Wnt5a Interaction with Intestinal Ror2

Regulates Villin Expression

by

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Abstract

Regulation of expression of the intestinal actin-binding protein, villin, a marker of intestinal epithelial differentiation, is poorly understood. Activation of the extracellular calcium-sensing receptor (CaSR) on sub-epithelial myofibroblasts stimulated the secretion of Wnt5a, while activation of the CaSR on intestinal epithelia increased expression of Ror2, a Wnt-family co-receptor. Immunocytochemistry has localized Ror2 expression in the epithelia lining the small intestine from the crypt base to the villus tip. The aim of this study was to determine whether Wnt5a binding Ror2 in intestinal epithelia stimulated transient increases in phospho-ERK1/2 (pERK1/2) which lead to increased expression of villin transcript and protein. To examine Wnt5a-Ror2 regulation of villin expression, we transgenically overexpressed wild-type, truncated, or mutant Ror2 constructs in HT-29 adenocarcinoma cells and nontransformed fetally-derived human intestinal epithelial cells (HIECs), added conditioned media containing Wnt5a and measured changes in ERK1/2 phosphorylation, villin amplicons and protein expression by RT-PCR and Western blot techniques. Wnt5a addition caused a transient increase in pERK1/2, which was maximal at 10 min but diminished by 30 min. Transient transfection with a siRNA duplex against Ror2 diminished Ror2 amplicons and protein and reduced the extent of pERK1/2 activation. Structure-function analysis revealed that deletion of the cysteine-rich, kringle, or tyrosine kinase domain or substitution mutations of tyrosine residues in the intracellular Ser/Thr-1 region of Ror2 prevented the Wnt5a-stimulation of pERK1/2. Deletion of the intracellular proline and serine/threonine rich regions of Ror2 had no effect on Wnt5a-stimulation of pERK1/2 in HT29 cells. Western blot analysis demonstrated that villin protein was increased by over-expression of wild-
type Ror2 in HT-29 cells and HIECs in the presence of Wnt5a. The increase in villin expression was blocked by pharmacological inhibition of MEK1&2 and casein kinase 1, but not by PKC and p38 inhibitors. Neither Wnt3a nor EGF addition increased villin protein. This work suggested that stromal Wnt5a will stimulate pERK1/2 via the Ror2 tyrosine kinase domain to generate increased villin protein. These findings suggested that Ror2 homeostasis and Wnt5a interaction with Ror2 are important determinants of the regulation of villin expression in the intestine.
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<th>Full Form</th>
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<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>BDB</td>
<td>Brachydactyly B</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAM KII</td>
<td>Ca(^{2+})/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CaSR</td>
<td>calcium sensing receptor</td>
</tr>
<tr>
<td>CDX2</td>
<td>caudal type homeobox 2</td>
</tr>
<tr>
<td>CK1</td>
<td>casein kinase I</td>
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<tr>
<td>CRC</td>
<td>colorectal cancer</td>
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<tr>
<td>CRD</td>
<td>cysteine rich domain</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
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<tr>
<td>DSS</td>
<td>dextran sodium sulfate</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>Elk-1</td>
<td>E-26 like protein 1</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal regulated kinase 1 and 2</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Frz</td>
<td>frizzled receptor</td>
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<tr>
<td>GRK2</td>
<td>G-protein receptor kinase 2</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>glycogen synthase kinase - 3 Beta</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIEC</td>
<td>human intestinal epithelial cells</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>LRP5/6</td>
<td>(low density) lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
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<td>MEK1&amp;2</td>
<td>mitogen activated protein kinase kinase 1 &amp; 2</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PDVF</td>
<td>polyvinylidene difluoride</td>
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<td>protein kinase C</td>
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<td>RRS</td>
<td>Recessive Robinow Syndrome</td>
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<td>RTK</td>
<td>receptor tyrosine kinase</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>rWnt3A</td>
<td>recombinant Wnt3a</td>
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</tr>
<tr>
<td>siRNA</td>
<td>short-interfering RNA</td>
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<tr>
<td>TBS-T</td>
<td>tris buffered saline</td>
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<tr>
<td>TCF/Lef</td>
<td>T-cell factor/ lymphoid enhancer factor</td>
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<tr>
<td>Wnt5a CM</td>
<td>Wnt5a conditioned media</td>
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<td>WT</td>
<td>wild type</td>
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Chapter 1
Introduction

WNT GLYCOPROTEINS

The patterning and continuous cycling of the intestinal epithelia after weaning in the mammal relies on Wnt signaling (Barker et al., 2007). Some of the processes regulated by the 19 members of the Wnt family include: embryonic induction, cellular proliferation, differentiation, migration, tissue polarity establishment and cytoskeletal arrangement (Kikuchi & Yamamoto, 2008). Wnt regulation of such a diverse set of developmental processes implies that disruptions in normal Wnt signaling can have some very profound effects (Nusse et al., 2008). Defects in Wnt signaling have been associated with colorectal cancers (Kinzler et al., 1991), bone density defects (Gong et al., 2001; Boyden et al., 2002) and degenerative vascular diseases (Little et al., 2002; Toomes et al., 2004; Nusse, 2005). Indeed, defective Wnt signaling is one determinant of colon cancer (van Es & Clevers, 2005; Segditsas & Tomlinson, 2006). Understanding the types of signal transduction cascades induced by Wnts will have a significant impact in improving our understanding of disease progression (Polakis, 2000; Clevers & Batlle, 2006; de Iongh et al., 2006; Fox & Dharmarajan, 2006; Krishnan et al., 2006).

Wnt Signaling: Non-canonical vs. Canonical Wnt Signaling

Following their synthesis, Wnt proteins are dynamically modified, undergoing elaborate post-translational processes including glycosylation and palmitoylation (Mason et al., 1992; Reichsman et al., 1996; Tanaka et al., 2000; Mikels & Nusse, 2006). Upon secretion, Wnts can interact with a number of receptors to induce either canonical (β-
catenin dependent) or non-canonical (β-catenin independent) signaling (Wong et al., 1994; Kuhl et al., 2000). The main components of Wnt signaling are summarized in Figure 1.

Canonical Wnt Signaling

In canonical Wnt signaling, the binding of Wnts to extracellular Frizzled (Frz) and LRP5/6 co-receptors initiates a signal cascade that induces the liberation of β-catenin from its destruction complex (Bhanot et al., 1996; Wehrli et al., 2000; Li & Bu, 2005; Li et al., 2008). Subsequently, free β-catenin accumulates in the cytoplasm, eventually translocating to the nucleus, where it binds and activates the transcription factors, T-cell factor/lymphoid enhancer factor (Tcf/Lef). The activated Tcf/Lef subsequently induces the transcription and expression of various proliferative genes associated with cell-cycle regulation, including c-myc or cyclin D1 (Hurlstone & Clevers, 2002).

Non-Canonical Wnt signaling

In contrast, in the absence of Wnts, or in non-canonical Wnt signaling, β-catenin is not liberated. In some instances, β-catenin is stabilized in a destruction complex and is phosphorylated sequentially by casein kinase Iα (CKIα) and glycogen synthase kinase-3β (GSK-3β), then ubiquitinated. The ubiquitinated β-catenin is eventually recognized and degraded by a proteosome (Ikeda et al., 1998; Liu et al., 1999; Kikuchi et al., 2007). In other cases, Wnts signaling is independent from β-catenin, activating downstream
Figure 1. Wnts can signal canonically or non-canonically. In canonical Wnt signaling (left), Wnts bind to the Frz receptor and co-receptor LRP5/6. This binding event triggers the release of β-catenin from its Axin/APC–associated stabilizing complex. This free β-catenin accumulates in the cytoplasm, translocating to the nucleus. Nuclear β-catenin binds to the TCF/LEF transcription factors, stimulating the expression of target genes such as c-myc and cyclin D. Non-canonical Wnt signaling (on right) typically occurs upon Wnt-Ror2 binding or in the absence of Wnts. β-catenin remains stabilized in its destruction complex, where it is ubiquitinated by GSK3β and CKIε. The phosphorylated β-catenin is furthermore recognized by a proteosome and is degraded. In other instances, JNK, CAM KII or PKC–mediated cascades may be induced.
effectors such as PKC (Weeraratna et al., 2002), calcium-calmodulin kinase 11 (CAM KII) (Ishitani et al., 2003), or JNK (Yamanaka et al., 2002).

Traditional ideas from developmental biology studies regarding Wnt signaling evolved to classify individual Wnts based on their potential to induce either canonical or non-canonical Wnt signaling (Wong et al., 1994). Typically Wnt1, 3, 3a, and 7a were considered canonical Wnts, while Wnt2, 4, 5a, 5b, 6, 7b and 11 were perceived as non-canonical Wnts. It is now understood that this classification may be artifactual (Mikels & Nusse, 2006; van Amerongen et al., 2008). However, mounting evidence suggests that receptor context, rather than the specific Wnt involved, may dictate the type of signaling cascade induced (Mikels & Nusse, 2006; van Amerongen et al., 2008).

Role of Wnt Receptors in Dictating the Type of Signaling Cascade Induced

Several recent studies have shown that the type of Wnt signaling induced is more likely a product of the specific receptor being activated, rather than the intrinsic properties of the individual Wnt protein itself (Mikels & Nusse, 2006; MacLeod et al., 2007; Pacheco & Macleod, 2008). The strongest evidence that Wnt signaling is mediated by receptor type was demonstrated by investigations using mouse fibroblasts and 293 human kidney cells (Mikels & Nusse, 2006). The authors found that Wnt5a treatment in Frz4-overexpressing cells upregulated levels of cytosolic β-catenin and increased β-catenin reporter activity. However, when similar cells were stably transfected with the Ror2 receptor, Wnt5a addition antagonized β-catenin dependent signaling. Results from this study suggested that canonical or noncanonical Wnt
signaling was not dictated by the intrinsic properties of the ligand. This important finding demonstrated that the receptor (or co-receptors) involved contributed to the type of signaling induced.

Furthermore, recent studies have also demonstrated that overexpression of Ror2 further increased extracellular calcium sensing receptor (CaSR)-mediated Wnt5a inhibition of β-catenin signaling in colon cancer cells (MacLeod et al, 2007). Our lab has reported that the extracellular calcium sensing receptor (CaSR) upregulated Wnt5a secretion from colonic myofibroblasts, which lie directly underneath the colonic epithelia. While on the overlying epithelia, CaSR activation increased Ror2 receptor expression. In this same study, Wnt5a-Ror2 interaction in the colon subsequently led to the activation of the caudal type homeobox transcription factor 2, CDX2. We have shown that the Wnt-stimulated CDX2 mediates the expression of a variety of intestinal-specific genes including sucrase isomaltase in the intestine (Pacheco & Macleod, 2008). Taken together, these studies highlight the importance of Wnt5a - Ror2 interaction for the induction of non-canonical signaling. These investigations have linked Wnt5a activation of Ror2 with the initiation of epithelial differentiation in the intestine.

ROR2: A RECEPTOR TYROSINE KINASE

Ror2, along with Ror1, constitute the highly conserved Ror family of receptor tyrosine kinases (RTKs) (Forrester, 2002). Members of the RTK superfamily share common domains, including a single membrane-spanning region and a conserved intracellular tyrosine kinase domain. The general mechanism of receptor activation
involves ligand binding to the extracellular domain of the receptor and subsequent receptor dimerization. These events result in the phosphorylation of tyrosine residues within the activation loop of the receptor. RTK phosphorylation elicits activation and recruitment of downstream effectors such as mitogen-activated protein kinases (MAPKs). RTK initiation of such signaling cascades mediate early developmental processes such as cellular proliferation, differentiation, migration and survival (McKay & Morrison, 2007).

