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Tissue Specific *Porcupine* Deletion Reveals a Novel Role for Ectodermal *Wnts* in Musculotendon Development

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Masters of Science

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ABSTRACT

Tissue Specific *Porcupine* Deletion Reveals a Novel Role for Ectodermal *Wnts* in Musculotendon Development

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The *Wnt* family of secreted proteins consists of 19 family members (in the mouse) and is known to signal through multiple pathways that regulate crucial processes in the development of almost all tissues. Dissecting the roles of individual *Wnts* has been hampered due to functional redundancy that exists between family members. We made use of a conditional allele of the O-acyltransferase, *Porcupine* (*Porcn*), that is required for the secretion of all *Wnt* ligands, and the *Msx2Cre* deleter to eliminate the secretion of all *Wnt* ligands from the ventral limb ectoderm, ventral abdominal ectoderm, and urogenital ectoderm. Phenotypically the limbs of these mice have several similarities with *En1* mutant mice which have a double-dorsal phenotype. However, we show that appropriate dorsoventral limb pattern is maintained at the molecular level and that the observed defects are due to a failure to appropriately execute ventral pattern. Additionally, newborn mice lack ventral digital tendons and the most superficial musculature in the regions of strongest and earliest deletion. Molecular analysis indicates that tendons are lost downstream of the absent musculature and are initially patterned correctly. Thus we show a role for ectodermal *Wnts* in the development of underlying musculature. We additionally examine the role of limb mesenchymal *Wnts* in the development of deeper limb musculature utilizing the *Prx1Cre* deleter. The deep musculature of the autopod and zeugopod is reduced or absent in mutants and the development of superficial musculature appears to proceed normally. Hence we show that superficial muscles require only ectodermal *Wnts* and deeper muscles require only mesenchymal *Wnts*.

Keywords: porcupine, wnt, muscle, development, limbs, anus, abdomen, tcf4

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INTRODUCTION

Wnt signaling

Wnt signaling has been shown to play a role in many developmental processes and cancers in a wide variety of model organisms. These roles have generally been uncovered through manipulation of *Wnt* response competence factors or a mix of *Wnt* ligands. Both of these approaches have their own unique weaknesses.

Knocking out or otherwise modifying competence factors of signaling pathways such as receptors or downstream transcriptional machinery has the severe limitation of failing to identify the specific function of a signaling center of interest. Additionally, the most commonly manipulated pathway member is *β-catenin* which also has a role in cell-adhesion (Peifer et al., 1993). Thus it can be difficult to show that *β-catenin*'s role in *Wnt* signaling is responsible for an observed defect. Additionally, *β-catenin* is only a member of the "canonical" *Wnt* signaling pathway. Other *Wnt* pathways include the *Jnk* dependent pathway, a Ca^{++} dependent pathway and a putative planar cell polarity pathway (Nusse 2012; Barrow 2011). *β-catenin* manipulation will not reveal the role of *Wnt* ligands in signaling through any of these other pathways.

However, *Wnt* ligand manipulation, which avoids those two issues, has its own set of difficulties. There are 19 different *Wnt* ligands, so there are often multiple *Wnts* expressed in a tissue or region of interest. These overlapping *Wnts* are often partly or fully redundant, with the result that knocking out just one of them yields a very minor phenotype or perhaps no phenotype at all. For instance, *Wnt2* and *Wnt2b* have similar expression patterns in the anterior mesoderm surrounding the region of gut that will give rise to the lungs. In *Wnt2b* mutants there is no apparent lung defect. In *Wnt2* mutants, lung hypoplasia manifests. In double mutants, complete lung agenesis occurs (Goss et al., 2009). We have also observed a similar effect when examining

Wnt5a mutants, *Wnt5b* mutants, and double mutants. These genes play overlapping roles in directed growth as manifested by defects in growth of structures that grow outward along an axis such as the body axis, skull, and limb as manifested in double mutants. However, *Wnt5a* mutants exhibit a defect primarily in the forelimbs and snout while *Wnt5b* mutants have no obvious defects at all (Fig. 1). Thus the full phenotypic magnitude is not manifested in single mutants.

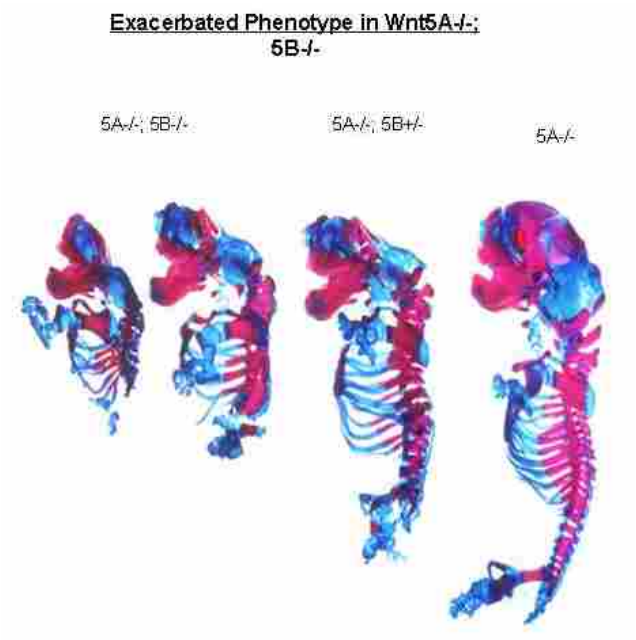

Figure 1- *Wnt5a/5b* double mutants have a far more severe convergent extension defect than *Wnt5a* mutants. (Unpublished data, Barrow lab)

These cases illustrate that it is sometimes possible to identify the redundantly acting *Wnts*, and determine their joint role. However, in some cases many *Wnts* act together or overlap and preliminary analysis cannot pinpoint which specific *Wnts* should be jointly ablated. Additionally, the difficulty of performing crosses to generate multiple mutants increases exponentially with each additional mutation.

Dorsoventral patterning

Wnt signaling plays a vital role in dorsoventral pattering in the vertebrate limb. *Wnt7a,* which is expressed in the dorsal ectoderm, induces a dorsal fate in underlying limb mesenchyme by activating expression of the dorsal fate marker *Lmx1b* (Riddle et al., 1995; Fig. 2). Appropriate ventral fate is maintained by the transcription factor *Engrailed1*. *En1* is expressed throughout the ventral surface ectoderm of the limb and represses expression of the dorsalizing paracrine factor *Wnt7a* (Logan et al., 1997; Fig. 2). As a result, *Wnt7a* expression is restricted to

the surface ectoderm of the dorsal limb in normal limbs. In *En1* mutants, *Wnt7a* is ectopically expressed in the ventral ectoderm and activates *Lmx1b* expression in the ventral mesoderm. These molecular changes cause a ventral to dorsal fate transition in the ventral limb, which results in a loss of footpads, rounded nails, a loss of ventral sesamoid bones, and inappropriate digit curvature. Sesamoid bones are found on the ventral side of digits and aid in the mechanical process of flexion in wild type mice. The digit curvature in *En1* mutants may have been related to the loss of those bones or perhaps result from the dorsalization and flattening of the ventral tendons. Presumably in either case the root cause is a loss of normal function in the ventral musculotendon contractile unit (Cygan et al., 1997).

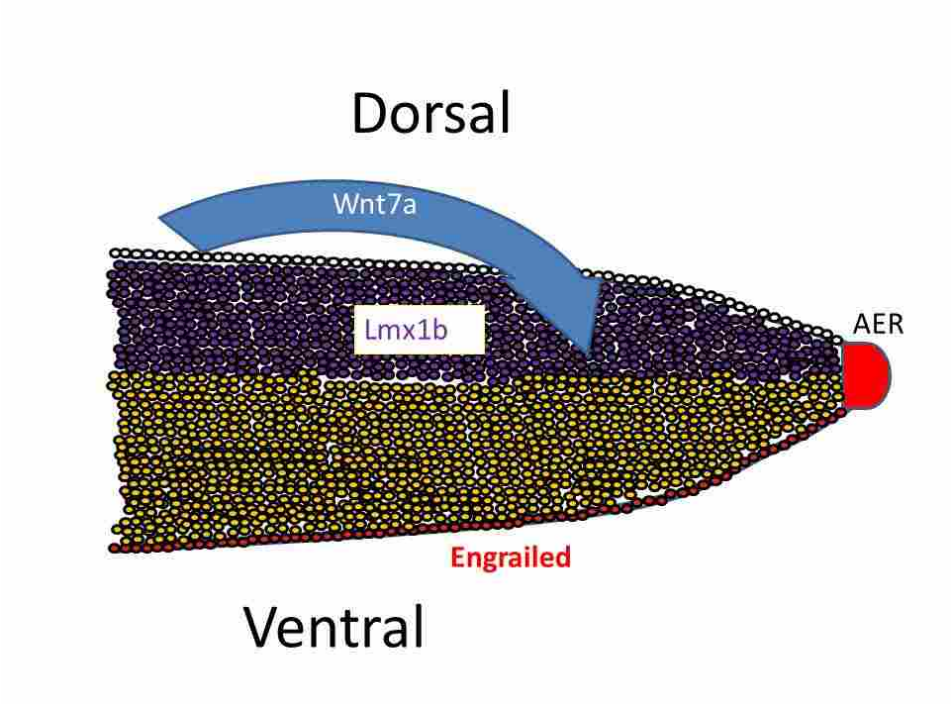

Figure 2- *En1* (expressed in red cells) is expressed in ventral limb ectoderm and prevents expression of *Wnt7a* in that region. *Wnt7a* is expressed in the dorsal ectoderm (white cells) and dorsalizes nearby mesenchyme by inducing *Lmx1b* (purple cells).

Development of the musculotendon contractile unit

Tendon development

Scx is the earliest known marker for tendon precursor cells and its expression in the limb is initially found at E10.0 in the limb lateral plate mesoderm (LPM) just deep to the surface

ectoderm (Fig. 3A). This first mass of induced tendons will eventually become the proximal tendons of the limbs. By E11.0 an intermediate set of tendon primordia begin to express *Scx.* These cells will give rise to the tendons of the zeugopod or middle limb compartment (Fig. 3C). The last primordia to appear are in the distal limb just deep to the ectoderm and superficial to the condensing digits. These tendon progenitors are first detected by *Scx* in situ hybridization at E12.5 (Fig. 3E) and are more fully individuated into flexor digitorum tendons by E13.5 (Schweitzer et al. 2001; Fig. 3F).

