Supporting Information

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DN A S



Fig. S1. (Continued)



Fig. S1. (Continued)

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DNAS



Fig. S1. Expression patterns of Wnts in neonatal trabecular bone (A), cortical bone (B), and perichondrium (C). (Scale bars: 20 µm.)



DapB DapB DapB (white) DAPI

Fig. S2. Negative controls for multiplex fluorescent ISH. Negative controls in the green, red and white channels using bacterial gene DapB in the cortical bone (*A*), trabecular bone (*B*), and perichondrium (*C*). The autofluorescence is because of erythrocytes and cell cytoplasm. CB, cortical bone; GP, growth plate; M, marrow; Pc, perichondrium; TB, trabecular bone. (Scale bars: 10 μm.)



Fig. S3. Initial AXCT2-labeled population does not contain Osteocalcin-expressing mature osteoblasts or osteocytes. Ocn-expressing mature osteoblasts (red) and osteocytes (embedded in the bone) are not labeled by AXCT2;Ai6-labeled cells (green) after 48 h of trace. Representative images from the trabecular bone (*A* and *B*), cortical bone (*C*), and perichondrium (*D*) are shown. The perichondrium does not contain osteocytes or Ocn-expressing osteoblasts. The antibody against Ocn routinely stains for a hematopoietic population (marked by asterisks), as suggested by their rounded morphology. CB, cortical bone; GP, growth plate; M, marrow; Pc, perichondrium; Ps, periosteum; TB, trabecular bone. (Scale bars: 25 μm.)

DNA C



Fig. 54. Progeny of AXCT2-labeled cells are ubiquitously found throughout the 1-y traced femur, and do not display regional biases. (*A*) Montage of a whole femur from a 1-y traced AXCT2; Ai6 mouse (*Left*), with magnified views of various parts of the bone (*B–D*). Note the large number of labeled osteocytes and cells lining the endostea throughout the femur. The muscle fibers surrounding the bone also contain ZsGreen-labeled cells. (Magnification: *A*, 4.5×.)



ZsGreen Perilipin DAP

Fig. S5. Some adipocytes are labeled with ZsGreen after 6 mo of trace. After 6 mo of trace, most adipocytes (marked by Perilipin expression in red) do not express ZsGreen (*B*), but some occasional adipocytes that carry the ZsGreen label can still be observed (*A*). (Scale bar: 25 µm.)

DNAS



Fig. S6. Spontaneous labeling is very rare in the bones of AXCT2;Ai6 mice. (*A*) Pulse-chase scheme for corn oil controls. (*B–F*) At all of the time points surveyed, there was a very low level of ectopic labeling in the trabecular bone (*Upper*) and cortical bone (*Lower*) regions. (*F*) Montage of a whole femur 1 y after corn oil injection at P0. Vast majority of the ectopically labeled cells have a dendritic morphology and reside in the marrow (*F*, *Inset*), distinct from osteolineage cells. (Magnification: *F*, *Inset*, 65×.) CO, corn oil; CB, cortical bone; GP, growth plate; M, marrow; Pc, perichondrium; TB, trabecular bone. (Scale bars: 25 μm in *B–E*.)



Fig. 57. Immunostaining against WIs. Control Osx-GFP::Cre;WIs^{fl/+} (*Upper*) and mutant Osx-GFP::Cre;WIs^{fl/+} (OW, *Lower*) samples stained with antibody against Wntless, which appear as punctae. Trabecular bones are outlined with white dotted lines, Osx-GFP⁺ nuclei are outlined in green solid lines. Left and right images within each panel are identical, except that the image on the right lacks GFP staining to better visualize the red punctuate Wntless staining. There are fewer red dots associated with Osterix-GPF⁺ nuclei in the mutant than in the control. Insets show magnified views of boxed regions. M, marrow; TB, trabecular bone. (Scale bars: 25 µm.) (Magnification: *Insets*, 2×.)



Fig. S8. Controls for ISH in OW mutants. (*A*) View of entire bone sections from Osx-GFP::Cre;Wls^{fl/+} control (*Left*) and Osx-GFP::Cre;Wls^{fl/fl} mutant (OW, *Right*) stained with hematoxylin, with the boxed regions shown at higher magnification in the lower panels. Black arrowhead points to constriction. (*B* and *C*) Negative and positive ISH controls for sample integrity using probes fort *DapB* (*Left*) and *Polr2a* (*Center*), respectively. *Axin2* expression in the skin (Fig. 5 *J*, *iii* and *iv*) and prehypertrophic chondrocytes (*Right*) in both control and mutant samples act as internal controls to show that Axin2 expression in nonosseous tissues were not affected in the OW mutants (n = 2 controls and 2 mutants). CB, cortical bone; M, marrow; TB, trabecular bone; aberrant bone is the bone found in constriction region in the mutants. (Scale bars: 200 µm in *A* and 20 µm in *B* and *C*.)

