REVIEW

A Comprehensive Overview of Skeletal Phenotypes Associated with Alterations in Wnt/ β -catenin Signaling in Humans and Mice

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The Wnt signaling pathway plays key roles in differentiation and development and alterations in this signaling pathway are causally associated with numerous human diseases. While several laboratories were examining roles for Wnt signaling in skeletal development during the 1990s, interest in the pathway rose exponentially when three key papers were published in 2001-2002. One report found that loss of the Wnt co-receptor, Low-density lipoprotein related protein-5 (LRP5), was the underlying genetic cause of the syndrome Osteoporosis pseudoglioma (OPPG). OPPG is characterized by early-onset osteoporosis causing increased susceptibility to debilitating fractures. Shortly thereafter, two groups reported that individuals carrying a specific point mutation in LRP5 (G171V) develop high-bone mass. Subsequent to this, the causative mechanisms for these observations heightened the need to understand the mechanisms by which Wnt signaling controlled bone development and homeostasis and encouraged significant investment from biotechnology and pharmaceutical companies to develop methods to activate Wnt signaling to increase bone mass to treat osteoporosis and other bone disease. In this review, we will briefly summarize the cellular mechanisms underlying Wnt signaling and discuss the observations related to OPPG and the high-bone mass disorders that heightened the appreciation of the role of Wnt signaling in normal bone development and homeostasis. We will then present a comprehensive overview of the core components of the pathway with an emphasis on the phenotypes associated with mice carrying genetically engineered mutations in these genes and clinical observations that further link alterations in the pathway to changes in human bone.

Keywords: Wnt signaling; mouse models; Lrp5/Lrp6; β -catenin; skeletal phenotypes

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Overview of Wnt signal transduction

Wnts are a family of 19 mammalian proteins characterized by a conserved pattern of cysteine residues(1). Wnts can activate several signaling pathways after binding to their cognate receptors, however, the bulk of this review will focus on the best characterized of these pathways, the so-called canonical pathway (Figure 1). This pathway results in the stabilization of the β -catenin

protein and subsequent transactivation of target genes (2). It is initiated by Wnt ligands binding to a receptor complex that includes a member of the Frizzled (Fzd) family of seven-transmembrane receptors and either Lrp5, or the related Lrp6 protein(3). Engagement of this complex results in the phosphorylation of the cytoplasmic domain of Lrp5 or Lrp6, creating a binding site for the Axin protein. In the absence of an upstream signal, Axin exists in a multiprotein complex that also includes Adenomatous polyposis coli (APC) and the serine/threonine protein kinase, Glycogen synthase kinase 3 α/β . This complex facilitates β -catenin for GSK3-dependent phosphorylation, targeting it for ubiquitin-depen-

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dent proteolysis. Binding of Axin to phosphorylated Lrp5/6 inhibits the ability of this complex to induce β -catenin degradation. The increased levels of cytoplasmic β -catenin can eventually enter the nucleus (4, 5) and increase target gene transcription.

Built onto this core pathway are a large number of proteins that modulate activation of the pathway. For example, several extracellular proteins such as Dkks (6),

Sclerostin (7), and Secreted frizzled-related proteins (Sfrps) (8) inhibit formation of the Wnt-Lrp5/6-Fzd receptor complex by various mechanisms. In addition, cadherin-dependent cell adhesion, which links cell-cell contacts to the cytoskeleton (Figure 1), may also play key roles in mediating Wnt/ β -catenin signaling due to the key role that β -catenin plays in this process (9).

Figure 1 Overview of Wnt/ β -catenin signaling. Production and secretion of Wnt ligands is dependent on the Porcupine-dependent lipid modification of Wnt and the presence of Wntless to facilitate the transport of lipid-modified Wnt to the plasma membrane(10). Once secreted, Wnt proteins bind to a receptor complex that includes either Lrp5 or Lrp6 and a member of the Frizzled family of seven-transmembrane receptors(3). In the absence of a Wnt ligand, a multiprotein complex, which includes GSK3, Axin, and APC, facilitates Casein kinase 1 (CKI)-primed and GSK3-dependent phosphorylation of β -catenin, targeting it for proteolytic degradation via the E3-ubiquitin ligase β -TrCP (11). Activation of the Wnt receptor complex leads to the activation of Dishevelled (Dvl) and the phosphorylation of the cytoplasmic domain of Lrp5/6 leading to the recruitment of Axin to the plasma membrane. This inhibits the processes, which induce the degradation of β -catenin, leading to increased accumulation in the cytoplasm. β -catenin can subsequently enter the nucleus where it can bind to members of the LEF/TCF family and activate target gene transcription via the recruitment of factors such as BCL9, Pygopus, and Parafibromin (a component of the PAF complex) to target gene promoters(12,13). In the absence of β -catenin nuclear localization, TCF/LEF proteins can associate with members of the Groucho family to facilitate transcriptional repression. β -catenin also plays a key role in mediating cellular adhesion via its interactions with Cadherins. Finally, several extracellular inhibitors of this process, such as Dkks, Sost, and potentially Kremen, can negatively regulate the formation of the Wnt receptor complex.

Finally, the mechanisms necessary for the production and secretion of Wnt from cells have recently become better defined (14). This process is dependent on at least two genes, Porcupine (Prcn) and Wntless (Wls). Por-

cupine is an acyltransferase that mediates the addition of a lipid modification to all mammalian Wnts, while Wls binds to lipid-modified Wnts and facilitates their transport to the plasma membrane for secretion.

Several key observations emphasized the importance of Wnt signaling in skeletal homeostasis

While several groups had been studying the role of Wnt signaling in skeletal patterning and development during the 1990s, it was arguably the demonstration that mutations in human genes encoding components of the pathway that intensified the interest of the skeletal biology field in this pathway. Due to their conceptual importance, we will briefly overview these developments from the early part of the last decade.

Osteoporosis pseudoglioma and high-bone mass phenotypes linked to LRP5 mutations

Osteoporosis pseudoglioma syndrome (OPPG) (259770, OMIM 2012) is a rare, recessive disorder characterized by severe juvenile osteoporosis and early-onset blindness related to hyperplastic primary vitreous (15). Additional abnormalities may include short stature, abnormal bone shape and frequent bone fracture. OPPG can be readily distinguished from other childhood bone-fragility disorders by normal collagen synthesis, hormone level, and calcium level (16). The OPPG gene was originally mapped to chromosome 17q12-13 (16), followed by the identification of the Wnt co-receptor, *LRP5*, as the target of the causative mutation underlying OPPG (17). Many subsequent studies have identified additional missense, nonsense, and splice-site mutations in *LRP5* that result in OPPG (18-24). The diverse causative mutations are consistent with OPPG being due to a loss of Lrp5 signaling, as opposed to a gain in function. Carriers of OPPG-related *LRP5* alleles have lower bone mineral density and increased incidence of bone fracture (17, 25).

Further support for the role of LRP5 in bone growth was provided in 2002 when two groups independently reported that a point mutation in LRP5 (G171V) was present in affected individuals in families displaying an autosomally dominant high bone mass trait (26,27). In one of these reports, data was presented suggesting that disruptions in the ability of Dkk1 to inhibit LRP5-induced canonical Wnt signaling may underlie the phenotypes (26).

Sclerosteosis

Sclerosteosis (269500, OMIM 2012) is a rare disorder most commonly seen in the Afrikaner population of South Africa. Sclerosteosis is characterized by osteopetrosis and syndactyly, with the latter being the diagnostic discriminating between sclerosteosis and other sclerosing bone dysplasias (28). Additional features of this disorder include gigantism, facial hypoplasia, resistance to bone fracture, and gradual deafness and facial paralysis due

to bony encroachment on the ear canal and cranial nerves with age (28). The first sclerosteosis gene was mapped to 17q12-21 (29), with subsequent identification of both nonsense and splice mutation alleles of a novel gene the authors named sclerostin (*SOST*); although the authors acknowledge a U.S. patent application, PCT/US99/27990, which had named this gene *BEER* (30). Additional nonsense (31), missense (32), and frameshift (33) mutations in *SOST* have been identified. While the original characterization of the *SOST* protein suggested that it could bind bone morphogenetic proteins (BMP), thereby functioning as a BMP antagonist (34, 35), another study revealed no influence of *SOST* on classical BMP signaling *in vitro* (36). *SOST* was then shown to inhibit Wnt signaling by interacting with LRP5/6 (37, 38) and potentially with LRP4 (39). The role of the *SOST*-LRP4 interaction in sclerosteosis may be particularly relevant because two LRP4 mutations were identified in unrelated sclerosteosis patients from the Mediterranean who had normal *SOST* gene structure (39-41) (614305, OMIM 2012). These mutations reduced the *SOST*-LRP4 interaction, and it was shown that LRP4 is necessary for *SOST*'s inhibitory effect on bone formation (39).

van Buchem syndrome

van Buchem syndrome (VBCH), also known as hyperostosis corticalis generalisata (239100, OMIM), is an autosomal recessive disorder most commonly seen in the Dutch. Like sclerosteosis, VBCH is a progressive bone dysplasia producing abnormal bone thickening that can lead to facial nerve palsy and hearing loss from bony encroachment on the cranial nerves and ear canal. Visually, the most pronounced feature of VBCH is the enlargement of the skull and mandible (42). Clinically, the best way to distinguish it from sclerosteosis is the absence of gigantism and syndactyly in VBCH (28, 43). Due to their clinical similarities, it is not surprising that VBCH and sclerosteosis were originally mapped to the same genomic location, 17q12-21 (42). However, unlike sclerosteosis, which results from mutations that disrupt the coding region of the *SOST* gene, the VBCH mutation is due to an Alu recombination-mediated, 52-kb deletion that is 35 kb downstream from *SOST* (44, 45). This region has been shown to regulate the transcription of *SOST* in osteoblast-like cells (46). While carriers of this mutant allele show lower serum levels of *SOST*, there does not appear to be an effect on bone mineral density (BMD) (47). A recent study identified an ECR5 enhancer element in this region, and Mef2C is the primary transcription factor binding this site to enhance *SOST* transcription (48).

It is worth noting that gain-of-function mutations in

LRP5 can result in sclerosing bone disorders that resemble VBCH and sclerosteosis (49). Patients may present with greatly elevated BMD, abnormal cranial bone growth, including enlarged jaw and torus palatinus, and bony encroachment on cranial nerves and ear canals. However, there is a clear difference in the pattern of inheritance between these patients and those with VBCH or sclerosteosis: while the *SOST*-related sclerosing bone disorders require two inactive alleles of *SOST*, excessive bone growth due to a gain-of-function mutation in *LRP5* is phenotypically dominant (26, 27, 49).

Evidence for specific functions of Wnt signaling components in skeletal development or disease

In this section, we discuss the phenotypes observed in mouse models, and in human patients, that are associated with alterations in many of the core components of the Wnt signaling pathway. In addition, this information is also summarized in Table 2, which is an extension of the information presented in an excellent review recently written by Westendorf and colleagues

(50). In addition, because of the widespread use of conditional deletions within the osteochondral lineage to assess Wnt pathway function in skeletal disease, a summary of several key transgenic mouse strains is provided in Table 1.

Wnts

Wnts are secreted, cysteine-rich glycoproteins that are highly insoluble due to a conserved palmitoylation site. While glycosylation may be dispensable for efficient Wnt secretion, palmitoylation by the acyl-transferase porcupine is essential for both Wnt secretion and function (51, 52). There are 19 WNT proteins in humans; they signal through binding of one of the frizzled class of receptors in combination with a co-receptor. The specific combination of Wnt + frizzled receptor + co-receptor determines whether the cellular response is β -catenin-dependent (canonical) or independent (non-canonical) (51). Because of their low solubility, Wnts diffuse poorly and are likely to have a limited range of signaling. Effective diffusion likely occurs by association of Wnts with lipoproteins (160) or exosomes (161), but whether either of

Table 1 Commonly Used Strains Driving Cre Expression in Skeletal Elements

Promoter Used to Drive Cre Expression	Expression Pattern	Ref
Prx1	Embryonic day 10.5 - budding limbs, throughout mesoderm.	(53)
Dermo1 (Twist2)	Embryonic day 9.5 - Mesenchymal osteochondro progenitor cells condensed mesenchyme (undifferentiated chondrocytes and osteoblasts)	(54)
Sox9	Embryonic day 10.5 all cells in cartilaginous primordia and perichondrium//spinal cord, intestinal epithelium, pancreas, testis	(55)
Collagen 2a1		
Mouse	Embryonic day 9 - notochord, cranial mesenchyme, somites optic vesicle// ED 11.5 - perinotochordal condensations and cranial mesenchyme// ED 14.5 - all cartilaginous elements	(56)
Rat	Embryonic day 15.5 - growth plate chondrocytes, cartilage of hind limb only	(57)
Collagen 10a1	Lower hypertrophic chondrocytes of the cartilage lineage, with a small amount of expression in the skin.	(58)
Osterix1	Embryonic day 14.5- bone perichondrium - Intramembranous and endochondral bony elements/sporadic expression within hypertrophic Chondrocytes	(59)
Collagen1a1		
2.3 kB (Mus)	Embryonic day 14.5 skull, long bone ossification centers, osteoblasts, odontoblasts, 16.5 -all bones	(60)
3.2 kB (Mus)	Tendon, facia fibroblasts, osteoblasts, odontoblasts, low expression in skin	(60)
Osteocalcin		
1.3 kB (Endogenous OG2)	Bone	(61)
1.3 kB -(Artificial OG2)	Bone and cartilage	(61)
2.3 kB	Osteoblasts	(61)
Human 10 kB	Embryonic day 17 -ossification centers, mature osteoblasts, and osteocytes	(62)
DMP1	Osteocytes and odontocytes	(63)

Table 2 Skeletal Phenotypes in Mouse Strains Carrying Genetic Modifications in Components of the Wnt Signaling Pathway. The following abbreviations are used: CKO (Conditional knockout), KO (full-body knockout), GOF (Gain of function), OE (Overexpression), CGOF (Conditional Gain of Function), OMIM (Online Mendelian Inheritance in Man), NA (Not applicable)

