

Supplementary materials

R-spondin 3 deletion favors Erk phosphorylation to enhance Wnt signaling and bone formation.

Kenichi Nagano¹, Kei Yamana¹, Hiroaki Saito¹, Riku Kiviranta¹, Ana Clara Pedroni¹, Dhairya Raval¹, Christof Niehrs^{2,3}, Francesca Gori^{1*} and Roland Baron^{1,4*}.

¹Division of Bone and Mineral Research, Harvard School of Dental Medicine and Harvard Medical School, ²Division of Molecular Embryology, DKFZ-ZMBH Alliance, Deutsches Krebsforschungszentrum (DKFZ), 69120, Heidelberg, Germany, ³ Institute of Molecular Biology (IMB), 55128, Mainz, Germany, ⁴Endocrine Unit, Massachusetts General Hospital.

*Co-senior Authors

Address correspondence to:

Francesca Gori

Division of Bone and Mineral Research

Department of Oral Medicine, Infection and Immunity

Harvard School of Dental Medicine

Boston, MA 02115

Tel: (617) 432-1940

Fax: (617) 432-1897

Email: francesca_gori@hsdm.harvard.edu

And

Roland Baron

Division of Bone and Mineral Research

Department of Oral Medicine, Infection and Immunity

Harvard School of Dental Medicine

Boston, MA 02115

Tel: (617) 432-7320

Fax: (617) 432-1897

Email: roland_baron@hsdm.harvard.edu

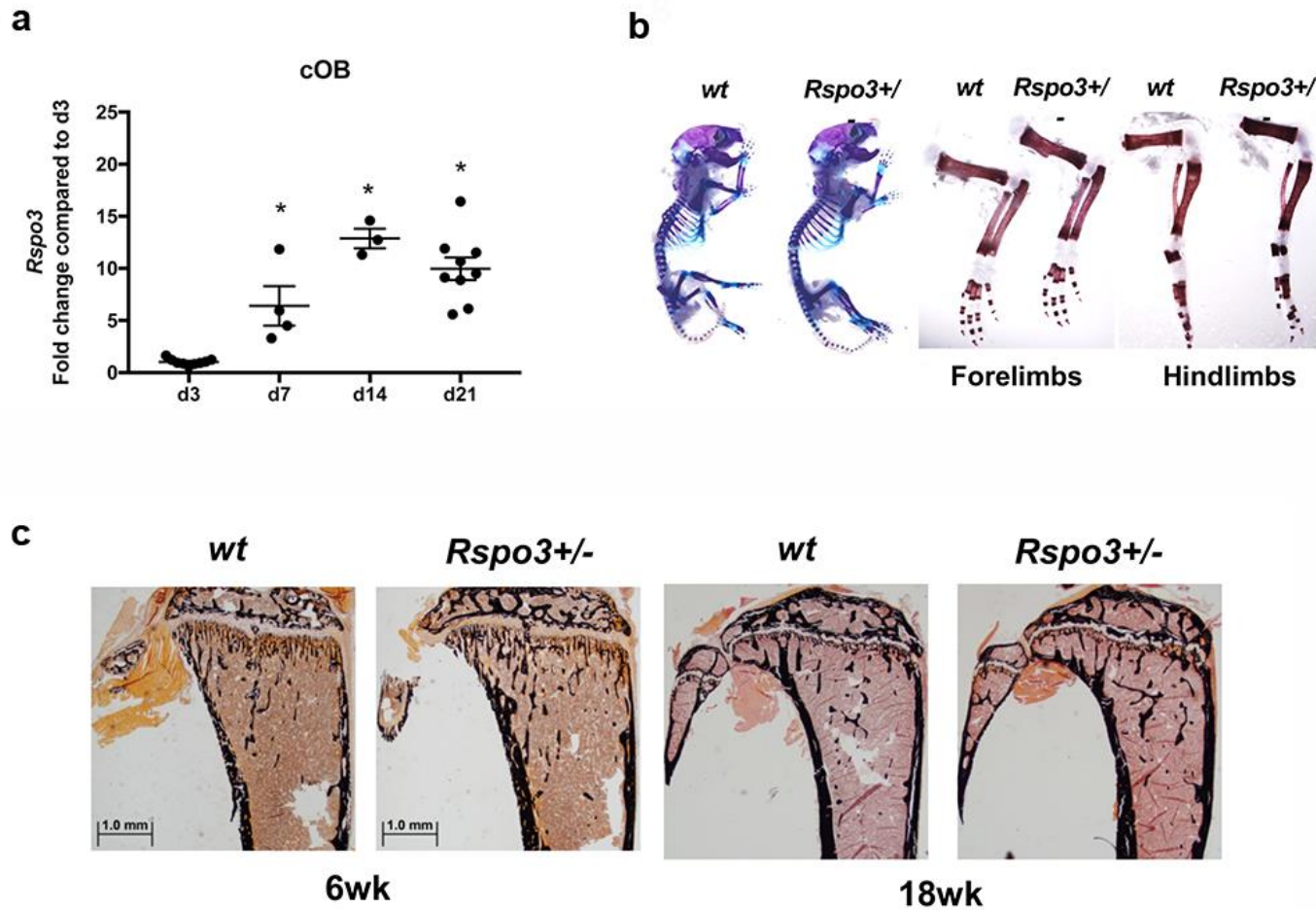


Figure S1. Skeletal phenotype of mice with *Rspo3* haploinsufficiency. a) *Rspo3* expression in calvarial OB (n=4-9). Data show all samples and the mean $\pm$ SEM (n=3-9) *p<0.05 by Student T test. b) Representative skeletal preparations of wt and *Rspo3*^{+/-} newborn (n=3-4). c) Representative images of Von Kossa staining of 6 and 18-wk old wt and *Rspo3*^{+/-} males.

