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WNT5A Mutations in Patients with Autosomal Dominant Robinow Syndrome

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Abstract

Robinow syndrome is a skeletal dysplasia with both autosomal dominant and autosomal recessive inheritance patterns. It is characterized by short stature, limb shortening, genital hypoplasia and

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

Genebank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for human *WNT5A* [NM003392], mouse *Wnt5a* [NM009524], human *ROR2* [NM004560], mouse *Ror2* [NM013846], and *Danio rerio* insulin [NM131056]).

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>

HapMap, <http://hapmap.org/>,

GeneCards™ genome, <http://www.stanford.edu/cgi-bin/genecards/index.shtml>,

UCSC Genome Browser <http://www.genome.ucsc.edu/>

Author Contributions

Study design by SCE, SB, JLL, LAS. Human subjects approval obtained by JLL and HGB. Sample retrieval and phenotyping done by JH, SB, JFM, JLL, and HGB. ADP, CMS, ANN, SH were involved in PCR reactions and sequencing of PCR products. Generation of constructs pT3TSWNT5A, pT3TSWNT5AC182R, pT3TSWNT5AC83S, and pT3TSdnWNT5A by ADP. Zebrafish experiments done by ADP and MER. Xenopus Experiments and qPCR were done by ANN, CMS, CJB and JLL. Mouse data and Fig. 1 by JRS, AP, and MER. Linkage analysis done by HGB and HVB. Manuscript written by ADP, SCE, JLL, HGB, HVB, LAS and JFM. All authors have agreed to all content in the manuscript, including the data presented.

Competing Interest Statement

The authors declare that they have no competing financial interests.

craniofacial abnormalities. The etiology of dominant Robinow syndrome is unknown, however the phenotypically more severe autosomal recessive form of Robinow syndrome has been associated with mutations in the orphan tyrosine kinase receptor, *ROR2*, which has recently been identified as a putative *WNT5A* receptor. Here we show that two different missense mutations in *WNT5A*, which result in amino acid substitutions of highly conserved cysteines, are associated with autosomal dominant Robinow syndrome. One mutation has been found in all living affected members of the original family described by Meinhard Robinow and another in a second unrelated patient. These missense mutations result in decreased *WNT5A* activity in functional assays of zebrafish and *Xenopus* development. This work suggests that a *WNT5A/ROR2* signal transduction pathway is important in human craniofacial and skeletal development, and that proper formation and growth of these structures is sensitive to variations in *WNT5A* function.

Introduction

The Wnt family of signaling proteins in humans has 19 identified members, with the major subgroup of Wnts (*WNT1*, *WNT3A*, *WNT8*) signaling through activation of β -catenin dependent transcription (Nusse, 2005). This canonical, or β -catenin dependent Wnt signaling pathway functions primarily to activate cell proliferation and cell fate change during development. A non-canonical, β -catenin independent signal transduction pathway that controls cell polarity or movement has also been identified (Heisenberg et al., 2000). *Wnt5a* has been shown to signal in a non-canonical fashion, modulating cellular movements independent of β -catenin (Heisenberg et al., 2000; Slusarski et al., 1997). Non-canonical Wnt signaling is necessary for the directional cell migration of pancreatic islet cell progenitors during pancreas formation in zebrafish and mice (Kim et al., 2005). In addition, non-canonical *Wnt5a* signaling regulates directional cell migration necessary for secondary palate fusion during mouse development (He et al., 2008). Non-canonical Wnt signaling is an area of active research in developmental biology, and it may involve multiple downstream pathways. Evidence exists for a *Wnt5a* signaling cascade involving the intracellular activation of calcium/calmodulin-dependent protein kinase and protein kinase C via the Frizzled2 transmembrane receptor, resulting in Ca^{2+} fluxes (Kohn and Moon, 2005). Other studies suggest that *Wnt5a* can signal through the orphan tyrosine kinase receptor, *Ror2*, but there may be multiple downstream mediators of this ligand-receptor complex (Mikels and Nusse, 2006; Oishi et al., 2003; Schambony and Wedlich, 2007). In the work presented here, we demonstrate that mutations in *WNT5A* are associated with human phenotypes similar to those found with loss-of-function *ROR2* mutations (Afzal et al., 2000; van Bokhoven et al., 2000), suggesting a role for this newly identified pathway in human development and disease.

In 1969, Meinhard Robinow and colleagues described a human syndrome characterized by short stature, mesomelic limb shortening, hypertelorism, mandibular hypoplasia, irregular dental alignment and hypoplastic external genitalia (Robinow et al., 1969). Based on the initial pedigree, Robinow syndrome was recognized as an autosomal dominant inherited syndrome [MIM 180700] with high penetrance. Over 100 patients with Robinow syndrome have since been identified in families with both autosomal dominant and autosomal recessive inheritance patterns (Patton and Afzal, 2002).

