2 knockdown cell lines exhibited

reduced rates of cell migration in comparison to 786-0 RC3 cells, in agreement with previous research using a scratch wound healing assay (13) (Figure 3.3A). We also examined the effects of overexpression of Ror2 in HEK293T cells, where we observed a mirrored increase in migration in Ror2 expressing cells relative to GFP control (Figure 3.3B).

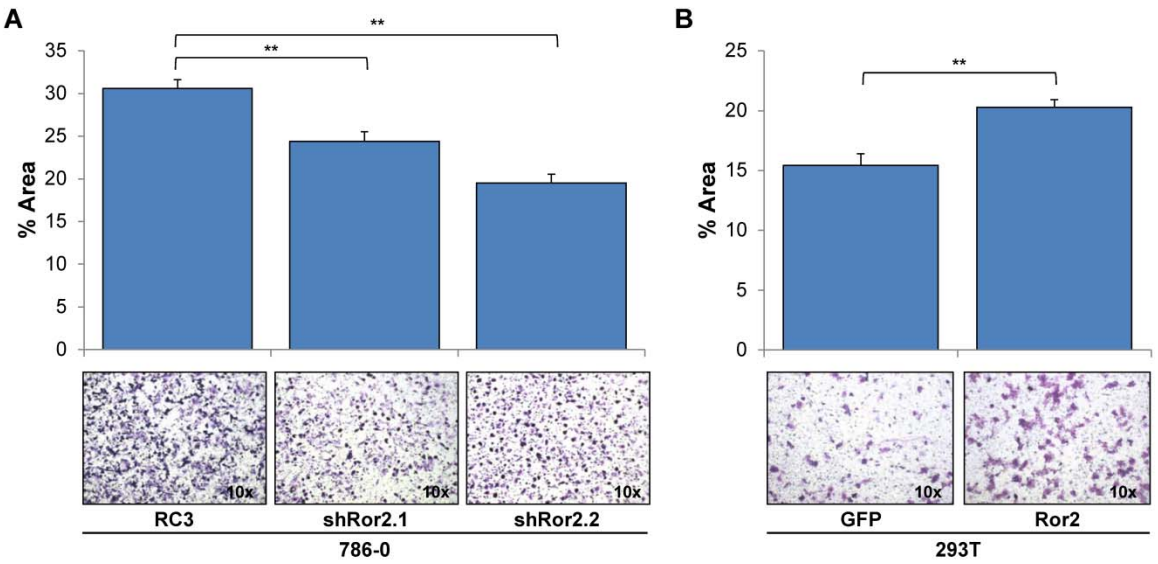

Figure 3.3: Migration in RCC cells is enhanced by Ror2 expression
Analysis of migration potential using Boyden chamber assays in **A)** 786-0 cells show a significant reduction in migration in both independent shRNA Ror2 knockdowns in comparison with control 786-0 RC3 cells, with representative images shown below. **B)** Concordantly, HEK293T cells exhibited a significant increase in migration in Ror2 expressing cells relative to GFP control under normal culture conditions, with representative images shown below. The percentage area covered was quantitated from 7 random fields using ImageJ, with representative images shown. *P*-values were calculated using an Anova one-way analysis with error bars representing SEM shown (**<.001).

Intact Ror2 kinase domain is required for MMP2 expression and migration in RCC cells

To further determine if Ror2's contribution to the migratory phenotype in RCC cells was dependent upon signaling capacity or solely upon expression, we used 786-0 cells which overexpressed either wild-type Ror2 or mutant Ror2-DM generated using site-directed mutagenesis. The Ror2-DM kinase domain was mutated in two key regions known to be critical for RTK signaling: the ATP binding pocket and the DFG (found as DLG in Ror2) loop heretofore described (29). We have previously shown that these mutations in Ror2 result in

loss of the enhancement of β-catenin stabilization and induction of downstream Axin2 transcription in RCC cells (29). Quantitative RT-PCR for Ror2 shows that Ror2 and Ror-DM were expressed at comparable levels in 786-0 cells, but expression of Ror2-DM failed to increase basal expression of MMP2 (Figure 3.4A). We examined the migratory potential of these same cells in a Boyden chamber assay under normal culture conditions and found that 786-0 cells expressing Ror2-DM exhibited a corresponding loss in migration compared with 786-0 Ror2 cells (Figure 3.4B).

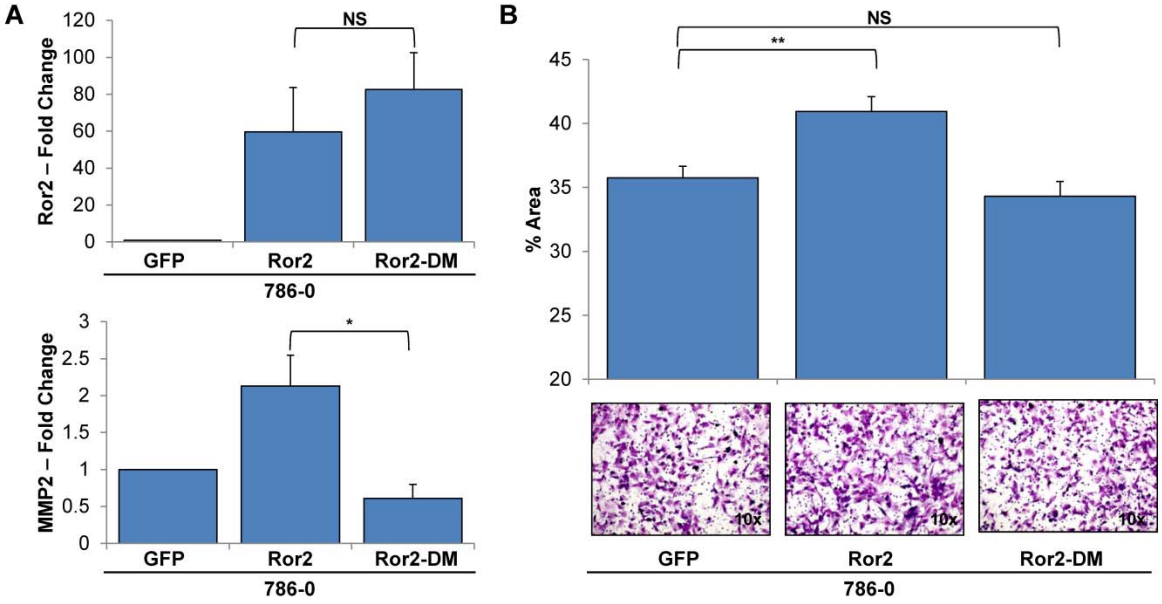

Figure 3.4: Intact Ror2 kinase domain is required for MMP2 expression and migration in RCC cells
A) Quantitative RT-PCR in 786-0 cells shows equivalent levels of Ror2 expression in Ror2 and Ror2-DM cells 24 hours post induction, with a significant decrease of MMP2 expression in Ror2-DM expressing cells. For all quantitative RT-PCR assays, transcript values were normalized to B-Actin RNA internal standard. Error bars represent SEM across triplicates in duplicate experiments.
B) Boyden chamber assays using 786-0 cells under normal culture conditions, show a significant reduction in migration with 786-0 Ror2-DM cells compared to Ror2 expressing cells, with representative images shown below. The percentage area covered was quantitated from 7 random fields using ImageJ. *P*-values were calculated using an Anova one-way analysis with error bars representing SEM shown (*<.05, **<.001).

SFRP2 expression is Ror2 dependent in RCC cells

In addition to MMP2 we have here shown the novel correlation between the Wnt antagonist SFRP2 and Ror2 expression in both RCC cells and primary tumors. To further validate this connection between SFRP2 and Ror2 we gauged expression of protein levels

by immunoblot in RCC- and renal-derived cells. Suppression of Ror2 in 786-0 cells showed a corresponding decrease in SFRP2 in both shRor2 knockdowns targeting separate domains of Ror2 (Figure 3.5A). Likewise, overexpression of Ror2 following induction with doxycycline in both 786-0 and HEK293T cells resulted in an increase in SFRP2 expression (Figure 3.5B&C).

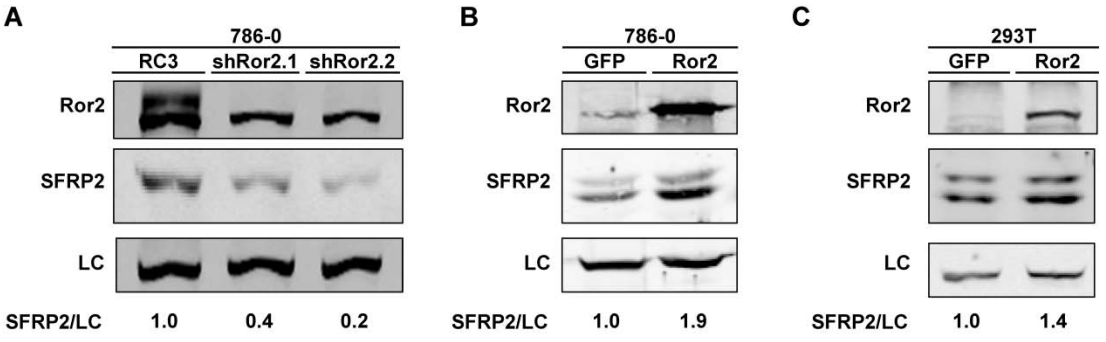

Figure 3.5: SFRP2 expression is Ror2 dependent in RCC cells
Immunoblot for the indicated antibodies of whole cell lysates in **A)** 7860-RC3 control cells show higher expression of SFRP2 compared to both stable shRor2 cells. **B)** 7860 cells or **C)** HEK293T exhibit increased levels of SFRP2 expression upon induction of Ror2 (48 hours) with Doxycycline (500ng/ml). Changes in SFRP2 relative to loading control (LC) are shown with densitometric quantification below the corresponding bands.