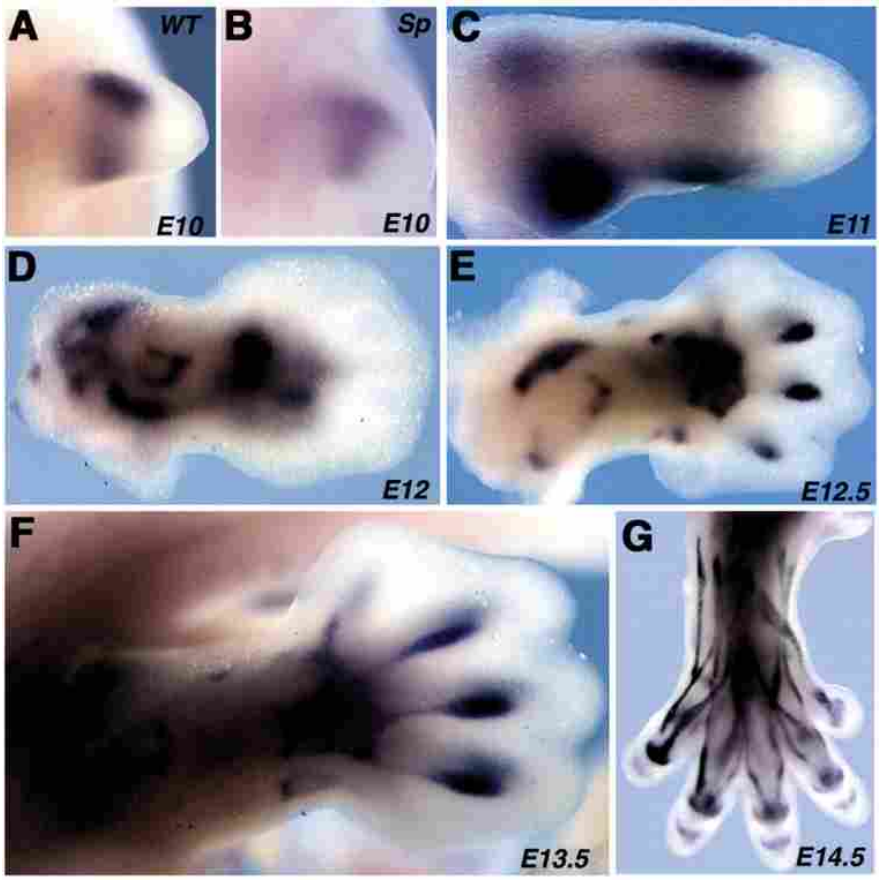

Figure 3- Scleraxis in situ hybridization reveals the location of tendons throughout early mouse limb development. Tendon progenitors are initially observed at E10.0 as paired proximal dorsal and ventral masses A. At E11 more distal dorsal and ventral masses appear C. At E12.5 the most distal tendon primordia form (Schweitzer et al. 2001)

A few signals and signaling centers have been identified that seem to be vital for limb tendon formation and differentiation. When limb ectoderm is removed, tendon primordia fail to form in the regions lacking ectoderm, indicating that an ectodermal signal is necessary for tendon induction. *BMP* signaling also controls tendon progenitor development as high levels of *BMP* signal prevent tendon progenitor induction. In the distal limb *BMP* signal is repressed by the newly forming cartilaginous elements which secrete *Noggin*. However, even with *BMP*

signaling blocked, tendon formation depends on surface ectodermal signals (Schweitzer et al., 2001; Fig. 4). Once induction has occurred, *TGF-β* signaling maintains and allows for further development of tendons. When *TGF-β* signaling is ablated, tendons initially form, but eventually degenerate, with much of the distal tendon primordia lost at E12.5 (Pryce et al. 2009).

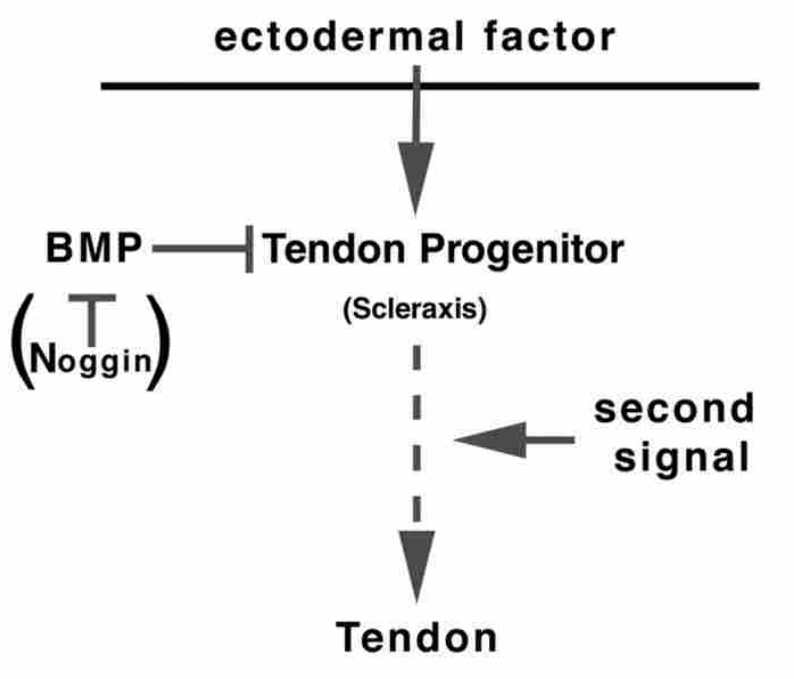

Figure 4- A figure illustrating the need for ectodermal and mesodermal signals to initially induce tendons. (Schweitzer et al. 2001)

Skeletal muscle specification and migration

Skeletal muscle is initially specified in the dorsal and lateral borders of the somite by signals from the overlying surface ectoderm (Chevallier et al., 1977; Christ et al., 1977; Brand Saberi and Christ 1999). At the level of the limbs *Pax3*, *Lbx1*, *cMet* and *Pax7* are induced in these regions of the somite and nearby LPM expresses *Scatter Factor/Hepatocyte Growth Factor* (*SF/HGF*). *SF*, signaling via the *cMet* receptor, induces an epithelial to mesenchymal transition (EMT) in *Pax3* expressing cells and these cells (called migratory myoblast progenitors or MMPs) then migrate out of the somite to enter the proximal limb bud. Currently it is unknown whether *SF* is directly recruiting the MMPs or simply inducing EMT. However, the chemokine receptor *CXCR4* is expressed in MMPs and its ligand, *SDF1* is expressed in the connective tissue of the limb bud. Preliminary work indicates that this signaling pair may be responsible for the guidance

of MMP migration (Vasyutina et al., 2005). Upon entry into the limb, *Lbx1* expressing MMPs continue to move progressively more distal in the limb (Brand-Saberi et al., 1996; Bladt et al., 1995). However, in *Lbx1* knockout mice the MMPs remain proximal in the limb and no distal limb muscles are formed (Gross et al., 2000).

A very different mechanism for muscle migration occurs in the inter-limb somites. The borders of these somites are not exposed to *SF* so EMT does not occur and the myoblast progenitors undergo early muscle development in place. Once they begin to express the master regulatory muscle transcription factor *MyoD*, they begin to migrate to their various destinations as a sheet (Christ et al., 1983; Brand Saberi and Christ 1999). This process is referred to as myotomal extension and occurs independently of *Lbx1* and *cMet/SF*. However, there is some speculation that the most ventral body wall musculature—the rectus abdominis—is formed by a subset of MMPs like those that populate the limbs (Evans et al., 2006).

Lastly, the skeletal muscles of the perineum—such as the external anal sphincter—migrate via a mechanism that has features in common with both limb musculature and body wall musculature. Prospective perineal myoblasts originate primarily in the same somites as the hindlimb musculature and undergo EMT under the influence of *SF*. They enter the hindlimb at the same time and in the same location as the hindlimb MMPs and begin to express *MyoD*. Up to this point their induction and migration has proceeded in exactly the same fashion as any other limb muscle progenitor. Then, under the influence of *SDF1,* expressed in the perineum, they migrate out of the hindlimb, split into their respective perineal muscles and enter the perineum (Rehimi et al., 2010; Valasek et al., 2005; Fig. 5). Since the muscle precursors enter the limb and then leave again to migrate towards the perineum, this muscle migration process has been nicknamed “in-out migration” (Evans et al., 2006). The external anal sphincter forms by this

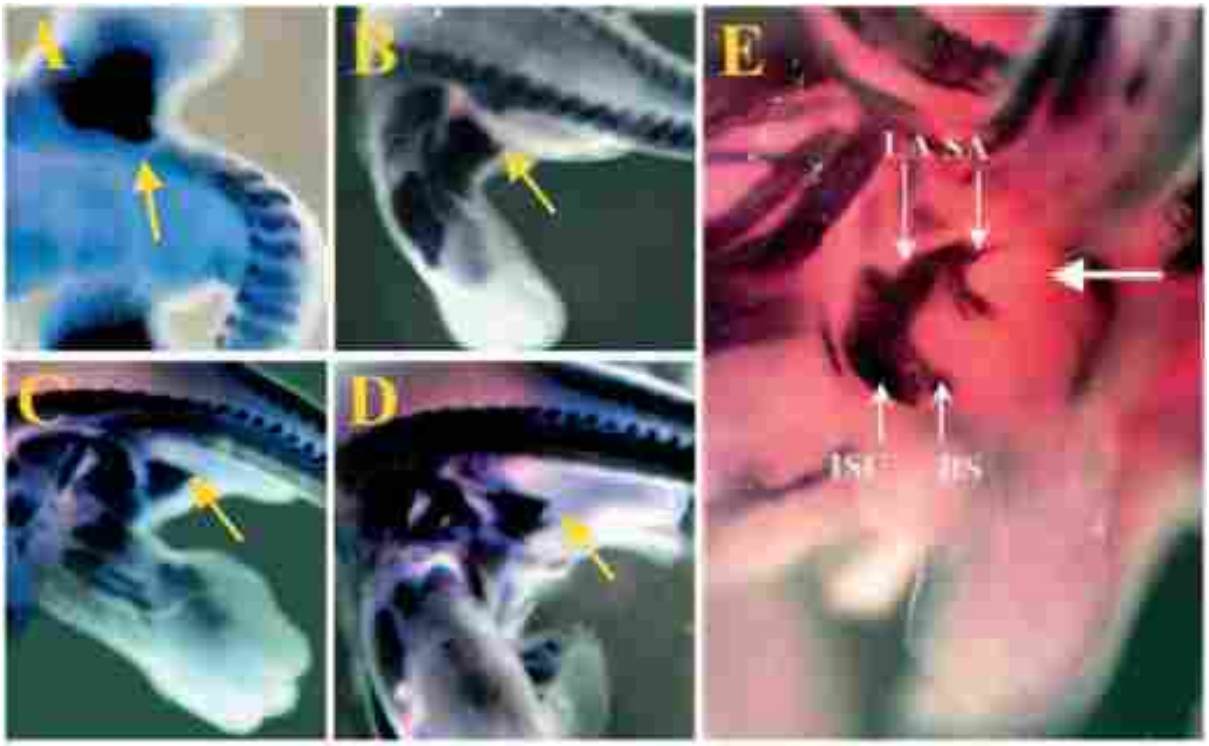

Figure 5- MyoD in situ to analyze mouse perineal muscle development. By E11.5 the ventral muscle mass of the hindlimb has formed A. Muscle migrates out towards the perineal region at E12.5, E13.0, and E13.5 (B-D). By E14.5 the migrating musculature has begun to form individual perineal muscles and has surrounded the anal opening which is indicated by the arrow (E). (Valasek et al., 2005)

process by about E14.5 and expresses *Myosin Heavy Chain (MyoHC)* by E15.5 (Yamaguchi et al., 2008). These myoblast progenitors migrate in an *Lbx1* independent fashion as they never migrate into or enter the distal limb and they also express *MyoD* during the second leg of their journey (Valasek et al., 2005). These two features are characteristic of myotomal extension so the perineal muscle progenitors seem to utilize both types of migration.

The role of muscle connective tissue

Simple guidance of MMPs into the limb is necessary for the formation of limb musculature, but is insufficient to instruct muscles in their specific, complex shapes. However, MMPs preferentially enter and develop in regions of LPM that express the canonical *Wnt* transcription factor *TCF4*, but do not express *Scx* (Bonafede et al., 2006). In fact, regions expressing *Scx* (i.e. tendons) are always devoid of myoblasts (Kardon 1998; Kardon et al., 2003).

These *TCF4* positive/*Scx* negative cells have been positively identified as muscle connective tissue fibroblasts and seem to provide shape and pattern to the newly arriving myoblast progenitors (Mathew et al., 2011).

