

Supplementary Information

**Noncanonical Wnt5a enhances Wnt/ $\beta$ -catenin signaling  
during osteoblastogenesis**

Masanori Okamoto<sup>1</sup>, Nobuyuki Udagawa<sup>2</sup>, Shunsuke Uehara<sup>2</sup>, Kazuhiro Maeda<sup>3</sup>, Teruhito Yamashita<sup>4</sup>, Yuko Nakamichi<sup>4</sup>, Hiroyuki Kato<sup>1</sup>, Naoto Saito<sup>5</sup>, Yasuhiro Minami<sup>6</sup>, Naoyuki Takahashi<sup>4</sup>, and \*Yasuhiro Kobayashi<sup>4</sup>

## Methods

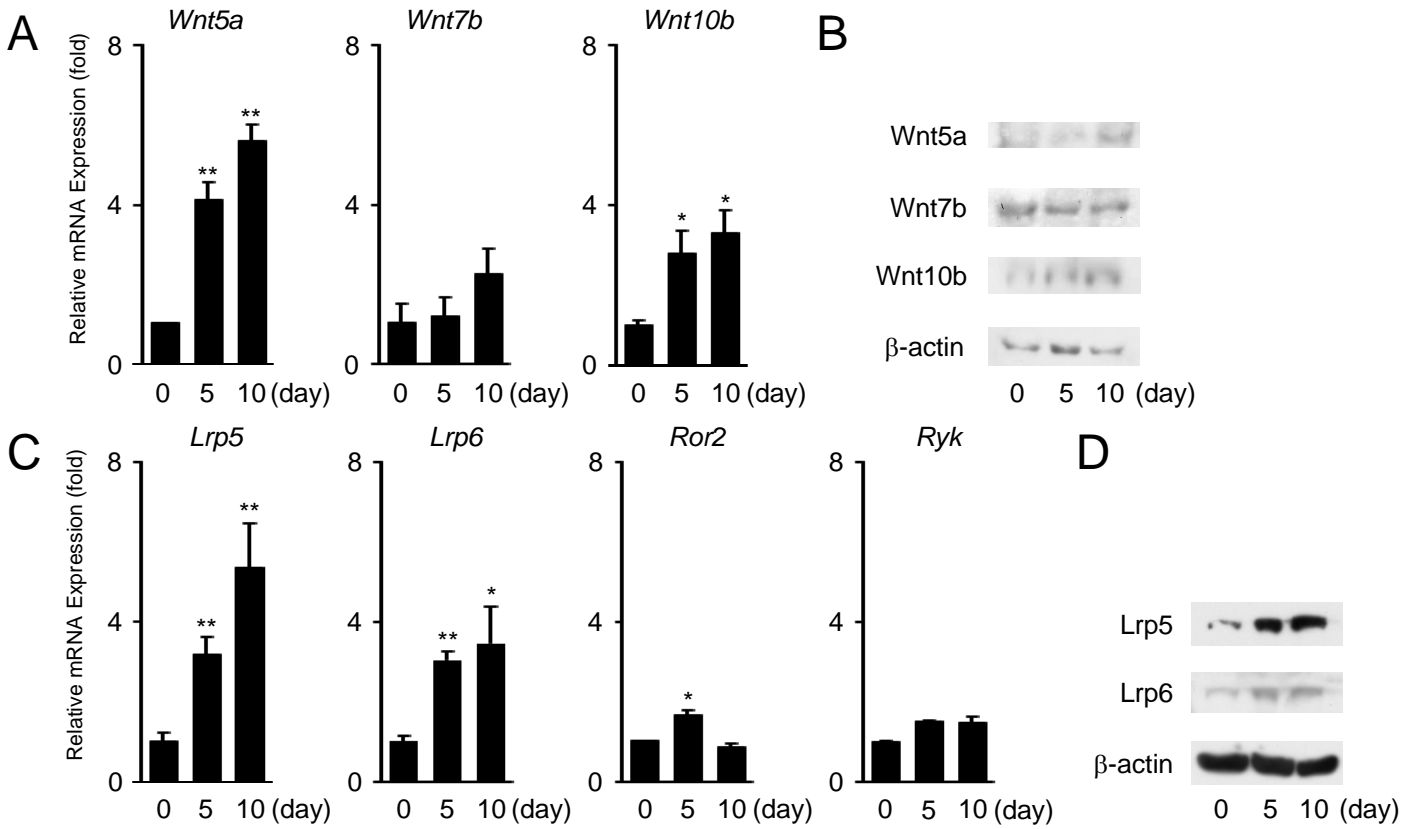
### Luciferase assay

After adenovirus-mediated gene transfer of *Lrp5* or *LacZ* cDNA, calvarial cells were cultured under osteogenic conditions for 10 days. Cells were harvested with the trypsin-EDTA treatment, and Super TOPFlash reporter vectors<sup>1</sup> with 8 x Tcf/Lef binding sites (5 mg Addgene, Cambridge, MA) and the pRL-TK Luciferase control reporter vector (0.05 mg, Promega, Madison, WI) were transfected into these cells ( $1 \times 10^6$ ) using a Nucleofector kit and a device (Lonza, Basel, Switzerland) according to the manufacturers' instructions. Two days after transfection, these cells were stimulated with 50 ng/ml recombinant Wnt3a (R&D systems, Minneapolis, MN) for 24 hours. Reporter activity was measured using the Dual-Luciferase Reporter assay reagents (Promega).