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
Wnt3	CKO	Exons 3/4	RAR β -Cre (Forelimb Ectoderm) or Msx2-Cre (Hindlimb Ectoderm)	Loss of limbs / digits in the forelimbs (RAR β -Cre) or hindlimbs (Msx2-Cre) with variable severity. No additional skeletal or bone specific Cre model assessments.	(64)	""
Wnt3a	KO	Exon 3	Germline	Homozygotes died by 12.5 dpc. Heterozygotes had decreased BMD, BV/TV, Tb N, and increased Tb Sp.	(65,66)	NA
	vt-Hypomorphic Mutation	Extragenic	Germline	Vestigial tail (vt): Reduced Wnt3a expression in developing embryo. Decreased ossification and number of caudal vertebrae. Phenotype was more severe in vt/- mice.	(67)	NA
Wnt4	KO	Exon 3	Germline	Homozygotes died at birth. Delayed chondrocyte maturation. Severe joint fusion with concomitant loss of Wnt9a.	(68,69)	NA
	OE	Transgene R26-Flox-Neo-Wnt4	Col2a1-Cre (Chondro-osteoprogenitors)	Dwarfism with increased hypertrophic chondrocytes in growth plates of long bones. Normal BMD.	(70)	NA
Wnt5a	KO	Exon 2	Germline	Homozygotes died at birth. Shortened skeletal elements and loss of digits. Decreased hypertrophic chondrocytes and ossification that was most severe in distal bones. Heterozygotes had decreased BMD, BV/TV, Tb N and increased Tb Sp.	(65,71,72)	Robinow Syndrome (OMIM: 180700)
	OE	Transgene Col2a-Wnt5a	Chondro-osteoprogenitors	Shortened skeletal elements and delayed ossification. Increased growth plate cartilage with a large zone of undifferentiated chondrocytes with a low index of proliferation.	(72)	""
Wnt5b	OE	Transgene Col2a-Wnt5b	Chondro-osteoprogenitors	Similar phenotype as Col2a-Wnt5a mice, but the undifferentiated chondrocytes were highly proliferative.	(72)	NA
Wnt7a	KO	Exon 2	Germline	Frequent loss of posterior digits and ectopic dorsal formation of sesamoid bones in paws.	(73)	Al-Awadi/Raas-Rothschild/Schinz el phocomelia syndrome (OMIM:276820)
	px-Inactivating Mutation	Exon 3 + Frameshift	Germline	Postaxial hemimelia (px): Same phenotype as Wnt7a KO.	(74)	""
Wnt7b	CKO	Exon 3	Dermo1-Cre (Mesoderm)	Homozygotes were viable. Decreased ossification. Abnormal chondrocyte and osteoblast zoning in growth plates.	(75)	NA
Wnt8a	OE-Xenoprotein	Transgene β -actin-Wnt8a (Gallus gallus)	Germline	Axis duplication during early embryo development.	(76)	NA

Cont.

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
Wnt9a/14	KO-LacZ	Exon 2	Germline	Homozygotes died at birth. Decreased size and mineralization of appendicular bones. Ectopic cartilage nodules in cranial sutures and joints. Fusions of wrist and ankle bones.	(69)	NA
	CKO	Exon 2	Prx1-Cre (Limb mesenchyme)	Same phenotype as Wnt9a/14 KO-LacZ.	(69)	NA
	OE	Transgene Col2a-Wnt9a	Chondro-osteoprogenitors	Homozygotes died by 16.5 dpc. Decreased cartilage.	(77)	NA
Wnt9b	CKO	Exon 2	CMV-Cre (Germline)	Homozygotes died at birth. Cleft palate. No skeletal or bone specific Cre model assessments.	(78,79)	NA
	clf1-Hypomorphic Mutation	IAP transposon downstream of Wnt9b	Germline	Cleft-lip1 (Clf1): Both clf1/clf1 and clf1/- mice were viable. Cleft palates. No skeletal assessments.	(80)	NA
Wnt10b	KO	Not published	Germline	Homozygotes were viable. Decreased BV, BMD, and Tb N at 8 weeks. Decreased serum OC. No change in TRAP5b.	(81)	Split-Hand/Foot Malformation 6 (OMIM: 225300)
	KO	Exons 2-5	Germline	Homozygotes were viable. Trabecular and cortical bone thickness normal at 1 month, but BV/TV and Tb N were increased. Expedited age related bone loss. Bone cell numbers were normal, but % mineralizing surface was decreased. Heterozygotes had a similar phenotype.	(82)	""
	OE	Transgene Fabp4-Wnt10b	Adipocytes (Adipose tissue and bone marrow)	Mice were viable. Increased Tb N and decreased Tb Sp. Increased mechanical resistance. Decreased rate of age related and ovariectomy induced bone loss.	(81)	NA
Wnt16	KO	Exons 1-3	Germline	Homozygotes were viable. Reduced appendicular cortical bone mass and strength, particularly females. Normal vertebral BV/TV.	(83)	SNPs within WNT16 associated with cortical bone thickness, BMD, and fracture risk (83)
Porcn	CKO	Exons 2/3	Sox2-Cre (Epiblast)	Heterozygous females were viable. Shortened or absent limbs and digits with variable severity. No skeletal or bone specific Cre model assessments.	(84)	Focal Dermal Hypoplasia (OMIM: 305600)
	CKO	Exons 2/3	Msx2-Cre (Hindlimb ectoderm) or Prx1-Cre (Limb mesenchyme)	Shortened hindlimbs and syndactyly of digits (Msx2-Cre). Shortened limbs and loss of digits, but no syndactyly (Prx1-Cre).	(84)	""
	CKO-Hypomorphic	Exons 3-7 + FRT-Neo	Chimeras	FRT-Neo cassette created a hypomorphic allele in chimeras. 9/17 resulting male and female chimeras had absent, fused, or shortened digits on limbs and vertebral abnormalities.	(85)	""

Cont.

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
Wls	CKO	Exons 3-7	Ella-Cre or Hprt-Cre (Germline)	Viable male and female mice in low yield with abnormal hair follicle development (Ella-Cre). Hprt-Cre resulted in heterozygous females in low yield. Frequent axial truncations and neural tube malformations in embryos. No skeletal or bone specific Cre model assessments.	(85)	""
	CKO	Exons 3-7	Prx1-Cre (Limb mesenchyme)	Similar to the Barrott <i>et al</i> mouse, but with syndactyly of soft tissue and no loss of digits.	(85)	""
	CKO	Exon 1	Oc-Cre (Osteoblasts)	Progressive decreases in BMD, trabecular and calvarial bone, as well as cartilage starting at 20 days of age. <20% of mice survived over 2 months. Massive decreases in BV/TV and cortical bone by 7 weeks. Decreased Serum OC and CTX-II. Increased TRAP staining.	(86)	""
	CKO	Exon 3	Prx1-Cre (Limb mesenchyme)	Homozygotes died at weaning. Limbs were hypoplastic, shortened, with truncated autopods. Ossification, chondrocyte hypertrophy, and osteogenesis were also impaired.	(87)	""
	CKO	Exon 3	Msx2-Cre (Hindlimb Ectoderm)	Truncated autopods in all limbs, but shortened zeugopods only in hindlimbs. Defective suture fusion and ossification of skull.	(87)	""
	CKO	Exon 3	Dermo1-Cre (Mesoderm)	Reduced mineralization, malformation and/or absence of skeletal elements during skeletogenesis. Loss of hypertrophic chondrocytes.	(88)	""
	CKO	Exon 3	(2.3kb)Col1a1-Cre (Mature osteoblasts) or Osx-Cre (Osteoblast precursors)	No defects in skeletogenesis or bone mineralization.	(88)	""
	CKO	Exon 3	Col2a1-Cre (Chondro-osteo progenitors)	Shortened long bones, loss of bone collar formation, decreased bone mineralization, and reduced chondrocyte maturation.	(88)	""
Fzd2	KO-LacZ	Exon 1	Germline	Homozygotes have reduced viability and cleft palate. Skeletal elements smaller, but normal shape.	(89)	NA
Fzd9	KO	Exon 1	Germline	Normal skeletogenesis. Decreased vertebral Tb N, but normal Tb thickness. Decreased osteoblast surface, matrix apposition, and mechanical resistance of long bones. Osteoclasts activity was normal.	(90)	Hemizygous loss of FZD9 may contribute to the overall phenotype of Williams-Beuren syndrome (OMIM: 194050)

Cont.

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
Lrp4	KO	Exon 1	Germline	Homozygotes died at birth. No skeletal assessments.	(91)	Cenani-Lenz Syndactyly Syndrome (OMIM: 212780)
	KO-Hypomorphic	Exon 36-Intron 36	Germline	Polysyndactyly with fusion and duplication of digits in all limbs. Decreased size of femurs. Total BMC and BMD were reduced. Vertebrae had reduced BV/TV, Tb N and increased Tb Sp. Cortical bone was normal. Elevated markers of osteoblast (OCN and ALP) and osteoclast (DPD) activity.	(92)	Sclerosteosis 2 (OMIM: 614305)
	dan	Retroviral insertion Intron 1	Germline	Digitation abnormale (dan): Homozygotes were viable. Fully penetrant brachydactyly and syndactyly on all limbs with duplications out of plane. Shortening and fusing of appendicular skeletal elements.	(93)	""
	mdig	Splicing mutation; Loss of exon 15	Germline	Malformed digits (mdig): Similar phenotype as Lrp4-dan mice	(93)	""
	mte	Asp1436Gly mutation in C-terminal YWTD domain	Germline	Mitaine (mte): Similar phenotype as Lrp4-dan mice, but homozygotes died at birth.	(94)	""
	mitt	Premature stop codon C-terminal to LDLR-A domain	Germline	Mitten (mitt): Similar phenotype as Lrp4-mte mice.	(94)	""
Lrp5	KO-Genetrap	Intron 1	Germline	Homozygotes were viable. At 26 weeks Lrp5 null mice weighed ~9% less than wildtype littermates. Decreased BMC, BMD, as well as cortical and trabecular bone mass. Decreases in both osteoclast and osteoblast surface area.	(95)	Osteoporosis-Pseudoglioma Syndrome (OMIM: 259770)
	KO-LacZ	Exons 7/8	Germline	No Lrp5 or Lrp5-LacZ transcripts were transcribed. Similar osteopenic phenotype as the Iwaniec <i>et al</i> mice, but there was no decrease in body size or weight.	(96)	Low bone mass (OMIM: 601884)
	KO	Exon 6	Germline	Similar phenotype as other LRP5-KO mice. Reduction in bone apposition rate. Osteoblasts had decreased proliferation and mineralization <i>ex vivo</i> , however Yadav <i>et al</i> , 2008 reported no change in osteoblasts <i>ex vivo</i> . Osteoclasts and chondrogenesis were normal. A truncated Lrp5 transcript and protein were produced with this allele.	(97,98)	

Cont.

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
Lrp5	KO-LacZ	Exon 1	Germline	No Lrp5 or Lrp5-LacZ transcripts were transcribed from the Lrp5 promoter. Both homozygotes and heterozygotes had decreased BV/TV. Lower BMD became apparent at approximately 4 weeks of age and persisted throughout life.	(99)	""
	CKO	Exon 2	CMV-Cre (Germline)	Similar phenotype to the other Lrp5-KO mice.	(100)	""
	CKO	Exon 2	Dermo1-Cre (Mesoderm)	Normal skeletogenesis. No other skeletal assessments.	(100)	""
	CKO	Exon 6	(2.3kb)Col1a1-Cre (Mature osteoblasts) or Villin-Cre (Intestines)	No change in bone mass with Col1a1-Cre, but reduced bone mass with Villin-Cre.	(98)	""
	CKO	Exon 2	Dmp1-Cre (Osteocytes) or Villin-Cre (Intestines)	No change in bone mass with Villin-Cre, but reduced bone mass with Dmp1-Cre.	(101)	""
	GOF	A213V (Mimics human Lrp5-A214V)	Germline	Increased cortical bone mass, BV/TV, Tb N, and Tb size with a decrease in Tb Sp. Increased mechanical resistance.	(101, 102)	Endosteal Hyperostosis, autosomal dominant (OMIM: 144750)
	CGOF	A213V (Mimics human Lrp5-A214V)	Dmp1-Cre (Osteocytes), Prx1-Cre (Limb mesenchyme), or Villin-Cre (Intestines)	Global increases in bone mass with Dmp1-Cre that mimicked global Lrp5-A214V expression. Increased bone mass in limbs only with Prx1-Cre. No change in bone mass with Villin-Cre.	(101)	Osteosclerosis, autosomal dominant (OMIM: 144750)
	GOF	G170V (Mimics human Lrp5-G171V)	Germline	Global increases in cortical bone mass, BV/TV, Tb N and trabecular thickness with a decrease in Tb Sp. Greater enhancement of endosteal bone formation during development and in response to load.	(101, 102)	Osteopetrosis, autosomal dominant 1 (OMIM: 607634)
	CGOF	G170V (Mimics human Lrp5-G171V)	Dmp1-Cre (Osteocytes), Prx1-Cre (Limb mesenchyme), (2.3kb)Col1a1-Cre (Mature osteoblasts) or Villin-Cre (Intestines)	Global increases in bone mass with Dmp1-Cre that mimicked global Lrp5-G171V expression. Increased bone mass in limbs only with Prx1-Cre. Villin-Cre showed no effect on bone mass in one study (Cui <i>et al</i> , 2011), but resulted in global bone mass enhancement in another (Yadav <i>et al</i> , 2008). Additionally, the latter group showed no enhancement of bone mass when Col1a1-Cre was used to activate Lrp5-G171V in bone.	(98, 101)	High bone mass (OMIM: 601884)
	KO-LacZ	Fusion of LacZ with first 321 amino acids of Lrp6	Germline	Homozygotes died between E14.5 and birth. Numerous abnormalities including truncation of the axial skeleton, limb defects, and urogenital malformation. Heterozygotes had normal skeletogenesis and BMD, but reduced BV/TV.	(99, 103)	Coronary artery disease, autosomal dominant 2 accompanied by low bone mass (OMIM: 610947)

Cont.