**Ror2 Expression**

Upon the discovery of the Ror2 receptor tyrosine kinase in the early 1990s, the specific functional role of, and the ligand(s) activating Ror proteins were not obvious. In fact, their name reflects their original and long-standing status as orphan tyrosine receptors (Forrester, 2002). Early work was thus directed at localizing Ror1 and Ror2 expression, to better understand their specific functions. Analysis of the highly conserved tyrosine kinase domain in this family of receptors indicated a strong amino acid homology to Trk family of RTKs (Masiakowski & Carroll, 1992). Initially identified by PCR-based studies, Ror1 and 2 have been localized in a wide range of organisms including: the fruitfly, *D. melanogaster* (Wilson *et al.*, 1993; Oishi *et al.*, 1997; Pacheco & Macleod, 2008); *C. elegans* (Koga *et al.*, 1999; Forrester, 2002); the mouse, *Mus musculus* (Oishi *et al.*, 1999; DeChiara *et al.*, 2000; MacLeod *et al.*, 2007); and in humans (Masiakowski & Carroll, 1992). The presence of Ror2 in murine small intestine and colon was first reported in 2008 by our laboratory (Pacheco & Macleod, 2008).
Expression studies in mouse models have been critical in solving the mystery that is Ror2 function. Studies of Ror2 knock out (Ror2\(^{-/-}\)) mice have revealed Ror2’s functions in embryonic development. Deletion of the Ror2 gene resulted in embryonic lethality (DeChiara et al, 2000). Though heterozygous Ror2 mutant mice thrived and resembled WT Ror2 littermates, homozygous Ror2 knock out mice died hours after birth (DeChiara et al, 2000). Ror2 has also been linked with processes driving bone development. Ror2\(^{-/-}\) mice exhibit obvious abnormalities in bone formation (Takeuchi et al, 2000). Subsequent expression analysis has revealed that Ror2 is selectively expressed in anlagen of bones, which undergo further ossification to become rib and limb bone structures, and in proliferating chondrocytes of the mouse. Not surprisingly, mutations in Ror2 resulted in incomplete or malformed bones originating from endoossification, such as: shortened proximal long bones of limbs, shortened digits, missing middle phalanges and shortened growth plates (DeChiara et al, 2000).

We have localized Ror2 receptor expression in murine intestinal epithelia from the base of the crypts to the villus tips (Pacheco & Macleod, 2008). (Refer to Figure 2). In contrast, in the murine colon, only the surface epithelia expressed Ror2. Ror2 was also found on some cell types, not identified, in the lamina propria of the small intestine (Pacheco & Macleod, 2008).

Unfortunately, work completed with human Ror2 is not as complete. Two Ror2-linked disorders have been identified clinically: Robinow Syndrome and Brachydactyly
Figure 2. Ror2 expression has been localized in epithelia lining the mouse small intestine and colon. A: Western blotting for Ror2 in epithelial cell lysates, isolated by standard calcium chelation from the jejunum, ileum and large colon. B: Ror2 protein expression, tagged in red, is localized in epithelia lining throughout the jejunum, from the bottoms of the crypts to the tips of villus. In the colon, Ror2 protein expression is restricted to the upper surface epithelia. Figure from Pacheco & Macleod, 2008.
type B (Afzal & Jeffery, 2003). These disorders are discussed in more detail, in later sections. It is worth mentioning that these disease states result from disruptions to putative regions of the Ror2 receptor (Afzal & Jeffery, 2003).

**Ror2 Structure**

Early studies were aimed at not only localizing receptor expression, but purifying and mapping out the specific domains associated with the Ror2 receptor. Like other RTKs, these receptors are single-pass membrane spanning proteins, characterized by an N-terminal ligand-binding extracellular domain and a common C-terminal, intracellular tyrosine kinase domain (Schlessinger, 2000). Ror2 is further characterized by three well conserved extracellular domains: the membrane distal Ig-like domain, the Frizzled-like cysteine-rich domain, and the membrane-proximal kringle domains. Ror2 also expresses well conserved intracellular domains: the membrane-proximal tyrosine kinase domain, and a membrane-distal proline-rich domain, flanked by two serine/threonine rich regions (Forrester, 2002). The domain architecture of Ror2 is summarized in Figure 3.

**Ror2 immunoglobulin domain**

The distal extracellular region of Ror2 is characterized by an Immunoglobulin (Ig) -like domain. Ig domains are often associated with secreted proteins in vertebrates and C. elegans. It is commonly believed that this domain contributes to the maintenance of protein-protein interactions (Forrester, 2002). However, the two Drosophila Rors, Dror and Dnrk lack the Ig-like domain (Oishi et al, 1997). Thus, the precise function, if any, of the Ig-like domain remains unknown (Forrester, 2002).
Figure 3. Schematic representation of the Ror2 constructs used in these experiments. Full length Ror2 is characterized by three extracellular domains (Ig-like, cysteine rich and kringle domains), as well as four conserved intracellular domains (tyrosine kinase, serine/threonine rich -1, proline-rich, serine/threonine rich-2 domains). The various Ror2 constructs are characterized by deletions of specific putative domains of Ror2. The ∆CRD Ror2 contains a deletion of the cysteine-rich domain. In the ∆K Ror2 construct, the kringle domain has been truncated. In RS Ror2, a majority of the tyrosine kinase domain, as well as regions distal to this domain have been deleted. The 5YF Ror2 construct contains five substitution mutations in the ser/thr-1 region, where 5 tyrosine residues have been replaced by phenylalanines. The BDB Ror2 construct contains a deletion of the ser/thr-1, proline and ser/thr-2 rich regions.
Ror2 cysteine rich domain

The cysteine rich domain (CRD) of Ror2, consisting of a conserved sequence of ten cysteines, lies membrane proximal to the Ig domain. A homologous CRD domain is expressed by other Wnt receptors and mediators such as: the Frizzled family and the large class of secreted fizzled related proteins (sFRPs) (Masiakowski & Carroll, 1992; Rehn et al, 1998; Xu & Nusse, 1998). Previously, structure-function studies have demonstrated that this region of the receptor maintained Wnt-receptor binding (Bhanot et al, 1996; Xu & Nusse, 1998).

Ror2 kringle domain

The membrane-proximal kringle domain of Ror2 receptor is also widely expressed in proteases of the blood clotting cascade (Patthy, 1985; Furie & Furie, 1988; Nakamura et al, 1989) and the Torpedo RTK (Jennings et al, 1993). Typically, this region is thought to mediate protein-protein interactions. It is speculated that the kringle domain may contribute to the stabilization of Wnt-Ror2 binding, however its precise function is still unknown (Forrester, 2002; Green et al, 2008).

Ror2 kinase domain

The kinase domain is the characteristic region which is shared among all members of the receptor tyrosine kinase superfamily. Ror2 is no exception; both Ror family members express a conserved YXXDYY sequence within the kinase domain (Pearson & Kemp, 1991; Forrester, 2002). Early characterization studies of the Trk and MuSK
RTKs, reported that this sequence was required for receptor activation (Songyang & Cantley, 1995; Forrester, 2002).

Further investigations have indicated that Ror2 kinase activity was a necessary component of Wnt5a-mediated activation of JNK-signaling in *Xenopus* convergent extension (Schambony & Wedlich, 2007). Nevertheless, other studies have suggested that Wnt5a stimulation may not be required for Ror2 kinase activity (Mikels & Nusse, 2006) while Wnt1 has been unequivocally shown to activate this kinase. There is a consensus that the cell type in which Ror2 is expressed is a determinant of whether Wnt5a stimulates Ror2 tyrosine kinase activity (Green et al, 2008).

**Ror2 proline and serine/threonine rich domains**

Mammalian Ror1 and 2 also contain a series of unique domains in the terminal end of the receptor membrane distal to the intracellular kinase domains. These regions are characterized by two serine/threonine–rich regions, separated by a proline-rich region. This region is not common in all Ror2 orthologs and its precise function, if any, is not known (Forrester, 2002; Green et al, 2008).

Current knowledge of how putative domains of Ror2 mediate Wnt signaling is limited. Though several laboratories have shown that the extracellular CRD facilitated Wnt-Ror interaction (Oishi et al, 2003; Nishita et al, 2006; Li et al, 2008) and others have reported that Ror2 kinase activity mediated Wnt signaling (Yamaguchi et al, 1999; Oishi et al, 2003; Billiard et al, 2005; Schambony & Wedlich, 2007), the specific
functions of other domains of Ror2 are not well characterized. As Ror2-mediated Wnt5a signaling becomes better characterized, such as in the intestine (MacLeod et al, 2007; Pacheco & Macleod, 2008), it is becoming increasingly important that the roles of the other Ror2 domains be examined.

**Disruptions to Ror2 domains lead to specific disease states**

Disruptions in specific domains of Ror2 can result in some serious disorders. At least two human genetic disorders have been linked to mutations in *Ror2*: Recessive Robinow Syndrome (RRS) and autosomal dominant Brachydactyly type B (BDB) (Afzal et al, 2000; Oldridge et al, 2000; van Bokhoven et al, 2000; Patton & Afzal, 2002; Afzal & Jeffery, 2003). Interestingly, though both disorders are the direct result of mutations in the same gene (*Ror2*), the genetic and phenotypic profiles associated with each disease states are quite different (Afzal & Jeffery, 2003). Patients with RRS exhibit short stature, stunted limbs, brachydactyly and abnormal morphogenesis of the face, which resembles that of a fetus (Afzal et al, 2000; van Bokhoven et al, 2000; Patton & Afzal, 2002). Meanwhile, symptoms of BDB include shortened fingers and toes (Afzal et al, 2000; Schwabe et al, 2000).

The genetic basis of RRS involves homozygous missense, nonsense and frameshift mutations scattered throughout Ror2 exons 5, 7, 8, and 9 encoding the regions associated with the cysteine-rich domain, kringle domain, the region lying between the kringle and tyrosine kinase domain as well as the tyrosine kinase domain, respectively (Afzal & Jeffery, 2003). These mutations result in either the premature termination of the
3’ end of Ror2 or truncations in the CRD, kringle, and tyrosine kinase domains (van Bokhoven et al, 2000; Afzal & Jeffery, 2003). These deletions typically result in loss-of function for the Ror2 receptor (van Bokhoven et al, 2000; Afzal & Jeffery, 2003).

In contrast, genetic truncation mutations associated with BDB are subdivided into mutations which cluster distal to the tyrosine kinase domain, and mutations which lie proximal to the TKD (Oldridge et al, 2000). Previous reports where RT-PCR analysis was performed on blood samples collected from patients suffering from BDB found that mutations occurred within a seven amino acid sequence found between the tyrosine kinase domain and the first serine/threonine-rich domain. These mutations subsequently truncated the protein prematurely at the C-terminus (Oldridge et al, 2000).