Through simply manipulating the *Wnt/β-Catenin* pathway in these fibroblasts it is possible to significantly change muscle pattern. For instance, LPM cells expressing a dominant negative version of *TCF4* that represses *TCF4's* normal transcriptional targets do not overlap with muscle and can prevent muscles from forming entirely in regions of high expression (Fig. 6 L,M). Conversely, cells expressing a dominant active form of *β-catenin* that activates its transcriptional targets in the absence of *Wnt* signaling ectopically express *TCF4* and are often surrounded by ectopic musculature (Kardon et al., 2003; Fig. 6 E-G). Lastly, *Wnt3a* soaked beads are surrounded by musculature even when placed in regions that would normally be cartilaginous, indicating that *Wnt* ligand can inhibit the normal cartilaginous fate of LPM and induce a muscle

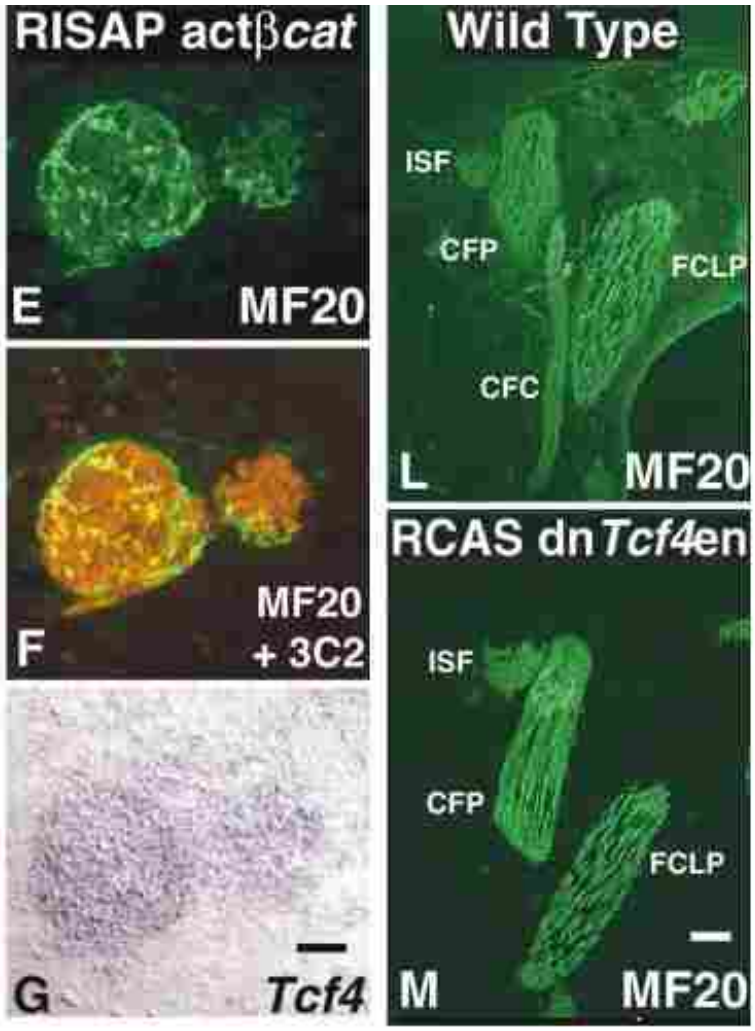

Figure 6- Dominant active β-catenin induces TCF4 cell autonomously and recruits muscle (E-G). Dominant negative TCF4 prevents formation of musculature in a regulative fashion resulting in loss of the CFC muscle (L, M) (Kardon et al., 2003)

connective tissue fate (ten Berge et al., 2008; Fig. 7).

These results demonstrate three principles: *TCF4* can be induced by canonical *Wnt* signaling in limb LPM, muscle connective tissue can be induced ectopically by *Wnt* signaling, and the *Wnt* transcription factor *TCF4* plays a direct role in the recruitment of MMPs by muscle connective tissue. These findings have led to the hypothesis that gradients of *Wnt* ligand and *Wnt* pathway inhibitors lay down a prepattern of *TCF4* expressing muscle connective tissue in the limbs that is filled in by MMPs.

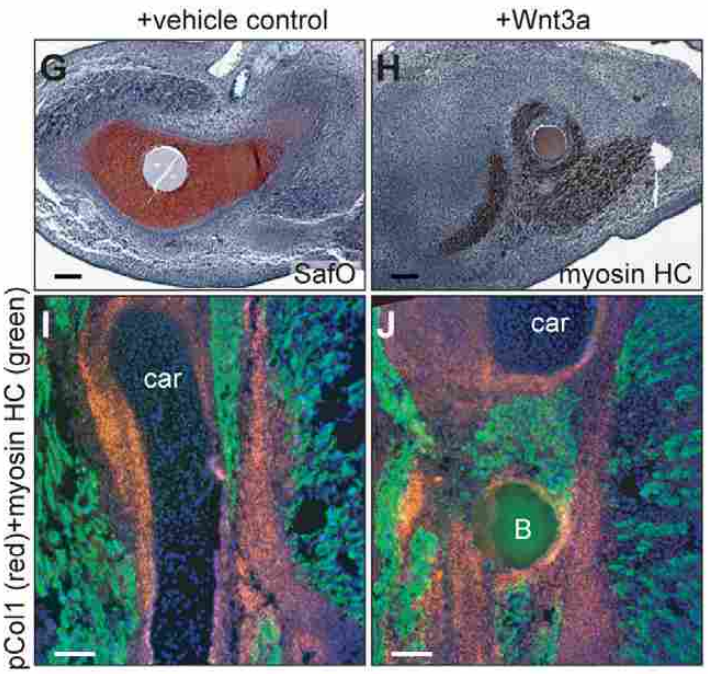

Figure 7- Wnt3a soaked beads repress the cartilaginous fate and recruit musculature into the region surrounding them. (ten Berge et al., 2008)

Connective tissue fibroblasts and/or the surface ectoderm may also play an active role in inducing the differentiation of musculature. Myoblasts cultured in isolation express very little *MyoHC* and do not develop into multinucleated muscle fibers. However, if these cells are cultured with *TCF4* positive connective tissue they develop into multinucleated myofibers as they would in situ (Mathew et al., 2011; Fig. 8). Another group has shown through transplantation experiments that somitic myotome cells contribute to nothing but limb vasculature if introduced into a region of limb where the ectoderm has been removed. Interestingly, introduction of *Wnt6* protein rescues this defect and a subset of the transplanted cells contributes to limb musculature instead (Geetha-Loganathan et al., 2006). However, limb

musculature can develop with minor defects while lacking *β-catenin* so it is unlikely that surface ectodermal *Wnts* play a direct role in myofiber induction and they likely work through an intermediary like connective tissue fibroblasts (Hutcheson et al., 2009). Alternatively, *Wnts* could act through a non-canonical pathway in this process. *Wnt11* secreted by the neural tube and acting through the PCP pathway has been shown to be necessary to correctly orient muscle fibers along the body axis (Gros et al., 2009.) Additionally, in vitro results indicate that *Wnt11*, *Wnt14*, *Wnt4*, and *Wnt7a* can upregulate *MyoHC* expression in limb micromass cultures (Anakwe et al., 2003). Nothing is certain about which pathway these *Wnts* signal through though and they may even be acting through *TCF4* expressing connective tissue fibroblasts.

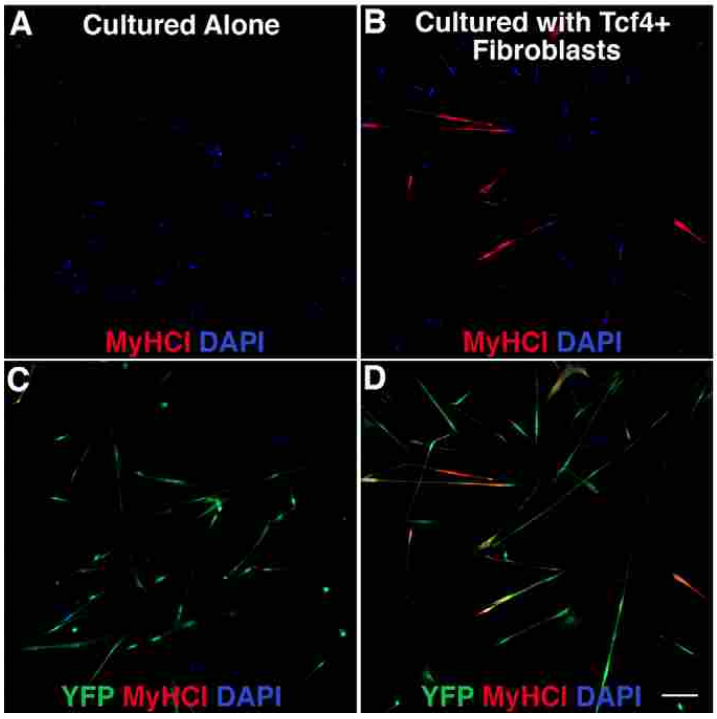

Figure 8- Myoblasts form myofibers and begin to express *MyoHC1* in the presence of TCF4+ fibroblasts, but do not differentiate when cultured alone. (Mathew et al. 2011)

In contrast, the work of some groups indicates that surface *Wnts* play a negligible role in the induction of *TCF4* expression and the patterning of muscle. When *BMP* expressing limb margins and the overlying surface ectoderm are removed, *TCF4* is still induced in the exposed LPM and myoblasts migrate into the region, indicating that surface ectodermal *Wnts* may be dispensable for these processes (Bonafede et al., 2006). On the other hand, the LPM may have been primed by surface *Wnts* to express *TCF4* and removal of *BMP* repression was sufficient to allow for *TCF4* expression. Another group misexpressing *Dkk1*

(a potent canonical *Wnt* inhibitor that binds to *Wnt* receptors) throughout the surface ectoderm with the *K14* (*Keratin14* gene) enhancer found no tendon or muscle defect in embryos. Transgene expression began in some regions as early as E9.5 so would presumably be early enough to reveal any role for ectodermal *Wnts* in *TCF4* induction. However, they did see a great deal of variability in *Dkk1* expression and phenotype between pups. This may have prevented the phenotype from being noticed or perhaps even manifesting (Andl et al., 2002). Thus although some evidence does point to a role for the surface ectoderm in musculotendon patterning, it has yet to be established.

RESULTS

Porcupine

Porcupine (*Porcn*) manipulation is an excellent alternative to other *Wnt* pathway manipulation approaches (Barrott et al., 2011). First identified as a segment polarity gene in *Drosophila*, *Porcn* is an X-linked putative membrane bound o-acyl transferase (MBOAT) necessary for the palmitoylation of *Wnt* ligands prior to secretion (Riggleman et al., 1990; Yang et al., 2008). *Porcn* is required for the palmitoylation of a serine residue (S209 in *Wnt3a*) that is conserved in all but one of the known *Wnts*. In drosophila, *Porcn* has been shown to be necessary for the secretion of multiple *Wnts*, but *WntD*, which lacks the serine, is secreted without any lipid modifications (van den Heuvel et al., 1993; Tanaka et al., 2002; Ching et al., 2008; Takada et al., 2006; Fig. 9). All mammalian *Wnts* contain a conserved serine homologous to S209 that one would predict would also be palmitoylated by *Porcn* prior to secretion (Galli et al., 2011).