The autosomal recessive form of Robinow syndrome [MIM 268310], which is characterized by more severe skeletal, vertebral and craniofacial abnormalities (Mazzeu et al., 2007b; Patton and Afzal, 2002) is often caused by loss-of-function mutations in the gene encoding the tyrosine kinase-like orphan receptor 2, *ROR2* (van Bokhoven et al., 2000), (Afzal et al., 2000). Recently, *Ror2* has been identified as a putative receptor for *Wnt5a* (Mikels and Nusse, 2006; Schambony and Wedlich, 2007). *Wnt5a* and *Ror2* are expressed in adjacent and partially overlapping domains during mouse embryogenesis, and *Wnt5a* can directly bind to the extracellular cysteine-rich domain of *Ror2* (Nomi et al., 2001; Oishi et al., 2003; Schleiffarth et al., 2007). *Wnt5a* null mice and *Ror2* null mice exhibit phenotypes grossly similar to those found in Robinow syndrome patients, including shortening of the anterior-posterior axis, facial dysmorphism, genital hypoplasia and cardiac defects (DeChiara et al., 2000; Oishi et al., 2003; Schleiffarth et al., 2007; Yamaguchi et al., 1999). *Wnt5a* null mice have a more pronounced phenotype compared to *Ror2* null mice (Oishi et al., 2003), but these differences can be explained by functional compensation by the related gene *Ror1* in *Ror2* nulls (Nomi et al., 2001). *Ror1/Ror2* double mutant mice exhibit a Robinow syndrome-like phenotype that more closely resembles *Wnt5a* null mice (Nomi et al., 2001; Oishi et al., 2003; Yamaguchi et al., 1999). *Wnt5a* null mice exhibit a phenotype that is more severe than human dominant Robinow syndrome patients with perinatal lethality, 100% penetrance of cardiac defects, and presence of rib fusions (Schleiffarth et al., 2007; Yamaguchi et al., 1999). Heterozygous mutations in *ROR2* that cause brachydactyly type B [MIM 113000] do not result in simple loss-of-function like those *ROR2* mutations described in recessive Robinow syndrome. Rather, the *ROR2* mutations reported in patients with brachydactyly type B are thought to result in dominant-negative mutations (Oldridge et al., 2000; Schwabe et al., 2000; Stricker et al., 2006). These studies suggest that mutations in other genes in a *ROR2* signal transduction pathway may be associated with dominant Robinow syndrome. We tested the hypothesis that mutations in *WNT5A* are associated with autosomal dominant Robinow syndrome, thereby identifying an important role for this signaling pathway in human craniofacial and skeletal development.

Results

Wnt5a null mice and *Ror2* null mice exhibit phenotypes similar to those found in Robinow syndrome patients, including shortening of the anterior-posterior axis, facial dysmorphism, genital hypoplasia and cardiac defects (Afzal et al., 2000; Mazzeu et al., 2007b; Oishi et al., 2003; Robinow et al., 1969; Schwabe et al., 2004; van Bokhoven et al., 2000). Figure 1 illustrates the phenotypic characteristics of this well described mouse model and patients with a clinical diagnosis of dominant Robinow syndrome (Mazzeu et al., 2007b; Oishi et al., 2003; Robinow et al., 1969; Yamaguchi et al., 1999). These observations led us to screen for *WNT5A* mutations in patients with dominant Robinow syndrome.

Our initial screening for *WNT5A* mutations in patients with dominant Robinow syndrome focused on the family in which the syndrome was initially described (Robinow et al., 1969). In that report, the skeletal, genital and craniofacial abnormalities of four living affected family members were described in detail. We obtained DNA from the original four patients initially described by Dr. Robinow and colleagues, and from all additional living family members both affected and unaffected (Fig. 2C). Family members not previously reported

(Generation V), who are offspring of the affected female sibling of the proband described in the original report (Robinow et al., 1969), manifested mesomelic limb shortening, short stature and characteristic facial features. The affected male family members have no children.

PCR amplification (Tables 1) and sequencing of the coding regions of *WNT5A* in this family revealed heterozygous missense mutations in two sequential base pairs in exon 4 of *WNT5A*. This mutation of *WNT5A*, 544-545CT→TC (Fig. 3D) results in an amino acid substitution, C182R, at a highly conserved cysteine residue (Fig. 3B). This mutation is present in all seven affected family members and not present in the only living unaffected family member, (III-3) (Fig. 2C and data not shown). This mutation is in a highly conserved region of the *WNT5A* protein (Figure 3D), and was not identified in 196 unaffected control samples by ARMS PCR or sequencing, or by review of human single nucleotide polymorphism (SNP) databases (<http://hapmap.org/>, GeneCards™ genome-<http://www.stanford.edu/cgi-bin/genecards/index.shtml>, and UCSC Genome Browser <http://www.genome.ucsc.edu/>), which showed no human SNPs at *WNT5A* 544-545. No other mutations in the coding regions of *WNT5A* were identified in this family (Fig. 2C).