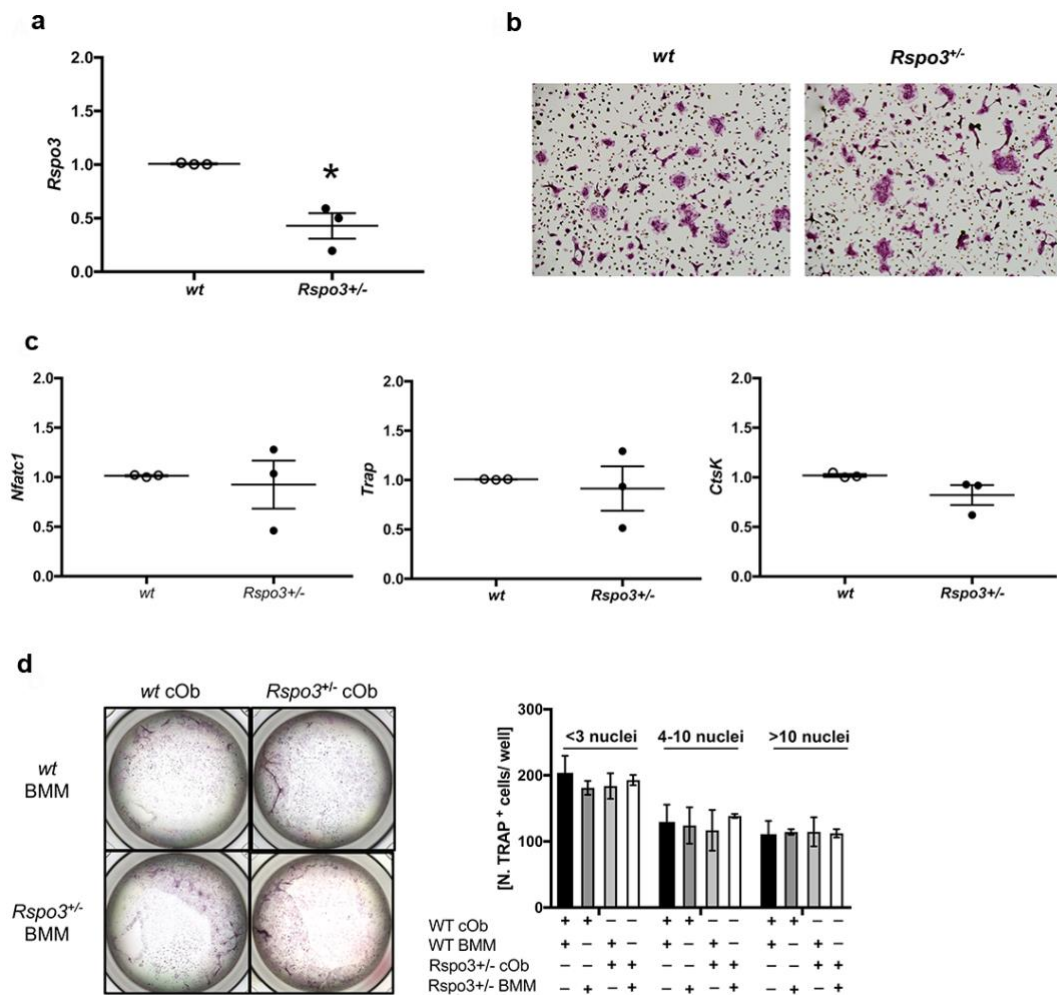


Figure S2. *Rspo3* haploinsufficiency does not affect osteoclastogenesis. a) *Rspo3* expression in BMM-derived osteoclasts. b) Representative images of TRAP staining of *wt* and *Rspo3*^{+/-} BMM-derived osteoclasts. c) *Nfatc1*, *Trap* and *Ctsk* expression in *wt* and *Rspo3*^{+/-} BMM-derived osteoclasts. d) Representative mix-matched experiment and quantification of TRAP⁺ BMM-derived osteoclast. Data show all samples and the mean $\pm$ SEM (n=3) *p<0.05 by Student T test.

Table S1. Histomorphometric analysis of *Rspo3*^{+/-} and *wt* males.

Parameters	6 wk		12 wk		18 wk		Two Way ANOVA		
	<i>wt</i> (n=8)	<i>Rspo3</i> ^{+/-} (n=7)	<i>wt</i> (n=8)	<i>Rspo3</i> ^{+/-} (n=9)	<i>wt</i> (n=8)	<i>Rspo3</i> ^{+/-} (n=6)	Genotype	Age	Interaction
BV/TV (%)	10.9±1.50	10.4±1.41	9.18±1.10	14.3±1.23**	8.18±0.56	10.7±1.19	0.0204	NS	NS
Tb.Th (μm)	30.9±1.94	29.0±1.74	34.7±2.01	41.7±1.96*	34.2±1.17	39.4±2.02*	0.0248	<0.0001	0.046
Tb.N (/mm)	3.45±0.30	3.51±0.30	2.61±0.19	3.41±0.22*	2.38±0.12	2.68±0.22	0.0476	0.0014	NS