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
Dkk1	CKO	Exon 2	CMV-Cre (Germline)	Identical phenotype as Lrp6 KO-LacZ mice.	(100)	""
	CKO	Exon 2	Dermo1-Cre (Mesoderm)	Homozygotes had mostly normal skeletogenesis. Severe skeletal defects and lethality were seen with concomitant loss of Lrp5.	(100)	""
	Cd	G494D in second YWTD-EGF domain	Germline	Crooked (Cd): Crooked tail and exencephaly with incomplete penetrance. Heterozygotes were viable with varying levels of vertebral malformations. Homozygotes had reduced viability. Mice that completed neurulation had severely misshapen and / or fused vertebrae. Delayed ossification of the digits, but no polysyndactyly.	(104)	""
	Gw	D549G in final YWTD-EGF domain of the 2nd beta propeller domain	Germline	Gwazi (Gw): Homozygotes and heterozygotes had ectopic sesamoid bone formation between caudal vertebrae causing kinked tail with incomplete penetrance.	(105, 106)	""
	Rs	R886W in second YWTD-EGF domain of the third beta propeller domain	Germline	Ringelschwanz (Rs): Frequent spine malformations. Delayed ossification of metatarsals and metacarpals with oligodactyly. Reduced BMD as well as cortical and trabecular bone mass. Increased markers of bone resorption with no change in osteoblasts.	(107, 108)	""
Dkk1	KO	Exons 1-4	Germline	Homozygotes died at birth. Severe craniofacial malformation as well as fused and ectopic digits. Heterozygotes had no overt phenotype. However, BV/TV, mineral apposition rate, osteoblast surface, and mechanical resistance were increased. There was no change in osteoclasts.	(109, 110)	SNPs in DKK1 associated with the extent of joint damage in rheumatoid arthritis (111)
	OE	Transgenes Col2a1-Dkk1, Col10a1-Dkk1, or Tie2-Dkk1	Chondro-osteoprogenitors, Hypertrophic chondrocytes, or Endothelial cells	There was no change in the overall pattern of cartilage or bone development in chondrocyte overexpression of Dkk1. Overexpression in endothelial cells resulted in smaller skeletal elements, abnormal hypertrophic chondrocytes, reduced Tb size, but an increase in Tb N. TRAP staining and osteoclast numbers at the hypertrophic chondrocyte - trabecular bone interface was decreased. The mineral apposition rate was unaffected.	(112, 113)	NA

Cont.

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
	OE	Transgene (2.3kb)Col1a1-Dkk1	Mature osteoblasts	Reduced BMD and trabecular BV. Osteoblast surface area and number were reduced..Osteoclasts were normal.	(114)	NA
Dkk2	KO	Exons 1-4	Germline	Homozygotes were viable. Osteopenia with major defects in mineralization rates. Increased osteoclast numbers, but no change in osteoblasts.	(115)	NA
	OE	Transgenes Col2a1-Dkk2, Col10a1-Dkk2, or Tie2-Dkk2	Chondro-osteo progenitors, Hypertrophic chondrocytes, or Endothelial cells	There was no change in the overall pattern of cartilage or bone development in chondrocyte or endothelial overexpression of Dkk2.	(112)	NA
Sost	KO-LacZ	Exons 1/2	Germline	Homozygotes were viable. High bone mass similar to Lrp5-A214V with primary enhancement of cortical bone. Fracture healing was also enhanced.	(102, 116, 117)	Sclerosteosis 1 (OMIM: 269500)
	CKO-Hypomorphic	Δ ECR5 (Mimics mutation in Van Buchem's disease)	(2.3kb)Col1a1-Cre (Mature osteoblasts)	Homozygotes were viable. Increased BV/TV and osteoblast surface with no change in osteoclasts. Similar phenotype as Sost-KO, but not as severe.	(48)	Van Buchem disease (OMIM: 239100) and Craniodiaphyseal dysplasia (OMIM: 112860)
	OE	Transgene Oc-Sost	Osteoblasts and osteocytes	Mice were viable, but osteopenic with disorganized bone architecture, thin cortices, reduced trabecular bone, and chondrodysplasia. Decreased osteoblast surface and reduced bone formation rate, with no change in resorption markers.	(34)	Several SNPs have been identified within SOST related to volumetric BMD of the spine in Caucasian males (118)
Sfrp1	KO-LacZ	Exon 1	Germline	Homozygotes were viable. Skeletal elements and bone accrual normal, but reduced age-related trabecular bone loss that was most pronounced in females.	(119)	NA
Sfrp2	KO	Exons 1/2	Germline	Homozygotes were viable. Shortened metacarpals / metatarsals and phalangeal bones with delayed ossification and a reduction of hypertrophic chondrocytes.	(120)	NA
Sfrp3 (Frzb)	CKO	Exon 1	Ela-Cre (Germline)	Homozygotes were viable. Increased BMD, cortical thickness, and stiffness. No change in trabecular bone. Enhanced bone formation in response to load.	(121)	SFRP3 SNPs associated with osteoarthritis, osteoporotic fracture, and hip shape (122)
Sfrp4	OE	Transgene SAP-Sfrp4	Various	Decreased rate of bone acquisition from 5-15 weeks after birth. Decreased BV/TV and Tb size.	(123)	NA

Cont.

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
Dvl2	OE	Transgene (2.3kb)Col1a1-Sfrp4	Mature osteoblasts	Similar phenotype as the SAP-Sfrp4 transgenic mice.	(124)	NA
	SAMP6-Hypomorphic mutation	Promoter mutation	Germline	Senescence accelerated mouse P6 (SAMP6): 40 fold increase in Sfrp4 expression in calvaria. Decreased osteoblast number and bone mass.	(124)	NA
	KO	Exons 2-6	Germline	Homozygotes had reduced viability. Surviving mice were predominantly female, lived 2+ years, with high frequency of kinked tails, forked / fused ribs, and abnormal vertebral bodies. No additional skeletal assessments.	(125)	NA
Axin1	KO (Fu-Tg1)	Exons 1-3	Germline	Homozygotes died at E9.5. Heterozygotes had tail bifurcation and rib fusion.	(126)	Caudal duplication anomaly (OMIM: 607864)
	Fu	IAP retrotransposon insertion Intron 6	Germline	Fused (Fu): Phenotype similar as Axin1-KO mice, but with incomplete penetrance.	(126)	""
	Fu-kb	IAP retrotransposon insertion Exon 7	Germline	Knobbly (Fu-kb): Similar phenotype as Axin1-KO.	(127)	""
Axin2	KO-LacZ	Exon 2	Germline	Homozygotes were viable, but commonly developed craniosynostosis due to enhanced mineralization and ossification of the cranial sutures. Protection against age related decreases in BMD and BV. Reduced hypertrophic chondrocyte zone.	(128-130)	Oligodontia-colorectal cancer syndrome (OMIM: 608615)
	canp-Hypomorphic mutation	V26D Enhances Axin2 protein stability	Germline	Canopus (canp): Homozygotes died during gestation. Shortened or doubled tails.	(131)	Numerous SNPs have been identified within AXIN2 associated with tooth agenesis and cleft palate (132)
Apc	CKO	Exon 15	Col2a1-Cre (Chondro-osteoprogenitors)	Homozygotes died perinatally. Shortened and truncated limbs, malformed craniofacial structures, severely malformed vertebrae, and a loss of chondrocyte and osteoblast differentiation. Heterozygotes had no change in bone measurements or mechanical properties.	(133)	""
	CKO	Exon 14	Oc-Cre (Osteoblasts)	Homozygotes had extreme osteopetrosis with greatly reduced viability. Interestingly, bones had a massive loss of both osteoblasts and osteoclasts at 2 weeks.	(134)	""

Cont.

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
Gsk3β	KO	Exon 2	Germline	Viable mice were recovered with complete loss of Gsk3 β in a heterozygous Gsk3 β background. These mice were dwarfed with shorter limb bones and vertebrae. Chondrocyte zoning was normal, however there was metachromasia and decreased Col2a1 expression. Cultured chondrocytes had no change in β -catenin protein levels, cellular localization, or signaling <i>ex vivo</i> .	(112)	NA
Gsk3β	KO	Kinase domain	Germline	Homozygotes died 24 hours after birth. Cleft palate. Decreased ossification of the skull, ear bones, and cranial base. In contrast, heterozygous mice had increased cortical and trabecular bone mass, with no change in growth plate morphology. Bone formation rates were increased.	(135-137)	NA
	CKO	Exon 2	Sox2-Cre (Epiblast)	Homozygotes died 24 hours after birth with cleft palate.	(138)	NA
	CKO	Exon 2	Col2a1-Cre (Chondro-osteoprogenitors)	Skeletogenesis, growth plate zoning and β -catenin staining was normal. There was a potentially compensatory two-fold increase in GSK3 β protein levels.	(139, 140)	NA
β-catenin	CKO	Exons 3-6	Col2a1-Cre (Chondro-osteoprogenitors) or Dermo1-Cre (Mesoderm)	Homozygotes died shortly after birth. Skeletal defects from Col2a1-Cre crossings include shortened limbs, loss of tarsal synovial joints leading to bone fusion, and domed skulls. Dermo1-Cre produced a more severe phenotype.	(77)	NA
	CKO	Exons 3-6	Brn4-Cre (Neural tube and hindlimbs)	Malformation of hindlimbs, including truncation or absence of tibia, fibula, and digits.	(141)	NA
	GOF	Exon 3	Col2a1-Cre (Chondro-osteoprogenitors) or Dermo1-Cre (Mesoderm)	Heterozygotes died around E18 with severe and generalized chondrodysplasia. Reduced endochondral bone size.	(142)	NA
	GOF	Exon 3	Brn4-Cre (Neural tube and hindlimbs)	Enlarged hindlimb buds during development.	(141)	NA
	GOF	Exon 3	(2.3kb)Col1a1-Cre (Mature osteoblasts)	Heterozygotes failed to thrive and died shortly after weaning. Skeletal assessments revealed greatly increased bone mass and cartilagenous deposits in long bones and vertebrae. Osteoblast numbers were normal, but Col1a1 expression was increased. Osteoclasts were decreased.	(143)	NA
	GOF	Exon 3	Prx1-Cre (Limb mesenchyme)	Heterozygotes died at birth with loss of limb and skull bones.	(144)	NA

Cont.

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
	GOF	Exon 3	Osx1-Cre-TetOff (Osteoblast precursors - Tetracycline inhibitable)	Heterozygotes died at birth. Shortened long bones with premature ossification and mineralization. Loss of hypertrophic chondrocytes.	(59)	NA
	GOF	Exon 3	Col2a1-Cre-ERT2 (Chondro-osteoprogenitors - Tamoxifen inducible)	Tamoxifen induced recombination at 3 or 6 months resulted in a loss of articular cartilage 2 months later.	(145)	NA
	GOF	Exon 3	Col2a1-Cre-ERT2 (Chondro-osteoprogenitors - Tamoxifen inducible)	Tamoxifen induced recombination at E13.5 followed by skeletal assessments at several embryonic time points. Disordered chondrocyte zoning. Increased thickness of the perichondrial bone collars in limbs. TRAP staining showed no change in osteoclast activity.	(146)	NA
	GOF	Exon 3	Axin2-rtTA (Wnt responsive cells) + TRE-Cre [functions as a Doxycycline inducible Axin2-Cre]	Expansion and increased ossification of cranial sutures. Fully mature osteoblast differentiation was inhibited. No additional skeletal assessments.	(147)	NA
	GOF	Exon 3	PPAR γ -tTA (Osteoclast progenitors) + TRE-Cre [functions as a Doxycycline inhibitable PPAR γ -Cre]	Severe osteopetrosis with a 27-fold increase in the BV/TV ratio. Trabecular bone and cortical bone mass were both increased. Histomorphometry revealed large decreases in osteoclast number and surface area, with no effect on osteoblasts. The osteopetrotic phenotype was already evident in 15 day old pups. Osteopetrosis could also be induced by removing Dox in adult mice.	(148)	NA
	CKO	Exons 2-6	(2.3kb)Col1a1-Cre (Mature osteoblasts)	Homozygotes were viable. Decreased bone mass. Increased osteoclast activity. No change in osteoblasts.	(143)	NA
	CKO	Exons 2-6	Ocn-cre (Mature Osteoblasts)	Homozygotes died within one month of birth with decreased bone mass. Increased osteoclast activity.	(134)	
	CKO	Exons 2-6	Prx1-Cre (Limb mesenchyme)	Mice died at birth. Appendicular bones were shortened, partially fused, with loss of distal structures and mineralization. Growth plate chondrocyte zoning was normal, but bone collars were absent.	(144)	NA
	CKO	Exons 2-6	Dermo1-cre (mesenchyme)	Homozygotes exhibited an inhibition of commitment to the osteoblast lineage with severe defects in skeletogenesis.	(144, 149)	NA

Cont.