Linkage analysis for this family (Fig. 2) was performed concurrent with *WNT5A* exon sequencing by using a total of 382 fluorescently labeled short tandem repeat (STR) polymorphic markers (ABI PRISM Linkage Mapping Set MD10.A). All of the affected family members (Fig. 2) showed linkage with a LOD score of 1.78 at chromosome 3 in a region containing the human *WNT5A* locus. A LOD score of 1.78 is the maximum predicted score achievable with the number of meioses (individuals) available for this pedigree. Importantly, linkage to other candidate genes implicated in *WNT5A* signaling, including *ROR2* and the *WNT5A* receptor *FRIZZLED2*, was excluded by this analysis.

We identified a second heterozygous missense *WNT5A* mutation 248G→C in an unrelated adult patient with a sporadic dominant Robinow syndrome phenotype using the same methods. This mutation predicts an amino acid substitution (C83S)(Fig. 3F) affecting a second highly conserved cysteine residue (Fig. 3A). This patient's phenotype included marked hypertelorism, short nose, short stature, and mesomelic shortening of the limbs. The parents were unaffected clinically, however no parental DNA is available for testing. Sequence data from 173 control DNA samples did not show the 248G→C missense mutation in exon 3, nor was it found in the SNP databases as described above. Coding regions of *WNT5A* were sequenced in 23 additional unrelated patients with clinical diagnosis of dominant Robinow syndrome, and no *WNT5A* mutations resulting in amino acid substitutions were identified. This suggests that mutations in the coding regions of *WNT5A* cause only a subset of this syndrome as defined by current clinical criteria (Mazzeu et al., 2007b).

Mutations of cysteines in *Drosophila wingless (wg)*, and mammalian Wnts negatively affect Wnt function in multiple assay systems (Mason et al., 1992; McMahon and Moon, 1989; van den Heuvel et al., 1993; Willert et al., 2003). We hypothesize that mutating any of the 24 conserved cysteines might inhibit *WNT5A* function by altering protein folding, secretion, palmitoylation, or receptor binding. PolyPhen (*Polymorphism Phenotyping*) protein

structure modeling (<http://genetics.bwh.harvard.edu/pph/>) supports our prediction that the *WNT5A* mutations identified in our study affect *WNT5A* function. Based on this modeling, the calculated PSIC software score difference of 3.151 and 4.513 for the *WNT5AC83S* and *WNT5AC182R* mutations, respectively, is characterized as “probably damaging”. Therefore, both the *WNT5AC83S* and *WNT5AC182R* mutations are predicted to, with a high level of confidence, affect protein function.

The functional relevance of the *WNT5A* mutations C182R and C83S was tested *in vivo* by overexpression of mRNA in zebrafish embryos. We have previously shown that *wnt5* signaling is required for normal pancreatic islet cell coalescence in zebrafish and mice, and that this process requires a gradient of signal along the anterior-posterior axis in zebrafish (Kim et al., 2005). Disruption of this signaling gradient by mRNA overexpression of human *WNT5A* results in a failure of insulin-expressing cell coalescence into a pancreatic islet (Fig. 4D, F) when compared to control embryos (Fig. 4B, E). Overexpression of *WNT5AC182R* mRNA (Fig. 4G, H) and *WNT5AC83S* mRNA (not shown) are both less effective at disruption of islet cell coalescence than overexpression of wild type *WNT5A* mRNA in this assay of Wnt signaling function (*WNT5AC182R* vs control, $p = 0.00005$; *WNT5AC83S* vs control, $p = 0.002$) (Fig 4L). This data suggests that both the C182R and C83S mutations create phenotypes due to expression of less active (hypomorphic) forms of *WNT5A*.

WNT5A can precociously activate canonical, β -catenin dependent Wnt signaling during early axis formation in frogs (He et al., 1997). Although this scientific scenario is an artificial test for *WNT5A* because *WNT5A* does not perform this function *in vivo*, it is nevertheless a test of biochemical potential for activation of canonical Wnt signaling. We tested the ability of both wild type *WNT5A* and *WNT5AC182R* mRNAs to induce duplicate axis formation upon injection into zebrafish embryos, and we noted a similar incidence of duplicated embryonic axes when assayed at 24hpf (Fig. 4). Interestingly, and in sharp contrast to the similarly reduced signaling ability in a non-cell fate Wnt pathway, injection of *WNT5AC83S* mRNA exhibited a significantly lower incidence of duplicated embryonic axes compared to wild type injected embryos ($p = 0.001$) (Fig. 4M), suggesting a significant change in the biochemical potential for canonical Wnt signaling in this *WNT5A* sequence variant. As we do not yet understand the rules whereby a cell distinguishes between the canonical and non-canonical signaling potential of Wnt proteins, this observation suggests a loss of a single cysteine residue can serve as a major delineating structural feature.