Expression of Ror2 promotes in vivo tumor growth & vascularity

Based on the expression findings of these microarrays we sought to further investigate the relationship of SFRP2 and Ror2 *in-vivo*, as expression of both have shown to be tumor-promoting in RCC cells. Suppression of Ror2 in RCC cells orthotropically injected into the kidney resulted in a dramatic reduction of tumor growth *in-vivo (13)*. Moreover, overexpression of SFRP2 in RCC cells has been shown to increase cell proliferation *in-vitro* and tumor growth *in-vivo* (18). To elucidate the effects *in-vivo* of overexpression of Ror2 in RCC cells, we utilized 786-0 cells.Ror2 overexpression in 786-0 cells had no effect on the rate of tumor occurrences, however a trend toward increased tumor growth was seen in 786-0 cells (Figure 3.6A&B).

To further characterize the effects of Ror2 and SFRP2 expression *in-vivo* we turned to another aspect crucial to increased tumor growth: the supporting vasculature for the

tumor. RCC tumors are known to be highly vascularized due to the shift in the VHL/HIF axis resulting in increased expression of VEGF. As SFRP2 has also been demonstrated to be an important angiogenic factor we stained for the endothelial cell marker CD31 to measure the microvessel density in paired tumors from 786-0 xenografts. Overexpression of Ror2 both in 786-0 cells resulted in a significant rise in microvessel density (Figure 3.6C), suggesting that Ror2 and resulting SFRP2 expression contributions to tumor promotion may be partially mediated through increases in the supporting vasculature for RCC tumors.

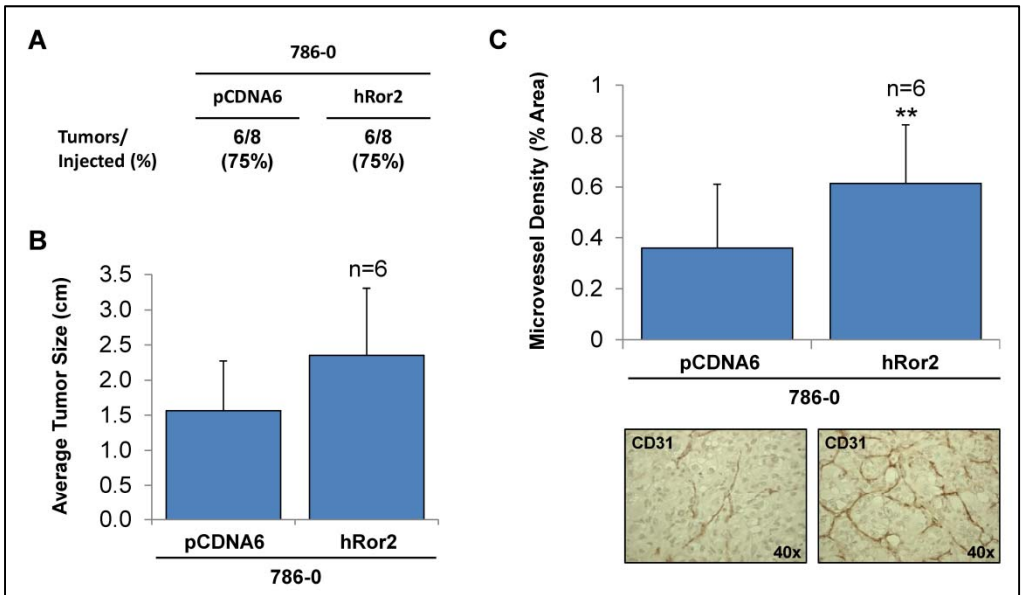

Figure 3.6: Expression of Ror2 promotes *in vivo* tumor growth & vascularity
A) Tabulated rates of successful injection and growth of all 786-0 xenograft tumors cells are shown.
B) Average tumor size (cm) with error bars showing stdev for resulting xenograft tumors following orthotropic injection of 786-0 cells containing either the control empty vector, pcDNA6 or hRor2 exhibit a trend to increased *in-vivo* tumor growth with Ror2 overexpression.
C) IHC staining for CD31 in the resulting tumors from injection of 786-0 cells show a significant increase in microvessel density with Ror2 overexpression in comparison to control cells with representative CD31 staining shown below the corresponding cell line. Positive CD31 staining was calculated across 4 random fields per each of the tumor pairs with error bars showing stdev. P-values were calculated using Anova one-way analysis (**<0.01)

Ror2 expression is higher in ccB tumors

Prior work by Brannon et al in further elucidating the underlying molecular biology of ccRCC led to the uncovering of two distinct molecular subtypes, ccA and ccB (26). A SAM

analysis of the gene expression of these two subtypes across 117 tumors showed distinct gene expression patterns (Figure 3.7A). As the two subtypes displayed quite divergent expression patterns Brannon et al further investigated whether these subtypes correlated with changes in survival outcome. Kaplan-Meier plots for cancer-specific survival displayed a highly significant survival difference between ccA and ccB with a median survival of 8.6 versus 2 years (*P*= 0.0002) and similar differences in overall survival (Figure 3.7B) (26). Because Ror2 had prior association with cell migration, tumor growth, and aggressive disease paralleling our findings here in RCC (3,8,12,13,15,17), we tested the differential expression levels of Ror2 in ccA and ccB tumors within our core dataset of 417 ccRCC tumors from The Cancer Genome Atlas (TCGA) (30). Ror2 expression was significantly higher in ccB tumors, suggestive of Ror2 playing a tumor-promoting role in RCC (Figure 3.7C).

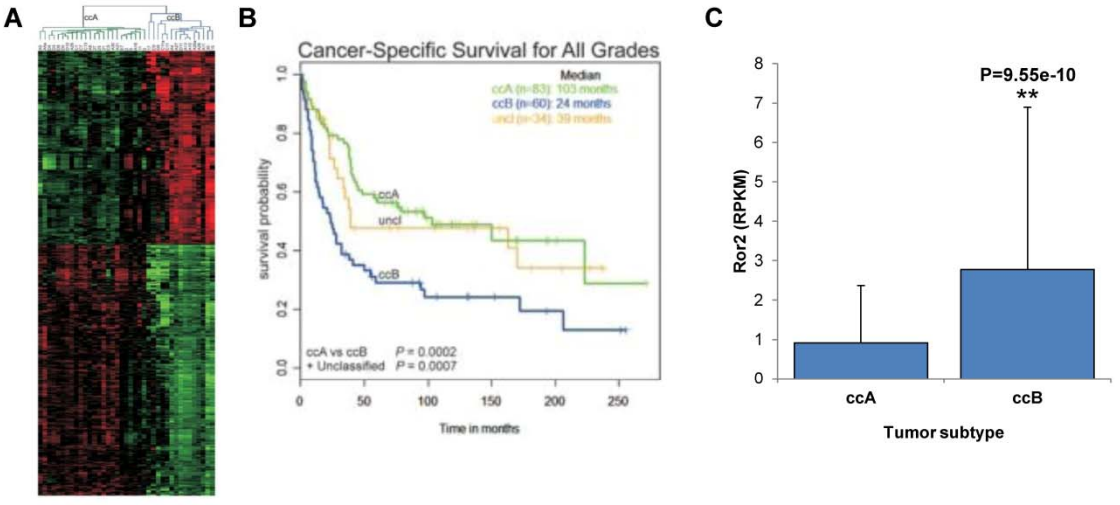

Figure 3.7: Ror2 expression is higher in ccB tumors
A) Heatmap of probes of differentially expressed between ccA and ccB tumors (Figure adapted from Brannon et al *Genes Cancer* 2010) **B)** Kaplan-Meier plots for cancer specific survival are displayed for ccRCC tumors following classification to ccA, ccB, or unclassified (uncl) subtype by LAD prediction analysis (n=177) , show ccB have significant reduction in survival (Figure adapted from Brannon et al *Genes Cancer* 2010) (**C)** Comparative analysis of Ror2 expression (RPKM) between ccA and ccB RCC tumors from the TCGA dataset shows ccB suptype have significantly higher expression of Ror2 (N=417) with error bars showing stdev. P-values were calculated using Anova one-way analysis (**<0.01)

Ror2 expression predicts poor clinical outcome in patients with RCC

The TCGA is a National Cancer Institute initiative designed to catalog multiple cancers through collaboration, creating a publicly available database of high-quality tumor samples with corresponding clinical information, pathology reports, DNA sequencing, DNA methylation, copy number, miRNA Expression, mRNA expression, and protein expression. The scope and size of the TCGA ccRCC dataset of over 400 tumors with complete clinical and molecular information provides sufficient statistical power to more deeply scrutinize the underlying molecular biology and provide a more complete picture of ccRCC.

Using the same core ccRCC TCGA dataset for determining Ror2 expression in ccA and ccB subtypes, we classified these tumors into Ror2-High (Z-score>0) and Ror2-Low (Z-score<0) expression categories dependent upon their normalized Z-score (30). The clinical and pathological characteristics of these patients in Table 3.1 showed significant increase in mean tumor size (P= 0.048) and a surprising shift away from the expected 2:1 male:female ratio (P= 0.047) to ~3:1 ratio with higher Ror2 expression. High Ror2 expressing tumors also trended toward being of a higher clinical stage, nuclear grade, and tumor stage (TNM) (Figure 3.8A-C). Further examination of these trends is needed to determine if they are truly significant.