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
Tcf1 (Tcf7)	CKO	Exons 2-6	Osx1-Cre-TetOff (Osteoblast precursors - Doxycycline inhibitable)	In the absence of pre-natal Dox exposure, embryos had severe skeletal defects due to a lack of fully differentiated osteoblasts, which resulted in no mineralization or ossification of bone. Results suggested a conversion of pre-osteoblasts to chondrocytes. If Dox was removed at 2 or 4 months of age, there was a dramatic loss of Tb bone with greatly enhanced bone marrow adiposity by 6 months of age.	(59, 150)	NA
	CKO	Exons 2-6	PPAR γ -tTA (Osteoclast progenitors) + TRE-Cre [functions as a Doxycycline inhibitable PPAR γ -Cre]	Heterozygotes were osteoporotic with decreased trabecular and cortical bone mass. Increased osteoclast number and surface area with no change in osteoblasts. Interestingly, homozygotes were osteopetrotic, similar to, but not as severe as the β -catenin GOF allele.	(148)	NA
	CKO	Exons 2-6	Dmp1-Cre (Osteocytes)	Homozygotes were viable. Growth retardation and early lethality at 3-5 months. Early onset cortical and cancellous bone loss in both the appendicular and axial skeleton. Osteoclast number and activity were elevated, with no change in osteoblasts.	(151)	NA
	CKO	Exons 2-6	Col2a1-Cre-ERT2 (Chondro-osteo progenitors - Tamoxifen inducible)	Reduced hypertrophic chondrocyte zone, disorganization of prehypertrophic chondrocytes, and a reduction of the primary ossification center. By E18.5 there was a failure of vascularization of the cartilage lacunae.	(146)	NA
	CKO	Exons 2-6	Osx1-Cre-ERT2 (Osteoblast precursors - Tamoxifen inducible)	Tamoxifen induced recombination was performed in 2-month-old mice. Osteopenia developed by day 21 characterized by reduced Tb N and increased Tb Sp, but no change in trabecular thickness. Decreased cortical bone by day 49. Osteoclast numbers and activity were elevated at day 21 and beyond. Serum PINP levels and osteoblast numbers were decreased at day 8, but elevated in the subsequent time points.	(152)	NA
	KO	Exon 7	Germline	Homozygotes were viable. Reduced bone mass accompanied by increased TRAP staining. No change in osteoblasts.	(143)	NA
	OE - Dominant negative	Transgene Col2a1-Tcf7 $\Delta\beta$ -catenin binding domain	Chondro-osteo progenitors	Reduced skeletal element size, endochondral ossification, chondrocyte maturation and proliferation.	(153)	NA

Cont.

Gene	Method	Exons	Cre line (Tissue)	Phenotype(s)	Ref.	Related Human Skeletal Disorder
Tcf4 (Tcf7l2)	KO	Δ HMG domain	Germline	Concomitant loss of Tcf7l2 and Lef1 resulted in craniofacial abnormalities not present in either single mutant. However, chondrogenesis and osteogenesis appeared normal.	(154, 155)	NA
Lef1	KO	Δ HMG domain	Germline	Homozygotes died within 2 weeks after birth. Reduced body size, poor hair development, and a pointed face. Jaw bones were normal, but teeth failed to develop. Concomitant loss of Lef1 and Tcf1 resulted in the failure to form limb buds. There was a sex-specific decrease in BV/TV and cortical bone mass in heterozygous females, with a decrease in osteoblast activity. Osteoclast activity was also decreased.	(156-158)	NA
	OE	Transgene (2.3kb)Col1a1-Lef1 Δ N isoform	Mature osteoblasts	Increased trabecular BV and osteoblast activity, but no change in osteoblast number. Osteoclasts were normal.	(159)	NA

these secretory modes influences receptor association and signaling is not known. The expression of all 19 Wnts in the limbs of developing mouse embryos has been documented, and it revealed highly regulated expression of each Wnt temporally and spatially(162). This information, combined with the abnormal axis phenotypes seen in numerous Wnt mouse models, highlights the critical importance of Wnt proteins in controlling body patterning during development.

Mouse models support crucial and non-redundant roles of most of the Wnt proteins during embryogenesis. For example, germline deletion of Wnts 1, 3, 3a, 4, 5a, 7b, 9a, 9b, and 11 are all lethal, and deletion of Wnt2 results in decreased viability (66, 68, 71, 163-166). Because Wnts were initially characterized for their importance in body patterning in *Drosophila* and *Xenopus* (167,168), it is not surprising that mutation of individual Wnt proteins in mice results in either failure of axial body patterning (Wnt1, 3, 3a, 5a, and 8a) (66, 71, 76, 163, 165) and/or abnormal limb development (Wnt3, 5a, 7a, 9a) (64, 69, 71, 74). However, most Wnts appear to have at best a redundant role in body patterning in the mouse; they may have more critical roles in the development of specific organs or tissues (Wnt2, 2b, 4, 5b, 6, 7b, 8b, 9b, 10b, 11, and 16) (78-81, 83, 166, 169-176). While many mice have been created carrying floxed alleles of individual Wnt genes for conditional deletion in specific tissues, most of these have not yet been crossed with mice carrying

bone-specific Cre recombinases (64, 69, 75, 78, 79, 169, 170, 177-182).

While not all Wnt models have been assessed for specific changes in bone structural qualities, several are clearly important for bone mechanical properties. For example, *Wnt3a*^{+/-} and *Wnt5a*^{+/-} mice have reduced BMD, bone volume to tissue volume (BV/TV) ratio, and trabecular number (Tb N) and increased trabecular spacing (Tb Sp) (65), which was traced back to abnormal chondrocyte and osteoblast function in *Wnt5a* mutants (72). Interestingly, overexpression of a *Wnt5a* or *Wnt5b* transgene in chondrocytes via the *Col2a1* promoter resulted in a skeletal phenotype similar to that of *Wnt5a*-null mice: shortened skeletal elements and delayed ossification(72). Although they have not been fully assessed, hypomorphic *Wnt3a-vt* mice have abnormal development and ossification of vertebrae (67). Interestingly, mice expressing a *Col2a1-Wnt4* transgene in cartilage have abnormal chondrocyte zoning and dwarfism but normal BMD (70).

When some Wnts are specifically deleted from bone tissue, there are often decreases in bone quality. For example, removal of Wnt7b in bone by *Dermo1-Cre* results in decreased skeletal ossification and smaller bones during embryonic development (75). The loss of Wnt9a results in decreased size and mineralization of appendicular long bones, as well as fusion of joints. Whereas *Wnt5a* seems to have the most profound

effect on distal skeletal elements (72), Wnt9a seems important for the development of proximal long bones. Global knock-out of Wnt4 results in no abnormal joint phenotype, but concomitant loss of Wnt4 and Wnt9a exacerbates the joint fusion phenotype seen in Wnt9a knock-outs (69). The role of Wnt9a in joint formation was further supported by the finding of loss of chondrocytes and reduced cartilage in mice when a Wnt9a transgene was overexpressed in chondrocytes via a Col2a1 promoter (77).

Overexpression of a Wnt10b transgene with the Fabp4 adipocyte-specific promoter enhanced trabecular bone formation and the mechanical properties of long bones. This is likely a consequence of expression from the bone marrow compartment (81). Further evidence to support Wnt10b's role in bone development comes from knock-out mice, which have a 30% reduction in bone volume and BMD at 8 weeks of age. This was coupled to a reduction in serum osteocalcin (OC) but no change in TRAP5b, which suggests a loss of osteoblasts and no change in osteoclasts (81). Wnt16-null mice, while appearing to have normal skeletal development and structure, have significantly reduced cortical bone thickness and strength at 24 weeks of age (83).

Several human disorders have been directly linked to mutations in various WNT proteins. Many of these reflect the abnormal skeletal development in the Wnt mouse models. A Gln83X mutation in WNT3 has been identified in tetraamelia, autosomal recessive (OMIM: 273395). This disorder produces malformed heads, failure to develop limbs, cleft palate, and pelvic hypoplasia (183). Two autosomal dominant mutations affecting WNT5a (Cys182Arg and Cys83Ser) have been identified in Robinow syndrome (OMIM: 180700). Robinow patients have hypertelorism, craniofacial dysmorphism, short stature, and mesomelic shortening of the limbs (184). Two mutations in WNT7a result in similar, but clinically distinct disorders. The first is Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome (Arg292Cys or Gly204Ser) (OMIM: 276820), which results in absence of the ulna and fibula and severe limb deficiencies including truncated limbs and loss of digits (185, 186). The second is Fuhrmann syndrome (Ala109Thr) (OMIM: 228930), which is less severe and causes bowing of the femurs, aplasia or hypoplasia of the fibula, and poly-, syn-, or oligo-dactyly (185).

While no mouse model currently exists for WNT10a, several mutations have been identified that result in odontoonychodermal dysplasia (OMIM: 257980) (187-190). These patients have several distinguishable malformations including dystrophic nails, malformed or undeveloped teeth, and hyperhidrosis of hands and

feet. Less severe mutations of WNT10a (Asp217Asn and Gly95Lys) lead to tooth agenesis, selective 4 (OMIM: 150400), which produces loss or truncation of the lateral incisors (189, 190). Mutations in WNT10b result in split-hand/root malformation 6 (OMIM: 225300). Both missense (Arg332-Trp) and frameshift (4-BP DUP, 458AGCA) mutations have been identified in this disorder, which presents with syndactyly, median clefts of the hands or feet, and aplasia or hypoplasia of the phalanges, metacarpals, and metatarsals. Additional abnormalities include mental retardation, ectodermal, craniofacial, and orofacial clefting (191, 192). While no specific disorder has been linked to WNT16, individual SNPs have been identified in the WNT16 gene that are associated with cortical bone thickness, BMD, and fracture risk (83).

Porcupine and Wntless

The mechanism of Wnt secretion has been a hot topic in the literature, and two proteins have been identified that are essential for the process. One is porcupine (Porcn), an ER-resident O-acyltransferase that is specifically involved in the palmitoylation of Wnts (193). The second is Wntless (Wls; aka Evi, Sprinter, or Gpr177) which serves as a Wnt chaperone (194). Proper function of both of these proteins is necessary for Wnts to be secreted; the absence of either Porcn or Wls results in the intracellular accumulation of Wnt proteins (51, 52, 193). Both Porcn and Wls mouse models produce severe phenotypes that are reminiscent of several Wnt knock-out mice, further supporting the concept that both Porcn and Wls have crucial and non-redundant roles in Wnt signaling (84-87, 195-197).

Porcn is a member of the MBOAT family, having a low sequence similarity (<35%) to lysophospholipid acyltransferases (198). The gene lies on the X-chromosome, and mutations that disrupt the function of PORCN are male embryonic lethal and cause the female X-linked dominant disorder known as focal dermal hypoplasia (305600, OMIM 2012) (199). The structure of Wls is not conclusively defined, but it is predicted to be a multi-pass transmembrane protein (194). Wls is found in both the Golgi and the ER, and it is recycled via endosomes (200). Recognition of Wnts by Wls depends upon palmitoylation of Wnt by Porcn (200). The palmitoleate-binding domain of PORCN is believed to reside within amino acids 43-200, because loss of these residues abolishes WNT-WLS interactions (201). The mechanism of how Wnts dissociate from Wls upon reaching the cell surface has not been determined, but one study suggests that changes in pH may play a role (201). Crystal structures would greatly assist in answering these questions.

The loss of one functional copy of *Porcn* during embryogenesis is highly lethal to female mice, but is completely lethal in male embryos by day E15.5. Females that do survive have a spectrum of abnormal phenotypes with varying degrees of penetrance. These results are not surprising due to stochastic epigenetic silencing of one copy of the X-chromosome during female development. Skeletal abnormalities that are frequently seen in these mice include loss or fusions of digits, shortened or truncated limbs and body axis, and cleft palate. Crossing *Porcn*-floxed mice with mice carrying *Prx1-Cre* or *Msx2-Cre* removes *Porcn* from the developing limb ectoderm, leading to viable male offspring that have severely shortened and malformed limbs (84, 85). No studies yet have used bone-specific Cre promoters to examine the role of *Porcn* in the maintenance of bone quality throughout life.

Homozygous deletion of *Wls* by homologous recombination, or by using Cre expressed early in embryogenesis to remove a floxed portion of the gene, is embryonic lethal before E10.5, with failure to develop mesoderm by E8.5 (195-197). However, heterozygotes develop without appreciable abnormalities. Interestingly, limb ectoderm-specific loss of *Wls* driven by *Prx1-Cre* is more severe than the related *Prx1-Cre-Porcn*-floxed model: these mice die during weaning due to such severe limb malformations that the mice are unable to acquire food or water. Their limbs are hypoplastic, shortened, and had truncated autopods. Further investigation revealed delayed cartilage hypertrophy and osteogenesis. The use of *Msx2-Cre* resulted in truncated autopods in all limbs, but also truncated zeugopods in hind limbs. Due to some expression of *Msx2-Cre* during craniofacial development, there was also a failure of fusion of the skull sutures (87).

Crossing the *Wls*-floxed mice with those carrying an *OC-Cre* allele resulted in osteoblast-specific loss of *Wls*. These mice have normal skeletal development and BMD at birth, but they are prone to spontaneous fractures at a young age. This likely has to do with deficits in bone repair, as there was a significant reduction in BMD by 20 days that became more significant with age. Less than 20% of these mice survive past two months, and only a handful make it to 4 months of age. There was a 90% decrease in BV/TV ratio by 7 weeks and a greater than 50% decrease in cortical bone of the femur. The mice showed a progressive loss of trabecular bone, calvarial bone, and cartilage. There was also evidence of decreased osteoblastic activity (fewer osteoblasts and decreased serum OC) and increased osteoclastic activity (increased TRAP staining of femur sections). There was an apparent increase in cartilage

degradation, because serum levels of CTX-II were markedly elevated (86).

Recently, mutations in *PORCN* have been identified as driving the X-linked dominant syndrome known as focal dermal hypoplasia (FDH) or Goltz syndrome (OMIM: 305600). These include both nonsense and missense mutations (13-BP DUP, NT1059; Gly60Arg; Arg124X; Trp74X; and Arg365Gln) (199, 202, 203). FDH is only seen in heterozygous females, which suggests that a complete loss of *PORCN* is embryonic lethal in humans. Stochastic X-linked inactivation leads to asymmetrical skeletal deformities such as syndactyly, absence or underdevelopment of digits or extremities, ectrodactyly, and polydactyly in various combinations. Additional abnormalities include osteopathia striata of long bones, scoliosis, and clavicular dysplasia (204). No human disease has yet been directly associated with WLS, but SNPs have been identified within an intron of *WLS* that are associated with BMD in Asian and European populations (205, 206).