It is obvious that specific domains of the Ror2 receptor serve critical functions in transducing extracellular signals, as disruptions in putative regions of the receptor have some very severe clinical consequences. Classical studies have focused on the effect of Ror2 overexpression in mediating cellular processes. Recent work has been aimed at elucidating the specific ligands and domains involved in Ror2 receptor activation. For instance, our primary focus has been the characterization of Wnt5a-induced activation of Ror2, and the resulting downstream signaling cascades in the murine and human colon (MacLeod et al, 2007; Pacheco & Macleod, 2008). Future studies will involve elucidating which functional domains of the receptors are required for Wnt5a-Ror2 signaling in the intestine.
EXTRACELLULAR-SIGNAL REGULATED KINASE 1 AND 2

Extracellular-signal regulated kinases 1 and 2 (p44ERK1 and p42ERK2, or known collectively as ERK1/2) are members of the mitogen-activated protein kinase (MAPK) family (McKay & Morrison, 2007). MAPKs are highly conserved mediators of vital biological developmental processes including cell growth, differentiation, and cell death (Murphy & Blenis, 2006). ERK1/2 is no exception (Torii et al, 2004). Previous investigations have demonstrated that ERK1 knock-out mice exhibited stunted thymocyte maturation (Pages et al, 1999; Torii et al, 2004). Furthermore, other studies have reported that ERK2-deficiency induced embryonic lethality in mice, as ERK2\(^{-/-}\) embryos failed to develop mesoderm (Yao et al, 2003) and the ectoplacental cone (Torii et al, 2004), structures necessary for placenta formation.

As its name suggests, ERK1/2 is stimulated by a variety of extracellular stimuli including growth factors, cytokines, and agonists of G-protein coupled receptors (GPCRs) (Chen et al, 2001). These ligands first induce the phosphorylation of small G protein Ras-Raf family members, such as Raf-1, A-Raf or B-Raf. This activated Raf subsequently activates MEK 1 or 2, direct mediators of ERK1/2 phosphorylation (Murphy & Blenis, 2006). Upon phosphorylation, ERK1/2 dissociates from its stabilization complex in the cytoplasm and translocates to the nucleus. Nuclear ERK1/2 binds to transcriptional factors, such as Elk-1 which can induce the transcription of a variety of genes (Torii et al, 2004). Recent studies using gastric cancer cells, which begin to differentiate and resemble enterocytes, have reported that Elk-1 activation triggers promoter activation of intestinal-specific gene, villin (Rieder et al, 2005).
Villin is a 92.5 kDa, actin-binding cytoskeletal protein associated with the apex and axial bundle structures of intestinal microvilli (Khurana & George, 2008). It is an epithelial cell protein that regulates cell migration, death and epithelial-to-mesenchymal transition (Khurana & George, 2008). Its specific expression and numerous functions underscore its significance in enterocyte cell function (George et al, 2007). Surprisingly, very little is understood about how villin is produced in the intestine.

Villin Expression

Villin has been localized in epithelial cells lining: exocrine glands, the thymus, renal and urogenital organs, as well as in intestinal mucosa lining the embryonic intestine. In the mouse, the villin gene is first detected at 9 days post coitum (dpc), in the developing hindgut ectoderm (Maunoury et al, 1988; Ezzell et al, 1989). Expression of villin rapidly migrates from the small and large intestinal ectoderm to the distal stomach. By day 14-15 dpc, a stage characterized by rapid intestinal epithelia remodeling and villus formation, villin gene expression is noticeably upregulated (Maunoury et al, 1988; Maunoury et al, 1992). By day 16 dpc and afterwards, a cellular monolayer which constitutes the pyloric monolayer is evident; at which point, levels of villin gene expression are undetectable in stomach cells, in contrast to heightened villin expression levels in the established intestine (Madison et al, 2002). In the developed small intestine, villin protein expression is detected along the entire crypt-villus axis, with levels of villin protein expression increasingly enhanced towards the villus tip (Maunoury et al, 1992). Interestingly, we have previously localized Ror2, which we presently argue is a mediator
of villin expression, in murine intestinal epithelia along the entire crypt-villus axis in the small intestine (Pacheco & Macleod, 2008). (Refer to Figure 2). Villin’s unique expression gradient in the developing intestinal epithelia reflects that as enterocytes differentiate, villin protein expression increases. Because villin is expressed at low levels in proliferative stem cells of intestinal crypts, it is commonly regarded an early marker for committed epithelial cells (Khurana & George, 2008). However, villin, in addition to being a marker of early differentiation plays several key roles in organizing actin filaments and apoptotic regulation in the developed intestine.

Traditional Roles of Villin: an actin severing and binding protein

Microvilli are unique apical cell surface extensions of epithelial cells which facilitate enhanced absorptive capacity of these cells. An accumulating body of evidence has shown that villin mediates cytoskeletal organization in the intestine (Coluccio & Bretsch, 1989). Purification and immunofluorescence has confirmed that villin is a major protein associated with the actin filaments stabilizing the microvillus core (Bretscher & Weber, 1978; Bretscher & Weber, 1979). Studies have shown that the knock down of endogenous villin expression in intestinal epithelial cells induced low rates of brush border assembly in murine models (Costa de Beauregard et al, 1995). Significant changes in microvilli formation were observed in villin knock-out mice: the intestinal villus structures were noticeably thicker and less organized (Pinson et al, 1998). Villin is also a necessary component of cytoskeletal remodeling. A recent investigation reported that intestinal mucosa of villin knock-out mice remain intact following Shigella flexneri-infection. S. flexneri is an invasive bacterium which induces destruction of
cytoskeletal proteins, such as actin, to penetrate through host tissue (Athman et al., 2005). These findings highlight a potential role for villin in cytoskeletal maintenance of an intact brush border.

Villin: A novel role in epithelial cell homeostasis

There is an emerging body of work showing that villin protein, in addition to maintaining actin organization, plays a role in the regulation of apoptosis in the developing intestinal epithelia (Wang et al., 2008). Intestinal epithelial cells undergo high rates of cellular proliferation accompanied by high rates of apoptosis. These processes must be balanced for intestinal epithelial cells to maintain their absorptive and structural functions.

Investigations involving homologous villin (−/−) knock out mice have suggested that villin functions as an anti-apoptotic protein. Profiles of villin−/− mice indicated that cellular apoptosis was increased in these animals compared to WT control mice (Khurana & George, 2008). In studies with mice and patients, decreased villin expression has been associated with the increased severity of dextran sodium sulfate (DSS)-induced colitis or inflammatory bowel diseases, respectively (Kersting et al., 2004; Wang et al., 2008). This correlation is thought to be due to heightened apoptosis in the gastrointestinal epithelium, leading to a compromised intestinal barrier (Khurana & George, 2008).

In contrast to decreased villin expression, enhanced villin expression has been linked to conditions such as chronic gastritis (Blaser & Parsonnet, 1994) and Barrett’s
esophagus (Chen et al, 2008). Both disease states are characterized by an inflammatory-mediated transformation from normal (gastric or esophageal) lining to simple columnar epithelia, which resemble cells found lining the intestine. This pathological phenotype is accompanied by the expression of intestinal-specific markers, including villin (Tsukamoto et al, 2004; Chen et al, 2008). Thus, elucidating the mechanisms which regulate villin expression in vivo will enhance our current understanding of the processes which mediate normal and disease development in the intestine.

In spite of the role of villin in regulating actin filament organization and apoptosis, very little is understood about how villin gene and protein expression are regulated in normal intestinal epithelia. In diseases where the tissue begins to differentiate as intestinal epithelia, such as H. pylori infection in gastric cells which can lead to gastric cancer, the expression of a villin promoter was increased by Elk-1, a downstream nuclear transcription factor which is activated by ERK1/2 phosphorylation (Rieder et al, 2005). This finding suggested to us that the activation of ERK1/2 might, in the appropriate conditions, influence villin expression in intestinal epithelia.

The intestinal epithelium is a model of choice to study the regulation of signal transduction pathways during differentiation because it is a constantly differentiating system with a rapid and orderly turnover of cells (Traber, 1994). Differentiation of cells starts with a sudden loss of their proliferative ability when they reach the upper third of the crypts; this process is characterized by marked changes in cell ultrastructure and by the expression of several new cell products that include expression of the gut
diasaccharidase sucrase-isomaltase (Vachon & Beaulieu, 1995). *In vitro* and *in vivo* analysis has suggested that pERK1/2 MAPK activities are necessary for both cell cycle progression and differentiation of intestinal epithelia (Aliaga *et al*, 1999; Boucher *et al*, 2005). Overexpression of wild type or constitutively active MEK-1, the immediate upstream activator of ERK1/2, in nonimmortalized human fetal-derived intestinal epithelial cells led to arrest of cellular proliferation through induction of the cyclin-dependent kinase inhibitors (Boucher *et al*, 2005). Recent analysis of the MAPK activities required for intestinal differentiation have focused on p38 MAPK rather than ERK1/2 since the former will phosphorylate and activate CDX2, a major transcription factor determinant of epithelial differentiation (Boucher & Rivard, 2003; Boucher *et al*, 2005).

**Hypothesis and Overview**

Based on the findings presented in these studies, we hypothesized that Wnt5a stimulates the Ror2 receptor expressed in intestinal epithelia, inducing ERK1/2 MAPK signaling and subsequent villin protein production. To test this hypothesis, we examined, by Western blotting, whether Wnt5a challenge in Ror2 overexpressing HT29 intestinal cells and fetally-derived, non-transformed human intestinal epithelial cells would stimulate ERK1/2 phosphorylation and villin protein. We went on to determine whether receptor silencing, mutations and deletions to conserved regions of Ror2; or the addition of various MAPK inhibitors, alternative Wnt family members and ERK1/2 agonists would affect villin protein production in these cells.
Chapter 2
Materials and Methods

Materials

Inhibitors such as PD 098059 (MEK-1 inhibitor), SB 203580 (a p38 MAPK inhibitor), Bisindolylmaleimide I (inhibitor of PKC isotypes: -α, -β, -γ, -δ, -ε) and D4476 (a casein kinase I inhibitor) were purchased from EMD Calbiochem-Novabiochem (San Diego CA). Other investigations have demonstrated that at the following concentrations: 10µM PD 098059 (Alessi et al, 1995; Aliaga et al, 1999), 10µM SB 203580 (Clerk et al, 1998; Zhou et al, 2005; Tu & Perdue, 2006), 1µM Bisindolylmaleimide I (Toullec et al, 1991; Vayro & Silverman, 1999) and 100µM D4476 (Rena et al, 2004; Bryja et al, 2007), ERK MAPK, p38 MAPK, PKC and CK1 and inhibited, respectively, in a variety of cell lines.

The restriction enzyme, xhoI, and appropriate buffer were purchased from Invitrogen (Burlington ON). Restriction digest was completed consistent with manufacturer’s instructions. Recombinant mouse Wnt3a and recombinant mouse Wnt5a were obtained from R&D Systems (Minneapolis MN).

Cell Culture

The human colorectal adenocarcinoma HT29 cell line and mouse L-cells were purchased from American Tissue and Cell Culture (Rockville, MD). Fetally-derived, non-transformed, human intestinal epithelial cells (HIEC) were obtained from Dr. Boudreau (University de Sherbrooke, Sherbrooke, QC). The HT29 and L-cells were
grown in Dulbecco’s modified eagle media (DMEM) supplemented with 10% fetal bovine serum (FBS), and 0.05% Penstrep (all purchased from Invitrogen, Carsbad CA). The non-transformed fetal HIECs were cultured in OPTI-MEM supplemented with 4% FBS (Invitrogen), 0.05% L-glutamine (Invitrogen), 0.02% EGF (Sigma, Oakville ON) and 0.05% HEPES (BioShop, Burlington ON).

All cells were maintained at 37°C, in 95% air, 5% CO₂ atmospheric conditions. HT29 cells and HIECs were passaged every 4-5 days, with 0.25% and 0.05% Trypsin (Invitrogen Carsbad CA), respectively. Only HT29 cells passaged within the first 6 weeks of initial thawing and passage 16-22 HIECs were used. Early-passage cells were used to prevent spontaneous differentiation and transformation that is often observed in late-passage carcinoma-derived intestinal epithelial cell lines (Pinto et al, 1983; Vachon & Beaulieu, 1992).