To specifically test the hypothesis that these human variants generate antimorphic proteins, we compared the injections of these human variants with injections of mRNA encoding a dominant-negative *WNT5A* protein form. Injections with a C-terminal truncation, dominant negative mutant, (*dn*) *WNT5A* (Sen et al., 2001), did not cause axis duplication, but instead produced phenotypes similar to the *Wnt5* loss of function embryos (*pipetail*) (Hammerschmidt et al., 1996) (Fig. 4J). This data suggests that the C83S and C182R mutations are not acting as dominant-negative inhibitors of Wnt signaling, but they are instead acting as partial loss of function or hypomorphic alleles of *WNT5A*.

Additional studies were performed to test the function of these mutations in altering convergent-extension movements during *Xenopus* gastrulation (Moon et al., 1993) and to

place them in a novel ROR2-mediated noncanonical Wnt pathway (Schambony and Wedlich, 2007). In 1993, Moon et al showed that *Xwnt5A* blocks activin-mediated elongation of blastula stage animal caps without altering mesoderm formation (Moon et al., 1993). In order to determine the effect of the *WNT5AC182R* and *WNT5AC83S* mutations on cell movement in *Xenopus* embryos, we compared control animal caps isolated at preblastula stages to control animal caps exposed to activin protein, and to animal caps harvested from embryos that had been injected at the two cell stage with either moderate dose (30pg) or high dose (200 pg) *HWNT5A*, or *WNT5AC182R* and *WNT5AC83S* mutant constructs (Fig. 5) and then exposed to activin at blastula stages. Non-activin treated uninjected (Fig 5A) and embryos injected with 200 pg *WNT5AC182R* mRNA (Fig. 5C) are also shown. These experiments demonstrated reduced ability of the mutant forms of *WNT5A* to block activin-mediated cell movement during gastrulation at moderate doses (Fig. 5 D,E,F), however at higher doses all three forms were effective at blocking activin-mediated cell movement and animal cap elongation (Fig. 5 G,H,I). This observation confirms that while both the *WNT5AC182R* and *WNT5AC83S* mutations have reduced activity in this assay system, some functional ability to activate *WNT5A* signaling is retained and supports our hypothesis that these mutations represent an alteration of *WNT5A* function due to a hypomorphic allele, and not a dominant negative mechanism of action.

Recent studies have demonstrated *Wnt5A/ROR2* mediated activation of noncanonical Wnt signaling pathways and of *Xenopus* paraxial protocadherin (XPAPC)(Schambony and Wedlich, 2007). We used a simple assay of XPAPC activation as evidenced by induction of mRNA expression to determine if the mutations described in patients with Robinow syndrome acted through the *WNT5a/ROR2* signaling pathway. We found that in this assay, the *WNT5AC83S* mutation had a significantly reduced ability to induce XPAPC expression when compared to wild type *HWNT5A* (Fig. 6) and that the *WNT5AC182R* mutation trended to reduced ability to alter XPAPC expression when compared to wild type mRNA, without reaching statistical significance (data not shown).

Discussion

The overlapping phenotypes displayed by the dominant and recessive forms of Robinow syndrome implicate *WNT5A* as a ligand for the tyrosine kinase receptor ROR2. Ror2 function in non-canonical Wnt signaling is newly described during *Xenopus* gastrulation (Schambony and Wedlich, 2007), as no prior experimentation has demonstrated a role for the highly conserved kinase domain of Ror2 in any known Wnt-dependent process (Kim and Forrester, 2003; Mikels and Nusse, 2006; Oishi et al., 2003). The array of mutations in *ROR2* associated with recessive Robinow syndrome demonstrates a role for the conserved kinase-like domain of ROR2 in normal human development (Schwabe et al., 2000). This work, describing *WNT5A* mutations in dominant Robinow syndrome, supports a non-canonical signaling model in which a Wnt ligand signals via a tyrosine kinase receptor, and implicates the *WNT5A/ROR2* pathway in human craniofacial, skeletal and genital development.

In this work, we describe *WNT5A* coding sequence variations in the original family in which Robinow syndrome was described and a second unrelated patient. Robinow syndrome