Frizzled (Fzd)

The Frizzled receptors form a family of 10 human genes. The proteins are characterized by seven transmembrane domains, an extracellular N-terminal domain that is N-glycosylated, a large extracellular cysteine-rich domain that is responsible for Wnt binding, three intracellular loops, and a cytoplasmic C-terminal tail that contains multiple phosphorylation sites. Fzds have a strong similarity to other G-protein-coupled receptors, and Wnt-mediated G-protein signaling through frizzleds may be important for some non-canonical Wnt signaling pathways (207). Crystallization of *Xenopus* Wnt8 with Fzd8 revealed a specific hydrophobic binding interaction between the conserved N-terminal lipid "thumb" domain on Wnt8 and a conserved site on Fzd8. The data suggested how specific Wnts may interact with specific Fzds due to interactions between the conserved hydrophobic C-terminal "index finger" domain and a less conserved binding groove on Fzd8 (1).

Although mouse germline deletions exist for Fzd1-6, 8, and 9 (90, 201, 208-216), only mouse models of Fzd9 deficiency have been thoroughly characterized for skeletal abnormalities (90). The individual Fzd receptor knockouts do not produce profound malformations in body patterning. However, most of the receptors are crucial for organ functioning: several knockouts result in late embryonic (Fzd5 (211)) or post-natal lethality (Fzd2, 3, 4 (201, 208, 209)). Two of the models do produce skeletal abnormalities; for example, craniofacial cleft palate and hypognathia in *Fzd2*-null mice (201), and curly tail in *Fzd3*-null mice (208). Fzd1 and Fzd8 germline deletions

produce no overt phenotype (201, 215), but further probing is likely to reveal subtle phenotypic consequences, such as the loss of *Fzd6* resulting in abnormal hair patterning (214).

Deletion of *Fzd9* results in no abnormal phenotype of skeletal development, nor is there a difference in bone volume by 6 weeks, but there is a 35% decrease in trabecular bone volume of the vertebrae by 24 weeks. This was due to lower trabecular number, not a change in trabecular thickness. No difference in osteoclast activity was apparent by TRAP staining, but calcein labeling revealed a significant decrease of bone anabolism and osteoblast surface. Both homozygous-null and heterozygous mutant animals had reduced cortical thickness, lower trabecular bone volume, fewer osteoblasts, decreased bone formation rates, and decreased mechanical resistance (90). As the study authors remarked, just because there is not an overt phenotype does not mean there is no consequence of *Fzd* loss on bone quality; further investigation into bone phenotypes of the other *Fzd* models is warranted. No human skeletal disorders have been directly linked to any of the frizzled receptors.

Low-density lipoprotein-related proteins 5 and 6 (Lrp5/6)

Lrp5 and *Lrp6* are part of the β -catenin-dependent (canonical) Wnt signaling pathway that is primarily involved in cell differentiation and proliferation (217). Intracellular PPP(S/T)P repeats in the cytoplasmic tail of *LRP5* and *LRP6* are phosphorylated during Wnt signaling (218, 219). These sites can be phosphorylated by numerous kinases including GSK3 (218), GPCR kinase 5 and 6 (220), protein kinase A (PKA) (221), mitogen-activated protein kinase (MAPK) (222), and cyclin-dependent kinase 14 (CDK14) (223). Phosphorylation of these sites then promotes further phosphorylation of neighboring serine residues in PPSPXS sites by members of the casein kinase I (CKI) family (224). This allows the binding of axin and recruitment of the β -catenin destruction complex to the cell membrane (217). Inhibition of canonical Wnt signaling by sclerostin (*Sost*) and Dickkopf-1 (*Dkk1*) is mediated by binding to *Lrp5/6* (37, 225).

Lrp4 does not have an overlapping function with *Lrp5/6*, and its role in modulating canonical Wnt signaling has only recently begun to be appreciated. *Lrp4* binds both *Sost* and *Dkk1* *in vitro* (92). In addition, *Lrp4* enhances the ability of *Sost* to inhibit canonical Wnt signaling, but it appears to have no influence on *Dkk1* activity. This is further supported by the identification of *LRP4* mutations in patients with sclerosteosis who have normal *SOST* function (39). *Lrp4* has also been shown to play an important role in bone development, and *Lrp4*

knock-out models have similar phenotypes as other Wnt pathway knock-outs (93, 226).

Several *Lrp4* mutant mice that display similar phenotypes have been identified from mutational screens. These mice all display brachydactyly and syndactyly on all limbs, along with shortening of distal segments and multiple fusions. The most interesting observation is that the abnormal digit duplications occur in the dorsoventral axis, which means they are out of plane. While producing similar gross anatomical consequences, *digitation anormale (dan)* and *malformed digits (mdig)* mice are viable as homozygotes, but *mitten (mitt)* and *mitaine (mte)* mice die at birth (93, 94). Mice that carry a mutation that results in the secretion of only the *Lrp4* extracellular domain have lower bodyweight, polysyndactyly, and abnormal tooth development with full penetrance. In-depth analyses of bone phenotypes revealed no change in bone shape, but femurs were shorter and have a narrower midshaft. There was a decrease in total BMD, but no change in cortical bone measurements. Vertebrae had reduced trabecular bone mass, and serum markers of osteoblast activity (OC and ALP) and osteoclast activity (DPD) were all elevated, suggesting increased bone turnover (92, 226). Interestingly, this model must have retained some *Lrp4* activity, because deletion of exon 1 causes perinatal lethality (91). A floxed mouse exists but has not been used to assess skeletal phenotype (227).

Mice carrying a germline homozygous deletion in *Lrp5* are viable and fertile. Skeletal development is normal, but low bone mass is seen in juveniles when mice begin developing spontaneous fractures. Heterozygotes have lower bone mass than wild types, but the decrease is not as severe as in the homozygous mutants. Bone formation rate and the number of osteoblasts are decreased, but there is generally no change in the number of osteoclasts or their activity (95-97, 99, 100). Studies using floxed alleles of *Lrp5* that are conditionally deleted in osteoblasts have yielded conflicting results. Deletion of *Lrp5* by *Col1a1-Cre* failed to mimic the low-bone-mass phenotype of *Lrp5* knock-out mice. The authors then went on to show that they could reproduce the low-bone-mass phenotype when *Lrp5* was conditionally deleted in the intestines via *Villin-Cre* (98); this was attributed to an increase in circulating serotonin. Another group removed *Lrp5* from osteocytes via *Dmp1-Cre*, which also resulted in a low-bone-mass phenotype. This study also removed *Lrp5* from the intestines using *Villin-Cre*, but there was no consequence on bone mass, nor was there any change in circulating serotonin following the use of either Cre (101). One potential difference between these two studies that may be influencing the

results is the location of the loxP sites in the *Lrp5* locus. In the first study, Yadav *et al* used an *Lrp5* allele that contained loxP sites around exon 6; previously this group had generated and characterized a germline mutant mouse lacking exon 6 and found that deletion of this exon still resulted in the production of a truncated protein product (97). The *Lrp5*-floxed mouse of Cui *et al* resulted in the loss of exon 2 as well as a frame shift after Cre recombination (101). It is possible that the truncated protein product produced after Cre recombination in the Yadav *et al* model has some confounding function. It is also possible that the different versions of the cre transgenic mice may account for some of the observed differences. Elucidation of the underlying reasons for these differences will be a key area of emphasis in the immediate future.

Knock-in models for gain-of-function mutations in *Lrp5* have also been produced. Mice containing A213V or G170V mutations mimic two high-bone-mass alleles seen in the human population (A214V and G171V, respectively). To avoid confusion with the literature, we will refer to the two lines by the names of their human homologs. Either mutation results in a global increase in cortical bone mass, BV/TV ratio, and trabecular number and thickness, with a decrease in trabecular spacing. However, while A214V promoted the greatest increase in cortical bone properties, G171V appeared to have the greatest effect on trabecular bone growth. The consequence of this was most apparent in mechanical tests, where the G171V mutation showed enhanced stiffness only in response to axial loading, but not to three-point compression; these characteristics are most influenced by changes to periosteal and endosteal bone structure, respectively (102). Furthermore, the A214V mutation favored increased periosteal bone formation, while the G171V mutation favored endosteal bone formation in response to mechanical loading (228). In further support of the local effect of *Lrp5* on bone mass, the use of *Prx1*-Cre to drive expression in the appendicular skeleton specifically resulted in high-bone-mass traits in limbs (101). Similar to the *Lrp5* conditional knock-out models, there are two conflicting results in the literature: one group found that the high-bone-mass trait is a consequence of intestine-specific expression of mutant *Lrp5* and changes in serotonin (98), while the other found that it was a result of mutant *Lrp5* expression in bone and that intestinal expression of mutant *Lrp5* had no influence on serotonin (101, 102).

There is also a mouse generated in an ENU mutation screen that produced a frameshift mutation in exon 23 of *Lrp5*, which resulted in the replacement of the last 39 amino acids of the C-terminal tail by 20 new amino

acids. There was a loss of the last three PPP(S/T)P motifs, which are crucial for *Lrp5*-mediated canonical Wnt signaling. These mice have been exclusively studied in exudative vitreoretinopathy (229), and they may be interesting in bone studies due to the retention of a fully functioning extracellular domain, which might compete for ligand binding with *Lrp6*.

Lrp6-null mice die between E14.5 and birth with numerous defects that mimic mutations in other Wnt signaling molecules, such as truncation of the axial skeleton, limb defects, and urogenital malformation (99, 100, 103). *Lrp6*^Δ mice have normal skeletogenesis and BMD but a reduced BV/TV ratio. *Lrp6*^Δ/*Lrp5*^Δ compound heterozygotes have limb abnormalities not seen in either single heterozygotic background, including loss of limbs, synostosis, or reduction of postaxial digits. *Lrp6*^Δ/*Lrp5*^Δ mice showed even more extreme limb abnormalities (99). Conditional deletion of *Lrp6* in the mesenchyme via *Derma1*-Cre revealed mostly normal skeletogenesis with only a slight delay in ossification of the skull at E17.5. A more severe skeletal phenotype became apparent when *Lrp5* was concomitantly deleted in the mesenchyme (*Lrp5*^{flox/flox} *Lrp6*^{flox/flox}), which resulted in perinatal mortality. These embryos had reduced body size, cranial malformation, global decreases in ossification, and failure of fusion in the sternum. There were also numerous areas of ectopic cartilage formation. The major skeletal defects were attributed to a lack of osteoblastogenesis characterized by a lack of osterix and osteocalcin expression. Growth plates revealed a loss of hypertrophic chondrocytes (100).

Three *Lrp6* mutant mice have been identified during mutational screens. Crooked tail (*Cd*) is a G494D mutation within the second YWTD-EGF repeat domain that is predicted to disrupt protein-protein interactions. It was shown to prevent Dkk-1-mediated inhibition of canonical Wnt signaling, but not Dkk-1's ability to bind *Lrp6*. The mutation also appeared to result in decreased interaction with *Mesd*, which might reduce the ability of the mutant receptor to be displayed on the cell surface. The mutation results in a crooked tail phenotype and exencephaly with incomplete penetrance. Those mice that complete neurulation have severe malformations of caudal vertebrae, including misshapen and fused vertebrae. Evidence suggests a delay in the ossification of digits, but no evidence of increased polysyndactyly (104).

Like *Cd* mice, *ringelschwanz* (*rs*) mutant mice are the result of a spontaneous mutation in *Lrp6* (R886W) located in the second YWTD-EGF repeat. As this lies in the third beta propeller region and this residue is highly conserved, this mutation is also predicted to disrupt Dkk

interactions with Lrp6. Approximately one-third of *rs* homozygotes die within a week after birth. Like *Cd* mice, the pups show malformations of the vertebral column and neural tube, but instead of exencephaly, spina bifida is the predominant disorder. Oligodactyly is also common, with loss of the fifth digit in one or more limbs. *rs* mice also show evidence of delayed ossification of metatarsal and phalangeal bones. Bone mass in the tibia is reduced, with a 16% decrease in BMD and 29% reduction in cortical bone thickness (107). Another study found a reduction in trabecular bone volume and increased bone resorption along with normal osteoblast number and mineralization.

The *rs* mutation, like the *Cd* mutation, decreased the ability of Lrp6 to interact with Mesd (108). A third identified mutation, known as *Gwazi* (*Gw*), results in heterozygous mice with crooked tails due to formation of a sesamoid bone between caudal vertebrae (105). The mutation is a D549G amino acid substitution in the last YWTD-EGF repeat of the second propeller domain of Lrp6. The tail kink phenotype is found in 65% of heterozygotes and 85.5% of homozygotes of the *Gw* allele. The phenotype is fully penetrant in *Dkk1^{+/-}Lrp6^{+/-Gw}* compound heterozygotes but drops to 21.5% in *Wnt3^{+/-}Lrp6^{+/-Gw}* compound heterozygotes, suggesting that the *Gw* mutation results in a gain of function (106).

As mentioned above, LRP5 function was linked to human skeletal disorders by three seminal papers (17, 26, 27). Subsequently, families with these and other mutations in LRP5 were identified. The originally identified families with high-bone mass carried Gly171Val mutations (26, 27). Another mutation at this codon, Gly171Arg, may be more detrimental, as the family reported severe headaches, which may be indicative of cranial nerve compression (49). Ala242Thr, Ala214Thr, Ala214Val, and Thr253Ile all lead to increased bone density with varied phenotypic consequences (49). Besides OPPG, inactivating mutations in LRP5 are also associated with Familial exudative vitreoretinopathy (FEVR) (230), a disease caused by incomplete development of the retinal vasculature. Some FEVR-associated mutations in LRP5, but perhaps not all, are also associated with low bone mass (231-233).

A missense mutation in *LRP6* (Arg611Cys) has been identified in *LRP6* that causes the complex, metabolic disorder coronary artery disease, autosomal dominant 2 (Arg611Cys) (OMIM: 610947). All individuals that carried a copy of the mutant allele had hyperlipidemia, hypertension, and lower bone density. The mutated residue is highly conserved across species and lies within one of the LRP6 EGF domains. *In vitro* studies have shown that this mutation results in a 48% reduction in canonical Wnt

signaling in response to WNT3a (234).