Preparation of Wnt5a Conditioned Media

Mouse L-cells, stably transfected with Wnt5a plasmid, were seeded on 10 cm plates and supplemented with 10% DMEM, as previously described. After 24 hours, cells were then incubated in 0.5mM Ca²⁺ DMEM containing 0.1% L-Glutamine and 0.05% Penstrep and supplemented with 0.2% bovine serum albumin. This media was collected 48 and 96 hours afterwards. Upon collection, media was centrifuged at 1000 RPM for 5 minutes and the remaining supernatant was isolated, divided into aliquots, and kept at -30°C for later use.
**Transient Transfections**

All DNA plasmid transfections completed for subsequent Western blot analysis and RT-PCR were performed using the Superfect transfection reagent, according to the manufacturer’s instructions (Qiagen, Mississauga, ON). The wild-type Ror2 plasmid (WT Ror2) and Ror2 constructs containing deletions of: the cysteine rich domain, aa 170-300 (ΔCRD Ror2); the kringle domain, aa 316-395 (ΔK Ror2); regions including and distal to the tyrosine kinase domain, aa 502-944 (ΔRS Ror2); regions including and distal to the tail of tyrosine kinase domain, aa 749-944, (BDB Ror2) and a Ror2 construct containing substitutions of phenylalanine for five tyrosine residues in the first serine/threonine rich domain, tyrosines 818, 824, 830, 833, 838, (5YF Ror2) were generous gifts from Dr. Minami (Kobe University, Kobe, Japan). The described constructs are summarized in Figure 3. The short interfering RNA duplex against Ror2 (siRNA<sub>Ror2</sub>) was comprised of 4 target sequences: 5′-GUCAUCGCUUGCCUGUUC-3′, 5′-ACCAACCCUUGAGCAUGA-3′, 5′-GCAAUGUGCUGGUGUACGA-3’, 5′-GAACCGGACUAUUUAUGUG -3’ and was commercially purchased (Dharmacon, Hornby ON).

**Western Blot Analysis**

HT-29 cells and HIECs were seeded on 6-well plates; 24 hours later, these cells were transfected with 2.0µg of a Ror2 plasmid construct (as previously described) or with 200nM of siRNA<sub>Ror2</sub> (previously described) using Superfect (Qiagen), according to manufacturer’s instructions. The plates were serum starved in 0.5mM Ca<sup>2+</sup> DMEM supplemented with 0.2% BSA, 0.1% L-glutamine and 0.05% penstrep, as described
previously. After 18 hours of serum starvation, the cells were incubated with Wnt5a conditioned media for 0, 2, 5, 10, 30, 60 minutes. Following incubation, cells were rinsed once with ice-cold PBS containing 1mM sodium orthovanadate and 25mM sodium fluoride. Cells were subsequently lysed with a buffer (consisting of 20mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 25mM NaF, 1% Triton X-100, 10% glycerol, 1mM dithiothreitol, 1mM sodium orthovanadate and a cocktail of protease inhibitors containing 10uM each of pepstatin, soybean trypsin inhibitor, leupeptin, aprotinin, calmodulin). Lysates were sonicated at 40% maximum amplitude (maximum voltage of sonicator: 117V, 20Hz, 4.0A) for 5s to liberate intracellular protein (Sonic Dismembrator Model 500, Fisher Scientific; Pittsburgh PA). Samples were then centrifuged at 14 000 RPM for 10 minutes at 4°C (MicroElectron 21L, Thermo Electron Corporation; West Sussex UK), divided into aliquots, and kept at -70°C.

Protein concentrations were quantified by measuring colour absorbance in lysates, with a spectrophotometer (EL 800 Universal Microplate Reader, Biotek Instruments Inc., Winooski VT), where Protein Assay Dye Reagent Concentrate had been added (Bio-rad, Hercules, CA). Total 40-60 µg amounts of lysates were loaded onto an 8% (for villin immunoblotting), 10% (for pERK1/2 or ERK1/2 immunoblotting), or 12% (for Ror2 or FLAG-tag, N-DYKDDDDK-C, immunoblotting) Bisacrylamide gel, along with a 250 kD dual colour protein ladder (Precision Plus, Bio-Rad). Proteins were transferred electrophoretically onto a polyvinylidene difluoride (PDVF) membrane (Millipore, Bedford MA).
Following transfer, membranes were blocked for 1 hour in 5% milk in 1X Tris Buffered Saline and 0.1% Tween-20 (1XTBS-T). To detect expression of phosphorylated-ERK1/2 (pERK1/2), total amounts of ERK1/2 (ERK1/2), Ror2, FLAG-tag and villin protein, membranes were incubated with a polyclonal rabbit antibody specific for pERK1/2, ERK1/2, Ror2, FLAG-tag, or villin in 1XTBS-T with 5% BSA in a 1:1000 dilution, (antibodies purchased from Cell Signaling Technology Inc., Danvers MA). Membranes were left at 4°C, shaking overnight. To detect β-actin protein expression, membranes were incubated with a mouse antibody specific for β-actin (Sigma, St Louis MO) diluted to 1:10000 in 1XTBS-T, under similar conditions. Membranes were subsequently washed 3 times for 5 minutes in 1XTBS-T and incubated with an anti-rabbit or anti-mouse secondary conjugated to horseradish peroxidase (Sigma, St Louis, MO) in 1XTBS-T containing 5% milk for 1 h. After (3) 5-minute washes in 1XTBS-T, protein bands were visualized by incubating the membrane with luminol and peroxide substrates from a commercially purchased chemiluminescence kit (SuperSignal, Pierce Chemical; Rockford IL).

Reverse Transcriptase – Polymerase Chain Reaction

A two-step RT-PCR for villin was performed to assess for differential gene expression. RNA was extracted from Wnt5a-challenged, Ror2-overexpressing HT29 cells and non-transformed fetal HIECs. RNA was isolated using TRIzol, according to manufacturer’s instructions (Invitrogen). RNA was isolated with chloroform and centrifugation at 10 000 RPM for 10 minutes at 4°C. An RNA precipitate was formed by storing the remaining aqueous solution in isopropanol at -30°C overnight. After washing
with 70% ice-cold ethanol, RNA pellets were resuspended in water, and final amounts were quantified using a spectrophotometer (Biophotometer, Eppendorf).

To synthesize cDNA, total 1.0 µg of RNA was combined with 5 mM dNTP mix, 10 µM oligo-dT primer, 10 units RNase inhibitor and 4 units Omniscript reverse transcriptase (from Omniscript kit purchased from Qiagen; Mississauga ON), and kept at 37°C for 1 h with the use of a Mastercycler (Eppendorf, Hamburg, Germany). Total 1.0 µg of cDNA, from the resulting cDNA synthesis, was combined with the appropriate primers for human villin along with a reverse transcriptase enzyme, Taq Polymerase, (from a purchased Reverse-Transcriptase kit by Qiagen; Mississauga ON). Primers for villin are sense 5’-TAG CTG TGG TTG TAA AGC AGT ACC – 3’ and antisense 5’-GGT ATC ATC TTT CTG AAG GAA TAG G-3’. The PCR cycle parameters were as follows: an initial 60s initial denaturation period at 104°C; 30 amplification cycles consisting of a 30s-denaturation period at 104°C; a 30s-annealing period at 55°C, and a 1-min elongation period at 72°C; a final 10-min elongation period at 72°C. The resulting PCR product was loaded onto a 1% agarose gel, stained with the visualizing agent, Ethidium Bromide, along with a 100 bp DNA ladder. After subjecting the gel with a 120mV current for 45 minutes, the DNA bands were visualized by exposing the gel to UV light.

Densitometry

Densitometry was performed on X-rays of developed Western blot experiments. X-rays were scanned by means of the EPSON Stylus CX4800 desktop scanner. Images
were converted to gray scale using Arc Soft Photostudio (version 5.5.0.58). The densities of individual bands were measured using ImageJ (version 1.40g). Measured experimental densities were normalized against the density of the appropriate loading control. These corrected densities were normalized to the most intense band in the given experiment.

Statistics

Data are presented as means +/- Standard Error of at least three separate experiments. Student t-tests were performed on data, where appropriate. Statistical significance was defined as: P < 0.05.
Chapter 3

Results

Wnt5a interaction with Ror2 stimulated ERK1/2 phosphorylation in adenocarcinoma-derived HT29 cells

We first determined whether Wnt5a and Ror2 would stimulate the ERK1/2 (p44/42) MAPK cascade in adenocarcinoma-derived HT29 intestinal epithelial cells (Figure 4). ERK1/2 activation is characterized by dual phosphorylation of both the ERK1 and ERK2 (p44 and p42) isotypes. Approximately 24 h after seeding on 6-well plates, cells were transfected with 2.0 µg of (full length) WT Ror2 DNA plasmid, and subsequently challenged with Wnt5a conditioned media (Wnt5a CM) for 0, 2, 5, 10, 30, 60 minutes. Upon screening for ERK1/2 phosphorylation via Western blot analysis, we found that levels of both p44 and p42 phosphorylation transiently and robustly increased between 2 to 30 minutes of Wnt5a CM treatment. Maximal ERK 1 and 2 phosphorylation occurred at 5-10 minutes, before diminishing to baseline levels at 60 minutes (Figure 4A, top panel). In contrast, in mock-transfected HT29 cells, which were Wnt5a-challenged in a similar manner, levels of ERK1/2 phosphorylation were substantially attenuated; dual-ERK1/2 phosphorylation was observed only at 10 minutes (Figure 4A, bottom panel).

Because ERK1/2 is an extracellular signal-regulated MAPK, and may be activated by a diverse set of agonists including cytokines, growth factors, and serum elements, we elected to determine whether the serum elements in the medium alone were sufficient to induce robust increases in ERK1/2 phosphorylation in these cells. Through
Figure 4. Wnt5a stimulation of Ror2 induced ERK 1/2 (p44 and p42) phosphorylation in HT29 cells. A: HT29 cells were grown in DMEM supplemented with 10% FBS, transfected with WT Ror2 or no plasmid. Cells were serum starved for 18 h. Cells were challenged with Wnt5a CM for 0, 2, 5, 10, 30, 60 minutes. ERK1/2 phosphorylation was robustly and transiently increased in cells overexpressing WT Ror2 upon Wnt5a conditioned media challenge over the course of 60 minutes. In mock-transfected cells, similar Wnt5a treatment induced low levels of ERK 2 (p42) phosphorylation. Western blot analysis screening for total (phosphorylated and unphosphorylated) ERK1/2 expression was included as loading control. B: Serum elements in the media alone were not sufficient to induce ERK1/2 phosphorylation. In cells where Ror2 receptor expression had been silenced, and subsequently treated with serum-free low-calcium media, very low levels of ERK 2 (p42) were detected. A similar pattern of low ERK2 phosphorylation was detected in untransfected HT29 cells. Western immunoblotting for total ERK1/2 was included as an internal loading control. Blots shown are representative of 3 separate experiments with similar results.
Western blot analysis screening for phosphorylated ERK-1 and -2 (pERK1/2), low levels of pERK2 were observed in cells where Ror2 receptor expression had been silenced and subsequently treated with minimum serum (0.2% BSA) media, which does not contain Wnt5a (Figure 4B, top panel), or where cells had been mock-transfected, and treated in a similar manner (Figure 4B; bottom panel).

To verify whether the Ror2 receptor is a necessary mediator of Wnt5a-induced ERK1/2 activation, we completed experiments employing the use of a short-interfering RNA duplex aimed specifically against the Ror2 (siRNA_Ror2). We have shown that in response to increasing transfection concentrations of siRNA_Ror2 in HT29 cells, Ror2 receptor expression was dose-dependently downregulated (Figure 5A). At 200nM concentrations of siRNA_Ror2, immunoreactive Ror2 expression was barely detectable in cell lysates; we used this concentration of siRNA_Ror2 for subsequent experiments.