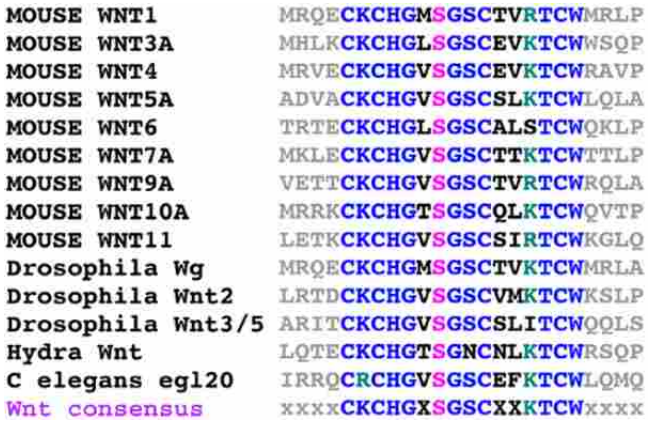

Figure 9- Consensus sequence surrounding the conserved serine palmitoylated only when *Porcn* is present. (Takada et al. 2006)

Direct evidence for *Porcn's* necessity for *Wnt* secretion has also been accumulating in mammals. In L cells, *Porcn* siRNA is capable of greatly reducing the secretion of *Wnt3a.* Further, in L cells transfected with *Wnt3a* expression constructs where S209 has been mutated, *Wnt3a* is not secreted (Takada et al. 2006). In a more recent experiment, mouse ES cells lacking *Porcn* were shown to be incapable of secreting *Wnt3a* (Barrott et al. 2011). Additionally, *Porcn* full and conditional knockouts in mouse recapitulate several known *Wnt* pathway defects. Embryos

mutant for *Porcn* exhibit a phenotype resembling a *Wnt3* knockout and fail to gastrulate (Biechele et al., 2011; Barrott et al., 2011; Barrow et al., 2007; Liu et al., 1999). *Porcn*$^{lox/Y}$; *Prx1Cre* mice recapitulate the limb defect seen in *Wnt5a* mutants and *Porcn*$^{lox/Y}$; *Msx2Cre* mice recapitulate the hindlimb AER loss defect seen in *Wnt3*$^{n/c}$*; Msx2Cre* mice (Barrott et al., 2011; Barrow et al. 2003; Yamaguchi et al., 1999; Fig. 10). These experiments provide evidence that *Porcn* is necessary for epithelial (*Msx2Cre*), mesenchymal (*Prx1Cre*), canonical (*Wnt3*), and non-canonical (*Wnt5a*) *Wnt* signaling. Additionally, although *Porcn* is not expressed ubiquitously, preliminary studies have shown its expression has not been lacking in any regions where *Wnt* ligands are known to be produced. Although *Nodal* and *Hedgehog* are both palmitoylated, *Porcn* plays no role in their palmitoylation or function (Biechele et al., 2011; Buglino et al., 2008).

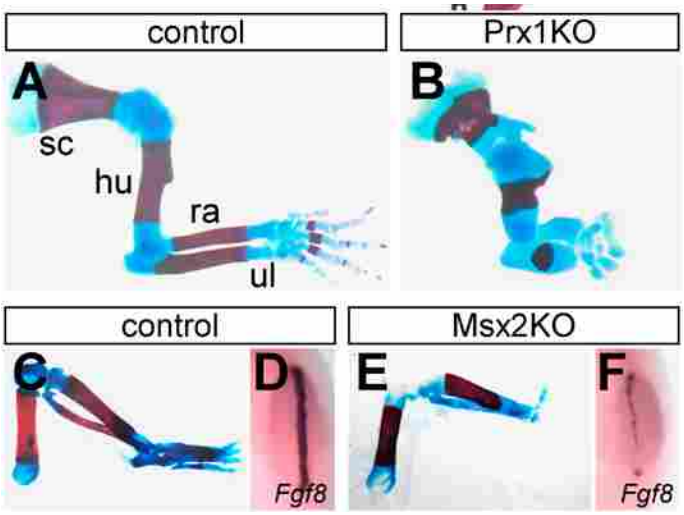

Figure 10- *Prx1Cre Porcn* ablation results in a phenotype similar to the *Wnt5a* KO limb skeletal phenotype (A, B). *Msx2Cre Porcn* ablation results in a skeletal (C, E) and genetic (D, F) phenotype similar to the *Wnt3*$^{n/c}$*; Msx2Cre* phenotype. (Barrott et al. 2011)

Porcn$^{lox/Y}$*; Msx2Cre* mice

These data mentioned above indicate that *Porcn* is a genetic bottleneck for the *Wnt* pathway and could therefore provide an excellent tool for dissecting the role of this pathway in different tissues in the developing mouse. Due to *Porcn*'s necessity in mouse gastrulation we have made use of a *Porcn* allele with loxP sites flanking exons 2 and 3 (Barrott et al., 2011) and two different *Cre* transgenes to remove the *Porcn* gene in a tissue specific manner. This approach has served us well in that we have been able to prevent secretion of *Wnt* ligand from

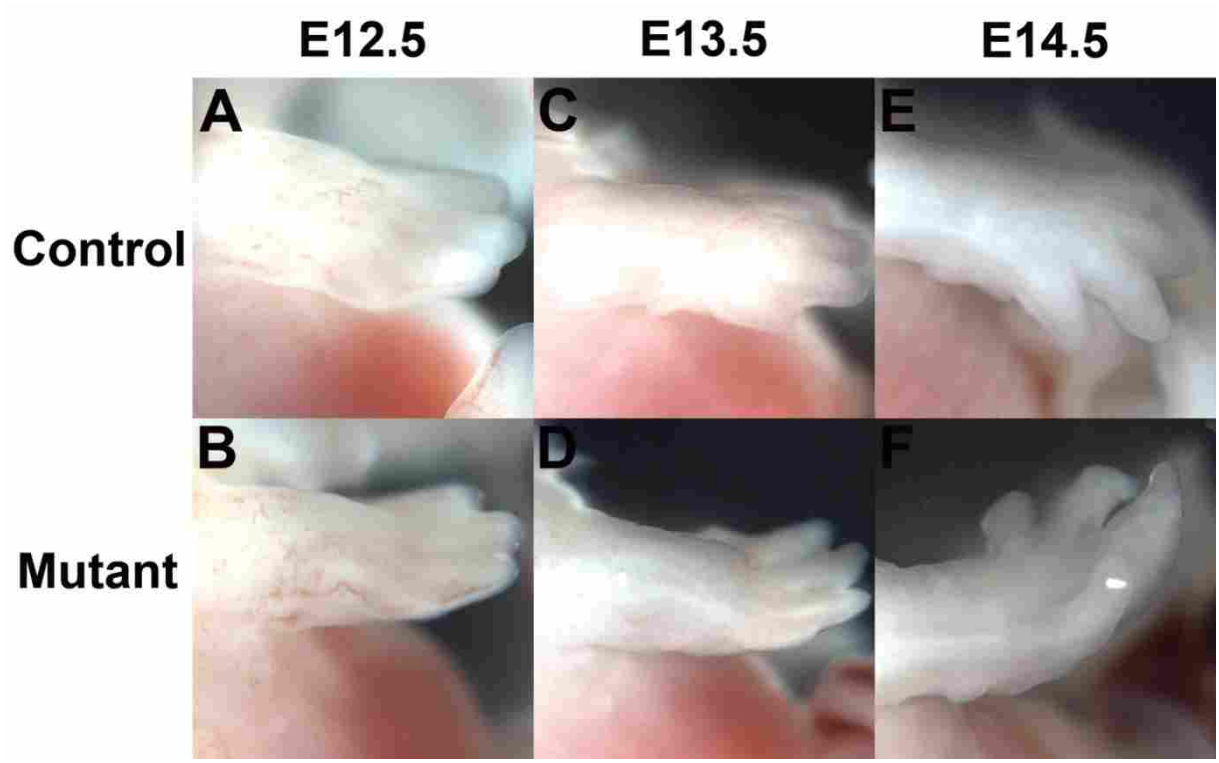

Figure 11- E12.5 mutants have dorsally curved autopods A, B. E13.5 mutants lack footpads and have even more dorsally curved autopods (C, D). By E14.5, mutants often have much more severe curvature and the nail defect is beginning to manifest (E, F).

putative signaling centers while leaving all other *Wnt* sources and signaling intact. Our limited *Porcn* conditional removal studies have demonstrated that this model can uncover defects that traditional approaches might miss. For example, we previously showed that conditional removal of *Wnt3* in the ventral ectoderm of the embryonic forelimb bud does not result in any observable defect (Barrow et al., 2003). In contrast, we have found that conditional removal of Porcn via the same *Msx2Cre* deleter results in several limb defects. $Porcn^{lox/Y}$*; Msx2Cre* mutants exhibit dorsally curved forelimbs, lack footpads, and have rounded deformed nails. Dorsal forelimb curvature is apparent at E12.5 (Fig. 11 A,B) and the defect is as serious as that observed in a newborn at E14.5 (Fig. 11 E,F).

To obtain this result, we crossed female mice homozygous for a conditional *Porcn* allele to males carrying the *Msx2Cre* transgene. All male offspring carry a single conditional copy of

the X-linked *Porcn* allele and all female offspring are heterozygous for the conditional *Porcn* allele. *Cre* carrying males are mutants and *Cre* carrying females are mosaic mutants. At birth the males have thin abdominal skin, lack footpads in the forelimb (Fig. 12 A,B), exhibit dorsal flexion of the forelimb autopod (Fig. 11 E,F), have rounded nails in the forelimb (Fig. 12 A,B), have AER loss defects in the hindlimb (Fig. 12 C,D), and have a nearly absent genital tubercle (Fig. 12 E,F). Since they are mosaics, females recapitulated these defects but to a lesser extent depending on the extent of cells with a deleted gene on the active X chromosome in a given area (Fig. 13 A-D). Some females exhibited an additional defect in that they were also missing nipples if a *Porcn* mutant clone corresponded with a region where a nipple would normally form and thin abdominal skin was commonly observed in them (Fig. 13 E,F). The skin was so thin in mutant males and mosaic females that it was possible to observe

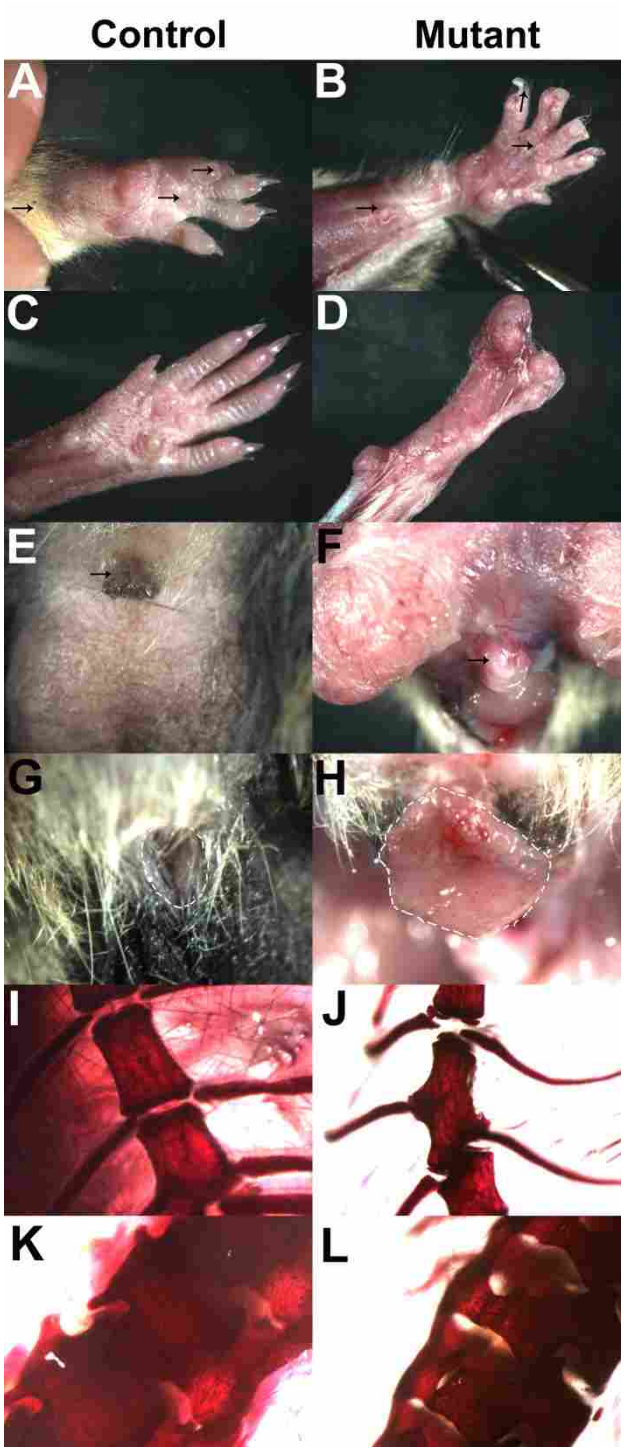

Figure 12- In the mutant male forelimb, footpads and ventral hair are absent. Additionally nails are rounded (A, B). In the mutant male hindlimb AER loss defects are apparent, generally the loss of parts of the autopod (C, D). Mutant males have greatly reduced genital tubercles (E, F). Mutant males have prolapsed rectums (G, H). 1/1 mutant males presented with a sternal defect and a vertebral closure defect (I-L)