Tb.Sp (μm)	273±27.1	270±29.2	365±32.1	265±24.8**	394±19.4	348±30.9	0.033	0.005	NS
MAR (μm/day)	1.58±0.14	2.01±0.13**	1.23±0.07	1.54±0.07*	1.07±0.05	1.21±0.06	0.001	<0.0001	NS
MS/BS (%)	23.3±1.17	20.7±2.6	45.3±3.60	43.0±2.65	26.1±2.58	29.4±2.30	NS	<0.0001	NS
BFR/BS (μm ³ / μm ² /year)	136.3±16.5	151±20.4	201±13.9	242±22.1	103±14.0	130±12.5**	0.029	<0.0001	NS
N.Ob/B.Pm (/mm)	4.55±0.37	5.17±0.21	4.94±0.61	7.39±0.97**	4.05±0.48	6.93±0.62**	0.0006	NS	NS
Ob.S/B.Pm (%)	7.00±0.57	8.80±0.38	6.39±0.92	10.4±1.58**	6.30±0.74	11.2±0.93**	0.0001	NS	NS
OS/BS (%)	2.87±0.38	3.18±0.29	4.62±1.10	6.81±1.02*	4.16±0.62	6.42±0.54	0.0186	0.0033	NS
O.Th (μm)	2.91±0.23	3.61±0.47	3.09±0.22	4.04±0.23*	3.65±0.40	4.44±0.29	0.0033	NS	NS
N.Oc/B.Pm (/mm)	0.95±0.08	0.95±0.15	2.29±0.11	2.12±0.26	1.33±0.07	1.39±0.15	NS	<0.0001	NS
Oc.S/B.Pm (%)	2.95±0.40	2.63±0.32	5.87±0.24	5.84±0.56	3.83±0.25	4.17±0.42	NS	<0.0001	NS
ES/BS (%)	4.27±0.52	4.10±0.53	1.61±0.33	1.65±0.26	4.38±0.27	4.93±0.35	NS	<0.0001	NS

Data are expressed as Mean±SEM. Two Way ANOVA followed by Fisher's LSD post-hoc test

*=p<0.05, **=p<0.005 compared to age-matched *wt* males.