## References

1. Veeman, M.T. *et. al.* Zebrafish prickles, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. *Curr. Biol.* **13**, 680-685, (2003).

Supplementary Figure S1



**Supplementary Figure S1. The expression of Wnt ligands (A, B) and co-receptors (C, D) in**

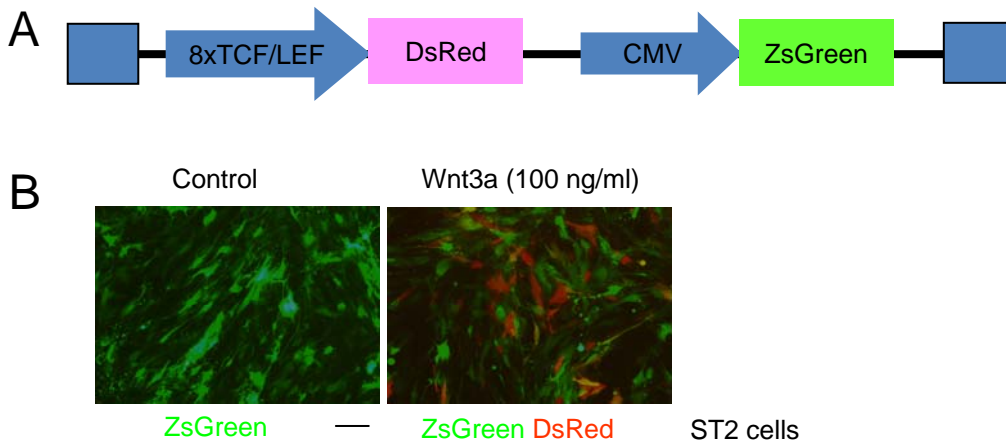
**calvarial cells in osteogenic medium.** Calvarial cells from wild-type mice were cultured in

osteogenic medium and subjected to real-time PCR analysis and Western blot analysis. In (B,D), 40

µg protein of cell lysates was used. In (A, C), data are expressed as the mean  $\pm$  SD ( $n = 3 - 4$  for

each group). \* $p < 0.05$ ; \*\* $p < 0.01$ . All  $p$  values based on the Student's  $t$  test.

Supplementary Figure S2

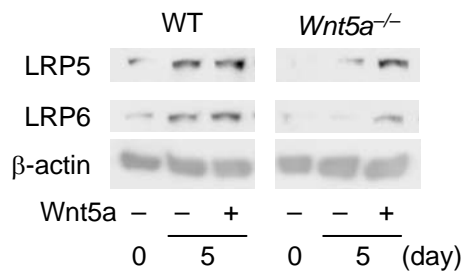


**Supplementary Figure S2.** A) Structure of the 8xTcf/Lef DsRed adenoviral reporter vectors.

B) Photographs of ST2 cells, in which the 8xTcf/Lef DsRed reporter was expressed.