characteristics include mesomelic shortening of the limbs, a flat facial profile, prominent forehead, and hypertelorism (Robinow et al., 1969). Recently, Wnt signaling has been shown to be a major factor controlling differences in facial development between chicken and mice (Brugmann et al., 2007). Comparisons of the dominant and recessive forms of Robinow syndrome suggest that during normal development the concentration of ligand governs the degree of limb outgrowth and continued development of facial structures. Humans with heterozygous *ROR2* loss-of-function mutations are unaffected carriers (Afzal et al., 2000; van Bokhoven et al., 2000). Heterozygous *Ror2* null mice are also phenotypically normal (DeChiara et al., 2000; Schwabe et al., 2004; Takeuchi et al., 2000). Loss-of-function mutations in both copies of *ROR2* are necessary for the more severe phenotypic manifestation of recessive Robinow syndrome (Afzal et al., 2000; van Bokhoven et al., 2000). One normal functioning copy and one copy of *WNT5A* with reduced function are associated with dominant Robinow syndrome. Despite this phenotype arising from one partially functioning *WNT5A* allele in humans, external examination of heterozygous mice in late gestation revealed none of the characteristic craniofacial, skeletal or growth abnormalities associated with the homozygous *Wnt5a*^{-/-} mutation. There is certainly precedent for a disease-causing heterozygous mutation in humans to be grossly undetectable in mice, and for mouse phenotype to vary with genetic background, as demonstrated in DiGeorge Syndrome (22q11 deletion/*TBX1* mutation)(Jerome and Papaioannou, 2001) and holoprosencephaly (*SHH* mutation)(Belloni et al., 1996; Roessler et al., 1996).

The molecular abnormalities described here in dominant Robinow syndrome suggest a model by which the *WNT5A/ROR2* signal transduction pathway is regulated by the local concentration of the ligand and the receptor concentration is not rate limiting. In this report we describe attenuation of *WNT5A* function associated with defects in limb outgrowth and abnormal craniofacial morphogenesis. This suggests that modest activity changes in *WNT5A* could be a mechanism for some normal variation of limb and facial development in humans.

A number of reasons may explain why *WNT5A* mutations were detected in only a subset of patients. First, since we used direct sequencing of the *WNT5A* coding regions, our analysis would not detect mutations in the promoter or other regulatory elements, or other large deletions or duplications. Subsequent to our sequence analysis an alternatively spliced form of exon 1 has been identified and confirmation and future sequencing of this exon would be appropriate in patients with clinically diagnosed dominant Robinow syndrome (Katoh, 2009). Second, it is likely that dominant Robinow syndrome is genetically heterogeneous, and mutations in other components of the *WNT5A/ROR2* signaling or regulatory pathways may also be responsible for dominant Robinow syndrome. Recently, Mazzeu, et al have identified abnormalities of Chromosome 1 associated with limb, skeletal, genital and craniofacial characteristics commonly found in Robinow syndrome (Mazzeu et al., 2007a). Potential genetic heterogeneity is confounded by difficulties in accurate clinical diagnosis of craniofacial and skeletal syndromes. Recessive Robinow syndrome has unique characteristic rib and vertebral anomalies and pronounced limb and craniofacial abnormalities that improve clinical diagnostic accuracy and are well correlated with *ROR2* mutations (Mazzeu et al., 2007a). In contrast, the phenotype of dominant Robinow syndrome is reported to be

more subtle, with less severe skeletal and craniofacial changes when compared to the recessive phenotype. Identification of a functional mutation in *WNT5A* in the initially described family with dominant Robinow syndrome sets a phenotypic standard with molecular correlation that will help us begin to sort out genotype/phenotype correlations and identify molecular etiologies in Robinow syndrome. The knowledge developed through the characterization of *ROR2* mutations in patients with recessive Robinow syndrome has been limited by the paucity of information about the role of this molecule in development. The identification of *WNT5A* mutations in dominant Robinow syndrome patients places this *WNT5A/ROR2* signaling pathway in an important role in craniofacial and skeletal development and disease.

Experimental Procedures

Human Studies

Approval for this study was obtained from the Institutional Review Boards of the participating institutions (University of Minnesota IRB Code #0603M83366 and Radboud University Nijmegen Medical Center). Patients with Robinow syndrome were identified based on clinical phenotype (Mazzeu et al., 2007b) and pedigree consistent with autosomal dominant transmission by one of our investigators (SB, JFM, HGB). All living members of the original family described by Dr. Robinow were enrolled. The patient with the 248G→C mutation is a female with a typical phenotypic presentation of the autosomal dominant Robinow syndrome phenotype. Informed consent was obtained from the patient, parent, or guardian in all cases, and 5 milliliters of blood were obtained by venipuncture. DNA was isolated with the VersaGene™ Genomic DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN) using a standard protocol for DNA extraction from whole blood. Control DNA for complete *WNT5A* coding sequencing was obtained from phenotypically normal unrelated individuals who consented to participate in this study and was isolated from whole blood as above.

Wnt5a Genomic PCR

100 nanograms of DNA were used in PCR reactions performed with intronic *WNT5A* primer pairs (Supplementary Table 1) spanning across each individual exon for all patient samples and controls. Negative control reactions for each primer set were run without DNA template to assay for DNA contamination.