We went on to compare ERK1/2 phosphorylation in HT29 cells, which had been transfected with WT Ror2 or siRNA_Ror2 and subsequently treated with Wnt5a CM. As previously shown, we observed that in WT Ror2-transfected cells, Wnt5a challenge induced a transient increase in dual ERK1/2 phosphorylation (Figure 5B, top panel). However, upon similar challenge with Wnt5a CM in siRor2-transfected cells, ERK1/2 phosphorylation was attenuated. The ERK1/2 phosphorylation was restricted to the ERK2 (p42) isotype. Similar low levels of ERK1/2 phosphorylation were observed previously in mock-transfected cells treated with Wnt5a CM (Figure 4B, bottom panel).
Figure 5. Wnt5a challenge in HT29 cells, where the Ror2 receptor expression has been silenced, attenuated ERK1/2 (p44/p42) phosphorylation. A: Immunoblotting for Ror2 demonstrated that increasing transfection concentrations of siRNA against the Ror2 dose-dependently downregulated Ror2 expression in HT29 cells. Screening for immunoreactive β-actin was used as an internal loading control. B: HT29 cells were transfected with siRNA (200nM) Ror2, and treated with Wnt5a CM, as previously described. Immunoreactive ERK1/2 was attenuated in siRNA Ror2-transfected cells, compared to control (Wnt5a CM-challenged WT Ror2 overexpressing) cells. Western blotting for total amounts of ERK1/2 was included to demonstrate equal loading. These results are representative of 3 separately performed experiments, which had similar results.
From these experiments, we conclude that activation of both ERK1/2 (p44/p42) occurred transiently in response to Wnt5a addition to Ror2 overexpressing epithelial cells. Furthermore, these findings suggest that medium change or knocking down expression of Ror2 have a comparable effect – stimulating p42 activation exclusively. We therefore elected to use the transient activation of both p44 and p42 as the index of ERK1/2 phosphorylation.

**Wnt5a-activation of ERK1/2 induced villin protein expression in adenocarcinoma cells**

Earlier studies using gastric cancer cells have reported that activation of the ERK1/2 MAPK cascade can induce Elk-1 mediated villin promoter activity (Rieder et al., 2005). Because Elk-1 is activated by phosphorlyated ERK1/2, we went on to determine whether Wnt5a-Ror2 induced ERK1/2 activation would enhance villin expression in HT29 cells. We assessed villin expression by RT-PCR and Western analysis, on HT29 cells overexpressing Ror2 and challenged with Wnt5a CM for 18 or 24 hours, respectively. The sequence of the RT-PCR product from the described experiment was:

5’-CGATATTAGTCCTACACCAATTGAAGTGAAATTTTGCAGATGTGCCTATGAGCACAAACTTCTGTGGCAAATGCCAGTTTTGTTTAATAATGTACCTATTCCTCAGAAAGATGATA -3’

Using the BLAST algorithm, this sequence was shown to be 100% homologous with human villin mRNA. In cells that were mock-transfected and/or left untreated, basal levels of villin transcript expression were low. In contrast, when WT Ror2-
overexpressing cells were challenged with Wnt5a CM for 24h, levels of villin amplicon were substantially upregulated. This upregulated villin transcript expression was lost in the presence of PD 098059 (10 µM), a specific MEK-1 inhibitor, which is directly upstream of the ERK1/2 MAPK cascade (Figure 6A).

There was a noticeable increase in villin protein production in WT Ror2 overexpressing cells challenged with Wnt5a CM for 24 hours. In contrast, villin protein expression was low in mock-transfected and/or untreated cells (Figure 6B). Again, this increased villin protein expression was lost in similarly treated WT Ror2-overexpressing cells, in the presence of PD 098059 (10 µM) (Figure 6B).

To assess whether other intracellular effectors could be involved in Wnt5a-stimulated villin protein production, we screened for immunoreactive villin in cells treated with inhibitors of PKC and p38 MAPK. Again, we observed low levels of villin protein in untreated or mock-transfected HT29 cells. Upon Wnt5a CM challenge in WT-Ror2 overexpressing cells, villin protein production was noticeably increased. In the presence of Bisindolylmaleimide I (1µM; an inhibitor of PKC -α, -β, -γ, -δ, -ε ), and SB 203580 (10µM; a specific inhibitor of all p38 isotypes), levels of villin protein production remained increased in Wnt5a CM-challenged Ror2-overexpressing cells. However, the addition of D4476 (100 µM; a CKI inhibitor) prevented Wnt5a-induced villin expression in WT Ror2 expressing cells (Figure 6C). Taken together, these results suggest that Wnt5a-Ror2 induced ERK1/2 activation, which enhanced villin protein
Figure 6. Wnt5a interaction with Ror2 upregulated villin transcript and increased protein expression via an ERK1/2 dependent mechanism. A: Villin transcript expression was normally low in cells which were: mock-transfected and untreated, mock-transfected and incubated in Wnt5a for 18 h, or transfected with WT Ror2 plasmid and left untreated. Wnt5a challenge in Ror2 overexpressing HT29 cells induced a substantial increase in villin transcript expression. This increased villin transcript expression was lost in the presence of a MEK-1 inhibitor (PD 098059; 1µM). RT-PCR analysis screening for GAPDH was included as an internal loading control. B: Villin protein expression was upregulated in WT Ror2–transfected cells treated with Wnt5a conditioned media for 24 h, compared to mock-transfected and/or untreated cells. In the presence of a specific MEK-1 inhibitor (PD 098059; 1µM), the increased villin protein expression was lost. Western blotting for β-actin was performed as an internal loading control. C: Inhibitors of PKC (Bisindolylmaleimide I; 1 µM) and p38 (SB 203580; 1µM) did not, but a CKI inhibitor (D4476; 100 µM) did prevent Wnt5a-induced villin protein increases. Immunoreactive villin, at levels similar to that of Wnt5a-treated WT-Ror2–transfected HT29 control cells, were detected in cells treated in a similar manner in the presence of a Bisindolylamlemide I and SB 203580. Wnt5a-stimulated villin protein was prevented with the addition of D4476. Screening for immunoreactive β-actin in cell lysates was conducted to show equal loading. These Western blots are representative of 3 individually performed experiments.
production in HT29 cells. This Wnt5a-induced ERK1/2 phosphorylation also required CKI involvement. However, PKC and p38 MAPK did not appear to mediate this effect.

**Wnt5a-induced ERK1/2 activation and subsequent villin protein production required specific functional regions of Ror2**

Having established Ror2 functionality in mediating Wnt5a’s induction of ERK1/2, we went on to elucidate the specific regions of the receptor necessary in mediating this affect. By restriction digestion with xhoI, we confirmed that the insert of the RS Ror2 plasmid construct contained fewer base pairs than that of the full length WT Ror2 plasmid insert (Figure 7A), consistent with previous reports (Kani et al, 2004). Western blot analysis screening for FLAG (N-DYKDDDDK-C) found that the expressed Ror2 receptor in RS Ror2 transfected cells had a lower molecular weight than the receptor expressed in WT Ror2 – transfected cells. These results were consistent with previous reports and confirmed the authenticity of the constructs used in this study (Kani et al, 2004).

To verify which domains of Ror2 are necessary for induction of ERK1/2 phosphorylation, we completed further Wnt5a time course experiments, assessing ERK1/2 phosphorylation via Western blot analysis, in HT29 cells overexpressing truncated or mutated Ror2 constructs. A set of control cells, which overexpress WT Ror2, were run concurrently with each experiment. Both experimental and control cells were treated with Wnt5a CM for 0, 2, 5, 10, 30 and 60 min. Experimental and control
Figure 7. Western blot and restriction digestion analysis of WT Ror2 and RS Ror2 construct. A: Restriction digestion of WT Ror2 and RS Ror2 constructs showed that insert size of the RS Ror2 plasmid contained fewer base pairs than that of the full length WT Ror2. B: Immunoblotting for FLAG tag showed that the expressed Ror2 was a lower molecular weight in RS Ror2 transfected cells compared to the expressed Ror2 in WT Ror2 transfected cells.
lysates were run concurrently on acrylamide gels and transferred onto PDVF membranes within the same apparatus and exposed on the same piece of film to ensure consistency. In cells expressing a Ror2 receptor where the cysteine rich domain has been truncated (ΔCRD Ror2), or the kringle domain has been deleted (ΔK Ror2), or a majority of the tyrosine kinase domain and regions distal to this domain have been deleted (RS Ror2), or where 5 tyrosine residues of the serine/threonine-1 domain had been substituted with phenylalanines (5YF Ror2), levels of Wnt5a-induced ERK1/2 phosphorylation were substantially attenuated compared to WT Ror2-transfected cells (Figure 8A, B, C, D). In all cases, ERK1/2 phosphorylation was restricted to the p42 isotype in these truncated or mutant Ror2-expressing cells, comparable to the ERK1/2 phosphorylation observed in similarly treated cells where the Ror2 had been knocked down by siRNA Ror2 (Figure 5B).

In contrast, in cells expressing a BDB Ror2 receptor, where only the distal proline and serine/threonine rich regions of the receptor had been deleted, levels of both ERK1/2 (p44 and p42) robustly increased over the course of an hour. The pattern of Wnt5a-induced ERK1/2 phosphorylation in these BDB Ror2-expressing cells was similar to that of control WT Ror2 – transfected cells (Figure 8E).

Densitometry confirmed that the intensity of ERK1/2 phosphorylation in WT Ror2-transfected control cells incubated with Wnt5a CM for 10 minutes was significantly increased compared to baseline. Additionally, the degree of ERK1/2 phosphorylation was significantly greater in Wnt5a-challenged WT Ror2 HT29 cells compared to similarly treated cells expressing ΔCRD, ΔK, RS or 5YF Ror2 (Figures 8 F, G, H, I).
Figure 8. Levels of Wnt5a-induced ERK1/2 phosphorylation were diminished or conserved in HT29 cells, depending on type of Ror2 construct expressed. A-D: ERK1/2 phosphorylation was attenuated in Wnt5a challenged cells transfected with: ΔCRD Ror2, (where the cysteine-rich domain of Ror2 was been deleted); ΔK Ror2 (characterized by a deletion of the kringle domain), RS Ror2 (which contained a truncation of regions distal to and including a major portion of the tyrosine kinase domain); or 5YF Ror2 (where five tyrosine residues of the ser/thr-1 region had been substituted with phenylalanines). E: However, Wnt5a-treated HT29 cells transfected with BDB Ror2 (where the entire proline
and serine rich regions was deleted), display a similar level and pattern of ERK1/2 phosphorylation compared to WT control cells. Screening for immunoreactive total ERK1/2 was included as an internal loading control. The blots discussed are representative of 3 separate experiments with similar results. F-I: Densitometry confirmed that maximal ERK1/2 phosphorylation in Wnt5a-challenged WT Ror2 control cells consistently occurred at 10 minutes. Additionally, maximal ERK1/2 phosphorylation was significantly more intense compared to baseline and compared to ERK1/2 phosphorylation in similarly treated cells expressing a truncated or mutated Ror2. J: The pattern and degree of ERK1/2 phosphorylation was conserved in BDB Ror2 transfected cells challenged with Wnt5a. Results are expressed as means +/- SD of three independently performed experiments. *P < 0.05 compared to 10min of Wnt5a CM incubation in truncated or mutant Ror2-transfected cells. †P < 0.05 compared to 0min of Wnt5a incubation in WT Ror2-transfected cells.
Densitometry on three separate ERK1/2 time course experiments confirmed that the
degree and pattern of ERK1/2 activation in Wnt5a-treated BDB Ror2 cells resembled that
of similarly treated control cells (Figure 8J).