Numerous individual *LRP4* mutations have been found in patients with Cenani-Lenz syndactyly syndrome (CLSS) (228). This disorder is characterized by mild face dysmorphisms including a prominent forehead, hypertelorism, and micrognathia. Limb malformations are more severe, including syndactyly of hands and feet and malformation (and frequent fusion) of the radius, ulna, metacarpals, and metatarsals. *LRP4* mutations have also been shown to lead to sclerosteosis 2 (OMIM: 614305) (39). Mutation in the third propeller domain of LRP4 favors sclerosteosis 2, which is less severe than CLSS, presenting with polysyndactyly and thickening of the cortical layer of the skull and long bones.

Dickkopfs (Dkks)

Four unique proteins of the Dickkopf family are encoded in the human genome. All contain an N-terminal signal peptide and two conserved cysteine-rich domains. The greatest conservation between Dkks is in the second CRD. Dkk1 has a single N-glycosylation site near the C-terminus. Dkk4 contains no N-glycans (235). Dkk1 can potentially form a complex on the cell surface between Lrp6 and Kremen, which results in the internalization of Lrp6 and subsequently inhibits canonical Wnt signaling (225). Dkk3 has been shown to have conflicting roles in regulating Wnt signaling depending upon cell type (236-238), and it does not bind or influence the level of Lrp5/6 as do the rest of the Dkk family. Dkk3 may exert its effects through Kremen interactions (239), but this interaction also seems inconsistent (225). For these reasons, Dkk3 is not usually considered a direct modulator of Wnt signaling. Dkk4 expression is a direct target of canonical Wnt signaling and may serve as a negative feedback mechanism (240). Dkk4 may inhibit the canonical WNT pathway, but may favor non-canonical Wnt signaling through JNK (241).

Dkk1-null mice die at birth and have some striking developmental abnormalities. Most malformations become apparent after E9.5, as these mice fail to develop eyes, olfactory placodes, the frontonasal mass, or mandible. All craniofacial features anterior to the ears fail to develop by late gestation. These mice also show fore- and hind-limb malformations, including fusion of digits and ectopic digits (110). *Dkk1* heterozygous mutant mice are viable, fertile, and are the same size as *Dkk1* wild-type littermates. However, the BV/TV ratio is increased, with an increase in trabecular number and size and a decrease in trabecular spacing. Histomorphometry has revealed that the mineralizing surface and matrix apposition rate are elevated two-fold in *Dkk1* heterozygous mice; the number of osteoblasts is in-

creased with no change in osteoclasts. Axial compression tests on the ulna show increased mechanical strength in *Dkk1* heterozygous mice, with a higher maximum load and energy (109). *Dkk1*-floxed mice do exist but have not yet been used to assess skeletal phenotypes with bone-specific expression of Cre (242). In opposition to what was seen in the *Dkk1* mouse models, deletion of *Dkk2* results in osteopenia, with major defects in mineral apposition rates. This has been attributed to an increase in the number of osteoclasts (115). No *Dkk4* mouse models have been described in the literature. No human skeletal disorders have been linked to any of the DKK family members.

Sclerostin

Sclerostin (SOST) is a secreted WNT inhibitor that prevents Wnt signaling by direct interaction with the LRP4/5/6 co-receptors (37-39). It is 190 amino acids long, glycosylated, and contains a cysteine-knot motif. SOST also contains a heparin binding site, and both the N-terminal (1-55) and C-terminal (145-189) ends are unstructured. It contains three loop domains, the second of which is bound by the SOST-inhibiting antibody Scl-Abl (243). This is the same site responsible for binding to LRP6 (244). Serum levels of SOST increase during fracture healing (245). Scl-Abl speeds fracture healing in mice (246) and non-human primates (116). SOST is expressed by osteocytes (36, 247) and articular chondrocytes (248). Polymorphisms in SOST are associated with BMD and fracture risk (249, 250). An ECR5 enhancer element downstream of the SOST coding region is critical for SOST expression and is lost in van Buchem syndrome (44-46, 48). This enhancer region is bound by the Mef2 family of transcription factors (48, 251).

Because SOST was originally discovered based on its role in sclerosteosis, it is not surprising that *Sost* mouse models have been well characterized for their bone phenotypes. *Sost* knock-out mice have accelerated fracture healing with faster callus maturation, mineralization, and enhanced stress resistance (116). There is also increased cortical bone area in long bones (102, 117). When compared with high-bone-mass-promoting alleles of *Lrp5*, *Sost*-null mice were found to have bone quality changes similar to those of the *Lrp5*A214V mice, but not to the *Lrp5*G171V mutation. Similar to what is seen in sclerosteosis, *Sost*-null mice have a 60-80% increase in cranial parietal thickness and reduced foramen size (102). Mice have also been made that mimic van Buchem syndrome by inserting loxP sites around the ECR5 regulatory domain downstream of *Sost*. Conditional deletion of this enhancer in osteoblasts by crossing these mice to *Col1a1*-Cre-expressing mice

resulted in a 41% increase in the BV/TV ratio by enhancing trabecular bone growth. These mice have an increased osteoblast surface with no change in osteoclasts. The high-bone-mass phenotype is not as extreme as that of *Sost*-null mice, supporting the idea that deletion of the regulatory element produces a hypomorphic allele (48). Mice have also been generated that over-express *Sost* in osteoblasts due to the expression of a transgene behind an osteocalcin promoter. These mice were osteopenic with disorganized bone architecture, thin cortices, reduced trabecular bone, impaired lamellar bone formation, and chondrodysplasia. Not surprisingly, these bones were more fragile and less resistant to fracture by compression and four-point bending. Calcein labeling revealed a smaller osteoblast surface and decreased bone formation rate, without a change in markers of resorption (34).

As discussed above, numerous mutations have been identified in *SOST* that lead to sclerosteosis (Gln24X; IVS1DS, A-T, +3 and/or IVS1AS, A-C, -67; Trp124X; and Arg126X) (30, 31), and van Buchem syndrome results from the deletion of an enhancer sequence approximately 35kb downstream of *SOST* (44). In addition to these well characterized but rare disorders, *SOST* has been implicated in the exceedingly rare cranio-diaphyseal dysplasia, autosomal dominant disorder (OMIM: 122860). Mutation of Val21 in the signal peptide of SOST to either Leu or Met significantly diminished SOST secretion. These mutations were documented in two unrelated individuals with this disorder but were not found in samples from healthy patients (252).

Due to the profound effect SOST has on bone mass and quality, this protein has garnered significant attention from scientists and clinicians as a potential therapeutic target for osteoporosis. The anti-SOST antibody (Scl-Ab) has shown very promising results in both human and animal models of low bone mass disorders and fracture healing. To avoid redundancy and to give full attention to the exciting results from Scl-Ab studies, we encourage you to read the recently published and highly thorough review by Ke *et al* (253).

Secreted frizzled-related proteins (sFrps)

Secreted frizzled-related proteins (sFRPs), like Dkk proteins, are secreted inhibitors of Wnt signaling. The first discovered was FRZB/SFRP-3, from the cartilage of mammalian long bones during embryonic and fetal development (254), and it was subsequently identified as a Wnt binding protein and an inhibitor of Wnt signaling (255, 256). In total, five sFRPs have been identified in mice and humans (257); all five are expressed during mouse limb development (162). The sFRPs and frizzled

receptors both have a cysteine-rich domain (CRD). Additionally, sFRPs contain netrin (NTR) domains, in common with netrin, complement proteins, and type I pro-collagen C-proteinase enhancer proteins (258). sFRPs and Wnts are generally expressed in polar opposite regions, which likely helps to establish a Wnt gradient (257). While sFRPs bind Wnts and can inhibit Wnt signaling, they may also be involved in enhancing the range of Wnt diffusion in the ECM when expressed together (257, 259). Further, specific sFRP-Wnt interactions control Wnt signaling (260). For example, while *Sfrp*-1 and -2 can inhibit Wnt3A-stimulated canonical Wnt signaling, *Sfrp*-3 cannot (261). FRZBb-2/SFRP-4 is highly expressed in bone cells, and its mRNA is highly up-regulated in osteoarthritis cartilage samples (262). SFRP-1 negatively regulates osteoblastogenesis and trabecular bone volume (119), and it also directly binds RANKL and can inhibit osteoclastogenesis (263).

Germline deletion of the *Sfrp1* gene in mice results in an interesting bone phenotype. Initial inspection revealed no overt gross anatomical phenotype, and the mice were fertile. Additionally, there was no difference at 52 weeks in overall BMD, femoral diaphyseal BMD, thickness, or periosteal and endosteal circumference; femur length was not affected. Most serum biomarkers of bone turnover were normal, except that phosphate was elevated at 20 weeks in males only (but returned to normal in older mice). However, histology of the proximal femur revealed a 149% increase in trabecular bone volume in females and 62% in males compared with wild-type littermates at 35 weeks, whereas there had been no difference at 20 weeks; this suggested a prevention of age-related bone loss. Mineral apposition rate was only significantly increased in females (32%), further supporting influence of sex on phenotype. There was a noticeable decrease in apoptosis rates of osteoblasts and osteocytes in calvaria as determined by TUNEL, with no increase in proliferation (119). *Sfrp2*-deficient mice were viable, fertile, and had a normal lifespan and no overt physical phenotype. Close inspection revealed shortened metacarpals/metatarsals and phalangeal bones, resulting in significantly shorter digits. Ossification of the phalanges was also delayed. This was determined to be a consequence of reduced hypertrophic chondrocytes, not influenced by changes in apoptosis (120).

Deletion of *Sfrp3* similarly resulted in no overt phenotype or changes in serum markers. Mice had no difference in subchondral bone mass, but both male and female mice had increased BMD, thickness, and stiffness in femurs and tibias; neither sex showed changes in measurements of trabecular bone. The most interesting consequence of *Sfrp3* deletion was an enhanced

anabolic response to ulna loading, even at loads below the threshold of response for wild-type mice (121).

While no skeletal assessment has been made on *Sfrp4* knock-out mice, increased expression of *Sfrp4* leads to decreases in bone mass. With a *Sap-Sfrp4* transgene, mice had smaller increases in BMD and BMC from 5 to 15 weeks relative to wild-type development. This resulted in lower areal BMD and BMC at 15 weeks. Unexpectedly, calcein injection did not show a difference in mineral apposition rates between the transgenic mice and wildtype. There was also no difference in serum OC or CTX levels (123). Similar results were seen with a *Col1a1-Sfrp4* transgene (124). The senescence-accelerated mouse P6 (SAMP6), which has lower peak bone mass at 4 months than other SAMPs, has mutations in the *Sfrp4* promoter that create a hypermorphic allele of *Sfrp4* with 40-fold increased expression. These mice have lower numbers of osteoblasts, and osteoblasts from these mice are less responsive to Wnt3A induction of canonical Wnt signaling *ex vivo* (264). No skeletal phenotypes have been assessed in *Sfrp5* knock-out mice (265).

No human skeletal disorders have been directly linked to any of the sFRP family members, but SNPs in *SFRP3*/*FRZB* have been associated with the risk of osteoarthritis (OA) and osteoporotic fracture (266-268), as well as with hip shape (122). However, the association of these SNPs with OA or osteoporotic fracture is not consistently observed in all studies (269-271). Serum levels of FRZB may also be a marker of rheumatoid arthritis and response to therapy (272). Similarly, SNPs in *SFRP4* were found to correlate with hip fracture risk, height, and body composition in Danish males, but the findings were not replicated in a Belgian population (273). Also the rs16879765 polymorphism was found to increase the risk of developing Dupuytren disorder nearly twofold in a Dutch population (274).

Dishevelled

Dishevelled (Dvl) proteins were originally discovered through its crucial role in cell polarity and identity. Loss of the *Dvl* gene phenocopies loss of the Wnt-1 homolog, Wingless, in *Drosophila*, supporting a critical role in downstream Wnt signaling (275). In mammals, three dishevelled homologs have been identified: Dvl-1, Dvl-2, and Dvl-3 (276-279). In canonical Wnt signaling, the three share mostly redundant roles (280), but they are involved in both β -catenin-dependent and independent Wnt signaling (281). All mammalian Dvl proteins contain three highly conserved domains. There is an N-terminal DIX domain that allows both homotypic and heterotypic interactions with other DIX-containing proteins such as axin1/2. This domain is necessary for Dvl's promotion of

β -catenin accumulation (282). Dvls also contain a PDZ/DLG domain that may promote interaction with APC and frizzled (283, 284), and a Dvl/egl-10/pleckstrin (DEP) domain found in several proteins that associate with G-proteins (285) and is critical for Dvl's promotion of JNK activation (286) and membrane localization (287). All three conserved domains, DIX, PDZ, and DEP, are necessary for β -catenin signaling through LEF1 (286).

The C-terminal of the PDZ domain is a conserved stretch of basic residues that functions as a nuclear localization signal in many proteins, but dishevelled has been found to be predominantly cytoplasmic (288). The N-terminal of PDZ contains a proline-rich, putative Src homology 3 (SH3) binding domain (289), which may recruit and promote Tyr654 phosphorylation of β -catenin by Src (290). Wnt/Wingless signaling results in the hyperphosphorylation of Dvl as well as its membrane localization (288, 291). The PDZ domain interacts with and is phosphorylated by casein kinase 2 (CK2) during Wnt signaling (292). Dvl promotes dephosphorylation of axin by recruiting protein phosphatase 2C α (PP2C) via its PDZ domain to the Dvl-axin- β -catenin complex, which promotes Wnt signaling (293). Dvl is also phosphorylated by casein kinase 1 (CK1) during Wnt signaling (294, 295), which enhances Dvl association with Frat-1 and activation of the Wnt pathway (296). Dvl is necessary for the recruitment of axin to the membrane during Wnt signaling (297). Several proteins inhibit Wnt signaling by interacting with Dvl, including Idax (298), Daple (299), and Dab2 (300). Dvl proteins are phosphorylated within the DEP domain by Abl kinases, which is critical for planar cell polarity function, but has no known role in canonical Wnt signaling (301). Dvl is ubiquitinated and targeted for proteasomal destruction by the HECT-containing, Nedd4-like, ubiquitin E3 ligase (ITCH), and this interaction is dependent upon Dvl's DEP domain (302).