PCR products were separated on a 1.5% agarose gel and amplicons were gel extracted using the S.N.A.P. Gel Purification Kit (Invitrogen, Carlsbad, CA). 30ng of purified DNA was then sequenced by our on site facility. A DNA ABI PRISM 3730xl DNA Analyzer was used in sequencing reactions of PCR products using dye-labeled terminator chemistry. Sequencing was conducted in both 5' and 3' directions using the same primers used for the PCR reactions (Table 1). Sequence variants were confirmed by re-sequencing in both direction three times, and compared to verified sequence of an additional one hundred seventy three controls for exon 3 obtained from phenotypically normal individuals (JLL and HGB). One hundred ninety six controls from normal banked DNA were also analyzed for the mutation identified in exon 4 by ARMS-PCR (179 controls)(Perrey et al., 1999) or

sequencing (17 controls). Primers used for ARMS-PCR recognized wild type *WNT5A* exon 4:

(5'-GGACTGGCTCTGGGGCGGCT-3', 5'-GAGGAGAGGACGGAGCTACA-3') and the C182R mutation in *WNT5A* exon 4:

(5'-GGACTGGCTCTGGGGCGGTC, 5'-GAGGAGAGGACGGAGCTACA-3').

40 ng of genomic DNA was used in ARMS-PCR analysis. PCR parameters were 94°C for 30 seconds, 62°C for 60 seconds, 72°C for 60 seconds, loop thirty times, 72°C for 10 minutes.

Linkage analysis was performed using a total of 382 fluorescently labeled short tandem repeat (STR) polymorphic markers (ABI PRISM Linkage Mapping Set MD10.A) with easyLINKAGE Plus pairwise and multipoint comparison. Data was analyzed using GeneHunter v2lr5 software, with parametric analysis for a dominant model of inheritance. We assumed complete penetrance, and a frequency of the disease allele of 1:10,000.

Genotyping of mice

Embryos used in this study were obtained by intercrossing *Wnt5a*^{+/-} mice (Yamaguchi et al., 1999). Wild type littermates were used as controls. Genomic DNA was PCR amplified as described (Kim et al., 2005). The study has been approved by the University of Minnesota Animal Care and Use Committee.

Skeletal preparation and histology

The skeletons of wild-type and *Wnt5a*^{-/-} embryos were stained with alizarin red and alcian blue as described (Hogan et al., 1986).

mRNA overexpression

WNT5A, *WNT5AC83S*, *WNT5AC182R*, and *dnWNT5A* were PCR engineered from cDNA clone (catalog # MHS1010-9204179, Open Biosystems, Huntsville AL) incorporating a 5' BglII site, a Kozak translation site (GCCACC), start codon, coding sequence, stop codon and a 3' SpeI restriction site. These constructs were ligated into pT3TS (Hyatt and Ekker, 1999) using BglII and SpeI engineered restriction sites. To make the *dnWNT5A*, a stop codon was inserted after the SPDYC motif as described (Sen et al., 2001). The *WNT5A* mutant constructs were generated by PCR using overlapping primers containing the mutated bases (base pairs 544-545, CT/TC) or (base pair 248, G/C) to incorporate the mutations into full-length constructs of *WNT5AC182R* and *WNT5AC83S* respectively. All clones were sequence-verified from both directions with primers flanking the mutation site (not shown). All four clones (pT3TS *WNT5A*, pT3TS *WNT5AC182R*, pT3TS *WNT5AC83S*, and pT3TS *dnWNT5A*) were linearized with XbaI and 5' (7-methyl guanosine) capped mRNA was transcribed with T3 RNA polymerase (T3 Message Machine, Ambion, Austin, TX).

Zebrafish maintenance and injections

Wild-type *Danio rerio* (Segrest Farms, Gibsonton, FL) and insulin-eGFP (Huang et al., 2001) embryos were raised at 30°C and spawning was performed as previously described

(Kimmel et al., 1995). *WNT5A*, *WNT5AC83S*, *WNT5AC182R*, and *dnWNT5A* mRNAs were injected into one-cell embryos. Axis duplication was scored at 24hpf in wild type embryos. Insulin-eGFP embryos were injected with mRNAs and scored for islet formation defects at 30hpf.

Whole mount *in situ* hybridization

pCRIITOPPO *Danio rerio* insulin clone containing base pairs 38-369 NCBI accession # NM_131056 was linearized with NotI and DIG labeled riboprobe was synthesized with T7 RNA polymerase (Roche, Mannheim, Germany). Whole mount *in situ* hybridization was performed as described (Jowett, 1999).