We then assessed levels of villin protein production in HT29 cells transfected
with a Ror2 receptor where the cysteine rich domain has been deleted (ΔCRD Ror2), five
tyrosine residues on the ser/thr-1 domain had been substitution-mutated (5YF Ror2), or
where the proline/serine rich regions of the receptor had been deleted (BDB Ror2).
Immunoblotting for villin demonstrated that in mock-transfected or unchallenged WT-
Ror2 expressing cells, levels of villin protein production were low. However, in WT
Ror2-overexpressing cells challenged with Wnt5a CM for 24 hours, levels of villin
protein production were increased. In contrast, in ΔCRD Ror2 and 5YF Ror2
overexpressing cells, treatment with Wnt5a CM did not enhance villin protein production
(Figure 9A). However, in cells where the proline and serine/threonine rich domains of
the Ror2 receptor had been deleted (BDB-Ror2), upon Wnt5a challenge, levels of villin
protein production were increased (Figure 9B).

Consistent with previous findings, our work suggested that the extracellular
cysteine rich domain is a necessary component of Wnt5a-induced ERK1/2 activation and
subsequent villin protein production. Additionally, these experiments also show that
despite the fact that both 5YF and BDB Ror2 rendered the ser/thr-1 domain of the
receptor inactive, either by mutation or deletion, respectively, these Ror2 constructs
Figure 9. Wnt5a-challenge attenuated villin protein expression in cells transfected with ΔCRD Ror2 and 5YF Ror2, but villin expression was increased in BDB Ror2-expressing cells. A: Wnt5a challenge induced a noticeable upregulation in villin protein expression in Ror2-overexpressing HT29 cells, as previously observed. In similarly challenged cells transfected with a ΔCRD Ror2 construct, where the cysteine rich domain of the receptor has been deleted, or with 5YF Ror2, a plasmid construct where five tyrosines of the ser/thr-1 region had been substituted with phenylalanines, villin protein expression was not increased. These low levels of villin expression resembled those found in mock-transfected and/or untreated control cells. β-actin immunoblotting was completed to show equal loading. Blots were representative of 3 separately performed experiments. B: In HT29 cells transfected with BDB Ror2, which is characterized by a deletion of the proline and serine/threonine rich domains of the receptor, levels of villin protein expression were increased upon Wnt5a CM challenge. This increased villin expression was comparable to that observed in similarly challenged WT Ror2 transfected cells. These results represent three individually performed experiments with similar results. Western blotting for β-actin was included as a loading control.
signaled in a different manner. It appears that the expression of the tyrosine residues in the ser/thr-1 was necessary for Wnt5a’s interaction with full length Ror2 to stimulate ERK1/2. However, deletion of regions including and distal to ser/thr-1 did not affect Wnt5a stimulation of ERK1/2 and subsequent increases in villin protein.

Recombinant Wnt5a, but neither Wnt3a nor EGF, stimulated villin protein production in adenocarcinoma-derived cells

To determine whether other forms of Wnt5a or other Wnt family members, besides Wnt5a, could stimulate villin protein production in HT29 cells, we challenged WT Ror2-overexpressing cells with recombinant mouse Wnt5a (rWnt5a) and recombinant mouse Wnt3a (rWnt3a), and assessed for villin protein production. In mock-transfected cells treated with Wnt5a CM, levels of villin protein were low, as observed in previous experiments. Upon addition of either Wnt5a conditioned media or rWnt5a (200ng/mL) for 24 h in Ror2- overexpressing cells, levels of villin protein were increased. Following similar addition of rWnt3a (200ng/mL) to WT Ror2-transfected cells, villin protein production was not increased (Figure 10).

To determine whether other ERK1/2 agonists could induce increased villin production, we stimulated cells with epidermal growth factor (EGF). EGF is a well-characterized activator of the ERK1/2 cascade. Western blot analysis screening for pERK1/2 confirmed that EGF (100nM) stimulated transient and robust increases in dual ERK1/2 (both p44/p42) phosphorylation between 2 to 30 minutes of incubation. (Figure 11A). Upon immunoblotting for villin protein, we found, as with previous experiments,
Figure 10. Incubation with recombinant Wnt5a, but not recombinant Wnt3a, enhanced villin protein expression. In WT Ror2 transfected HT29 cells, villin protein was slightly increased compared to Wnt5a CM-treated mock-transfected cells. There was a noticeable increase in similarly treated WT Ror2-overexpressing cells. Incubation with rWnt5a (200ng/mL) for 24 h enhanced villin expression to a similar degree. Treatment with rWnt3a (200ng/mL) for 24 h did not induce any immunoreactive villin expression. These experiments were performed three separate times, with similar results. β-actin immunoblotting was included as a loading control.
Figure 11. Epidermal growth factor (EGF) did not enhance villin protein expression. A: Challenge with EGF (100nM) for 0, 2, 5, 10, 30, 60 minutes induced transient and robust increases in ERK1/2 phosphorylation in HT29 cells. In contrast, weak pERK1/2 immunoreactivity was detected in cells which had been challenged with low serum media, which did not contain EGF, over similar time points. B: As previously observed, in HT29 cells which were mock-transfected or left untreated, low levels of villin protein expression were detected. In WT Ror2-overexpressing cells challenged with Wnt5a conditioned media for 24 h, villin protein expression was increased. In WT Ror2 overexpressing cells, challenged with EGF for a similar 24 h time period, low levels of villin expression were detectable, comparable to that found in mock transfected or untreated control cells. β-actin immunoblotting was included as a loading control. These results are representative of three separately performed experiments.
that the levels of villin protein expression in unchallenged or mock-transfected cells were not increased. In contrast, in WT Ror2-transfected cells challenged with Wnt5a CM for 24h, villin protein expression was noticeably increased, within the same experiment. However, in WT Ror2-transfected cells treated with EGF for a similar 24 h time period, immunoreactive villin was low (Figure 11B).

From these experiments, we conclude that both Wnt5a CM and recombinant Wnt5a treatment stimulated villin protein production. However, treatment with either another Wnt family member, Wnt3a, or a separate ERK1/2 agonist, EGF, did not induce enhanced villin production.

Wnt5a induced ERK1/2-dependent villin protein production in Ror2-transfected fetally-derived non-transformed human intestinal epithelial cells.

From our previous experiments, we observed that adenocarcinoma-derived HT29 cells expressed increasing basal levels of villin protein with passage. Thus, we elected to repeat similar experiments in fetally-derived non-transformed human intestinal epithelial (HIECs), which normally express very little or no villin protein.

To assess whether villin protein production is stimulated by Wnt5a-Ror2 in fetal non-transformed HIECs, we completed immunoblotting for villin in cells overexpressing the Ror2 receptor and had been challenged with Wnt5a CM for 24 h. As seen in previous experiments, in unchallenged and mock-transfected cells, levels of villin protein were low. In Ror2 overexpressing cells, without Wnt5a stimulation, there was a slight increase
in villin expression. With Wnt5a addition to WT Ror2-overexpressing cells, there was a noticeable increase in villin protein production (Figure 12A).

To assess if this Wnt5a induction of villin was ERK1/2-mediated, we challenged WT Ror2–overexpressing fetal derived nontransformed HIECs with Wnt5a CM for 0, 2, 5, 10 and 60 min and measured levels of ERK1/2 phosphorylation. We found that there was a transient and robust increase in dual ERK1/2-phosphorylation over the course of an hour. Maximal ERK1/2 phosphorylation occurred between 5-10 minutes (Figure 12 B). This pattern of ERK1/2 phosphorylation resembled that of similarly challenged WT Ror2-overexpressing HT29 cells (Figure 4A; top panel).

To determine which intracellular signaling cascades mediated this Wnt5a-stimulated villin production, we challenged WT Ror2 expressing cells with Wnt5a CM in presence of MEK-1 inhibitor (10µM; PD 098059), Bisindolylmaleimide I (1µM; an inhibitor of PKC-α, -β, -γ, -δ, -ε), SB 203580 (10µM; a specific inhibitor of all p38 isotypes) and D4476 (100 µM; an inhibitor of CKI isotypes), and assessed for levels of villin protein. In the presence of either PD 098059 or D4476, levels of villin protein were not increased upon Wnt5a addition (Figure 12 C and D). However, incubation with Bisindolylmaleimide I or SB 203580 did not prevent the Wnt5a-stimulated increases in villin protein (Figure 12 D).

From these experiments, we conclude that villin protein expression is enhanced upon WT Ror2 overexpression but villin expression was further increased with Wnt5a
Figure 12. Wnt5a challenge stimulated villin expression, via an ERK1/2 dependent mechanism in WT Ror2-overexpressing human intestinal epithelial cells (HIECs). A: Villin protein was barely detectable in mock transfected and/or unchallenged HIECs. Upon Wnt5a treatment, levels of villin transcript expression were slightly increased. Over-expression of WT Ror2 in these cells induced faint villin expression. Treating WT Ror2-cells with Wnt5a CM for 24 h noticeably enhanced villin protein expression. B: Wnt5a addition in Ror2 overexpressing cells transiently and robustly increased dual ERK1/2 phosphorylation over the course of an hour. C: The Wnt5a-enhanced villin protein expression was prevented in similarly treated cells, in the presence of PD 098059 (10µM). D: However, increased villin protein levels were maintained in Wnt5a-challenged WT Ror2 cells, despite the presence of either Bisindolylamlemide I (1 µM) or SB 203580 (10µM). Yet, in the presence of D4476 (100µM), Wnt5a-induced villin protein expression was prevented. For all experiments described, Western blotting for immunoreactive β-actin was performed to demonstrate equal loading. These experiments reflect results from similar experiments performed separately.
treatment. This Wnt5a-stimulated villin protein was likely mediated by an ERK1/2 MAPK cascade and/or by casein kinase I.

**The ser/thr-1 domain of the Ror2 receptor differentially mediated villin protein production in HIECS**

To determine which regions of the Ror2 receptor were necessary to mediate Wnt5a-induced villin protein production in fetal non-transformed HIECs, we completed Western blotting for villin in lysates collected from Wnt5a-treated cells where five critical tyrosine residues in the serine/threonine-1 region of Ror2 had been substituted with phenylalanine (5YF Ror2) or where the proline and serine/threonine-rich regions of the receptor had been deleted (BDB Ror2) (Figure 13). As previously observed, in unchallenged WT Ror2 (control) cells, we found low, but detectable levels of villin protein expression. Upon Wnt5a treatment in WT Ror2 overexpressing cells, levels of villin protein were increased. In contrast, in both untreated or Wnt5a CM – challenged 5YF Ror2-transfected cells, immunoreactive villin protein was undetectable. In untreated BDB Ror2 expressing cells, we observed low levels of immunoreactive villin protein, comparable to levels seen in untreated WT Ror2 –transfected HIECs. In Wnt5a-challenged BDB Ror2 overexpressing cells, the amount of villin protein was increased, comparable to that seen in similarly challenged WT Ror2 overexpressing cells. The results from these experiments were consistent with data collected from previous experiments in HT29 cells (Figure 9A and B). Though both 5YF Ror2 and BDB Ror2 eliminated tyrosine phosphorylation in the ser/thr-1 domain, upon Wnt5a treatment, these mutant/truncated Ror2 constructs signaled in a different manner. The tyrosine
Figure 13. Wnt5a treatment did not stimulate villin protein expression in 5YF Ror2 -expressing HIECs but did induce villin protein expression in BDB Ror2 -expressing HIECs. In untreated WT Ror-expressing cells, villin protein expression was not detectable; upon Wnt5a challenge for 24 h, villin expression was substantially increased. Levels of villin protein were low in untreated and Wnt5a-treated 5YF Ror2 –expressing cells. Similar Wnt5a treatment upregulated the amount of immunoreactive villin in cells transfected with BDB Ror2. β-actin immunoreactivity was assessed to confirm equivalent loading. These results are representative of 3 separate experiments.
phosphorylation in ser/thr-1 domain was necessary in full length Ror2 but not in a truncated version Ror2 where the regions including, and distal to, the ser/thr-1 region were eliminated. Thus, in both transformed (HT-29 cells) and fetally-derived non-transformed (HIECs) human intestinal epithelial, Wnt5a signaled differently in 5YF mutations compared with the BDB deletions of Ror2.