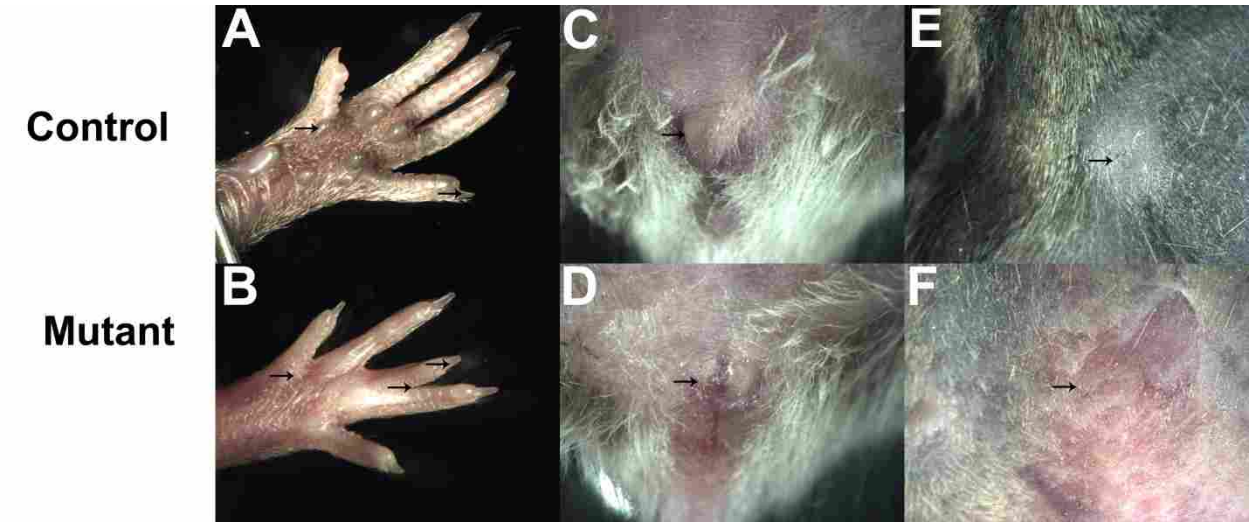

Figure 13- Female mosaic mutants exhibit mild AER loss defects in the hindlimb like syndactyly and brachydactyly and lose footpads (A, B). They also have reduction of the genital tubercle and lose nipples in regions of heavy deletion (C-F).

internal organs through it in regions of strong deletion (data not shown). All mutant males that survived to adulthood presented with rectal prolapse and grew no fur in regions of strong *Msx2Cre* activity (Fig. 12 G,H). They had essentially normal mobility but could not mate or grasp objects with their forelimb digits. Skeletal preps revealed sternal abnormalities in two of six mutants (Fig. 12 I,J) and vertebral abnormalities were observed in both adult mutant skeletal preps (Fig. 12 K,L).

Loss of the nipples, fur, and footpads is likely due to the well-documented requirement for *Wnt* signaling in the development of ectodermal appendages. Ectodermal *Wnt10b* is one of the earliest markers of each of these structures (Chu et al., 2004; Mikkola et al., 2006; Andl et al., 2002). The genital tubercle has also been shown to rely on ectodermal *Wnt* signals for proper development so the reduction in the penis and clitoris in adult male and female mutants respectively is unsurprising (Miyagawa et al., 2009).

Time-course of deletion

To better understand the basis of these defects, we performed *ROSA26* reporter assays to determine when and where *Cre* is active in embryos (as demonstrated through *β-galactosidase*

activity). We found that *Cre* activity is complete throughout the forelimb ventral ectoderm, abdominal ectoderm and urogenital ectoderm by E10.5 (Fig. 14 A-C). Additionally, the ectoderm that lines the anus comes from the urogenital ectoderm and *Porcn* is deleted by at least the time those cells enter the future anal canal at E14.5 (Fig. 14 G-I). We next utilized in situ hybridization of the canonical *Wnt* reporter gene *Axin2* to pinpoint the timing of the actual loss of *Wnt* signaling (Al Alam et al., 2011). At E10.5, mutant expression of *Axin2* in the ventral forelimb looks like it could be slightly reduced (Fig. 15 A,B), but by E11.5 *Axin2* expression is almost entirely absent

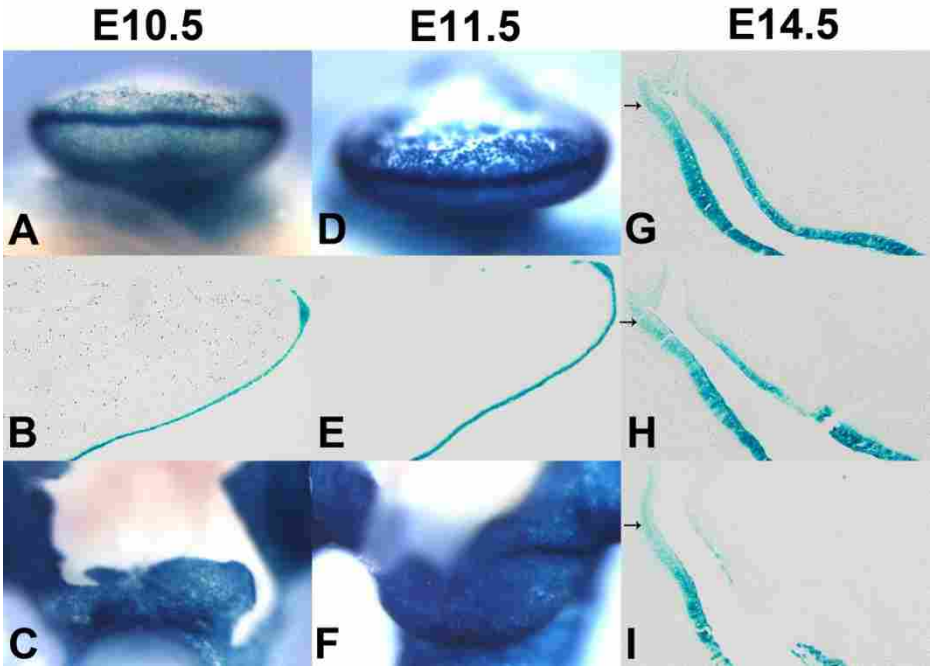

Figure 14- *Rosa*$^{lacZ/lacZ}$ reporter reveals recombination is complete in the ventral ectoderm of the forelimb by E10.5 (A,B). Deletion is complete in the urogenital ectoderm by E11.5 (F). The ectoderm that contributes to the anal canal (marked by arrows) is deleted in all cases although deletion in other parts of the perineum at E14.5 is variable (G-I).

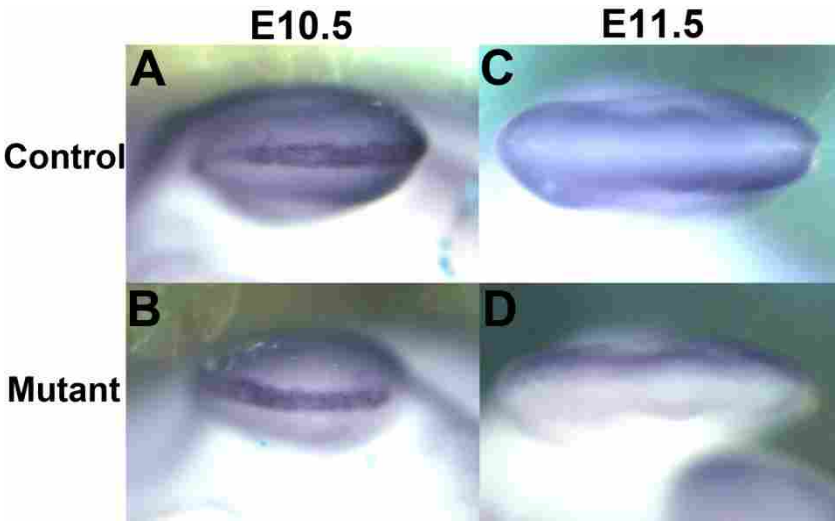

Figure 15- E10.5 mutants have similar ventral *Axin2* staining relative to WT (A, B). E11.5 mutants have almost absent ventral staining relative to WT (C, D).

from that region (Fig. 15 C,D). Additionally, by E12.5 mutant forelimbs have already begun to curve dorsally (Fig. 11 A,B). Thus our phenotype must be downstream from a *Wnt* ligand or ligands that are expressed in the ventral limb ectoderm between E10.5 and E12.5. The candidate genes are *Wnt3*, *Wnt4*, *Wnt6*, *Wnt7b*, *Wnt9b*, and *Wnt10b* (Witte et al. 2009; Parr et al. 1999). However, no forelimb phenotype has been reported for *Wnt4* mutants, *Wnt3*$^{n/c}$*; Msx2Cre* mice, *Wnt6* mutants, *Wnt7b* mutants, *Wnt9b* mutants, or *Wnt10b* mutants (Vainio et al., 1999; Barrow et al., 2003; Potok et al., 2008; Parr et al., 2001; Shu et al., 2002; Carroll et al., 2005; Vertino et al., 2005; Bennett et al., 2005). This result is an excellent example of how the ligand redundancy inherent in *Wnt* signaling can mask phenotypes in traditional knockout strategies. No less than six *Wnt* ligands are expressed at the time and place of interest and none of them are solely responsible for the phenotype uncovered by preventing all *Wnt* secretion.

Dorsoventral pattern is normal in *Porcn*$^{lox/Y}$*; Msx2Cre* mutants

The loss of *En1* resulting in the "double-dorsal" phenotype seemed an attractive candidate to examine as the potential cause of the *Porcn*$^{lox/Y}$; *Msx2Cre* phenotype as these mice also lacked footpads, had deformed nails, and the digits had inappropriate dorsal flexion. However, unlike *En1* mutants where ectopic ventral *Wnt7a* expression in the ventral ectoderm is

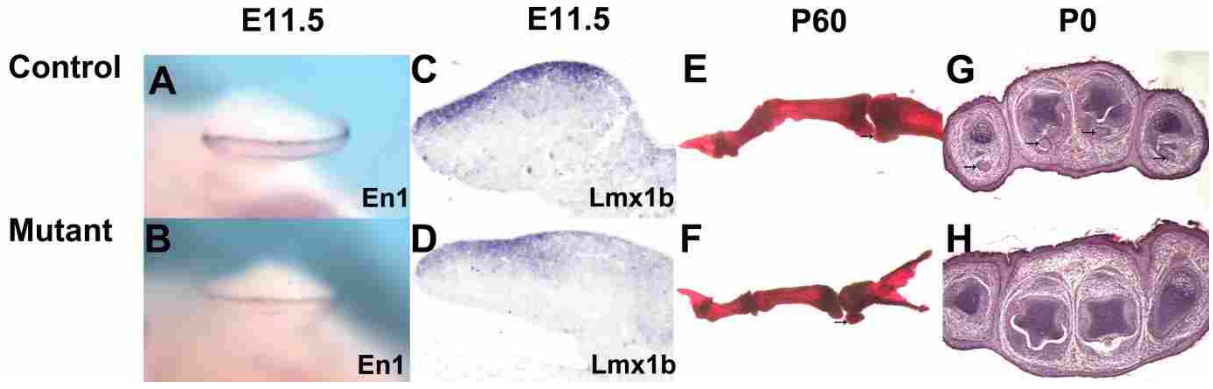

Figure 16- En1 is present ventrally in E11.5 mutants (A,B) and Lmx1b is absent ventrally in E11.5 mutants (C,D). Ventral arrows identify sesamoid bones, present in adult mutant mice similarly to control mice (E, F). Arrows indicate the presence of ventral digital flexor tendons in newborn controls and absence in newborn mutants (G, H).

the root cause of the double dorsal phenotype, the *Porcn*$^{lox/Y}$; *Msx2Cre* mutants should be unable to secrete *Wnt7a* in the ventral ectoderm. This made it unlikely that ectopic activation of dorsal pathways in the ventral limb was responsible for our phenotype.