Table 3.1: Summary of clinical characteristics of patients in the TCGA ccRCC dataset

	TCGA Dataset	Ror2-High (Z-score> 0)	Ror2-Low (Z-score <0)
Sample (n)	417	119	298
Age at Death	61 (26-90)	61 (26-90)	61 (29-90)
Gender			
Male	270 (65%)	90 (76%)	180 (60%)
Female	147 (35%)	29 (24%)	118 (40%)
Prior Tumor			
Yes	61 (15%)	20 (17%)	41 (14%)
Clinical Stage			
Stage I	195 (47%)	49 (41%)	146 (49%)
Stage II	40 (10%)	11 (9%)	29 (10%)
Stage III	111 (27%)	34 (29%)	77 (26%)
Stage IV	71 (17%)	25 (21%)	46 (15%)
Grade			
G1	5 (1.2%)	1 (0.8%)	4 (1.3%)
G2	175 (42%)	43 (36%)	132 (44%)
G3	170 (41%)	43 (36%)	127 (43%)
G4	66 (16%)	32 (27%)	34 (11%)
GX	1 (0.2%)	0	1 (0.3%)
Tumor Size (cm)	6.59	7.13	6.38
Staging (TNM)			
T1	200 (48%)	51 (43%)	149 (50%)
T2	49 (12%)	12 (10%)	37 (12%)
T3	162 (39%)	52 (44%)	110 (37%)
T4	6 (1.4%)	4 (3%)	2 (0.7%)
Nodes			
Node – (N0)	197 (47%)	56 (47%)	141 (47%)
Node + (N1)	12 (3%)	4 (3%)	8 (3%)
Node unknown (NX)	208 (50%)	59 (50%)	149 (50%)
Metastasis			
Mets – (M0)	346 (83%)	95 (80%)	251 (84%)
Mets + (M1)	71 (17%)	24 (20%)	47 (16%)

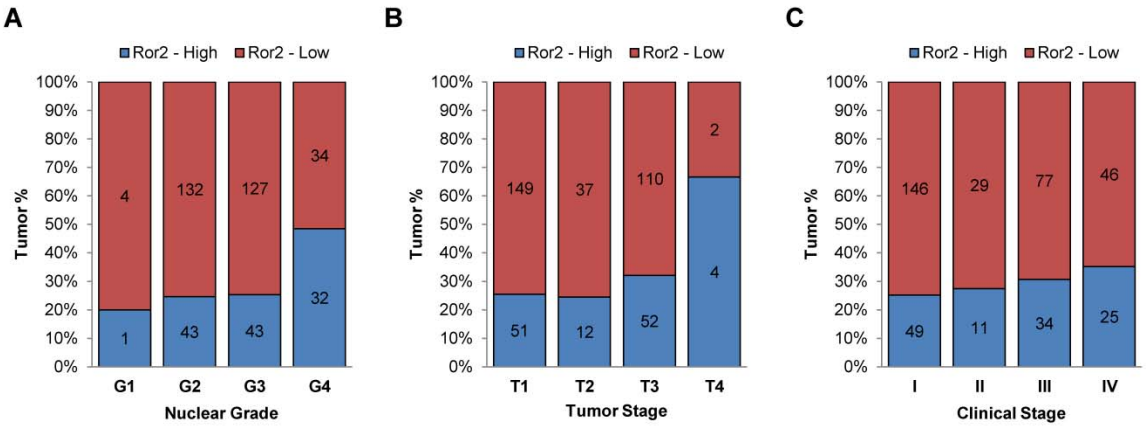

Figure 3.8: High Ror2 expression trends towards more aggressive ccRCC
Tumor percentage breakdowns of TCGA ccRCC cases (417) stratified by Ror2-High (Z-score>0) and Ror2-Low (Z-score<0) expression for exhibits trends with high Ror2 expression correlating with increased **A)** nuclear grade **B)** tumor stage **C)** and clinical stage.

ccA and ccB classification within ccRCC has been shown to be an impressive prognostic predictor in relation to survival; however, this finding required gene expression data across 120 probes (Figure 3.7B). Because higher Ror2 expression correlates with the more aggressive ccB subtype and has a prior-mentioned ability to serve as an independent biomarker in prostate, melanoma, and osteosarcoma, we sought to determine whether Ror2 expression could serve as an independent prognostic factor in RCC. This information would not only be a valuable contribution to prognostic nomograms currently employed within the clinic, but has the potential to develop into a predictive biomarker upon advent of Ror2 treatments in RCC.

We first analyzed overall survival for either Ror2-High or Ror2-Low expression from the ccRCC TCGA tumors with 95% confidence intervals and the median survival provided in Table 3.2. Kaplan-Meier plots showed the Ror2-Low subtype had a highly significant survival advantage over patients with high Ror2 expression (P = 0.005) (Figure 3.9A). Next, we examined overall survival in patients with no metastases, which likewise showed a significant survival difference between Ror2 expression subtypes (P = 0.004) (Figure 3.9B).

However, in an analysis of overall survival in ccRCC patients who developed metastases, Ror2 expression made no difference in survival time (P = 0.878) (Figure 3.9C). Finally, we assessed recurrence-free survival for patients with no metastases; our results exhibited a trend toward low Ror2-expressing tumors having a higher survival time (P = 0.292) (Figure 3.9D), but insufficient tumor samples within in this category prevent a firm determination. Even so, these results show Ror2 can serve as a prognostic biomarker in ccRCC, with high Ror2 expression correlating with increased tumor severity.

Table 3.2: Median Survival times with 95% confidence intervals

Survival Analysis	Subtype	Median Survival (months)	95% CI for median survival (months)
Overall Survival	Ror2 Low	90.4	75.5 – N/A
	Ror2 High	62.8	44.1 – 74.1
Overall Survival – No Mets	Ror2 Low	N/A	90.4 – N/A
	Ror2 High	69.2	52.9 – N/A
Overall Survival – Mets	Ror2 Low	25.2	18.4 – 47.0
	Ror2 High	19.6	11.3 – 51.5
Recurrence Free Survival	Ror2 Low	77.2	15.0 – N/A
	Ror2 High	19.6	16.2 – N/A

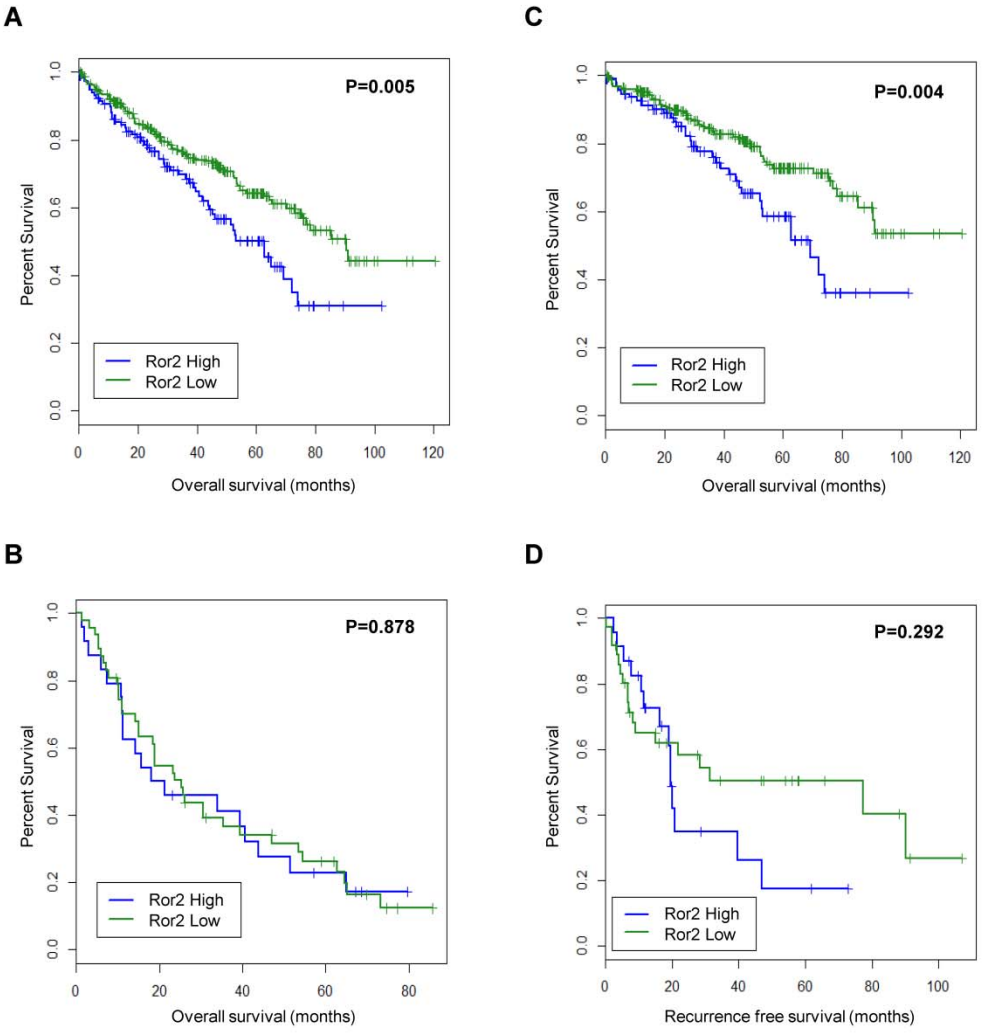

Figure 3.9: Ror2 expression predicts poor clinical outcome in patients with RCC
Kaplan-Meier survival curves for RCC cases from the TCGA were stratified by Ror2 expression. High Ror2 expression in RCC predicted significant lower overall survival **A)** with all RCC bearing patients (N=417) and **B)** metastasis free patients (N=346). No significant differences where seen in relation to Ror2 expression in **C)** metastatic RCC patients (N=71) and **D)** in recurrence free survival in metastatic free patients (N=59).