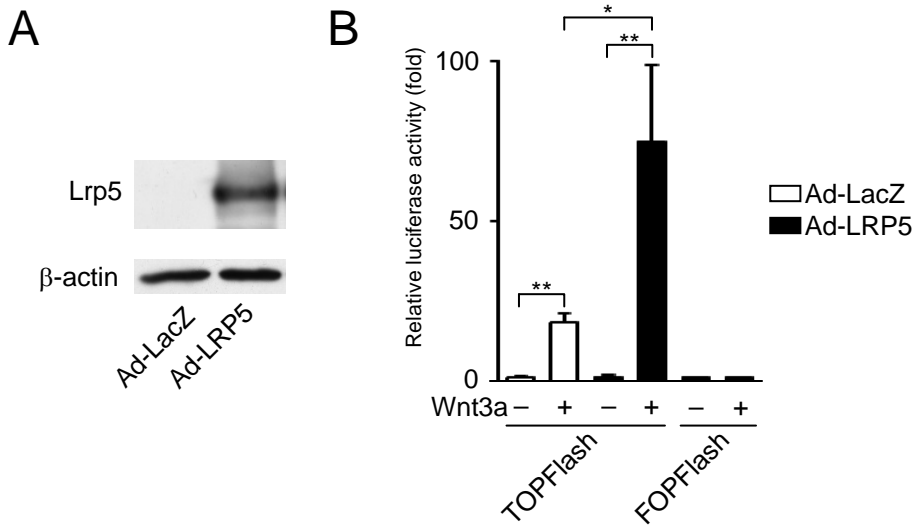
ST2 cells were treated with Wnt3a (100 ng/ml) for 48 hours. Bar, 15  $\mu$ m.

Supplementary Figure S3



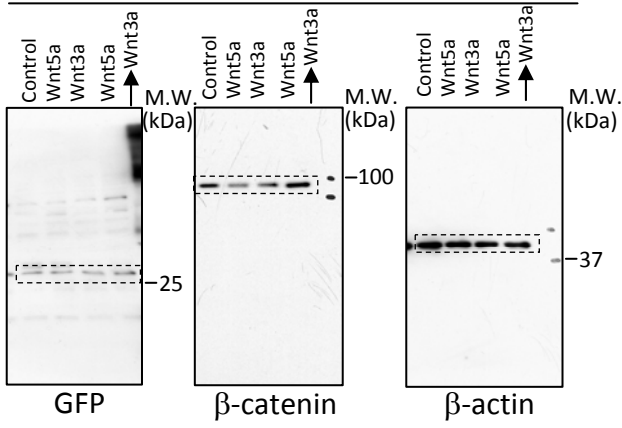
**Supplementary Figure S3. Western blot analysis of Lrp5/6 expression in wild-type (WT) and  $Wnt5a^{-/-}$  calvarial cells.** Cells were cultured in the presence or absence of Wnt5a (500 ng/ml) under osteogenic conditions for 5 days.

Supplementary Figure S4

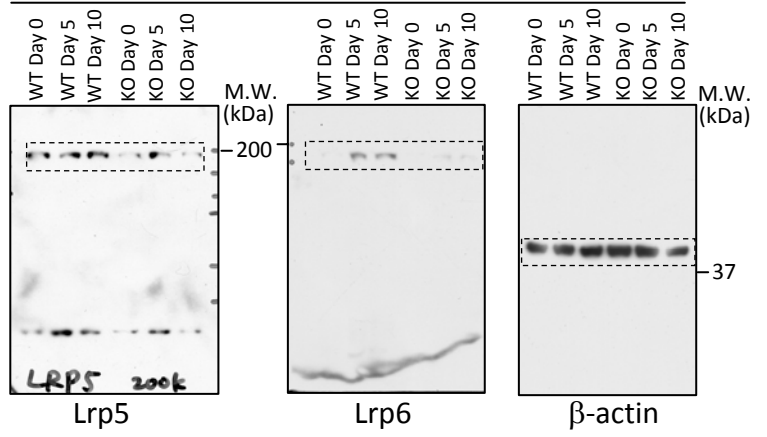


**Supplementary Figure S4.** Western blot analysis of Lrp5 expression (A) and Tcf/lef transcriptional activity (B) in calvarial cells infected with LacZ- and Lrp5-adenovirus. Data are expressed as the mean  $\pm$  SD ( $n = 6$ ). \* $p < 0.05$ ; \*\* $p < 0.01$ . All  $p$  values are based on the Student's  $t$  test.