To further investigate this possibility we performed several experiments to determine whether the dorsal curvature was a component of inappropriate dorsalization of the ventral limb. First, we utilized in situ hybridization to examine expression of the dorsal marker *Lmx1b* and the ventral marker *En1*. Mutants were indistinguishable from controls in expression of those genes and in all cases *En1* was present in the ventral ectoderm (Fig. 16 A,B) and *Lmx1b* was absent in the ventral mesenchyme (Fig. 16 C,D). This showed that any dorsalization that might have been occurring must have been occurring downstream of the major genetic pathways that regulate dorsoventral pattern. We next scrutinized the gross phenotype more carefully through skeletal preps and H&E staining of paraffin sections. Skeletal preps revealed that ventral sesamoid bones were still present, indicating that wild-type dorsoventral skeletal patterning was intact (Fig. 16 E,F). Additionally, our histological examination revealed that the flexor digitorum superficialis and profundus tendons were entirely absent in the autopod rather than simply flattened and dorsalized as they would have been in "double-dorsal" mice (Fig. 16 G,H). These experiments showed that the observed defects were not due to dorsalization of the ventral limb but were instead due to a failure of ventral limb characteristics to form. We then chose to further investigate the tendon defect and determine what role ectodermal *Wnts* play in tendon formation.

Tendon induction and loss

Due to defective ventral autopod tendons, *Scx* mutants have a similar limb curvature to *Porcn*$^{lox/Y}$; *Msx2Cre* mutants (Murchison et al., 2007) and as we mentioned previously, an ectodermal signal is known to be necessary for tendon induction. It is therefore possible that the

dorsal digital curvature observed in mutants is due to an early absence of ventral tendons which is in turn due to the absence of ventral *Wnt* signals. Utilizing wholemount *Scx* in situ hybridization we examined the time-course of tendon development in these mutants. At E12.5, tendon pattern in mutants is indistinguishable from controls and the tendon primordia of the digits appear as a long wide band along the ventral surface of the ectoderm just superficial to the digital condensations (Fig. 17 A,B). However, by E13.5 the flexor digitorum superficialis and profundus tendons are almost entirely absent in the digits (Fig. 17 C-F). The normal initial patterning of the tendons indicates that ectodermal *Wnts* play no role in the induction or patterning of limb tendons. The later loss of tendons could indicate a vital role for ectodermal *Wnts* in the maintenance of tendon development. Alternatively, these results could be explained by previous work, which demonstrated that although the initial patterning and induction of the

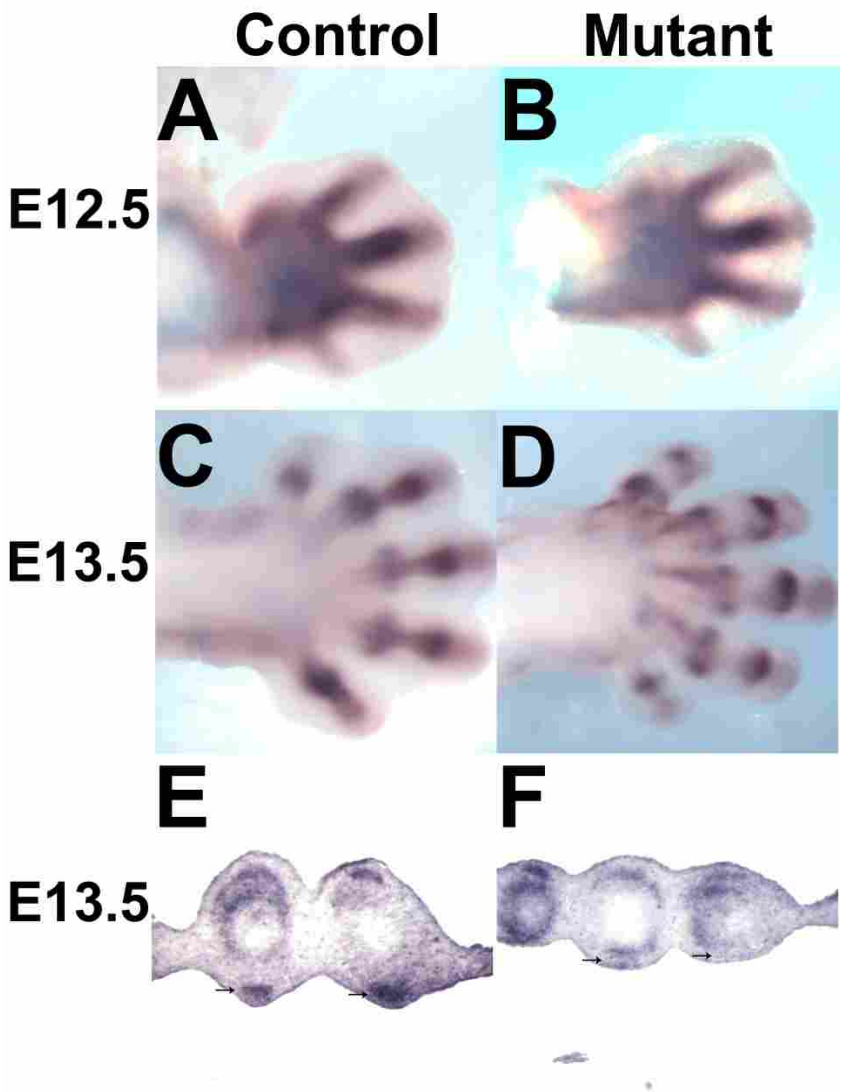

Figure 17- Scx wholemount in situ reveals no differences between mutants and controls at E12.5 (A, B). By E13.5, Scx in situ indicates a loss of the flexor digitorum tendons in wholemount (C, D) and in section (E, F).

digital tendons occurs independent of the limb musculature, tendons are lost later in development if the associated musculature is absent (Kardon 1998).

General loss of musculature in *Porcn*$^{lox/Y}$; *Msx2Cre* mutants

In all three locations in the body where *Cre* deletion is earliest and most complete there are indications that nearby musculature is absent. For instance, the loss of distal forelimb tendons after the patterning stage indicates that associated limb muscles may be absent. We were able to confirm this loss of musculature through immunohistochemical staining of myosin heavy chain with the pan-myosin heavy chain antibody, A4.1025, which marks all musculature. We found a reduction and/or loss of the lumbrical muscles (Fig. 18 A,B), the thenar (thumb) muscles, and the hypothenar (pinky) muscles in the autopod (Fig. 18 C,D). We additionally found a loss of the

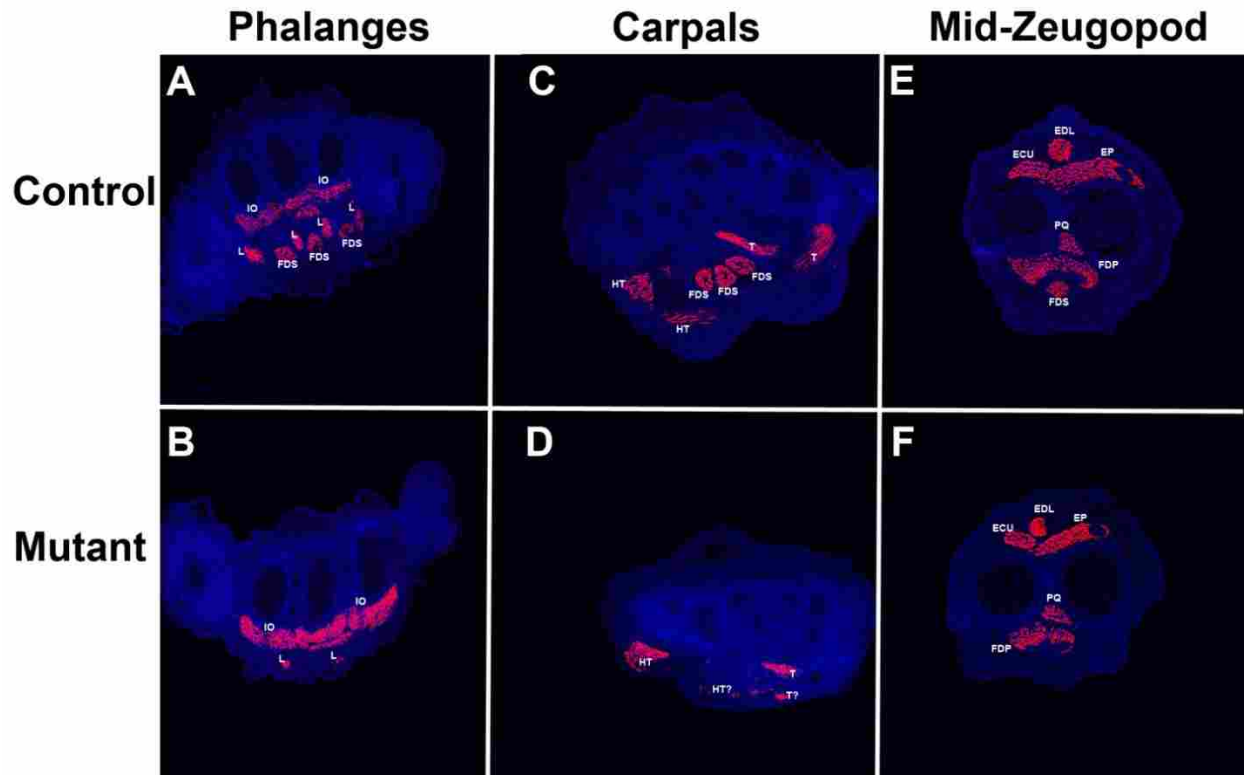

Figure 18- At E14.5 multiple distal limb muscles are missing or reduced in mutant males. In the distal limb interosseus (IO) muscles seem to be unaffected, but the branches of the flexor digitorum superficialis (FDS) are absent and the lumbrical (L) muscles are either reduced or absent (A, B). In the carpals hypothenar (HT) and thenar (T) muscles are reduced or absent and the FDS branches are absent (C, D). In the mid-zeugopod the flexor digitorum profundus (FDP) and pronator quadratus (PQ) are normal but the FDS is absent (E, F).

flexor digitorum superficialis (FDS) muscle in the autopod and zeugopod (Fig. 18 E,F). The reduction in thenar and hypothenar musculature provides a basis for the abnormal dorsal curvature found in the thumb and pinky and the loss of the FDS explains the curvature present in the middle three digits (Fig. 11 C-F). Additionally the loss of these muscles provides a mechanistic basis for the loss of the associated digital tendons. In contrast to those highly affected muscles, there are some muscles that seem to be unaffected by the deletion of ectodermal *Porcn.*

Most notable among these are the interosseus (IO) muscles (Fig. 18 A,B) and the pronator quadratus (PQ), (Fig. 18 E,F).