Discussion

Despite advancements in the care of RCC, there remains considerable room for improvement through identification of novel therapeutic targets and prognostic factors for assessing risk for patients. Ror2 has been shown to play a role in tumor progression in an increasing assortment of cancers including RCC through mediation of signaling cascades,

resulting in increased cell migration, invasion, and tumor growth. Prior work in RCC showed the ECM remodeling protein MMP2 expression to be Ror2-dependent, in congruence with our findings here using gene expression microarrays (13).

We sought to further elucidate the mechanisms of Ror2-dependent regulation of MMP2. Although Ror2 has been primarily been identified as receptor for the non-canonical Wnt5a ligand, Ror2 expression in the context of RCC has been shown to result in a heightened state of canonical β-catenin signaling which is further enhanced by the addition of Wnt3a (29). Furthermore, because MMP2 was shown to be induced downstream of the Wnt3a/β-catenin signaling cascade, we tested the effects on Wnt3a on MMP2 expression in RCC (31,32). Using both shRor2 knockdown and overexpression in 786-0 cells, we found that MMP2 expression mirrored the previous findings with the canonical Wnt target gene Axin2, suggesting that Ror2's migratory/invasive phenotype is not limited to Wnt5a/Ror2 signaling. Although the consistent increased expression of MMPs has been noted with Ror2 expression in a multitude of cell contexts, here we have uniquely established the need for the Ror2 kinase domain in maintaining heightened MMP2 expression. These results suggest that Ror2 kinase activity is required to mediate regulation of downstream target genes such as MMP2.

MMP2 has a well-established role in mediating cell migration and invasion through degradation of the ECM and basement membrane, allowing for increased cell motility. In keeping with previous findings, suppression of Ror2 results in decreased migration in 786-0 cells. Here we also show that overexpression of Ror2 in HEK293T and 786-0 results in increased migration. However, as the Ror2-DM was unable to alter MMP2 expression, migration of RCC cells was likewise unaffected, strengthening the argument that Ror2 kinase activity is required for migration.

In addition to the Ror2-dependent expression of MMP2, the discovery of the Wnt antagonist/agonist SFRP2 regulation by Ror2 is an intriguing discovery. Although SFRP2 is

typically thought of as a Wnt signaling antagonist, a recent study showed that SFRP2 expression in RCC cells enhances β-catenin-dependent signaling (18). As Ror2 expression likewise contributes to a poised state of β-catenin-dependent signaling in RCC, these findings together raise the possibility that Ror2 effects on B-catenin-dependent signaling are partially mediated through SFRP2 (29). RCC is a highly vascularized cancer with anti-angiogenic therapies forming the backbone of RTK-targeted therapies currently used in the clinic. The development of resistance to RTK therapeutics, possibly through upregulation of other angiogenic factors, represents a continuing challenge to clinicians in the treatment of RCC. SFRP2's additional function as an angiogenic factor presents a novel target for angiogenesis in RCC as previous studies have shown it to be expressed in the vasculature of RCC tumors (18,19). Our results which showed significant increases in tumor vascularity with overexpression in Ror2 xenografts *in-vivo* strengthens the proposal of SFRP2 as a potential target in RCC. Although overexpression of Ror2 only resulted in a trend towards increased tumor growth in xenografts, this trend was validated with the significant increase in tumor size observed with high Ror2 expression in primary RCC tumors highlighting Ror2's role in promoting tumor growth and progression.

A key clinical characteristic of RCC is the 2:1 male:female ratio, but the biology driving this difference remains unresolved. Interestingly, we observe a shift between low- and high-expressing Ror2 tumors from the expected 2:1 male:female to ~3:1, respectively. Indeed, earlier work toward understanding this gender bias showed that men tend to present with larger, higher stage, higher grade RCC than women with a poorer overall survival (33). High Ror2 expression likewise exhibited a trend towards higher nuclear grade, clinical stage and tumor stage in RCC tumors. These results exhibit that Ror2 contribution to a more aggressive disease may also help drive differences seen between genders in RCC. Further studies are needed to more fully explore the interplay between Ror2 and gender in RCC.

Our analysis of Ror2's potential as a prognostic biomarker for RCC shows that Ror2 expression is capable of independently predicting overall survival. The inability of Ror2 to serve as a distinguishing factor for metastatic RCC is not unsurprising as Ror2 correlates with the aggressive ccB subtype of ccRCC. These findings highlight the role of Ror2 in promoting a more aggressive RCC and its ability to serve as a prognostic factor in RCC.

Materials and Methods

Cell Culture

786-0 and derivative cell lines, 786-0 RC3 and 786-0 WT8 (kindly provided by Dr. W. Kaelin, Boston, MA), 786-0 scramble, stable monoclonal knockdown 786-0 shRor2.1 and shRor2.2 cells, 786-0 pCDNA6 and hRor2 cells (13), 786-0 GFP, Ror2, and Ror2-DM (29) were grown in DMEM with 10% FBS, nonessential amino acids, L-glutamine, and penicillin/streptomycin. HEK293T and derivative cell lines, 293T GFP, Ror2, Ror2-DM (29) were grown in DMEM with 10% FBS, nonessential amino acids, L-glutamine, and penicillin/streptomycin. Expression of all GFP tagged constructs was visually confirmed 24-48 hours after induction with doxycycline (500ng/mL).

Quantitative RT-PCR

Total RNA was extracted from cells using Qiagen RNeasy Mini Kit (Valencia, CA, USA). cDNA was made from 500ng of total RNA using Random Primers (Invitrogen, Carlsbad CA) and Superscript II RT-PCR reagents (Invitrogen, Carlsbad CA) and analyzed using the ABI 7900HT Fast Real-Time PCR System with the following proprietary FAM labeled primers: Ror2, MMP2, SFRP2, 18S, and β-actin (Applied Biosystems, Foster City CA).

Microarray Analysis

Gene expression for 786-0 scramble, shRor2.1, and shRor2.2 cell lines and 95 primary human RCC tumors was downloaded and prepared as previously described (34). Collection and analysis of these tumors was approved by the institutional biomedical ethics review committee. Relative gene expression for 786-0 shRor2.1 and shRor2.2 was calculated in reference to 786-0 scramble with significance of microarrays (SAM) used to determine the most significant probes positively correlated with Ror2 (FDR < 0.05). Unsupervised clustering of the Ror2 correlating probes and the average gene expression of all probes for Ror2, SFRP2, and MMP2 across the 95 primary tumors was done using Cluster v3.0 and visualized with Java Treeview v.1.1.5r2.

Immunohistochemistry

Prepared slides were deparaffinized, rehydrated in graded ethanol, boiled in citrate buffer for 30 min for antigen retrieval, and incubated with 1% H_20_2 for 15 min to quench endogenous peroxidase activity. Slides were then incubated with the primary rat anti-CD31 monoclonal antibody at a dilution of 1:50 (ab7388, AbCam, Cambridge, MA) overnight. Detection of the primary antibody was performed using Vectastain Elite ABC kit (Rat IgG) kit (Vector Laboratories INC., Burlingame, CA) and di-amino benzidine (DAB). All slides were counterstained with Hemotoxylin prior to mounting.

Xenograft Analysis

500,000 786-0 or 786-0 WT8 cells were injected orthotopically with control pCDNA6 and hRor2 expressing cells into opposing kidneys in a small cohort of athymic nude (*nu/nu*) female mice and aged 2.5-4 months. The resulting xenograft tumors were harvested and measured prior to being formalin-fixed and paraffin-embedded. Slides were prepared from serial sections from the prepared tissue blocks by the UNC Animal Histopathology Core.

The slides were immunostained for CD31 expression (ab7388, AbCam, Cambridge, MA) as detailed above.

Immunoblotting

Cells were lysed in 10mM Tris, pH 7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 20mM Na4P2O7, 2mM Na3VO4, 1% Triton X-100, 10% glycerol, 0.1% SDS , 0.5% deoxycholate, 1mM PMSF and a protease inhibitor cocktail (Roche Diagnostics, Indianapolis IN) and quantitated using Bradford reagent to measure absorbance at 595nm. Lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes (GE, Pittsburg, PA) and evaluated using the following antibodies: Ror2 (AF2064, R&D Systems, Minneapolis, MN), SFRP2 (sc-13940, Santa Cruz, Santa Cruz, CA), and the loading control Ku80 (ab1273, AbCam, Cambridge, MA). For all immunoblots following incubation with corresponding secondary antibodies conjugated to IR dye 680 or 800, membranes were scanned using the Odyssey IR imager (LI-COR Biosciences, Lincoln, NE) with densitometric analysis performed using Odyssey v3.0.21.

Transwell Migration Assay

For migration under basal conditions, HEK293T cells were induced with doxycycline (500ng/mL) 24 hours prior to be plated at $1x10^5$ cells, $5x10^4$ 786-0 cells for shRor2 cell lines, and $1x10^4$ 786-0 cells expressing Ror2 and Ror2-DM per well respectively in the upper chamber of an uncoated 6.5mm 8-μm pore size transwell chamber (Corning, Corning, NY) with normal culture media in both top/bottom wells and allowed to migrate for 6 hours or 18 hours. Following migration, cells were removed from the top of the transwell by gentle swabbing. The remaining cells were fixed in 3.7% formaldehyde, washed in PBS, and stained with Crystal Violet. The percentage area covered with migrated cells was calculated across 7 random low power fields using ImageJ.

ccA/ccB Assignment

All ccA and ccB assignments were made as described previously (26).