Neither Wnt3a nor EGF enhanced villin protein production in non-transformed human intestinal epithelial cells

To determine whether another Wnt family member (Wnt3a) or another ERK1/2 agonist (EGF) would stimulate villin protein production in fetally-derived non-transformed HIECs, we treated cells with either rWnt3a or EGF and probed for immunoreactive villin. Consistent with previous experiments, in mock-transfected HIECs villin protein production was faint. Upon WT Ror2-transfection, villin protein production was slightly increased. In Ror2 overexpressing cells challenged with Wnt5a CM for 24 hours, villin protein expression was substantially increased. However, when WT Ror2-transfected cells were challenged, similarly for 24 h, with rWnt3a protein (200ng/mL), levels of villin protein production were unchanged, comparable to levels observed in untransfected and untreated control cells (Figure 14). Furthermore, in experiments where we challenged HIECs with an alternate ERK1/2 agonist, EGF (100nM), we did not observe villin protein increases (Figure 15). From these experiments, we conclude that neither another Wnt family member, Wnt3a, nor another ERK1/2 agonist (EGF) was sufficient to induce enhanced villin protein production in fetal non-transformed Human Intestinal Epithelial Cells.
Figure 14. Incubation with recombinant Wnt3a protein did not stimulate villin protein expression in Ror2 overexpressing HIECs. Villin protein expression was not detectable in untreated mock-transfected HIEC, but was slightly enhanced in untreated WT Ror2-expressing cells. Villin protein expression was noticeably increased with Wnt5a CM-challenge for 24 h in WTRor2-overexpressing cells. In similarly transfected cells, rWnt3a incubation for a 24 h period did not induce increases in villin protein. β-actin immunoreactivity was assessed to confirm equivalent loading. These results are representative of 3 separate experiments.
Figure 15. Addition of EGF did not stimulate villin protein expression in HIECs. As previously shown, villin protein expression was substantially increased in HIECs which overexpressed Ror2 and had been subsequently challenged with Wnt5a CM. In contrast, in cells treated with EGF (100nM), an alternative ERK1/2 activator, villin protein levels are barely detectable. β-actin immunoreactivity was assessed to confirm equivalent loading. These results are representative of 3 separate experiments.
Chapter 4
Discussion

The major findings from this study are summarized in the schematic Figure 16. These studies are the first demonstration that Wnt5a interaction with the Ror2 receptor stimulated ERK1/2 activation and subsequent villin protein expression in adenocarcinoma-derived HT29 and fetally-derived non-transformed human intestinal epithelial cell lines.

Evidence that Wnt5a stimulated Ror2 to induce ERK1/2 activation was demonstrated by three different experimental approaches. First, Western blotting for ERK1/2 showed that Wnt5a conditioned media treatment induced transient and robust increases in ERK1/2 phosphorylation in Ror2 overexpressing HT29 cells and fetally-derived, non-transformed HIECs. Second, media change alone, with media which did not contain Wnt5a, in WT Ror2-transfected or mock transfected cells failed to stimulate ERK1/2 phosphorylation. These experiments demonstrated that the growth factors and serum elements contained in media were not sufficient to stimulate ERK1/2.

We have also demonstrated that Ror2 receptor expression was a necessary component of Wnt5a-induced ERK1/2 activation. In cells where Ror2 receptor expression had been knocked down by transient transfection with interfering RNA, Wnt5a-stimulated ERK1/2 phosphorylation was attenuated. Results of structure-function studies suggested that functionality of the cysteine rich, kringle and tyrosine kinase domains of Ror2 were necessary for Wnt5a-mediated activation of the ERK1/2 MAPK
Figure 16. Model of Wnt-induced villin protein expression in adenocarcinoma-derived HT29 cells and fetally-derived, untransformed Human Intestinal Epithelial Cells. Wnt5a interaction with Ror2 is mediated by the cysteine rich domain. Phosphorylation of the tyrosines within in the ser/thr-1 region, possibly by Src, induces ERK1/2 phosphorylation. Stimulated ERK1/2 upregulates villin transcript expression; this subsequently increases villin protein. This Wnt5a-induced villin expression is also mediated by CK1. As well, CDX2, acting downstream of, or in parallel with, the activated ERK1/2 MAPK cascade could be mediating Wnt5a-induced increases in villin protein.
cascade in these cells. Furthermore, in cells which expressed a Ror2 receptor with five substitution-mutated tyrosines in the ser/thr-1 rich region (5YF Ror2), Wnt5a addition did not stimulate ERK1/2 activation. However, in Wnt5a-challenged cells, which expressed a Ror2 receptor where the serine/threonine and proline rich regions were deleted (BDB Ror2), there was a robust and transient increase in ERK1/2 phosphorylation. This was the first evidence that though both the 5YF and BDB Ror2 constructs eliminated tyrosine phosphorylation function in the ser/thr-1 domain, Wnt5a interacted with these Ror2 constructs to signal in a different manner.

We also found that Wnt5a-induced ERK1/2 activation stimulated subsequent villin protein production in these cell lines. Western blotting for villin protein demonstrated that Wnt5a challenge in Ror2 overexpressing cells subsequently increased villin transcript and protein expression. Villin protein expression was blocked in the presence of a specific inhibitor of MEK-1, an upstream mediator of the ERK1/2 MAPK signaling cascade. Furthermore, we also report that in the presence of a casein kinase 1 (CK1) inhibitor, Wnt5a-enhanced villin expression was prevented. However, enhanced villin protein expression was not blocked in the presence of a general PKC or p38 inhibitor. These results suggested that Wnt5a-activation of ERK1/2 was highly specific, as another Wnt family member, Wnt3a, and EGF, a separate ERK1/2 agonist, failed to stimulate villin protein production. Together, these findings suggested a new Wnt5a-Ror2 regulated pathway mediating intestinal epithelial differentiation.
Defective Wnt signaling is one determinant of colon cancer (van Es & Clevers, 2005; Segditsas & Tomlinson, 2006). Mutations leading to constitutive Wnt signaling have been strongly associated with human colorectal cancer (CRC) as well as increased polyp formation in mice (Logan & Nusse, 2004). Elucidating the precise signaling cascades initiated by Wnts has become increasingly important in the development of new chemoprotective therapies for CRC (Polakis, 2000; Logan & Nusse, 2004; Sancho et al, 2004; Nusse, 2005).

Traditionally, Wnts have been classified based on their capacity to elicit either canonical (β-catenin dependent) or non-canonical (β-catenin independent) cascades. From recent studies, it has become apparent that the type of Wnt signaling induced was heavily dictated by receptor context. Previous reports have shown that, in addition to the Frizzled family of receptors, Wnts alternatively signaled through tyrosine kinases, such as Ror2 (Xu & Nusse, 1998; Schwabe et al, 2000; Forrester, 2002; Oishi et al, 2003). Several have gone on to demonstrate that both Wnt5a (Oishi et al, 2003; Mikels & Nusse, 2006; Nishita et al, 2006; Schambony & Wedlich, 2007; Li et al, 2008) and Wnt3a (Mikels & Nusse, 2006; Li et al, 2008) stimulate the Ror2 receptor. When Wnts signaled through the single-pass Ror2 receptor, non-canonical signaling pathways were typically triggered (Oishi et al, 2003; Schambony & Wedlich, 2007).

The most convincing evidence that Wnt signaling is dependent on specific receptor activated was summarized in a recent study, where Wnt5a was added to transfected HEK 293 cells. When these Ror2 overexpressing HEK 293 cells were
challenged with Wnt5a canonical signaling was inhibited. However, in similar cells, where Frz4 was overexpressed, Wnt5a treatment triggered a canonical signaling cascade (Mikels & Nusse, 2006). This study was the first demonstration that Wnt-induced signaling was dependent on receptor subtype rather than intrinsic properties of the specific Wnt ligand. Moreover, the results from this investigation suggested that Wnts which stimulated the Ror2 receptor typically induced non-canonical signaling cascades (Oishi et al, 2003; Mikels & Nusse, 2006; Li et al, 2008). Similarly, in our study, we have described how Wnt5a addition to Ror2 overexpressing intestinal epithelia cells triggered non-canonical signaling.

Interestingly, others have reported that Wnt-Ror2 signaling is also mediated by the functional domains of the Ror2 receptor. For instance, Wnt5a interaction with Ror2 expressed in HT-29, HEK, L, H441 and A549 cells antagonized β-catenin TopFlash reporter activity (Mikels & Nusse, 2006; MacLeod et al, 2007; Li et al, 2008). These effects were mediated by the intracellular domain of the receptor (Mikels & Nusse, 2006). In contrast, Wnt1 challenge in UROS human sarcoma cells stimulated the tyrosine kinase domain of Ror2 and subsequent β-catenin reporter activity (Billiard et al, 2005). We have described herein how Wnt5a activation of Ror2 induces a non-canonical signaling pathway in intestinal epithelial cells in vitro. In fact, we are the first to demonstrate that Wnt5a-Ror2 interaction stimulates an ERK1/2 MAPK cascade. Furthermore, we have demonstrated that the cysteine rich, kringle and tyrosine kinase domains, and likely tyrosine phosphorylation within the serine/threonine-1 region, are involved in mediating this effect.
A substantial amount of work originating from our laboratory has been directed at understanding how Wnt5a signaling is regulated in the intestine, as well as the type of signaling cascades elicited by Wnt5a’s interaction with Ror2. We have previously demonstrated that the extracellular calcium sensing receptor (CaSR) inhibited defective Wnt signaling, and stimulated non-canoncial Wnt signaling in the colon (MacLeod et al, 2007). Furthermore, we have described a novel paracrine relationship between the colonic epithelia and the supporting colon myofibroblasts, cells which lie directly underneath the epithelia. Upon activation of the CaSR in colonic epithelia, Wnt5a synthesis and secretion was induced. Meanwhile, similar CaSR activation in the overlying epithelia concurrently stimulated the expression of Wnt5a’s receptor Ror2. Colonic Wnt5a-Ror2 binding triggered the activation of the caudal type 2 homeobox domain transcription factor, CDX2, enhancing the expression of intestinal specific genes, such as sucrase isomaltase (Pacheco & Macleod, 2008). Our focus has since shifted to understanding the regulation of other markers of epithelial differentiation, such as villin, in the intestine.