Dvl mouse models have not been thoroughly assessed for skeletal phenotypes. *Dvl1* knock-out mice display no overt physical phenotype, but have abnormal behaviors including lower social interaction, not barbering, subordination, not sleeping huddled, and not building nests (303). *Dvl2* deletion results in 50% perinatal lethality due to cardiac anomalies. Interestingly, the surviving homozygous knock-outs are predominantly female and these mice have a normal lifespan. Some physical malformations in *Dvl2* mutant mice were documented, including kinked tails, scoliosis, and vestigial tail, and 90% had mild abnormalities of vertebral bodies and ribs. The number of ribs were normal, but they were often forked and fused. The physical abnormalities were exacerbated if *Dvl2* mutants were crossed with *Dvl1*-null mice

(125). The majority of *Dvl3* knock-out mice die before weaning, likely due to cardiac abnormalities, but no skeletal defects have been noted in these mice (304). None of the DVL family members have been directly linked to any human skeletal disorders.

Axin

The axin-1 protein is 832 or 868 amino acids in length, depending upon splicing, and is crucial for axis development (305). Axin negatively regulates Wnt signaling by interacting with GSK3 and β -catenin and by enhancing GSK3 phosphorylation of β -catenin. Axin contains RGS (regulator of G-protein signaling) and Dvl (DIX) domains. Axin is phosphorylated by GSK3 (306, 307), which may positively promote the association between β -catenin and axin (307). As a small-molecule inhibitor, axin appears critical in restraining β -catenin's function and preventing it from activating Wnt signaling and promoting osteoblastogenesis from mesenchymal stem cells (308). Axin interacts with APC through the RGS domain (309) and with protein phosphatase 2A via an N-terminal domain (310) that may regulate APC phosphorylation (311). Axin can dimerize and interact with Dvl via the DIX domain, which appears to be important for inhibiting Wnt signaling (282, 312), the heterotypic interaction by Dvl likely acting in a dominant negative fashion (313).

Axin forms a complex with β -catenin and casein kinase I to promote Ser-45 phosphorylation of β -catenin, thus priming β -catenin for further phosphorylation by GSK3 (314). Sumoylation on the C-terminal end appears to be critical for axin promotion of JNK activation (315) and enhances axin's stability (316). Lys505 of axin is ubiquitinated by the E3 ubiquitin ligase Smurf2, which promotes axin's proteasomal degradation (317). The stability of axin is increased by protein arginine methyltransferase (PRMT1) via decreased ubiquitinylation and enhanced interaction with GSK3 (318). Axins are polysylated by tankyrase 1 and 2, which promotes axin polyubiquitinylation (319) by the RING-domain E3 ubiquitin ligase RNF146 (320,321). Axin contains six RXL cyclin/CDK2 binding motifs spread throughout its structure, as well as numerous S/TP consensus target sequences for phosphorylation by CDKs (322). Most notably, CDK2 (322) and GSK3 (307) can both phosphorylate Thr609, Ser614, and Ser621 of axin, and phosphorylation of these sites enhances the axin- β -catenin interaction. Ser80, Ser82, and Ser222 can be phosphorylated by CKI, which enhances the axin-GSK3 interaction, and these residues can be dephosphorylated by protein phosphatase I (PPI) (323). Axins may mediate cross-talk between TGF β and Wnt signaling (324). Activation of Wnt signaling results in the recruitment of axin to the cytoplasmic tail of LRP5/6

(325, 326). Axin may also be involved in the nucleocytoplasmic shuffling of β -catenin (327,328).

Research suggests that axin-1 and axin-2/conductin are functionally redundant (329, 330), albeit, with different expression patterns during development (330, 331), and axin-2 functions in a negative feedback loop during Wnt signaling (331, 332). Axin-2 is 840 amino acids in length, shares 44% homology with axin-1, and retains the RGS and DIX domains, as well as binding sites for β -catenin, GSK3, and APC (329).

Axin was originally discovered as being the causative gene producing the “fused” phenotype in early mouse studies. Three alleles were identified that produced identical phenotypes with different levels of penetrance: Fused, Knobbly, and Kinky. Homozygosity for these mutations typically results in embryonic lethality by E9.5, with forebrain and neural tube defects as well as axis duplications. Heterozygotes survive, but often have rib fusions and tail bifurcation. Fused and Knobbly both result from IAP retrotransposon integrations, into intron 6 and exon 7 of *Axin1*, respectively. Kinky has been mapped to the *Fused* locus, but the causative mutation has not been identified (126, 127). To support that concept these mutations result in null alleles, two separate models with germline deletions of *Axin1* produced identical phenotypes to the fused mice with full penetrance (126, 333). Some very interesting knock-in models have also been developed. Knock-in of *Axin2* behind the *Axin1* promoter demonstrated that the functions of these two proteins are highly redundant, as the mice had no overt phenotypes and were fertile (330). However, it would not be surprising if subtle differences remain to be discovered. Such studies could potentially reveal unique roles of axin-1/-2.

Mouse models have also been used to assess the importance of the RGS and KVEKVD domains in axin-1 function. Removal of either of these domains results in phenotypes identical to the complete knock-out of axin-1. However, in the Δ KVEKVD model, it is difficult to determine whether the embryonic lethal phenotype is a really a consequence specific to the loss of this domain, because this mutation resulted in a 3- to 4-fold decrease in axin-1 protein (334). A very interesting mouse model that allows both temporal and tissue-specific overexpression of *Axin1* is the *TRE2-Axin1-GFP* transgene. *Axin1* can be overexpressed in specific tissues via tissue-specific promoter control of the reverse tetracycline transactivator (tTA), and temporally by the use of doxycycline to promote transcription via the tetracycline response element promoter (TRE). For example, crossing *TRE2-Axin1-GFP* mice with mice carrying the *MMTV-tTA* transgene resulted in abnormal mammary gland develop-

ment with decreased Wnt signaling in the glands (335). *TRE2-Axin1-GFP* mice have also been crossed with *Axin2-tTA* mice to assess the consequence of overexpressing axin-1 during canonical Wnt signaling. This resulted in abnormal neural development, but there were no in-depth assessments of skeletal phenotypes (336).

Fewer models have been developed for *Axin2*, but there have been some interesting studies published. Homozygous deletion of *Axin2* results in skull defects at early postnatal periods, including craniosynostosis (premature fusing of cranial sutures) due to alterations in intramembraneous ossification. The mice show evidence of aberrant activation of Wnt signaling, which likely accounts for the increased osteoblast proliferation and differentiation seen *ex vivo*, and ultimately for the enhanced bone mineralization and ossification *in vivo* (130). Further assessment of these mice revealed an important role of axin-2 in mature bone: significant differences in bone volume and BMD are seen at 6 and 12 months when compared with wild-type mice, but no differences in bone volume or BMD are observed at 2 months of age (129). These mice also show an abnormal chondrocyte phenotype due to expedited maturation, resulting in a shorter hypertrophic zone in the growth plate of long bones (128). An *Axin2* mutation was also identified in the canopus (canp) mouse: mice that were homozygous for the V26D missense mutation in *Axin2* were arrested at midgestation with abnormal heart development and shortened or doubled tails. *In vitro* analyses revealed that this mutation greatly enhances the stability of the axin-2 protein, tripling its half-life (131).

Axin1 and *Axin2* have both been implicated in cases of developmental disorders. *Axin1* was found to be methylated in a pair of discordant monozygotic twins with caudal duplication anomaly (OMIM: 607864) (337). Supporting the critical role of *Axin2* in craniofacial development, multiple studies in humans have identified polymorphisms/mutations in *Axin2* related to tooth agenesis (338-341) and oral clefts (132, 340). Mutations in *Axin2* are also associated with oligodontia-colorectal cancer syndrome (Arg656X) (OMIM: 608615) (338).

Adenomatous polyposis coli (APC)

The name of the protein APC, adenomatous polyposis coli, originates from an autosomal dominant condition known as familial adenomatous polyposis (FAP), which is characterized by the presence of numerous pre-cancerous polyps of the colon and other regions of the gastrointestinal tract (MIM:175100). Affected individuals may develop hundreds to thousands of adenomatous polyps within the colon and rectum, which progress to carcinomas. FAP is also associated with several skeletal

defects including dental abnormalities (342, 343), osteosarcoma (344), osteoma of the jaw (345), and desmoids tumors (346).

The human APC gene is found on chromosome 5q22 and has 15 exons encoding a 2843-amino acid protein (347) containing two N-terminal coil-coiled regions, seven internal armadillo repeats, and several other functional domains (348). APC interacts with a diverse set of proteins and other cellular components to regulate cell migration and adhesion, cytoskeleton regulation, and chromosome segregation. Within the β -catenin destruction complex, APC joins the scaffolding protein axin by binding β -catenin through its armadillo repeats in order to assist glycogen synthase kinase 3 (GSK3) in phosphorylating β -catenin. Phosphorylated β -catenin is rapidly degraded through recognition by the F-box/WD40-repeat-containing protein β -TrCP, targeting β -catenin to the proteasome (349). APC may also be a nucleocytoplasmic shuttle protein (350), which has been speculated to chaperone β -catenin out of the nucleus (351, 352) and thus preventing β -catenin from binding to TCF/LEF (350, 353, 354). As a negative regulator of β -catenin, defects in or loss of APC results in increased bone mass: FAP patients with APC mutations within the β -catenin binding domains have increased bone mineral density (355).

Consistent with APC being a negative regulator of β -catenin, *Ocn-Cre*-mediated deletion of *Apc* within mature osteoblasts and osteocytes (*Apc* CKO) produces early-onset, severe osteopetrosis leading to animal death at an early age (134). Osteoclasts were entirely absent in bones of these mice by 2 weeks of age. *Ocn-Cre*-mediated deletion of *Apc* is associated with elevated expression of the osteoclast inhibitory factor osteoprotegerin (Opg) within serum and in calvarial osteoblast cultures. Interestingly, no quantifiable differences were observed in proliferation or density during *in vitro* osteoblast differentiation. Deletion of APC via the *Col2a1*-driven Cre promoter resulted in impaired skeletogenesis due to the inability of chondrocytes and osteoblasts to terminally differentiate (133). From these data, it is clear that the role of APC in regulating β -catenin activity is crucial for both cell autonomous and intercellular regulation of skeletal cell types.

Glycogen synthase kinase 3

Glycogen synthase kinase 3 (GSK3), originally found to phosphorylate and inactivate the enzyme glycogen synthase, is a negative regulator of several signaling pathways involved in osteogenesis, such as insulin/IGF, hedgehog, and Wnt/ β -catenin. Within mammals, two GSK3 genes encode the α and β isoforms that differ

mainly due to the presence of an N-terminal glycine-rich extension within the α isoform; the isoforms share 98% sequence identity within the kinase domain, which makes up the majority of the protein (356).

GSK3 β has several substrates within the Wnt pathway, including Lrp5/6, β -catenin, axin, and APC, each of which play distinct roles in regulating the Wnt signal (357). GSK3 kinases typically prefer prephosphorylated or primed substrates by recognizing the consensus sequence Ser/Thr-X-X-X-Ser/Thr-P. Within the β -catenin destruction complex, casein kinase I (CKI) primes β -catenin by catalyzing Ser45 phosphorylation, in turn, facilitating sequential phosphorylation of three adjacent GSK3 consensus sites (Ser41, Ser37, Ser33) and promoting rapid degradation of β -catenin (314, 358-361). Additionally, GSK3 β -mediated phosphorylation of APC and axin increases the stability of the complex and its affinity to β -catenin (282, 306, 307). GSK3 phosphorylation of Lrp5/6 facilitates Wnt receptor complex internalization to form multiunit signalosomes, sequestering GSK3 and promoting β -catenin stabilization and Wnt target gene transcription (2, 362, 363).

There is a plethora of data indicating that Gsk3 β is an inhibitor of bone formation in several contexts and biological pathways. Germline deletion of Gsk3 β results in embryonic lethality due to high hepatocyte apoptosis (135). However, Gsk3 β heterozygote mice develop without gross anatomical abnormalities and have a higher trabecular bone volume density, an increased number of osteoblasts, and an accelerated rate of bone formation (136). This phenotype was attributed to the loss of Gsk3 β -mediated phosphorylation and inhibition of Runx2, an important transcriptional regulator throughout osteoblast differentiation. However, inhibition of Gsk3 β via oral lithium chloride treatment of *Lrp5*^{-/-} mice results in increased bone mass formation and decreased bone marrow adiposity, indicating that β -catenin stabilization favors osteoblast differentiation independent of Runx2 activity (136). Lithium chloride treatment of *Lrp5*^{-/-} primary calvarial osteoblast cultures restores β -catenin TCF reporter activity to wild-type levels (96). Lithium chloride treatment via oral gavage also increases bone mass in C57BL/6 and SAMP6 mice. Other specific dual Gsk3 (α & β) inhibitors induce expression of osteoblast markers such as osteocalcin, collagen I and V, runx2, and TCF reporter activity *in vitro*, as well as increased bone mass, strength, and other markers of osteoblastic differentiation and function within ovariectomized rats (364). Deletion of Gsk3 β within chondrocytes via *Col2a1*-Cre results in a compensatory up-regulation of the Gsk3 β isoform with a minimal effect on skeletal growth or development, indicating the importance of cell type

specificity (140).

Lithium chloride treatment, which has been used for decades to treat bi-polar disorders (365-367), increases bone formation in the distal femurs of mice regardless of physical activity (368). While some studies have shown a reduced fracture risk (369), others were unable to observe this protective effect (370). Chronic treatment with lithium results in prolonged β -catenin stabilization, yet doesn't result in increased susceptibility to several cancer types, in which elevated Wnt signaling is implicated, such as colon, breast or prostate (371-373). The importance of Gsk3 in bone formation is evident, but its mechanisms appear to be numerous and complex. Future cell-based and *in vivo* approaches are needed to elucidate cell autonomous and intercellular effects that GSK3 has on bone turnover.