Survival Analysis

Survival analysis was performed with the Survival library in R v 2.14, using the TCGA mRNA sequencing data (30) and associated clinical data compiled July 31, 2012. Ror2 expression was classified as high for Z-score normalized RPKM (reads per kilobase per million) values above 0. Overall survival was calculated as time to death or last follow-up, the latter being censored. Overall survival Kaplan Meier graphs were produced and log-rank scores calculated for all 417 patients, metastatic negative patients (n=346), and patients with metastatic disease (n=71). Recurrence free survival analyses were performed for metastatic free patients (n=59).

Statistical Analysis

One-way ANOVA analysis was used to generate *p*-values in the comparison of each experimental condition with the control. A *p*-value of <0.05 was considered significant and <0.001, highly significant. All error bars shown are the calculated standard deviation (stdev) or standard error of the mean (SEM) across duplicate or triplicate experiments.

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Chapter Four

CONCLUSIONS AND DISCUSSIONS

Overall summary

Renal cell carcinoma (RCC) remains difficult to treat despite the advent of receptor tyrosine kinase (RTK) therapies; only 5-10% of advanced RCC patients survive to 5 years (1,2). Though significant research has focused on RTKs as therapeutic targets for various cancers, over half of the kinome remains largely uncharacterized, with only a small percentage translating into successful utilization in the clinic (Figure 4.1) (3). Prior work by Wright et al. identified the RTK receptor tyrosine kinase-like orphan receptor 2 (Ror2), expressed in RCC in a VHL/hypoxia inducible factor-2α (HIF-2α)-dependent manner and determined that suppression caused loss of cell migration, anchorage-independent growth, and tumor growth *in-vivo* (4,5). However, this work left many questions concerning Ror2's signaling mechanisms and its ability to serve as a prognostic biomarker in RCC.

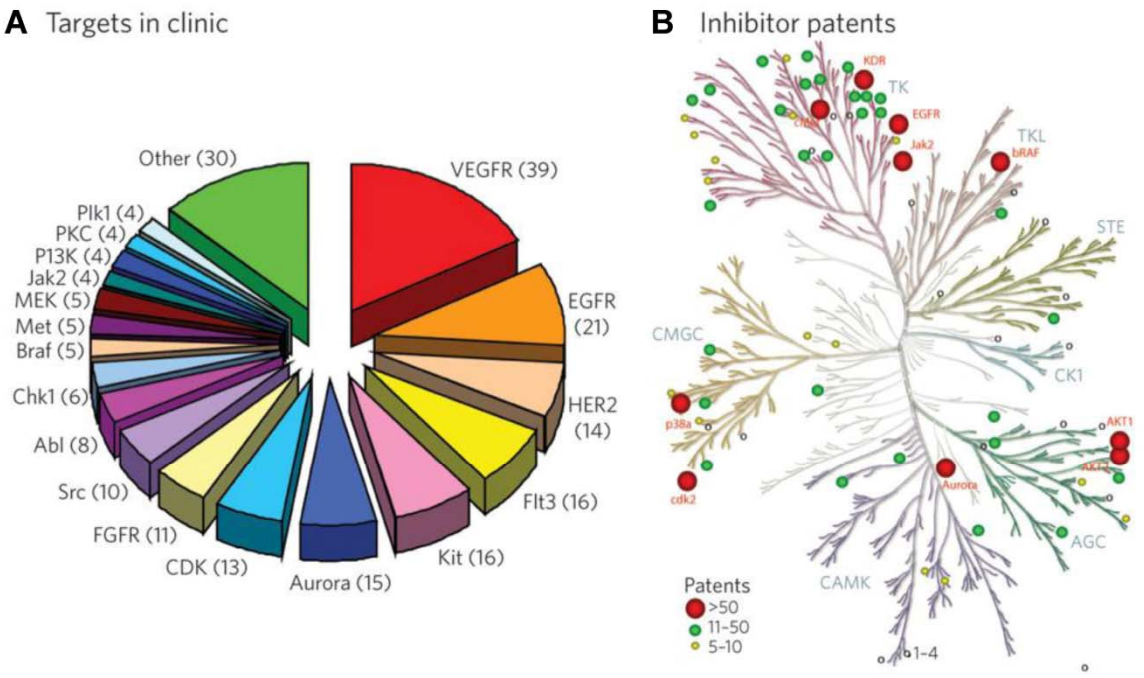

Figure 4.1: Kinase targets in clinical trials and the currently targeted kinome
A) Kinases targeted in clinical trials as of 2010. The chart does not consider cross-reactivity and groups closely related kinases, such as Aurora A and Aurora B, together. **B)** Kinases for which inhibitors have been claimed in the patent literature. Number of patents per target is reflected by the radius of the dot used.
Figure has been adapted from Fedorov et al. *Nat Chem Biol* (2010)

It is crucial in every area of science that one determines if one's research is novel and yet also takes previous works into account in order to build a greater level of understanding within the field. The studies herein presented meet these criteria. We have demonstrated the unique function of Ror2 in mediating β-catenin-dependent signaling in RCC, its expression resulting in a poised state independent of lipoprotein receptor-related protein 6 (LRP6). We have shown the need for an intact kinase domain in Ror2 for proper enhancement of β-catenin-dependent signaling, matrix metalloprotease 2 (MMP2) expression, and cell migration. Furthermore, we have illustrated Ror2's role in RCC tumorigenesis, promoting increased cell migration, tumor vascularity, and tumor growth. Finally, we have shown that Ror2 is capable of serving as a prognostic biomarker in RCC,

high expression in patients correlating with a more aggressive disease leading to a significant decrease in overall survival.

Comparisons to previous work

Ror2 research is still relatively young in comparison with more established areas. As such there is considerable room for new discoveries, but a necessity to analyze one's findings in reference to the fragments scattered within the field. With the majority of the literature describing Ror2 as a receptor for the non-canonical Wnt5a ligand, we began our investigation by first examining the non-canonical or β-catenin-independent Wnt signaling cascades, the planar cell-polarity (PCP) and Ca^{2+} pathways in RCC cells (6-10). To investigate the Wnt/PCP signaling cascade we looked first to changes in the activity of the GTPases RhoA, Rac1, and the downstream target of Rac1, c-Jun N-terminal kinase (JNK) as functional readouts. Surprisingly we saw no differences between cell lines in reference to the expression status of Ror2. This finding was not without precedent as Ho et al. has also shown the activation of JNK with Wnt5a to be independent of Ror2 (11). Likewise, we saw no changes in Wnt/ Ca^{2+} signaling with suppression of Ror2 in RCC cells transfected with a nuclear factor associated with T cells (NFAT)-driven luciferase reporter. However, these results are not a comprehensive analysis of Ror2 interactions with β-catenin-independent signaling. There are several possibilities that can account for this discrepancy of our findings with previous studies. First, the possibility that our choices of concentration of Wnt5a and time window were unable to capture Ror2-dependent differences. Second, it is also possible that we missed the pathway mediating this Ror2-dependent migration effect. Further studies to determine how these Wnt5a-dependent migration effects are being mediated in RCC cells is still needed.

Because the primary literature has focused on Ror2/Wnt5a signaling, some intriguing aspects of Ror2's effects on canonical or β-catenin-dependent signaling have been largely unnoticed or ignored. Our attention was first to drawn to Ror2's possible mediation of β-catenin-dependent signaling after our microarray gene expression analysis in RCC tumors identified several enriched gene ontologies from genes with correlating Ror2 expression. Previous studies had examined specific members of the Wnt signaling family either through RT-PCR or tissue microarrays to determine possible correlations with Ror2. Our approach improved on this several ways: first, our use of microarrays versus investigation of specifically chosen panels of genes provided a more unbiased method to examine the effects of Ror2 expression, thus allowing us to better infer the underlying biology of the tumors without assumptions of previous findings; second, our use of a large cohort composed of 95 primary tumors increased our statistical power to determine Ror2-dependent gene expression patterns; finally, our examination of primary human RCC tumors, which although more complicated can be more informative than RCC-derived cell lines which may have accrued additional characteristics through the subculturing process. The use of primary tumors also allowed us to take into account not only the effects of autocrine signaling of the tumor cells, but potential paracrine signaling between the tumor and the surrounding microenvironment. Unsurprisingly, we found enrichment for genes involved with skeletal development and extracellular matrix remodeling in concordance with Wright et al findings using a smaller set of RCC tumors (4). However, established Wnt signaling components and downstream target genes exhibited a positive correlation with Ror2 expression in primary RCC tumors including the canonical feedback target gene Axin2 and Wnt antagonist/agonist SFRP2. These results were validated using an RT-PCR array focused exclusively on Wnt signaling components and target genes in RCC cells with suppression and overexpression of Ror2.