Xenopus Embryos and Injections

Xenopus embryos were obtained by in vitro fertilization after induction of ovulation in female frogs using human chorionic gonadotropin (Sigma). Animal cap experiments were performed as described in (Moon et al., 1993). Briefly, the animal poles of both blastomeres were injected with 30 or 200 pg of *HWNT5A*, *WNT5AC83S* mRNA or *WNT5AC182R* mRNA at the two cell stage. The upper one fourth of the embryo was harvested using an eyebrow knife at blastula stage (stage 8) and cultured in 1/3 strength modified Ringer's solution and 5 ng/ml activin protein (R & D Systems, Minneapolis, MN). Embryos were assayed for elongation when control embryos were at stage 40. For *XPAPC* induction, the two dorsal blastomeres were injected with 200 pg of mRNA at the four cell stage and embryos rapidly frozen at stage 10.5 (early gastrulation) for RNA isolation, cDNA synthesis and quantitative PCR. (Methods were adapted from (Schambony and Wedlich, 2007).

XPAPC Induction by Quantitative PCR

XPAPC induction in injected Xenopus embryos was assessed by qPCR. Total RNA was isolated from embryos using TRIzol reagent (Invitrogen Life Technologies). cDNA was synthesized and amplifications were performed in triplicate with 1x RT2 Real-Time SYBR green mix (SuperArray) on a Stratagene Mx3000P Real-Time PCR system with the following parameters: 45 cycles, 95 °C for 30 s, 55°C for 60s, and 72°C for 60 s. qPCR data were normalized using ornithine decarboxylase (*ODC*) as a control. Primer sequences (Schambony and Wedlich, 2007) for *XPAPC* were: 5'- cccagtcggtctcttctctttg 3' (forward), 5'-ttgctgatgctgctcttggttag 3' (reverse), and *ODC* 5'- gccattgtgaagactctctccattc3' (forward), 5'- ttcgggtgattccttccac 3' (reverse). Data are presented as the mean $\pm$ s.e. using GraphPad Prism4.

Statistics

The transgenic insulin-eGFP experimental values are the individual scores from each embryo. The transgenic insulin-eGFP positive cell coalescence experimental mean was then calculated for each experiment. The mean value of the means of each individual experiment was calculated [(sum of individual experiment means)/number of individual experiment means=mean of the means]. The mean variance of the means was also calculated. The % of embryos injected with wild type *WNT5A* mRNA in pancreatic islet coalescence and axis duplication assays was normalized to 100% activity and the % activity of *WNT5AC182R* and

WNT5AC83S mRNA injected embryos was compared to wild type *WNT5A* mRNA injected activity.

We used Student's t-test to compare inhibition of insulin-eGFP cell coalescence in embryos injected with *WNT5A* mRNA compared to embryos injected with *WNT5AC83S*, or *WNT5AC182R* mRNA. *p* values <0.05 were considered significant.

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We would like to dedicate this manuscript to the memory of Dr. Ian N. Jongewaard.

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Figure 1. *Wnt5a*^{-/-} mice have anatomical defects phenotypically similar to patients with autosomal dominant Robinow syndrome

Wnt5a^{-/-} mouse embryos at E16.5 (B) have widely spaced eyes (hypertelorism) and a triangular mouth compared to controls (A). Lateral views (C, D) reveal flattening of the facial profile, micrognathia, and small, low set ears in the *Wnt5a*^{-/-} embryos (D) compared to control littermates (C). Skeletal preparations at birth show mandibular hypoplasia in *Wnt5a* mutants (F) compared to controls (E). G) Percentage of reported findings in dominant Robinow syndrome patients compared to phenotypes in *Wnt5a*^{-/-} mice. ^aData presented in

this figure, ^b (Yamaguchi et al., 1999) ^c(Yang et al., 2003) ^d(Schleiffarth et al., 2007). ^ePercentages of dominant Robinow syndrome (DRS) patients with each abnormality was adapted from ((Mazzeu et al., 2007b).

Figure 2. Index Family – Robinow syndrome

A) Photograph of an unaffected male (II-2) and his wife (II-1) with dominant Robinow syndrome. Features evident in the affected female include short stature, hypertelorism, and mesomelic limb shortening. B) Proband (Robinow et al., 1969), siblings and grandmother, who is the female pictured in panel A. Photograph in panel B from left to right, top row: male IV-4, male IV-1 (*proband); bottom row: female IV-2, and female II-1. Robinow syndrome features (described above) are evident in all four individuals. Additional images of all four individuals have been reported previously (Robinow et al., 1969). C) Pedigree of the index family with dominant Robinow syndrome originally described in (Robinow et al.,

1969). An additional generation (V) is shown in this pedigree. ** Original pedigree reported male I-2 and not female I-1 had dominant Robinow syndrome (Robinow et al., 1969). We confirmed female I-1, and not male I-2, had dominant Robinow syndrome by consulting the family.