β -catenin

β -catenin, encoded by the *CTTNB1* gene, is the component of the Wnt pathway that enters the nucleus to drive Wnt target gene expression. Upon binding of a Wnt to the co-receptors Lrp5/6 and frizzled, the β -catenin destruction complex is inhibited, leading to cytoplasmic accumulation and nuclear translocation of β -catenin. Target genes including *AXIN2* and *DKK1* (Dickkopf-related protein 1), which are antagonists of the Wnt pathway, act as negative feedback regulators (331, 374). This process is regulated through a cascade of post translational modifications of β -catenin and the molecules that regulate it. Destruction of β -catenin occurs via the GSK3-dependent process described above. Nuclear β -catenin binds to TCF/LEF DNA binding proteins to elicit the transcription of Wnt target genes. The deletion of exon 3 of β -catenin removes key residues and results in a non-degradable, stable form of β -catenin (375). Use of the knowledge of these mechanisms in concert with the Cre-lox system has generated several genetic studies into the role of β -catenin, Wnt signaling, and their role in bone formation.

β -catenin controls the fate of mesenchymal cells, from which chondrocytes, osteoblasts, and osteocytes originate. Mouse embryos lacking *Cnntb1* within mesenchymal progenitors (via *Dermo-Cre* or *Prx1-Cre*) have severely diminished osteogenesis and form a truncated cartilaginous skeleton (59, 144, 149, 376). This loss of β -catenin within skeletal precursors also causes ectopic cartilage formation, indicating that β -catenin is a positive regulator of the osteoblast lineage and an inhibitor of chondrogenesis (377). The importance of β -catenin in maintaining the osteoblast identity was further supported by removing β -catenin in pre-osteoblasts using the Dox-inhibitable *Osx-Cre*. Pre-natal deletion of *Cnntb1*

resulted in a lack of skeletal ossification and mineralization with formation of ectopic chondrocytes from osteoblast precursors (59). Intriguingly, if Dox was given until 2 or 4 months post-natally, there was a dramatic loss of trabecular bone with a concomitant increase in bone marrow adiposity. Additionally, *ex vivo* analyses revealed enhanced adipocyte differentiation of adult BMSCs in culture, suggesting that β -catenin suppresses adipogenesis (150). Genetic deletion of *Cnntb1* within mature osteoblasts (using *Ocn-Cre*) results in dramatic reductions in trabecular and cortical bone mass due to defective osteoblast differentiation (62, 134). This phenotype was attributed to a decrease in osteoprotegerin, resulting in elevated RANK ligand-stimulated osteoclast activation and increased bone resorption (134). *Cnntb1* deletion via *Collagen I-Cre* results in a low-bone-mass phenotype, despite having normal numbers of osteoblasts. Bones from these mice display more TRAP-positive osteoclasts, suggesting that this phenotype is due to increased resorption and that β -catenin signaling in osteoblasts regulates osteoclast differentiation (143). Osteocyte-specific deletion of *Cnntb1* via *Dmp1-Cre* causes progressive bone loss and premature lethality. This low-bone-mass phenotype is associated with an increased number of osteoclasts and higher osteoclast activity due to disruption of the OPG/RANK axis, although osteoblast function and the osteocyte density is normal (151). These results indicate that β -catenin is a master regulator of the osteochondral lineage, directing embryonic bone formation and postnatal bone homeostasis.

Overactivation of Wnt signaling within limb buds (*Prx1-Cre*-mediated) results in abnormalities of the appendicular skeleton, with only undifferentiated, Sox9-expressing cell types remaining. This is a further indication that β -catenin activity must be spatially and temporally controlled to direct bone formation (144). Stabilization of β -catenin within osteoblast precursors (via *osterix-Cre*-mediated excision of the third exon of β -catenin) results in premature mineralization during endochondral ossification but a loss in osteocalcin-expressing osteoblasts, signifying an inability to terminally differentiate (59, 143). Although the number of osteoblasts was not affected in this model, the blockade of osteoblast differentiation reduced the osteoclast population, perhaps due to alteration of the RANK/RANKL regulatory axis. From these results, the cessation of β -catenin signaling appears to be required for terminal differentiation of osteoblasts and for initiating subsequent programs of bone formation such as osteoclast and chondrocyte differentiation and activity.

The word "catenin"—from the Latin "catena", which means chain, was based on the original identification of

α -, β -, and γ -catenin (plakoglobin) as molecules that linked cytoplasmic domains of cadherins at cell-cell junctions with the actin cytoskeleton (378-380). This Wnt-independent role for β -catenin may be important in regulating bone mass accrual in response to mechanical stress. Mechanical deformation within calvarial osteoblasts induces β -catenin nuclear localization and activation of the Tcf/Lef reporter, independent of Lrp5 (381, 382). This phenotype is enhanced with the addition of lithium chloride treatment, which further inhibits GSK3 β (383). However, this mechanism is exclusively cell autonomous, as mechanical loading *in vivo* reduces SOST expression by osteocytes, resulting in elevated Lrp5/6- Δ -catenin signaling within osteoblasts. Conversely, mechanical unloading via tail suspension produces the opposite effect (384). From these data, it is apparent that there are multiple overlapping systems—from developmental cues to the response to physical stimuli for regulating β -catenin activity within osteoprogenitor cells in order to control cell fate and the deposition of bone.

Human skeletal diseases are more commonly caused by mutations in genes that regulate β -catenin and the Wnt pathway (Section I) than by mutations within *CTNNB1* itself. However, a meta-analysis of five human genome-wide association studies of the femoral neck and lumbar spine bone mineral density found an association between *CTNNB1* polymorphisms and altered BMD (205). Other developmental diseases, including cancer, have been attributed to defective regulation of β -catenin signaling (385), but β -catenin mutations alone do not appear sufficient for their pathogenesis. The regulation of β -catenin is dynamic via the integration of multiple points of regulation, from cell surface receptors and cytoplasmic modifiers to its nuclear translocation and transcription.

TCF/LEF

The TCF/LEF HMG box-containing family of transcription factors originated from the discovery of T-cell factor 1 (TCF1) and lymphoid enhancer factor (LEF-1) through investigations of the transcriptional regulation of the *CD3E* and *T cell receptor (TCR)* genes, respectively (386-390). TCF family members bind to the DNA consensus sequence YCTTTGWW (391) via their C-terminus and contact β -catenin through their N-terminus (392-394). There are four genes in this family: *LEF1*, *TCF1*, *TCF3*, and *TCF4*. *TCF1* and *LEF1* have overlapping expression patterns throughout development. *Tcf3* is ubiquitously expressed during early murine embryogenesis (E6.5) and gradually declines by day E10. *TCF4* is expressed later during development (E13.5) and is restricted to the

diencephelon, mesencephelon, and the intestinal epithelium (154). Of the three TCF genes, only *TCF1* and *TCF4* are reported to be expressed within differentiating osteoblasts (143, 395).

Tcf1^{-/-} mice develop without any gross anatomical abnormalities other than a severe defect in T lymphogenesis (396), but they have slightly lower BMD at one month of age (143). Although osteoblast numbers were not affected within this model, the low-bone-mass phenotype was attributed to reduced expression of the target gene and osteoclast activating factor, *Opg*. Mice carrying heterozygous mutations in *Ctnnb1* and *Tcf1* also have decreased bone mass with a decreased expression of *Opg*. Through chromatin immunoprecipitation, both Tcf1 and Tcf4 were found to co-occupy with β -catenin on the *Opg* promoter in D10 calvarial osteoblasts, indicating that *Opg* is a direct transcriptional target of β -catenin/TCF activity within the osteoblasts (143).

Lef1^{-/-} mice die perinatally and lack hair follicles, teeth, mammary glands, and portions of the trigeminal nerve (158). Mice lacking both *Tcf1* and *Lef1* die at birth and have an arrest of cell differentiation within α and β T cells at the CD8⁺ single positive stage (397). These mice also have abnormal neural tube formation and defects in differentiation of the paraxial mesoderm, closely resembling the phenotype of Wnt3a-deficient mice (156). Mice heterozygous for an inactivating mutation in *Lef-1* may show a gender and age-dependent role for *Lef-1* during bone mass regulation. Three to four month-old *Lef1*^{+/-} females have reduced bone mass, whereas males do not. This *Lef1* haploinsufficient low-bone-mass female phenotype can be rescued by crossing with *Gsk3 β* ^{+/-} mice. Male *Lef1*^{+/-} mice have normal bone mass, but show a reduction in bone mass upon androgen receptor inactivation (157). Loss of the β -catenin binding domain within *Lef-1* results in a similar overall phenotype, having severely reduced trabecular bone volume not seen within heterozygotes (50).

Mammalian *Tcf1* and *Lef1* genes harbor alternative promoters which produce truncated isoforms lacking the β -catenin binding domains to act as dominant negative co-repressors (398). Additionally, the *Tcf1* gene contains two promoters, four alternative exons, and three splice acceptors within the last exon encoding an extended CTD, yielding up to 96 different mRNA transcripts (399). Although full-length *Lef-1* expression was not detected within differentiating osteoblasts (143), *Lef-1 Δ N* is driven by *Bmp2* within mature osteoblasts to act as a negative regulator of Wnt transcription (400). Overexpression of *Lef1* inhibited osteoblast differentiation (401), yet expression of *Lef-1 Δ N* allowed maturation

(402). This dominant negative form of Lef-1 is known to inhibit β -catenin transcription (398, 403). Lef-1 Δ N retains low affinity to β -catenin through a second domain, and expression within mature osteoblasts (2.3Col1a1) results in slightly higher bone formation rates, trabecular bone, and an increase in serum osteocalcin (159). Future work is needed to tease out the temporal expression of different Tcf/Lef transcription factors in order to determine their roles in gene expression within osteoblasts during the formation of bone and how other “dominant negative” transcription factors such as *dnTcf1* regulate bone formation.

Other components of the nuclear β -catenin transcriptional complex

In the absence of nuclear β -catenin, Tcf/Lef proteins bind the transcriptional repressor Groucho/TLE on Wnt responsive elements (WREs) in order to facilitate HDAC-mediated repression of Wnt target genes (404-406). Upon entry of β -catenin to the nucleus, it binds to Lef or Tcf through its central armadillo repeats and initiates the formation of nuclear transcription complexes tethered to WREs via DNA-bound Tcf/Lef proteins. The N-terminus of β -catenin binds the transcriptional co-activators BCL-9 and pygopus, which associate with activated chromatin (marked by methylation of H3 [H3K4me]) to further decode histone tails via its PHD fingers and facilitate Wnt-induced transcription (407, 408). The C-terminal domain (CTD) of β -catenin binds several chromatin remodeling complexes, such as MLL1/2, TIP60, and CBP/p300. In particular, a core member of the polymerase associated factor (PAF) complex, parafibromin (CDC73), binds to the β -catenin CTD in order to recruit the PAF complex and RNA polymerase II (Pol II) to WREs (409-411). Binding of β -catenin/parafibromin may be mediated by protein tyrosine phosphatase, PTPN11 (Shp2), which dephosphorylates parafibromin and thus allows it to bind β -catenin, recruit the PAF complex, and activate transcription (13). Interestingly, loss-of-function mutations within the coding sequence of parafibromin lead to parathyroid adenomas and ossifying fibromas of the jaw (412-414). Further work using cell-specific knock-outs are needed to help determine how these regulators of β -catenin/TCF-mediated transcription govern bone formation.

β -catenin target genes

Ultimately, activation of the Wnt pathway leads to the expression of distinct sets of genes that drive progenitor cell proliferation, differentiation, or survival. Several Wnt/ β -catenin target genes have been identified and validated; they are enumerated on the Wnt home page

(<http://www.stanford.edu/group/nusselab/cgi-bin/wnt/>). However, validating Wnt target genes has proven difficult due to the diverse set of cell types, states, and stimuli that may differentially activate the transcription of specific target genes through different Tcf/Lef transcription factors. For example, *Lef1* expression is mainly restricted to developing lymphoid tissues and absent in osteoblasts, where *Tcf1* and *Tcf4* are expressed within differentiating osteoblasts. Within the pluripotent mesenchymal cell line C3H10T1/2, Wnt3a treatment up-regulates genes involved in adipocyte and osteoblast differentiation. An Affymetrix oligonucleotide genome-wide expression analysis performed on these cells identified 447 Wnt3a-regulated genes (415). Several of these are genes involved in the regulation of the Wnt pathway, such as an up-regulation of *Axin2* and *Fzd7*, or a down-regulation of *Tcf3*, *Sfrp2*, and *Fzd4*. Wnt3a induces expression of 13 osteoblast-regulated genes including *Bmp4*, *Opg*, *Col1a1*, *Hspg2*, and the transcription factors *Atf4* and *Twist*, but fails to elevate *Runx2* expression (415). From gene expression profiling studies and the evaluation of osteoblast-specific knockouts of β -catenin, it is clear that Wnt target genes are required for osteochondral fate decisions and the expression of lineage-specific transcription factors such as *Atf4* and *Twist*. Yet, down-regulation of Wnt signaling is required for osteoblast maturation, mineralization, and regulation of osteoclastogenesis. Further work is needed to identify the temporal expression of lineage-specific Wnt target genes to better understand the role of Wnt signaling in bone formation.

Summary

The demonstration that alterations in components of the Wnt/ β -catenin signaling pathway were causally associated with dramatic changes in bone mass in humans has stimulated extensive interest in developing methods to activate this pathway to treat bone diseases such as osteoporosis (reviewed in (253)). Several therapeutics based on the concept that activating this pathway can increase bone mass are currently in clinical development and preliminary results appear promising (253). Pharmaceutical companies are also evaluating therapies to inhibit Wnt signaling to treat several tumor types in which activation of the pathway is associated with tumorigenesis (for examples, see (416, 417)). In this context, it will be important to carefully monitor patients for deleterious effects on bone health. Overall, the possibility that millions of patients may be treated with compounds designed to modulate this pathway makes a continued emphasis on understanding the precise

mechanisms associated with Wnt-mediated control of normal bone health a critical research focus.

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