From these results we examined more closely the function of Ror2 in β-catenin-dependent signaling in RCC cells. The most intriguing of our finds was that, independent of exogenous Wnt3a, Ror2 expression resulted in increased dishevelled 2 and 3 (DVL 2 and 3) activation, stabilization of β-catenin, and induction of transcriptional activity measured using RT-PCR for Axin2 and a TopFlash luciferase reporter. This Ror2-dependent enhancement of the β-catenin signaling cascade resulted in a poised state, leading to further enhancement of canonical signaling consequent to the addition of Wnt3a. Prior works by Li et al. and Billiard et al. both noted this augmentation of canonical Wnt signaling upon expression of Ror2; the augmentation was observed but received no comment in the works of Mikels et al., possibly due to its inconsistency among a few experiments (12-15). Our findings highlight the need to not rely solely on the standard TopFlash luciferase reporter, as the increased sensitivity of RT-PCR more distinctly showed the basal enhancement of Axin2 transcription with Ror2 expression. In agreement with Li et al. we also observed inhibition of β-catenin-dependent transcription with siRNA targeting LRP6, but our results have uniquely shown that we do not see suppression of the increased stabilized pool with expression of Ror2 (15,16). These findings highlight the necessity of LRP6 in initiating the signaling cascade in response to Wnt3a. Li et al. proposed kinase activity was not necessary for the enhancement of β-catenin signaling because Ror2 construct containing a K507R mutation within the kinase domain didn't decrease the activity but deletion of the CRD or intracellular domains did (15). In our study we utilized a Ror2 double mutant (Ror2-DM) that mutated the same K507 residue to a M507 residue along with additional mutation of the gatekeeper residue of the DFG (DLG in Ror2) motif D633 to A633, altering two key areas for kinase activity as seen in Figure 4.2A. Expression of the Ror2-DM resulted in an abrogation of the stabilization of β-catenin, Axin2, MMP2 transcription and cell migration observed with expression of the wild-type Ror2 protein (16). These differences most likely can be attributed

to our differing approaches in mutating Ror2, as ours resulted in significant changes in the natures of the amino acids and affected both the DFG loop and putative binding pocket.

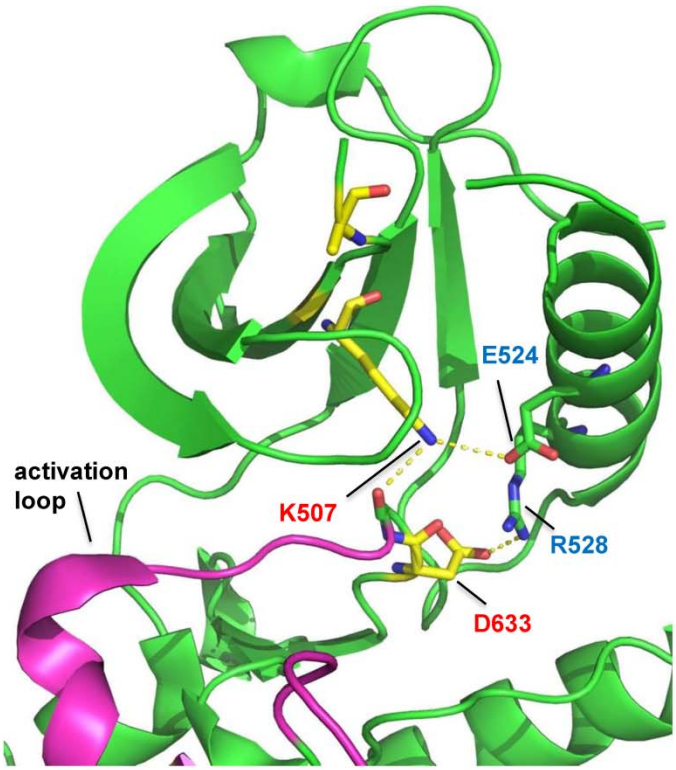

Figure 4.2: Positions of induced Ror2 mutations within the kinase domain
Positions of artificial mutations (red) and interacting residues (blue) are highlighted within the published Ror2 kinase domain crystal structure. The K507 and D633 residues (red) shown in red are key residues that have been used for site-directed mutagenesis in Ror2.
Figure adapted from image generously provided by Dr. Natalia Jura.

In Chapter 3, we presented Ror2's ability to serve as a tumor promoting factor and a prognostic biomarker for RCC. The work presented in these chapters builds on the growing literature showcasing Ror2's promotion of aggressive cancer in osteosarcoma, metastatic melanoma, gastrointestinal stromal tumor (GIST), and leiomyosarcoma (LMS) (17-19). A consistent feature of Ror2 expression in these cancers is the upregulation of matrix metalloproteases (MMPs) and increased cellular migration and invasion (4,20-23). In

accordance with previous work in RCC we observed a significant enrichment of extracellular remodeling proteases (MMPs and ADAM) and their regulators (TIMP) with Ror2 expression (16). In contrast to other cell contexts in which Wnt5a/Ror2 signaling drove expression of MMPs, MMP2 expression in renal cells increased with the addition of Wnt3a. Nonetheless, that we still saw decreased cell migration in 786-0 to both Wnt3a and Wnt5a with suppression of Ror2 suggests that Wnt5a/Ror2 signaling is still contributing to cell migration in RCC cells.

A truly novel find we identified from the many genes that correlated with Ror2 expression in RCC primary tumors was the secreted Wnt regulator secreted Frizzled-related protein 2 (SFRP2). We validated the Ror2 regulation of SFRP2 expression at both transcriptional and translational levels utilizing HEK293T and 786-0 cells. Our choice to follow up further on Ror2's regulation of SFRP2 expression was motivated by two recent studies showing novel functions of this protein as a potent angiogenic factor, a tumor growth promoting factor, and as a Wnt agonist in RCC cells (24,25). Akin to our results with Ror2, SFRP2 induction of β-catenin-dependent signaling was done in the absence of any exogenous Wnt. These results suggest that one of the mechanisms Ror2 employs leading to increased stabilization of β-catenin may be partially mediated through SFRP2. Additionally, our result with Ror2 overexpression leading to increased tumor vascularity in xenografts could be due to SFRP2's function as an angiogenic factor.

SFRP2 modulation of Wnt signaling centers on its interactions with Wnt ligands or Fzd receptors and has primarily been modeled as an antagonist which sequesters Wnts or prevents interactions with ligand receptors. However, a recent study showed that SFRP2's agonistic role was mediated by its binding to Fzd receptors (26). SFRP2's induction of angiogenesis was shown to be mediated through the non-canonical Wnt/Ca^{2+} pathway with the activation of calcineurin and NFAT (25). Upon use of monoclonal antibody targeting SFRP2 in MDA-MB-231 cells there was reduction in both nuclear β-catenin and NFAT (27).

Effects in both canonical and noncanonical pathways with inhibition of SFRP2 are suggestive that it may be independent of its role in binding Wnts and could be mediated through binding of Fzd receptors. Additional work is needed to examine SFRP2's contributions to Ror2-mediated Wnt signaling and angiogenesis in RCC cells.

Prior work by Brannon et al. using gene expression data from multiple data sets of primary human tumors resulted in a probe set of 120 genes, capable of discretely separating ccRCC into two molecular subtypes, ccA and ccB. Comparison of gene expression patterns between these subtypes showed ccA to have features of classical ccRCC, while ccB tumors were characterized as more aggressive due to the significant reduction of overall survival and expression of genes regulating cell adhesion, extracellular matrix remodeling, cell cycle progression, and epithelial-mesenchymal transition (EMT) (28). Given Ror2's tight link to many of these same features in RCC and other cancers, the natural extension was to ascertain if Ror2 was differentially expressed between these subtypes. As we predicted, Ror2 showed much higher expression in ccB tumors than ccA within TCGA tumors. Yet the simple addition of Ror2 into the ccB phenotype is not enough. Our goal was to determine if Ror2 could serve as an independent prognostic biomarker in RCC as had been done previously in gastrointestinal stromal tumor (GIST) and leiomyosarcoma (LMS) (17).

The data in the Cancer Genome Atlas (TCGA) for ccRCC is a valuable tool for investigating the underlying molecular biology of ccRCC tumors in relation to their known clinical features. We examined the effects of Ror2 expression in primary tumors from the published TCGA ccRCC dataset, which provided a significantly larger dataset of 417 ccRCC tumors with complete clinical features (including tumor size, nuclear grade, clinical stage, tumor stage, and survival) for each tumor. From this analysis were able to confirm our trend of increased tumor growth in xenografts with significant increase in tumor growth in high Ror2-expressing tumors. In agreement with aggressive phenotypes seen in metastatic melanoma, osteosarcoma, GIST, and LMS, we saw that higher Ror2 expressing tumors

trended to having increased clinical stage, nuclear grade, and tumor stage. A more rigorous statistical analysis is needed to determine if these relationships are truly significant. Finally, an analysis of overall survival for all patients with and without metastasis showed significant decrease with high Ror2 expression. Our findings show that Ror2 can serve as an independent prognostic biomarker in RCC and is an attractive therapeutic target in RCC.