As the PQ and IO muscles are unaffected in our *Msx2Cre* mutants, we decided to test whether they rely upon mesodermal *Wnts* for their appropriate patterning rather than ectodermal *Wnts*. To accomplish this, we generated *Porcn*$^{lox/Y}$; *Prx1Cre* mutants and examined their limb musculature. *Prx1Cre* drives

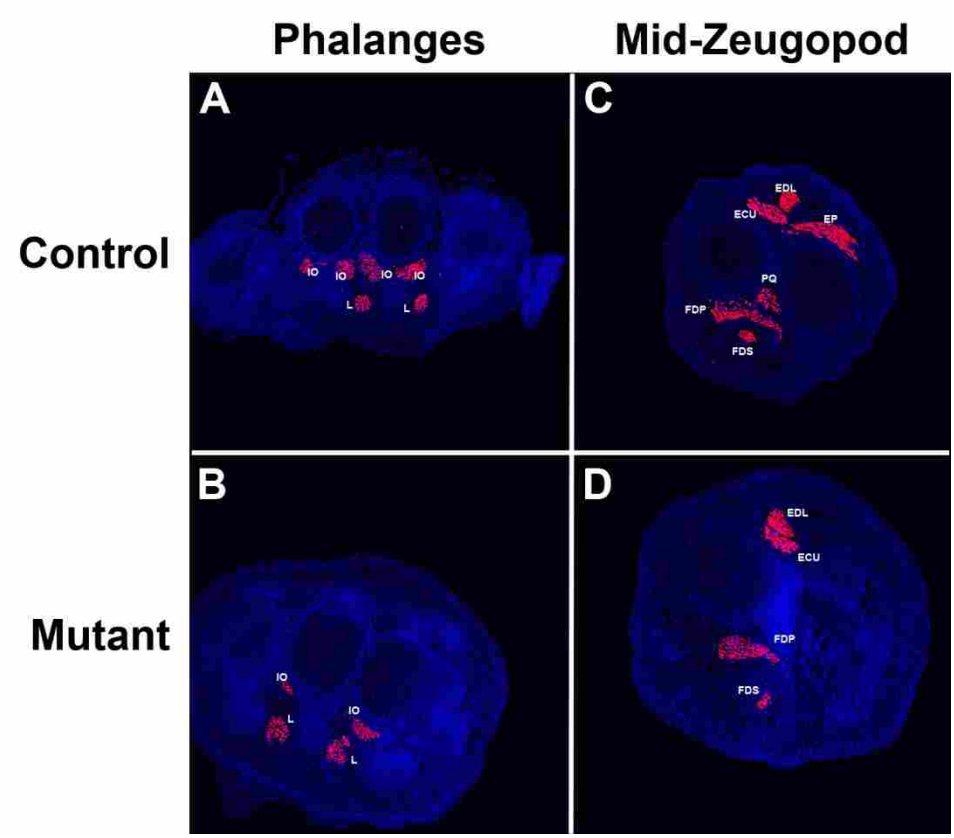

Figure 19- Muscles that were unaffected by the deletion of ectodermal Porcn seem to be reduced or lost in *Porcn*$^{lox/Y}$; *Prx1Cre* mutants at E14.5. The interosseus (IO) muscles (A, B) and the pronator quadratus (PQ) (C, D) are two of the best examples of this. Additionally, the flexor digitorum superficialis (FDS) is essentially unaffected in these mutants while it was the most heavily affected muscle in the *Porcn*$^{lox/Y}$; *Msx2Cre* mutants.

deletion at a very high rate in the limb mesenchyme by E10.5 and generates recombination of floxed genes in essentially every limb mesenchymal cell (Logan et al., 2002). Thus it is the *Cre* standard for knocking out genes specifically in the limb mesenchyme. In these mutants we found that the IO muscles are patchy or absent throughout their normal range (Fig. 19 A,B) and that the PQ muscle is absent or reduced in size throughout the mid-zeugopod (Fig. 19 C,D). Additionally, the FDS muscle in these mutants is as robust as in controls and seems to be unaffected (Fig. 19 C,D).

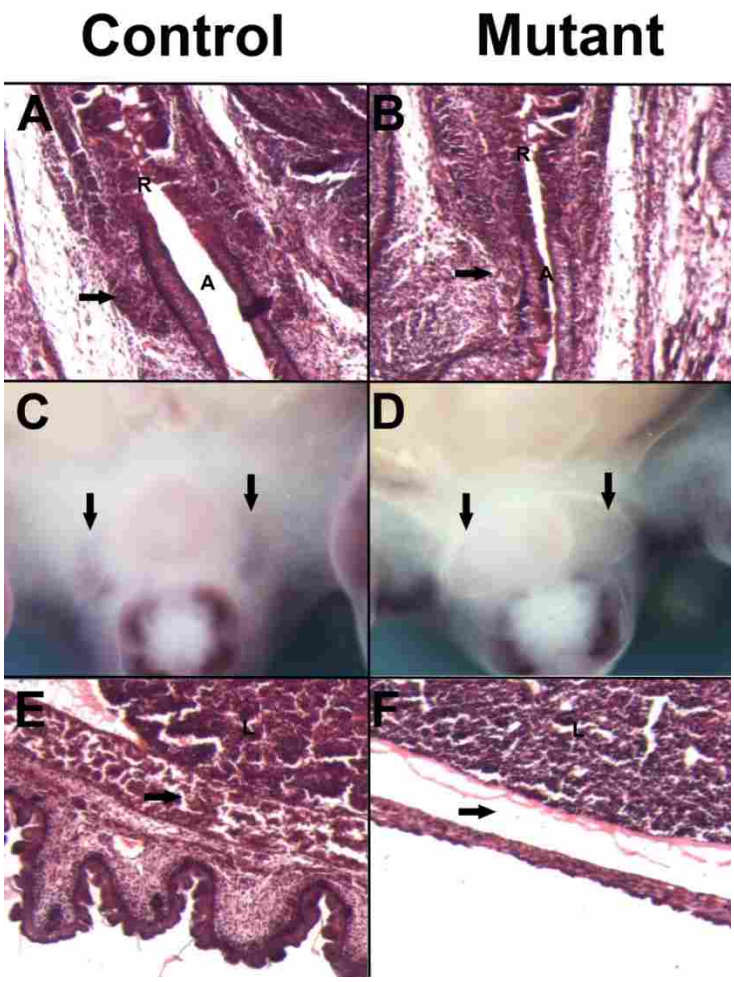

Figure 20- Arrows indicate the presence in controls and absence in mutants of the external anal sphincter. R is rectum and A is anus (A, B). Arrows show the absence of perineal muscle in mutants (C, D). Arrows indicate the loss of musculature just deep to the abdominal ectoderm of mutants. L is liver (E, F).

We found additional evidence that ectodermal *Wnts* play a critical role in the patterning of underlying muscles in the rectal prolapse that manifests in all adult mutants. A common cause of rectal prolapse is weakness, loss, or failure of the external anal sphincter or its associated nerves, tendons, or connective tissue. Indeed, H&E staining of sagittal E16.5 sections indicates that the external anal sphincter is entirely absent in mutants at that stage (Fig. 20 A, B). Additionally, *MyoD* in situ reveals that perineal musculature fails to migrate into the perineum

by E13.5 in mutants, while controls have robustly staining perineal muscle precursors. (Fig. 20 C,D). Our last clue is found in the transparent nature of the abdomen. This indicates that underlying abdominal musculature may be absent. Our preliminary histological analysis of the abdomen indicates that this is the case. Not only is the skin exceptionally thin, but the entire layer of muscle normally separating the skin from the liver is absent in mutants at E16.5 (Fig. 20 E,F).

DISCUSSION

We have shown that ventral ectodermal *Wnts* play no role in the establishment of initial dorsoventral pattern and that they are not required for the initial patterning and induction of tendons. Ectodermal *Wnts* do however play a role in the patterning and development of muscles that develop by very different means and in very different locations. Our results indicate that muscles that develop through myotomal extension, muscle precursor migration, and "in and out" muscle migration need ectodermal *Wnts* and may all rely upon *TCF4* expressing muscle connective tissue to take on the correct shape in the correct location (Fig. 21). Future research could demonstrate the role of *TCF4* expressing cells by looking at muscle connective tissue pattern in each of the regions that have perturbed muscle pattern. Although alterations in the pattern of muscle connective tissue lead to alterations in the pattern of musculature, the converse

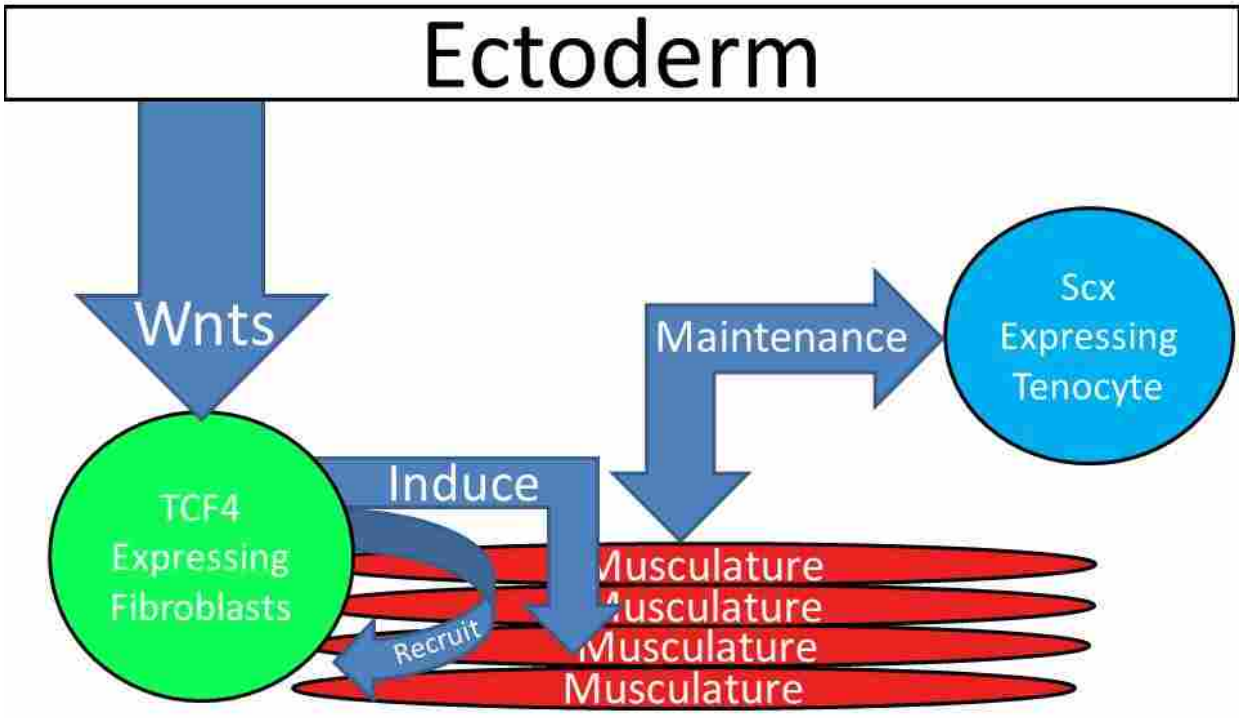

Figure 21- Ectodermal *Wnts* signal to *TCF4* expressing fibroblasts which in turn induce muscle differentiation in myoblasts and recruit myoblasts. The musculature in turn maintains *Scx* expressing tenocytes while being patterned in turn by tenocytes.

is not true. In mice lacking limb musculature due to a migration defect, the *TCF4* pattern is essentially normal at E13.5. Additionally, in chick embryos with limbs grafted to the coelomic cavity where MMPs cannot enter, the *TCF4* pattern is also normal (Kardon et al., 2003). Thus a simple way to determine if a muscle connective tissue or *TCF4* defect is upstream of any other defects would be to simply probe for *TCF4* mRNA or protein at E13.5. Perturbations in this pattern would indicate that ectodermal *Wnts* were likely involved in the establishment of the *TCF4* prepattern.