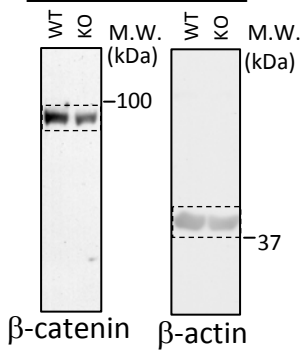
**Fig. 1C**



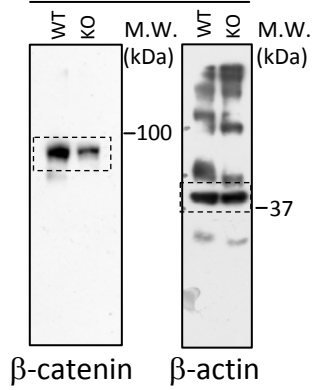
**Fig. 2B**



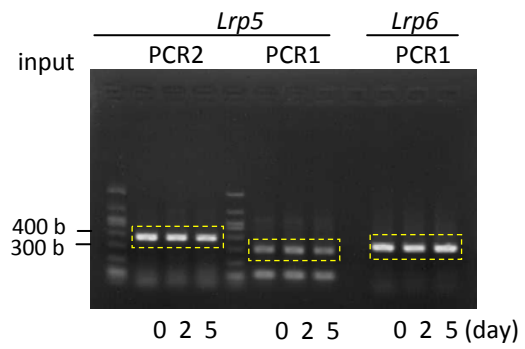
**Fig. 3D**



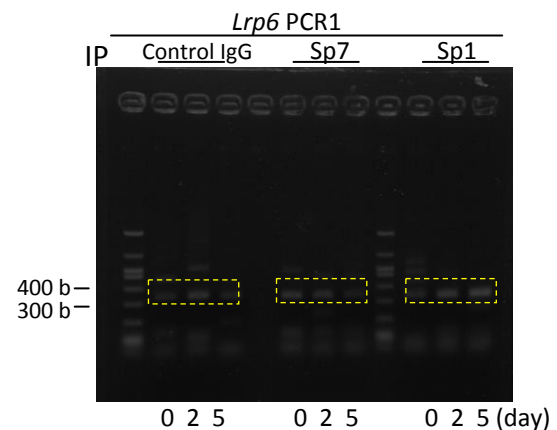
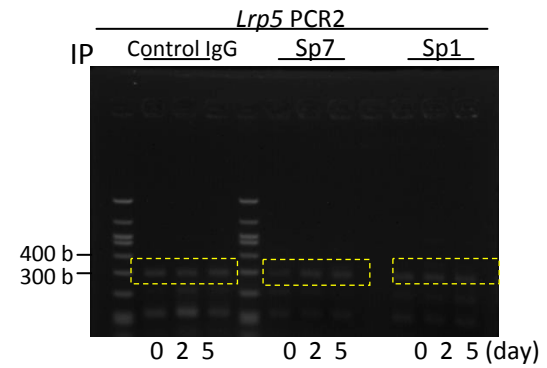
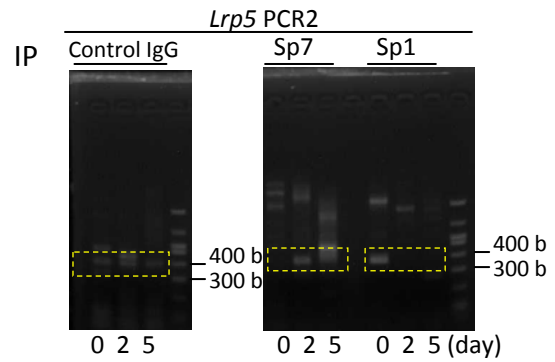
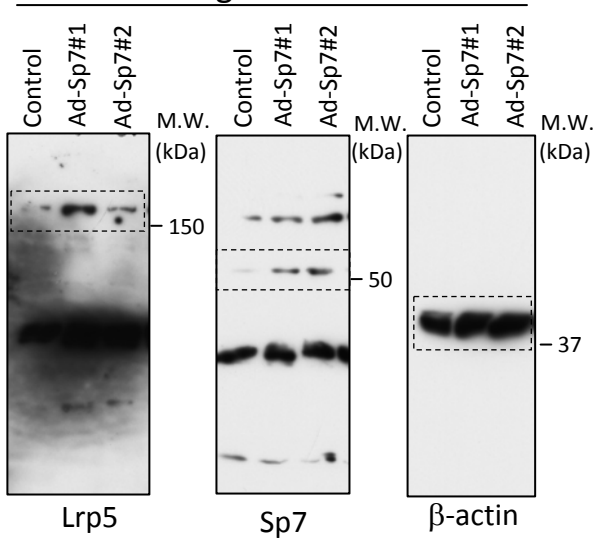
**Fig. 3F**