Ror2 activities promoting cancer

As previously noted, the majority of the field investigating Ror2 has placed it solely as a receptor for non-canonical Wnt5a. This paradigm was first established with the observed phenotypic overlaps between Wnt5a and Ror2 null mice (29-31). Yet, immunoprecipitation experiments of *xRor2* found it bound not only Xwnt5a, but Xwnt8 and Xwnt11 as well. Additionally *in-vitro* immunoprecipitation of Ror2 is capable of engaging multiple Wnt ligands including Wnt 1, Wnt3, Wnt3a, Wnt5a, and Wnt5b (6,14,32,33). However, the ability of Ror2 to bind various Wnts can only infer potential for activity in response to each of these ligands. Thus looking at changes in downstream Ror2 signaling in response to Wnt ligands is essential to determining the breadth of Ror2's role as Wnt receptor. An examination of Ror2 expression in osteosarcoma cell lines shows this potential for differential signaling in response to various Wnt ligands, with Ror2 potentiating β-catenin-mediated transcription with Wnt1 and inhibiting it in response to Wnt3. (14). In addition both Wnt5a and Wnt5b contributed to Ror2-dependent migration in osteosarcoma cells (32). This expanded profile of Wnt ligands interacting with Ror2 is also potentially reflected in our RCC tumors as both Wnt5b and Wnt4 showed significant correlating expression with Ror2. Surprisingly we saw that although Wnt5a did not result in any activation of non-canonical signaling in a Ror2-dependent manner, Ror2 expression did lead to an enhancement of canonical β-catenin signaling in RCC cells. These results suggest that Ror2 may serve as a

dual receptor and thus drive tumor progression. The question becomes, what are the mechanisms that regulate and confer Ror2's differential Wnt signaling?

Wnt signaling pathways have shown to be highly dynamic despite the repeated usage of core components in multiple pathways. Earlier paradigms had suggested that this dynamic nature was only due to the variety of pairings possible between the 19 Wnt ligands and the 10 Frizzled (Fzd) receptors, LRP5 and 6, and multiple other receptors. Although this is true, a precedent of multi-functionality is seen throughout the members of Wnt signaling. Wnt5a is dependent upon receptor context and was shown to be able to activate or inhibit canonical Wnt signaling *in-vitro* and *in-vivo* (12,34). Sato et al. showed Fzd2 is capable of initiating both canonical and non-canonical Wnt planar cell polarity (PCP) signaling depending upon its engagement of either Wnt3a or Wnt5a respectively (35). SFRP2 primarily has been shown to act as an antagonist of Wnt signaling through competitive binding of Wnt ligands, but expression of SFRP2 in RCC cells resulted in increased canonical Wnt signaling (24). DVL also serves to mediate both canonical and non-canonical Wnt pathways.

Mechanisms regulating the dual nature of Ror2 may function in several ways. First, Ror2 may have differing binding affinities between the Wnt ligands. In both Li et al. and Mikels et al. the concomitant addition of Wnt5a with Wnt3a CM with no resulting dilution changes resulted in a significant decrease of canonical signaling in Ror2-expressing cells. These results suggest that Ror2 may have a greater affinity for Wnt5a than Wnt3a, leading to the decreased activity as a result of Wnt3a being unable to bind and/or Wnt5a leading to downstream inhibition of β-catenin-dependent signaling. Sato et al. demonstrated that the receptor Fzd2 is capable of responding to both Wnt3a and Wnt5a, yet immunoprecipitations of Fzd2 with both ligands revealed its slightly increased affinity for Wnt5a (35). The same approach could be well used to investigate this possibility of differing affinities of Ror2 and Wnt3a. Additionally the use of Shisa, which specifically inhibits Fzd translocation to the

membrane by retaining it in the endoplasmic reticulum, would allow a determination of the necessity of Fzd coreceptors for Ror2 signaling (36). Second, Ror2 has been shown to interact with both Fzd2 and Fzd7 via its CRD domain, with Wnt5a engagement of Ror2-Fzd2 and Ror2-Fzd7 initiating the Wnt/PCP signaling cascade and enhanced β-catenin-dependent signaling through Ror2-Fzd2 with Wnt3a (10,15,35). This observed dichotomy of Fzd-Ror2 signaling would serve to explain Ror2 correlating canonical Wnt targets in primary RCC tumors, as we also see Fzd2 (16). Thus regulation of the dual nature of Ror2 is likely accomplished through a combination of the mechanisms of tight regulation of Wnt expression, Ror2's differing affinities for Wnt ligands, and expression of Fzd co-receptors.

A critical process in solid tumor progression and metastasis is the epithelial to mesenchymal transition (EMT), which converts cells from an epithelial, nonmotile morphology to become migratory and invasive (37). Wright et al. have shown previously in RCC that several key EMT genes, Twist1, Twist2, Snail2 and MMP2, correlated with Ror2 expression (4). In particular, Snail has been shown to mediate EMT of both epithelial cells and carcinomas. Recent work investigating EMT has shown expression of Snail induces expression of Ror2 and MPP2 in A431 cells, and suppression of endogenous Snail in SaOS-2 resulted in a loss of Ror2 and MMP13 expression (21). Interestingly, this same process has been noted in renal fibrosis with induction of Ror2 and MMP2 in damaged kidneys. Li et al. found that Ror2 was predominantly expressed in tubular epithelial cells that have undergone EMT, as noted by the expression of Snail and other mesenchymal markers (38). The importance of these findings is illuminated upon taking into account that the tubule cell is thought to be the cell of origin for RCC and that Snail is a HIF target (39,40). Prior work with Ror2 by Wright et al. in RCC cells demonstrated its HIF dependence but hypoxia independence, showcasing that the reactivation of Ror2 expression in RCC cells may be mediated by Snail upon its reactivation by injury or HIF.

Is Ror2 a pseudokinase?

Receptor tyrosine kinases (RTKs) are generally activated through ligand-induced oligomerization, typically homodimerization, resulting in trans-autophosphorylation of the kinase domain leading to the recruitment of downstream signaling partners. However, among the 58 human RTKs approximately 10% have been predicted to be catalytically inactive, termed pseudokinases (41). Despite alterations in several highly conserved residues both Ror1 and Ror2 were predicted to be active by Manning et al. (41). Yet, Ror1 was recently categorized as a pseudokinase by Gentile et al., due to its lack of catalytic activity *in-vitro* and autophosphorylation in cells. A plausible case for Ror2 inclusion as pseudokinase is made more clear upon examination of Ror1 and Ror2 kinase domains which exhibit a very high degree of amino acid identity (>70%) and share several key alterations in regions conserved in most RTKs (Figure 4.3). Among these conserved regions in RTK is the glycine-rich loop which associates with phosphate groups of ATP. Both Ror1 and Ror2 have significant substitutions in the second of the conserved glycine residues, most likely distorting the ATP-binding site. The DFG motif is another key conserved region playing a key role as metal binding site and in both Ror1 and Ror2 contains a change from DFG to DLG. This phenylalanine residue plays an important role in positioning the catalytic aspartate residue, yet an examination of the crystal structure for Ror2 kinase domain shows the leucine residue replacing the phenylalanine does not spatially interfere with the ATP binding pocket region (42). These similarities between Ror1 and Ror2 sequences show the possibility of Ror2 being pseudokinase.

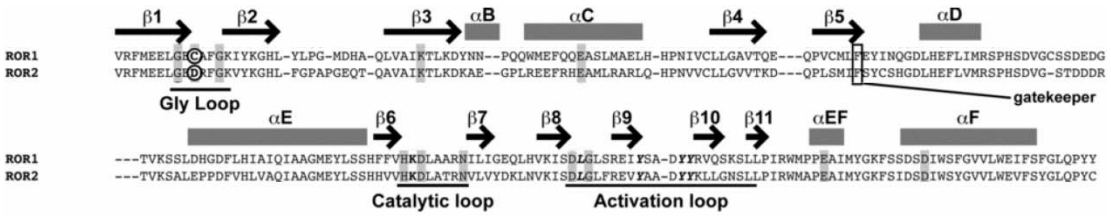

Figure 4.3: Sequence alignment of TKD domains from Ror1 and Ror2
Amino acid sequence alignment of Ror1 and Ror2 with secondary structure elements being noted. Catalytic residues conserved in all known kinases are shaded grey, and are circled in black (bold text) when altered. Uncircled residues in bold text are 'unusual', but found in some active kinases. The 'gatekeeper' residue, mutated to generate analogue-sensitive kinases, is boxed in black.
Figure has been adapted from Mendrola et al. *Biochem Soc Trans* (2013)

However, there is opposing evidence to Ror2's classification as a pseudokinase. The original examination by Gentile et al. of Ror1 began by cataloging Ror1 tyrosine phosphorylation in 43 cancer cell lines, of which only 3 were positive. Wright et al. in her original reporting of Ror2 expression in RCC cells showed that Ror2 did exhibit a basal level of phosphorylation which was labeled as autophosphorylation (4). Following this Mikels et al. examined Ror2 utilizing an *in-vitro* kinase activity, showing that immunoprecipitated mRor2 was capable of transphosphorylating the kinase domain of mRor2 and this capability was enhanced by pretreating cells with Wnt5a (13). Additionally, forced dimerization using a Ror2-targeted antibody in U2-OS osteosarcoma cells resulted in increased tyrosine phosphorylation of endogenous Ror2. Our findings using the Ror2-DM also demonstrate the importance of the tyrosine kinase domain in mediating β-catenin-dependent signaling, MMP2 expression and cell migration. Taken together these results suggest that Ror2 is an active RTK. However without the gold standard of directly showing Ror2's ability to bind and catalytically use ATP we are unable to place Ror2 in either camp of pseudokinase or active RTK.

Regulation and activation of Ror2 in RCC

The dysfunctional signaling by mutated or overexpressed kinases has been intimately linked as a potent driving force in cancer (3). The disregulation and constitutive activity of many RTKs are the result of mutations within the kinase that enhance or stabilize enzymatic activity such as epidermal growth factor receptor (EGFR). Mutations vary in their method of activation of kinases, from deletions of extracellular receptors, gain of function mutations, or mutations resulting in loss of auto-inhibitory function. EGFR is characterized by a series of deletions in the extracellular domain causing constitutive activation. Discovery of EGFR expression in non-small cell lung cancer and development of targeted therapeutics was thought would result in dramatic responses, however, these EFGR RTK inhibitors have been plagued by low response rates and quick turnover to resistance due to additional mutations (43,44). A previous study examining mutations of RTK in gastric cancer by Edris et al. identified a non-synonymous mutation in 1 of 52 samples resulting in the D644N substitution within the kinase domain of Ror2, possibly altering its activity. An analysis of the Ror2 mutations observed within the available TCGA datasets suggests that Ror2 mutations are, to varying degrees, rare in most cancers, as noted by a mere 0.2% ccRCC tumors (Figure 4.4) (17).