Little is known about how villin protein synthesis is regulated in the mammalian intestine. It is well understood that villin is as an actin-remodeling and stabilizing protein which serves crucial functions in stabilizing and remodeling the cytoskeletal microvilli (Coluccio & Bretscher, 1989). Its functions are known to be mediated by Src tyrosine phosphorylation (Mathew et al, 2008). Because villin is detected during the initial stages of differentiation, it is considered a marker of cells committed to differentiating into
intestinal absorptive epithelia (Pinto et al, 1999). Recent studies have implicated villin in the regulation of apoptosis, though its precise role remains unclear. Investigations have demonstrated that gelsolin, a homologous villin family member, -knock out mice display a decreased rate of apoptosis in neuronal and liver tissues, compared to their wild-type littermates (Harms et al, 2004). Furthermore, others have reported that in villin-gelsolin double knockout mice, villin functioned as an anti-apoptotic protein in vivo by preserving mitochondrial membrane integrity, and preventing the cleavage of caspase -9 and -3 (Wang et al, 2008). Decreased rates of apoptosis have some very serious clinical implications. Gastrointestinal disease states, including villus atrophy, epithelial hyperplasia and tumorigenesis have been associated with defects in apoptotic progression (Rieder et al, 2005; Zavros et al, 2005; Khurana & George, 2008). Because of the intestinal epithelia lining the lumen are constantly renewed, this area of the colon is characterized by increased rates of proliferation, differentiation, and apoptosis (Khurana & George, 2008). Disruptions in the homeostatic balance can impact the absorptive and secretory functions of the intestinal lining (Khurana & George, 2008). Elucidating exactly how villin expression is turned on and off in vivo may have some significant impacts in gastrointestinal physiology.

The current experiments are the first to demonstrate that Wnt5a stimulation of Ror2 in two different intestinal epithelial cell lines induced ERK1/2 phosphorylation and subsequent villin production. This Wnt5a-Ror2 signaling was specific as an alternative ERK1/2 agonist, EGF, and Wnt family member, Wnt3a, failed to increase villin protein.
As previously described, Wnts stimulate specific functional domains of the Ror2 receptor to trigger precise signaling cascades. In our experiments, we observed that Wnt5a stimulated ERK1/2 phosphorylation in cells expressing BDB Ror2 (where the serine/threonine and proline-rich regions had been deleted), but not in cells expressing a 5YF Ror2 (where five tyrosines of the serine/threonine-1 region had been substitution-mutated). Insight into the function of the C-terminus (ser/thr and proline regions) of Ror2 has been shown in a single study which documented that the C-terminus was required for the recruitment of the non-receptor kinase Src (Akbarzadeh et al., 2008). Endogenous and wild type Ror2 was a target for Src-mediated phosphorylation in the C-terminus region. Additionally, pharmacological inhibition of Src suppressed Ror2 activation (Akbarzadeh et al., 2008). One interpretation of these results was that the BDB Ror2 was defective in kinase activation via failure to recruit Src. Our current results showing 5YF Ror2 prevented pERK1/2 stimulation and villin protein increases, while BDB Ror2 had no effect, suggest to us that recruitment of Src and phosphorylation of the 5 tyrosines in the BDB region were a necessary and sufficient event for Wnt5a activation of Ror2 to generate transient pERK1/2 stimulation leading to villin production. Clearly, Wnt5a interaction with 5YF Ror2 and BDB Ror2 was different.

Further experiments are required to delineate how Src is mobilized by Wnt5a stimulation, as well as the role of Src in villin protein regulation. While it is known from in vitro experiments that Src tyrosine phosphorylated villin, leading to increased intestinal cell migration as well as a villin-mediated anti-apoptosis, these effects were observed in models of villin overexpression (Mathew et al., 2008). The current
experiments focused on how Wnt5a interaction with Ror2 led to increases in villin. There are no studies which have compared the functions of 5YF Ror2 and BDB Ror2 following Wnt5a stimulation. Earlier work has demonstrated that 5YF Ror2 failed to phosphorylate G-protein receptor kinase 2 (GRK2) on tyrosine residues by ectopic expression of CKI\(\varepsilon\), but that work did not test whether or not BDB Ror2 stimulated GRK2 (Kani et al., 2004). Indeed, these studies did not examine whether Wnt5a stimulated 5YF Ror2 and BDB Ror2 differently. For example, it will be important to understand how putative Src activation of the 5YF sites in the ser/thr-1 region alter function compared with the absence of the ser/thr-1 region altogether. Are other tyrosine sites in the intracellular domain Src phosphorylated in the regions deleted in BDB Ror2? Future studies could address whether pharmacological inhibition of Src kinases or transient expression of either constitutively active or dominant negative Src influence the production of villin mediated by Wnt5a interaction with Ror2. Nevertheless, our current results strongly suggest that tyrosine phosphorylation, likely mediated by Src kinases, at sites within the proline-serine/threonine rich regions of Ror2 are essential for ERK1/2 MAPK stimulation and villin protein production.

Previous work has concentrated on the roles of MAPK cascades in mediating proliferation and differentiation in intestinal epithelia but studies involving villin have not been reported. Several studies have explored the role of p38 MAPK in activating CDX2, a master transcription factor of markers of intestinal differentiation. Earlier work also implicated ERK1/2 phosphorylation in stimulating intestinal epithelial differentiation (Houde et al., 2001). Furthermore, others have shown in \textit{H. pylori} – gastric cancer cells,
that villin promoter activity is stimulated by Elk-1, a transcription factor which is
downstream of the ERK MAPK cascade (Rieder et al, 2005). These studies were
consistent with our findings that Wnt5a interacts with Ror2 to stimulate ERK1/2
phosphorylation. This event likely mediates villin protein production. We have shown
that pharmacological inhibition of MEK1/2 prevented Wnt5a-mediated increases in villin
protein in two distinct epithelial model cell types. However, pharmacological inhibition
of PKC isoforms α, -β, -γ, -δ, -ε and p38 MAPK isoforms had no effect on Wnt5a-
induced villin protein expression in HT-29 adenocarcinoma cells or in non-transformed
fetal-derived human intestinal epithelia. Previous studies using Ror2 have shown that
Wnt5a stimulates JNK (Yamanaka et al, 2002) and GSK-3β (Yamamoto et al, 2008),
while the receptors which mediate p38, PKC or CK 1/2 following Wnt5a stimulation
have not yet been identified.

Little is understood about the role of CKI in the intestine. CKI has been described
as a positive regulator of canonical Wnt signaling. However, others have shown that
CKIε abundance and activity increased in transmissible murine colonic hyperplasia
(Umar et al, 2007). This suggests that CKIε modulated hyperpoliferation in mouse
epithelia (Umar et al, 2007). Recent work has demonstrated that Wnt5a-mediated
dopaminergic neuronal differentiation was blocked in the presence of D4476, the same
CKI inhibitor used in the experiments described in this thesis. However, the specific
receptor mediating this effect was not investigated (Bryja et al, 2007). Our work is
consistent with previous findings that CKI mediates Wnt signaling in the intestine, as
Wnt5a-induction of villin protein was prevented by pharmacological inhibition of CKI
(Bryja et al, 2007). However, future experiments are required to deduce the precise function of CKI in the intestine. For instance does CKI phosphorylation precede or follow ERK1/2 activation, or does this event occur in parallel?

Though others have demonstrated that villin promoter activity may be induced by an Elk-1 transcription factor, which are downstream of the ERK1/2 cascade, recently, the villin promoter has been shown to contain sites which may be activated by CDX2 (Yamamichi et al, 2009). This is an important link with the original findings described in this thesis. Our laboratory reported that Wnt5a stimulation of Ror2 overexpressed in HT-29 and Caco-2 adenocarcinoma cells, profoundly increased both CDX2 transcript and protein. These changes led to activation of a sucrase-isomaltase promoter. Addition of recombinant human Wnt5a to these cells caused a 15 fold increase in CDX2 protein within 1h which declined to 3 fold over basal level by 2h (Pacheco & Macleod, 2008). The determinants of CDX2 protein half-life are largely unknown, but phosphorylation by p38 MAP kinase has been reported to increase CDX2 half-life (Houde et al, 2001). Because the increase in CDX2 transcript and protein occurred quite rapidly after Wnt5a addition to Ror2, but at longer times compared with the activation of pERK1/2 described in the current studies, it may be that the Wnt5a stimulation of pERK1/2 is an important determinant of the upregulation of CDX2 transcript or the substantial increases in CDX2 protein. The current studies have not investigated whether Wnt5a-induction of CDX2 protein is a necessary determinant of the subsequent stimulation of villin. Further experiments could reduce the CDX2 increases using interfering RNA techniques and address whether a) Ror2-stimulation of pERK1/2 is influenced and b) if villin transcript
and protein changes occurred. Well defined activators of the same transient increases in pERK1/2, such as EGF, could be used to assess if CDX2 increases will be manifested. For example, the current studies show that EGF caused the same transient stimulation of pERK1/2 in Ror2 overexpressing cells but did not cause an increase in villin protein. Is this because no CDX2 has been generated? Alternatively it could be that while both CDX2 and Elk-1 can activate the villin promoter, either factor alone is sufficient for villin production. Thus, we cannot conclude that Wnt5a-stimulation of Ror2 increases CDX2, which then activates villin transcription. Our current evidence strongly supports, however, a causal axis of Wnt5a-interaction with Ror2 on intestinal epithelial stimulating pERK1/2 to increase villin transcript and protein.

Thus far, we have proposed that Wnt5a-Ror2 induction of ERK1/2 and villin protein is by mediated by Src, CKI and CDX2. However, we have not yet explained how Ror2 overexpression, in the absence of Wnt5a, stimulates faint villin expression in intestinal epithelia. In our experiments we observed that overexpression of Ror2, alone, induced faint villin protein expression. This could likely be mediated by Ror2 homodimerization triggering kinase activity independently from Wnt5a. Several studies have shown that though Wnt5a addition induced activation of wild type Ror2 kinase (Billiard et al, 2005; Akbarzadeh et al, 2008). Additionally, in the absence of Wnt5a, a constitutively dimerised form of Ror2 exhibited tyrosine kinase activity (Akbarzadeh et al, 2008; Liu et al, 2008). Our lab has previously reported that Ror2 is expressed in intestinal epithelia throughout the crypt-villus axis (Pacheco & Macleod, 2008). Interestingly, Ror2 and villin share similar expression profiles. Villin protein is also
widely expressed in intestinal epithelia, though its expression is progressively increased as cells migrate towards the tip of the villi (Dudouet et al, 1987). Additionally, Ror2 and CDX2 also share similar expression profiles: both are localized to intestinal epithelia throughout the crypt-villus axis (Mallo et al, 1997). Whether Ror2 induces villin expression, independent of Wnt5a stimulation, has not been elucidated. It would be interesting to assess whether villin protein expression can be blocked in Ror2 overexpressing cells in the absence of Wnt5a and in the presence of ERK1/2, Src and CKI pharmacological inhibition. Though we cannot fully explain exactly how Ror2 overexpression mediates villin protein expression, our results clearly show that Wnt5a stimulation of Ror2 induces ERK1/2 phosphorylation and subsequent villin protein expression.

We conclude, based on our findings, that Wnt5a signals through Ror2 to stimulate ERK1/2 and subsequent villin protein expression in adenocarcinoma-derived intestinal HT29 cells and nontransformed, fetally-derived Human Intestinal Epithelial Cells. We have also shown that Wnt5a signals differently in 5YF Ror2-expressing cells compared to BDB Ror2 – expressing cells, though both Ror2 constructs eliminate tyrosine phosphorylation in the ser/thr-1 regions. Thus, it is likely that Src phosphorylation of the ser/thr-1 domain is mandatory for full length Ror2 to mediate Wnt5a-induction of ERK1/2 and villin protein. Additionally, we have demonstrated that CKI is required for Wnt5a-induced villin protein expression. As well, others have reported that CDX2 may induce villin promoter activity. However, the precise functions of Src, CKI, and CDX2 in the regulation of villin protein expression are not well characterized and must be
investigated in future experiments but may justifiably begin by considering Wnt5a stimulation of intestinal Ror2.
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