**Fig. 5A**



**Fig. 5B**



**Supplementary Figure S5. Uncropped images of Western blots and agarose gels. M.W.; molecular weight.**

Supplementary Table S1

Primers for real time PCR

		Forward	Reverse
	GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
	Wnt1	ATAGCCTCCTCCACGAACCT	GGAATTGCCATTTGCACTCT
	Wnt2	GGTCAGCTCTTCATGGTGGT	ATCTCTGTCCAGGGTGTTC
	Wnt2b	TCAACGCTACCCAGACATCA	ACCACTCCTGCTGACGAGAT
	Wnt3	AGGAGTGCCAGCATCAGTTC	ACTTCCAGCCTTCTCCAGGT
	Wnt3a	CTGGCAGCTGTGAAGTGAAG	TGGGTGAGGCCTCGTAGTAG
	Wnt4	CTGGAGAAGTGTGGCTGTGA	CAGCCTCGTTGTTGTGAAGA
	Wnt5a	CAAATAGGCAGCCGAGAGAC	CTCTAGCGTCCACGAACTCC
	Wnt5b	CTGCTTGCGTAATGAGACCA	AAAGCAACACCAGTGGAACC
	Wnt6	TCAGTTCAGTTCGGTTTCC	CATGGAACAGGCTTGAGTGA
	Wnt7a	GGTGCAGCATCATCTGTAA	TCCTTCCCGAAGACAGTACG
	Wnt7b	TTCTGGAGGACCGCATGAA	GGTCCAGCAAGTTTTGGTGGTA
	Wnt8a	AGCACAGAGGCTGAGCTGA	TCTGCTCTCCTCTCCTCCAC
	Wnt8b	CTGCTTGGAATTGCCTCTC	CTGCTTGGAATTGCCTCTC
	Wnt9a	TGCTTTCCTCTACGCCATCT	CCTTGACAAACTTGCTGCTG
	Wnt9b	TGGAGCGCTGTACTTGTGAC	GCACTTGCAAGTTGTTCTCA
	Wnt10a	CATGAGTGCCAGCATCAGTT	ACCGCAAGCCTTCAGTTTAC
	Wnt10b	CGAGAATGCGGATCCACAA	CCGGTTCAGGTTTTCCGTTA
	Wnt11	CAGGATCCCAAGCCAATAAA	GTAGCGGGTCTTGAGGTCAG
	Wnt16	GAGCTGTGCAAGAGGAAACC	TGAATGCTGTCTCCTTGGTG
	Frizzled1	CCCAACTCCCTCCTTGCTAC	CAAACCTTGTCGTTGCACACC
	Frizzled2	ATCTTTCTGTCCGGCTGCTA	GATGGCTAGGCTCTTGCACT
	Frizzled3	TTCCATGTCCGTACCAGGTT	ATGCTGCCGTGAGGTAGTCT
	Frizzled4	GATATCCCGCACATTCTCGT	TGGCACATAAACCGAACAAA
	Frizzled5	CACTCAAGACTCCGGAGAGG	GGTAGCGGCTTGTGGTAGTC
	Frizzled6	GTGCTGCAAGAGTCCTGTGA	CGCTGCTCTTTGGACTTACC
	Frizzled7	AAGACTTGCAAGACGATGCT	TGTATCTCCCACTCGCCTTC
	Frizzled8	ACTACAACCGCACCGACCT	ACAGGCGGAGAGGAATATGA
	Frizzled9	CGATGAACTGACTGGGCTCT	CACCACCAAGGACATGAAGA
	Frizzled10	CAGTCCTGGCAACACGTATG	TTTATGCACAGGCAGCAAAG
	LRP5	AATCAACAAGCCACCCTCTG	GGCTCCACCAACATACTCGT
	LRP6	ACCCTGCAGCACTGATGTCT	GTTCTCCTCCGCTGACAAGT
	Ror2	CCCAACTTCTACCCAGTCCA	TGTCCGCCACAGATGTATTG
	Ryk	GGCATGCAAAGTCCAAGG	CATGCCCATAGCCACAAAGT
MA024599	Alpl	ACACCTTGACTGTGGTTACTGCTGA	CCTTGTAGCCAGGCCCGTTA
MA081113	Col1a1	CCTGGCAAAGACGGACTCAAC	GCTGAAGTCATAACCGCCACTG
MA053008	Bglap1	AGCAGCTTGGCCCAGACCTA	TAGCGCCGAGTCTGTTCACTAC
MA073758	Runx2	GACGTGCCAGGCGTATTTC	AAGGTGGCTGGGTAGTGCATTC
MA079534	Sp7	CGCATCTGAAAGCCCCTTG	CAGCTCGTCAGAGCGAGTGAA
MA114855	Axin2	ATGTCCTGTCTGCCAGCGTTC	CAAGCACTAGCCAGTGGGTCAA