Although RTKs are the most commonly mutated genes in cancer, this is by far not the only mechanism in which they are activated. Many kinases are upregulated either through genetic amplification events or alterations within their regulatory mechanisms. In addition to the aforementioned deletion mutants, EFGR is often found to be amplified in breast, gastric, ovarian, and non-small cell lung cancer, the resulting overexpression leading to increased dimerization and constitutive activation (45). The works of Wright et al. concerning Ror2 beautifully laid out the aberrant expression of Ror2 to be dependent upon the inactivation of Von-Hippel Lindau protein (pVHL) and hypoxia inducible factor (HIF) expression (4,5). These findings placed Ror2 regulation as part of the program resulting

from the defining loss of *VHL* in RCC. As Ror2 expression has now been observed in a multitude of cancers beyond RCC, how Ror2 expression, notably missing from most adult tissues, becomes reexpressed is an interesting question. The low rate of mutation of Ror2 in cancer suggests that its expression in many of these cancers may be mediated through either gene amplification or changes in Ror2 regulatory genes such as *VHL*.

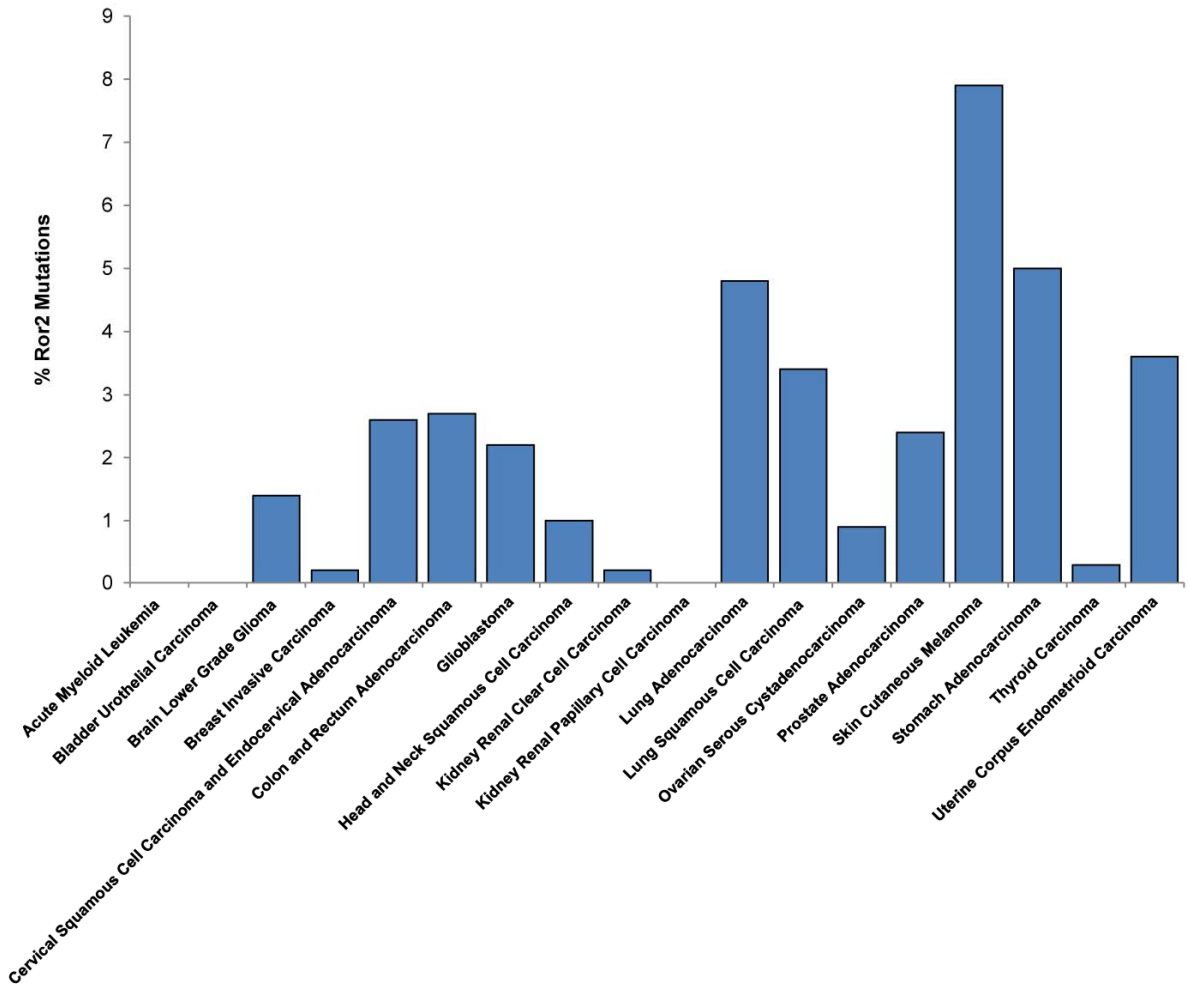

Figure 4.4: Ror2 maintains a low mutational rate in TCGA cancer datasets
Mutation rates for Ror2 in TCGA datasets downloaded from cBio portal (http://www.cbioportal.org).

Ror2 inhibition in cancer

Our findings presented in previous chapters showed Ror2 expression to result in a more aggressive RCC, marked by increased cell migration, tumor growth, tumor vascularity, and decreased survival, highlighting its great potential as a therapeutic target in RCC. In addition Ror2 represents an extremely attractive RTK target due to its very limited expression profile within adult tissues and the ever-growing list of cancers in which it is expressed. As such the question has become, what is the best avenue for the development of Ror2-based therapeutics?

Akin to Ror2, Ror1 has been shown to have a tumor-promoting role in a variety of cancers, but has been noted for its discriminating expression between normal and tumor cells in chronic lymphocytic leukemia (CLL) (46). Ror1 targeted monoclonal antibodies (MAB) have shown efficacy in both melanoma and breast cancer cells inducing apoptosis and reduction of cell migration and invasion (47,48). An additional MAB approach coupled with immunotoxin targeting Ror1 resulted in a significant increase in apoptosis in Ror1-expressing CLL cells (49,50). A translation of these MAB approaches for targeting Ror2 therapeutically has great potential.

Dependent upon Ror2's activity as an RTK, the development of a small molecule inhibitor for Ror2 may prove to be very bright avenue. The consistent drawback and sometimes saving grace of RTK inhibitors is their lack of specificity due to the highly conserved nature of ATP-binding pocket and catalytic loops governing kinase activity between kinases (3). However, as the Ror2 kinase domain is marked by a few departures from conserved sequences, including a switch from DFG to DLG in the catalytic loop may aid in the specificity of any identified small molecule inhibitors of Ror2 (51) (Figure 4.3). However, prior to any screening of potential inhibitors of Ror2 additional work is needed to further clarify the kinase activity of Ror2. If it is found that Ror2 truly does exhibit intrinsic

tyrosine kinase activity then it represents a prime target for the development of small molecule inhibitors.

Conclusions

The research presented within this dissertation describes the function of Ror2 as a member of the Wnt signaling network within the context of RCC, and reveals a novel role of Ror2 as a mediator of β-catenin-dependent signaling in RCC. Prior studies have demonstrated Ror2's dual role in Wnt signaling and its ability to mediate canonical Wnt signaling. However, for the first time we have demonstrated that Ror2 expression independent of Wnt3a and LRP6 leads to a poised state of β-catenin-dependent signaling via activation of DVL2/3 and stabilization of β-catenin. In addition we have also shown this poised state through stabilization of β-catenin required the kinase domain of Ror2.

Likewise, we more thoroughly examined Ror2's role in RCC tumorigenesis. Building off of previous works we have shown that Ror2 promotes increased cell migration, tumor growth, and for the first time, increased tumor vascularity. Finally we have shown the ability of Ror2 to serve as an independent prognostic biomarker, with high Ror2 expression in patients correlating with a more aggressive disease marked by increased tumor size, leading to significant decreases in overall survival. These findings have demonstrated the complexity of Wnt/Ror2 signaling in RCC, along with Ror2's capability to serve as a prognostic biomarker and possibly as a predictive biomarker with the advent of Ror2-targeted therapeutics.

Following these findings several key aspects of Ror2 signaling need to be addressed in order to allow the field to advance. First and foremost is the need to determine if Ror2 belongs to the class of pseudokinases or is an active RTK. This aspect of Ror2's catalytic activity is central to our understanding of its signaling, its interaction with other proteins, and how we can best develop Ror2-targeted therapeutic options for RCC and other cancers.

Second, although we have made strides in elucidating Ror2 signaling in RCC, additional work is needed to illuminate contextual differences in its signaling. Determination of what additional coreceptors or factors are influencing Ror2's differential signaling among the Wnt pathways will provide us a with better understanding of its differences between cancers and how these might influence phenotype and clinical outcome. Finally, with our establishing Ror2 as a prognostic biomarker in RCC and its known tumor-promoting role in many other cancers, there now is a great need for the development of diagnostic assays for Ror2 and targeted therapeutics.

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