Gene	TB endosteum	TB osteocytes	CB endosteum	CB osteocytes	Perichondrium	Periosteum	Total score
Wnt1	++	_	++	++	+	_	7
Wnt2	+	_	+	_	++	+	5
Wnt2b	++	_	+	+	++	++	8
Wnt3	+	_	+	_	+	+	4
Wnt3a	++	_	++	+	+	+	7
Wnt4	++++	_	+++	_	+++	+	11
Wnt5a	++++	+	+++++	++	++++	+++++	21
Wnt5b	++	++	+++	++++	++	++	15
Wnt6	+	_	+	_	+	_	3
Wnt7a	++	_	+	+	+	+	6
Wnt7b	+	+	+	+	+++	+	8
Wnt8a	+++	++	+	+	+	+	9
Wnt8b	++	_	+	+	+	+	6
Wnt9a	+++	+	++	+	++	+++	12
Wnt9b	+	_	_	+	+	+	4
Wnt10a	+	_	_	+	++	++	6
Wnt10b	++++	++++	+++	+++++	++++	++	22
Wnt11	+++	+	++	+	++	+++	12
Wnt16	+	_	+	_	+++	+++++	10
Axin2	++++	++	++++	++	++++	++++	20

Table S1.	Relative	expression	levels of	Wnts	and	Axin2

The level of gene expression was qualitatively determined by comparing the intensity of ISH signal of each gene across the various anatomical locations as well as with the other genes surveyed (Fig. 1 and Fig. S1). "—" means not expressed; "+" to "+++++" reflect increasing level of gene expression. Total score is the sum of all of the "+"s in all of the regions for that particular gene. CB, cortical bone; TB, trabecular bone.

Neonatal ISH present study		E18.5 and 1-wk-old qPCR, Andrade et al. (1)				Adult RNASeq, Ayturk et al. (2)	
Gene	ISH score	Gene	Pc	CB	Total	Gene	Score
Wnt10b	22	Wnt5a	72.5	22.5	95.0	Wnt10b	8.442
Wnt5a	21	Wnt5b	60.8	33.9	94.7	Wnt4	6.004
Wnt5b	15	Wnt4	14.7	39.2	53.9	Wnt16	4.273
Wnt9a	12	Wnt16	31.6	7.1	38.7	Wnt5b	4.235
Wnt11	12	Wnt10b	20.2	4.0	24.2	Wnt5a	1.535
Wnt4	11	Wnt11	7.5	1.2	8.7	Wnt2b	1.239
Wnt16	10	Wnt9a	2.7	0.3	3.0	Wnt9a	0.840
Wnt8a	9	Wnt1	ND	2.7	2.7	Wnt1	0.666
Wnt2b	8	Wnt2b	0.9	1.0	1.9	Wnt11	0.187
Wnt7b	8	Wnt2	0.58	ND	0.58	Wnt7b	0.170
Wnt1	7	Wnt3	ND	ND	ND	Wnt6	0.129
Wnt3a	7	Wnt3a	ND	ND	ND	Wnt10a	0.092
Wnt7a	6	Wnt6	ND	ND	ND	Wnt2	0.081
Wnt8b	6	Wnt7a	ND	ND	ND	Wnt9b	0.036
Wnt10a	6	Wnt7b	ND	ND	ND	Wnt3a	0.012
Wnt2	5	Wnt8a	ND	ND	ND	Wnt8b	0.011
Wnt3	4	Wnt8b	ND	ND	ND	Wnt8a	0.008
Wnt9b	4	Wnt9b	ND	ND	ND	Wnt3	0.002
Wnt6	3	Wnt10a	ND	ND	ND	Wnt7a	0.000

Table S2.Comparison of relative Wnt expression levels in ISHsurvey with published transcriptome profiling studies

The Wnts are ranked in descending order of expression level in each of the three studies. Left columns, current study; center columns, adapted from Andrade et al. (1); right columns, adapted from Ayturk et al. (2). The seven most highly expressed Wnts from our ISH survey are the top ranked genes in the published studies (in boldface), thus validating our approach. CB, cortical bone; Pc, perichondrium; ND, not determined.

Andrade AC, Nilsson O, Barnes KM, Baron J (2007) Wnt gene expression in the post-natal growth plate: regulation with chondrocyte differentiation. Bone 40(5):1361–1369.
Ayturk UM, et al. (2013) An RNA-seq protocol to identify mRNA expression changes in mouse diaphyseal bone: Applications in mice with bone property altering Lrp5 mutations. J Bone Miner Res 28(10):2081–2093.

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