Some muscles seem to be refractory to the absence of ectodermal *Wnts*. For instance, in the zeugopod of the limb the pronator quadratus and many other zeugopodal muscles form normally and only the flexor digitorum superficialis is absent. Perhaps the muscle connective tissue for these other muscles was induced by ectodermal *Wnts* prior to completion of the knockout. However, muscles develop in a proximal to distal fashion in the limb and the interosseus muscles found far distally in the autopod do not seem to be reduced or defective at all (Kardon 1998). Thus it may be less a matter of timing of the knockout and more a question of the source of the inducing *Wnts*. The muscles that are unaffected may be patterned under the control of mesenchymally expressed *Wnts* such as *Wnt5a* or *Wnt11*. Our study of musculature in *Porcn*$^{lox/Y}$; *Prx1Cre* mutants indicates that this may be the case. Muscles that are unaffected or mildly affected in *Porcn*$^{lox/Y}$; *Msx2Cre* mutants seem to be the most heavily affected muscles in *Porcn*$^{lox/Y}$; *Prx1Cre* mutants. Additionally, muscles that are robustly affected in *Porcn*$^{lox/Y}$; *Msx2Cre* mutants seem to be essentially normal in *Porcn*$^{lox/Y}$; *Prx1Cre* mutants. However, *Porcn*$^{lox/Y}$; *Prx1Cre* mutants recapitulate the *Wnt5a* skeletal phenotype in the limb which results in an extreme shortening of the skeleton and the loss of some skeletal elements. The skeleton also plays a role in the patterning of musculature so the changes in the skeletal elements of the

limb in *Porcn*$^{lox/Y}$; *Prx1Cre* mutants may be the primary cause of the observed muscle defects. In the future we hope to compare *Wnt5a* mutant limbs to *Porcn*$^{lox/Y}$; *Prx1Cre* mutant limbs as they have the same skeletal structure. Thus we could distinguish between muscle pattern changes due to skeletal structure changes and muscle pattern changes due to the loss of mesenchymally secreted *Wnts*.

Another unanswered question can be found in the perineal musculature. We have only shown that the external anal sphincter fails to form and that these muscles fail to enter the perineum. Further study should be done to examine whether the perineal muscle precursors ever leave the hindlimb. There are two other reasons that muscle could have failed to migrate. First, migration may not have occurred due to a loss of expression of *SDF1*.This ligand is not only expressed in the perineum but is also expressed in the connective tissue fibroblasts of the limb bud so it could potentially be the signal induced by ectodermal *Wnts* (Vasyutina et al., 2005). Alternatively, they could fail to migrate because the ventral muscle compartment in the hindlimb was so reduced due to the hindlimb *Wnt* defects that there were no myoblasts that could migrate out. However, this is also unlikely as most manipulations that affect hindlimb development do not affect the formation of perineal musculature. Only the chick limbless defect where no limb is formed at all and a complete surgical removal of the hindlimb bud have been sufficient to prevent perineal musculature formation (Valasek et al., 2005). The hindlimbs in our mutant mice, while often lacking much of the autopod, are very functional and essentially normal in the zeugopod and stylopod regions. Additionally, some distal autopod musculature does form in these mutants indicating that the hindlimb muscle mass was relatively normal in size (data not shown). Thus it is unlikely that the external anal sphincter defect stems from disruptions to the hindlimb. However, only further study of this process through *MyoD* and *SDF1* in situs can

definitively answer this question. Further research on this path would be enlightening and perhaps reveal additional roles for ectodermal Wnts in the patterning of musculature.

Porcn mutations have also been documented in the human population. In females, X-linked mosaicism of *Porcn* leads to Goltz syndrome/Focal Dermal Hypoplasia. Males can also be afflicted with this syndrome if they lose *Porcn* function chimerically (Grzeschik et al., 2007). As *Porcn* is involved in so many processes in development and its loss can result in defects chimerically, it is an attractive candidate to explain other human disorders. For instance, Poland Syndrome is a congenital defect of unknown etiology in which parts of the pectoralis major and other nearby muscles are absent with an ipsilateral deformation of the autopod. Ipsilateral axillary hair loss, thin skin, and nipple loss are also commonly reported as components of this syndrome (Urschel 2009). Late clonal loss of *Porcn* during development could explain all of these defects if *Porcn* were lost in the ectoderm that contributes to the chest epidermis and the epidermis of the ipsilateral limb. A simple genetic test of skin from the affected region of a Poland Syndrome sufferer could answer this question and is a future experiment we would like to do. If *Porcn* is the root cause of Poland Syndrome then our work will provide a great deal of help in elucidating the causes of the various associated defects, especially the muscle loss defect.

EXPERIMENTAL PROCEDURES

Histology

We utilize paraffin sectioning and Hemotoxylin and Eosin (H&E) staining to examine histology. We follow the Reynolds lab's H&E staining protocol and use the Reynolds lab's embedding machine for the embedding of embryos. For sagittal sections of E16.5 mice we use Bouin's fixative as it preserves the tissue better. We utilize 4% PFA in PBS for all other fixation as it is better for preserving antigenicity.

In Situ Hybridization

We utilize section and wholemount in situ hybridization to examine gene expression of Axin2, En1, Scleraxis and Lmx1b. We followed the McMahon Lab protocol, "Digoxigenin-labeled *in situ* Hybridization for P1 Mouse Kidney Sections" by J Bielagus and A Majumdar from May 3, 2002 for the section in situ hybridization. For wholemount in situ we used the 1997 McMahon lab protocol.

Rosa$^{lacZ/lacZ}$ Reporter

Our *Rosa*$^{lacZ/lacZ}$ mice have *lacZ* inserted in the *Rosa* locus which drives expression in every cell. The neomycin resistance gene flanked by *loxP* sites is inserted upstream of *lacZ* preventing its translation. Cre recombinase removes neomycin resistance by recombining at the *loxP* sites and allows for transcription and translation of *lacZ* (Soriano 1999). After recombination embryos will turn Xgal into a blue substrate in any region where recombination has occurred. Thus blue substrate marks the regions where Cre has been active. We utilize standard *lacZ*/XGAL staining procedures and paraffin sectioning to examine Cre deleter activity. We used the Krumlauf lab "β-Galactosidase Staining of Whole Embryos" protocol for all *Rosa*$^{lacZ/lacZ}$ staining.

Porcn Conditional Mice

Our *Porcn* conditional mice have had *loxP* sites inserted adjacent to exons 2 and 3 so that both of those exons will be deleted from the genome in the presence of *Cre* recombinase (Fig. 22 A). Exon 2 contains the translation start site so no protein is expected to be produced after recombination. ESCs that have undergone recombination lack protein according to a Western blot with a *Porcn* C-terminus reactive polyclonal antiserum (Fig. 22 B) (Barrot et al. 2011).

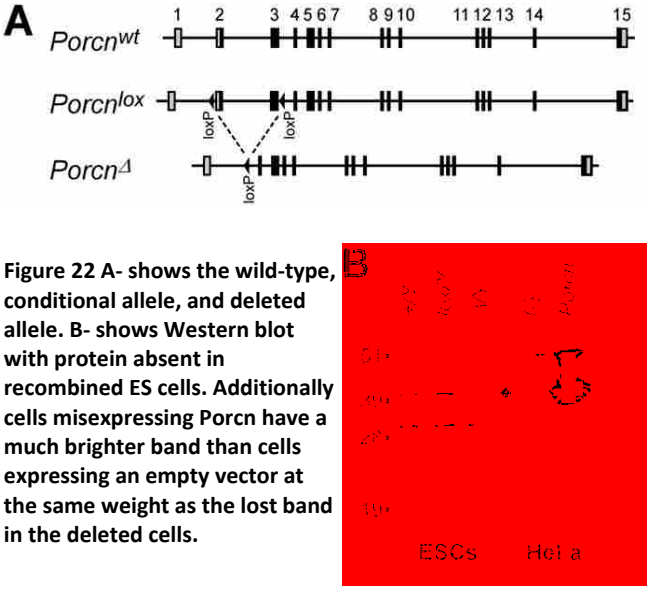

Figure 22 A- shows the wild-type, conditional allele, and deleted allele. B- shows Western blot with protein absent in recombined ES cells. Additionally cells misexpressing Porcn have a much brighter band than cells expressing an empty vector at the same weight as the lost band in the deleted cells.

Immunohistochemistry

Antibody	Type	Source	Product Number	Working Concentration
MyoD	Mouse IgG1	Santa Cruz Biotechnology	Sc-32758	4 ug/mL
MyoHC	Mouse IgG2a	Developmental Studies Hybridoma Bank	A4.1025	2.5 ug/mL
Pax7	Mouse IgG1	Developmental Studies Hybridoma Bank	PAX7	2.4 ug/mL
TCF4	Mouse IgG2a	Millipore	05-511	10 ug/mL

Table 1

For our immunohistochemistry we utilized antibodies according to Table 1. We use sodium citrate antigen retrieval for all antibodies. The *TCF4* antibody marks all muscle connective tissue

and the *MyoHC* antibody marks all mature and maturing musculature and all myosin heavy chain. The *MyoD* antibody marks myoblasts from their initial specification until their maturation. The *Pax7* antibody marks myoblast progenitors until they begin to differentiate. For immunohistochemistry we followed the Stark lab protocols: "Embryo embedding/sectioning" and "Immunohistochemistry on Frozen Sections."

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CURRICULUM VITAE

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RESEARCH INTERESTS

Vertebrate Limb Development
Muscle Migration and Development
Placode Development

ACADEMIC PREPARATION

2010-Present **MS in Physiology and Developmental Biology:** *Laboratory of Dr. Jeffery Barrow,* Brigham Young University, Provo, UT
'Development emphasis'

2004-2010 **BS in Physiology and Developmental Biology:**
Department of Physiology and Developmental Biology,
Brigham Young University, Provo, UT

PROFESSIONAL EXPERIENCE

2010-Present **Undergraduate Research Mentor**
Dr. Jeffery Barrow's Laboratory
Brigham Young University, Provo, UT

Undergraduate Mentoring Projects:
-Placodes rely on ectodermal Wnts
-Ectodermal Wnts are necessary for limb muscle recruitment

2010-Present **Teaching Assistant,** Developmental Biology
Physiology and Developmental Biology Department
Brigham Young University, Provo, UT

Spring 2011 **Teaching Assistant,** Cell Biology

Physiology and Developmental Biology Department
Brigham Young University, Provo, UT

2009-2010 **Undergraduate Research Assistant**
Dr. Jeffery Barrow's Laboratory
Department of Physiology and Developmental Biology
Brigham Young University, Provo, UT
'Clonal ROR2 knockout prevents migration of clones toward the AER'

FELLOWSHIPS AND AWARDS

January 2010 **ORCA Grant** ($1500)
Office of Research and Creative Activities
Brigham Young University, Provo, UT

August 2010 **Teaching Assistantship** ($5000)
Department of Physiology and Developmental Biology
Brigham Young University, Provo, UT

January 2011 **Teaching Assistantship** ($2500)
Department of Physiology and Developmental Biology
Brigham Young University, Provo, UT

January 2011 **Research Assistantship** ($2500)
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April 2011 **Teaching Assistantship** ($2500)
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June 2011 **Research Assistantship** ($2500)
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August 2011 **Teaching Assistantship** ($5000)
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January 2012 **Research Assistantship** ($5000)
Department of Physiology and Developmental Biology
Brigham Young University, Provo, UT

April 2012 **Research Assistantship** ($3750)
Department of Physiology and Developmental Biology
Brigham Young University, Provo, UT

March 2012 **James B. Duke Fellow** ($20,000)
Department of Graduate Studies

Duke University, Durham, NC

March 2012 **Chancellor's Scholar** ($5000)
Department of Graduate Studies
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PUBLICATIONS

Barrott JJ, Cash GM, **Smith AP**, Barrow JR, Murtaugh LC. Deletion of mouse Porcn blocks Wnt ligand secretion and reveals an ectodermal etiology of human focal dermal hypoplasia/Goltz syndrome. Proc Natl Acad Sci U S A. 2011 Aug 2;108(31):12752-7.

RESEARCH PRESENTATIONS

July 2011 **Poster: SDB 70th Annual Meeting,** Chicago, IL
'Ectodermal Roles of Wnt Secretion'

LABORATORY SKILLS

I have experience in the following:

E10-E12 age mouse embryos
Sectioning: frozen and paraffin
H&E preps
Skeletal preps
In situ hybridization
PCR and genotyping
DNA preps
Transformations
Restriction enzyme DNA analysis
DNA sequencing
Sonoporation
Mouse husbandry
72-148 hr chick embryos

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