Figure 3. Dominant Robinow syndrome is associated with C83S or C182R WNT5A mutations
A, B) Sequence alignments show the cysteines (C83 and C182, red arrows) are conserved from human to hydra. C) Sequence data of a *WNT5A* exon 4 PCR product showing the wild type sequence of base pairs 537–552. D) Exon 4 PCR sequence data of a patient IV-1 showing a missense heterozygous mutation 544-545CT→TC (C182R). E) Control PCR sequence data of *WNT5A* bases 240–255. F) Sequence of an individual with autosomal dominant Robinow syndrome with a missense heterozygous mutation 248G→C (C83S). G) Schematic of the human *WNT5A* protein with a signal peptide (SP), 24 cysteines shown in

red with the C83S and C182R mutation sites * and the palmitoylation site on C104 (Kurayoshi et al., 2007).

Figure 4. Cysteine mutations in WNT5A reduce the ability of this protein to activate non-canonical Wnt signaling in zebrafish embryos
 Uninjected brightfield (A) and fluorescent images of insulin-eGFP transgenic zebrafish embryos (B) at 30hpf showing insulin-positive cells have coalesced to form an islet. Insulin-eGFP positive cells in embryos injected with *WNT5A* mRNA (C, D) do not coalesce properly, providing an *in vivo* assay for non-canonical Wnt5a activity. *In situ* hybridization showing insulin-expressing cells coalescing to form the zebrafish islet at 30hpf (E). Injections with *WNT5A* mRNA (F), *WNT5AC182R* mRNA (G, H) or *WNT5AC83R* mRNA

(not shown) show scattered insulin expressing cells at 30 hpf, but the efficacy of this effect is reduced in *WNT5AC182R* ($32\% \pm 3\%$ $n=93$ $P=0.00005$) and *WNT5AC83R* ($55\% \pm 9\%$ $n=152$ $P=0.002$) mRNA injected embryos compared to *WNT5A* mRNA injected embryos ($100\% \pm 6\%$ $n=260$) (L). I) Uninjected zebrafish embryo at 26hpf. J) Zebrafish embryo at 26hpf injected with *dnWNT5A* mRNA (25pg) showing a bent tail phenotype similar to *pipetail* (*Wnt5*) mutants. Injections of *WNT5A* mRNA (K) or *WNT5AC182R* mRNA (not shown) into zebrafish embryos at the one-cell stage results in duplications of the embryonic axis, providing an overexpression-based *in vivo* activity assay for Wnt signaling competency by the *WNT5A* sequence variants. Normalizing the axis duplicating activity of *WNT5A* mRNA injected embryos to $100\% \pm 13\%$ $n=196$, we noted indistinguishable axis duplicating activity $96\% \pm 16\%$ $n=65$ in *WNT5AC182* mRNA injected embryos (M). Injections of 3pg of *WNT5AC83S* mRNA resulted in a significantly different ($P=0.001$), low percentage of axis duplication activity $3\% \pm 0\%$ $n=102$ compared to the full duplicating activity of *WNT5A* mRNA at the same dose (3pg) (M). In contrast, *dnWNT5A* injections did not duplicate the embryonic axis (J).

Figure 5. *WNT5AC182R* and *WNT5AC182S* have reduced ability to block activin-mediated cell movements during *Xenopus* gastrulation

Control (uninjected) blastula stage animal caps round up in normal culture conditions (A) and elongate when cultured in activin protein (B). Embryos injected with 200 pg of *WNT5AC182R* mRNA into each blastomere at a two cell stage form normal appearing animal caps under control conditions (C). Moderate dose (30 pg) *WNT5A* mRNA (D) and High dose (200 pg *WNT5A* mRNA) (G) block activin-induced animal cap elongation. In contrast, moderate dose (30 pg) *WNT5AC182* mRNA (E) and *WNT5AC83S* mRNA (F) do not inhibit activin-induced elongation fully. In contrast, higher doses (200 pg) of *WNT5AC182R* mRNA (H) and *WNT5AC83S* mRNA (I) injected at a two cell stage do inhibit activin-induced animal cap elongation.

Figure 6. *XPAPC* induction after wild type and mutant *WNT5A* overexpression as assessed by qPCR

Control embryos at stage 10.5, and embryos injected with the *WNT5A*C83S mRNA or wild type *HWNT5A* mRNA at the four cell stage were harvested at 10.5 and were assayed for *XPAPC* expression normalized to *ODC* expression. There is a greater than two fold increase in *XPAPC* expression after injection with wild type *HWNT5A*. There is significantly less induction of *XPAPC* expression by the *WNT5A*C83S mutant than *HWNT5A*.

Table 1*WNT5A* Primer Sequences

Exons	Forward Primer (5'-3')	Reverse Primer (5'-3')
1	CGCTTCAGCTCCGGTTCAC	CGACGCTGGAGTTCAGCTT
2	CGGATGCTTCCGTTGTTT	CCAGCATTAAATATTGCCGC
3	GGGACTGCACAATACCTCCG	GAGCTGGAAGGCATCCTCCT
4	CTCCCTACCCTCTACCCCAA	AGGACGGAGCTACAGGGAGA
5	CTCCATGAATGTGTGTATTG	GCTGCGTGCTGGGTGGCA