Table S2. Histomorphometric analysis of the tibia midshaft in 12 wk-old *wt* and *Rspo3^{+/-}* males.

Parameters	<i>wt</i> (n=5)		<i>Rspo3^{+/-}</i> (n=5)		P value
	Mean	SEM	Mean	SEM	
Ct.Ar[mm ²]	0.964	0.082	1.025	0.085	0.619
Ma.Ar[mm ²]	1.181	0.085	1.087	0.070	0.418
Tt.Ar[mm ²]	2.145	0.149	2.112	0.142	0.875
Ct.BV/TV (%)	44.85	1.804	48.39	1.517	0.171
Ct.Th (mm)	0.203	0.012	0.214	0.013	0.551

Table S3. Histomorphometric analysis of the tibia midshaft in 12 wk-old *wt* and *Rspo3*^{+/-} females.

Parameters	<i>wt</i> (n=4)		<i>Rspo3</i> ^{+/-} (n=4)		P value
	Mean	SEM	Mean	SEM	
Ct.Ar[mm ²]	0.369	0.019	0.404	0.023	0.293
Ma.Ar[mm ²]	0.614	0.052	0.648	0.052	0.664
Tt.Ar[mm ²]	0.983	0.048	1.052	0.075	0.469
Ct.BV/TV (%)	37.85	2.734	38.53	0.588	0.171
Ct.Th (mm)	0.199	0.011	0.212	0.010	0.426

Table S4. Histomorphometric analysis of 8 wk-old *Rspo3^{fl}* and *Rspo3^{OB}* mice.

Parameters	Male		Female	
	<i>Rspo3^{fl}</i> (n=10)	<i>Rspo3^{OB}</i> (n=10)	<i>Rspo3^{fl}</i> (n=7)	<i>Rspo3^{OB}</i> (n=9)
BV/TV (%)	6.18±0.37	10.5±0.74**	4.59±0.52	6.61±0.52*
Tb.Th (μm)	21.0.4±0.61	25.3±1.16**	19.8±0.05	21±0.6
Tb.N (/mm)	2.94±0.18	4.15±0.18**	2.32±0.27	3.11±0.17*
Tb.Sp (μm)	333.4±22	220±10.5**	451±56.5	311±21*
MAR (μm/day)	1.03±0.05	1.7±0.07**	1.32±0.11	1.73±0.05**
MS/BS (%)	31.2±1.16	40.4±2.02**	35.2±3.24	39.0±1.38
BFR/BS (μm ³ / μm ² /year)	118.3±9.08	251±17.3**	167±16.4	246±11.66**
N.Ob/B.Pm (/mm)	8.71±1.01	15.8±2.16*	8.71±1.51	13±0.1.06*
Ob.S/B.Pm (%)	7.50±0.94	14.2±2.44*	8.33±1.59	12.7±1.05*
OS/BS (%)	4.42±0.73	10.2±1.88**	8.33±1.29	11.9±1.52
O.Th (μm)	1.99±0.18	2.72±0.20*	1.99±0.19	2.29±0.10
N.Oc/B.Pm (/mm)	3.45±0.27	2.8±0.27	3.83±0.31	3.22±0.20
Oc.S/B.Pm (%)	6.54±0.41	5.46±0.39	7.83±0.46	6.39±0.43*
ES/BS (%)	1.67±0.23	1.53±0.41	2.15±0.31	1.92±0.32

Data are expressed as Mean±SEM. *=p<0.05, **=p<0.01 by Student's T-Test compared to age-matched *Rspo3^{fl}* mice.

Table S5. Histomorphometric analysis of 6 wk-old *Control*, *Rspo3^{+/-}*, *TgDkk1* and *Rspo3^{+/-};Dkk1-Tg* females.

Parameter	<i>Control</i> (n=5)	<i>Rspo3^{+/-}</i> (n=5)	<i>Dkk1-Tg</i> (n=5)	<i>Rspo3^{+/-};Dkk1-Tg</i> (n=6)	Two Way ANOVA		
					<i>Rspo3</i>	<i>Dkk1</i>	Interaction
BV/TV (%)	5.02±0.57	7.83±0.75 ^{ac}	2.67±0.44 ^{ab}	4.64±0.5 ^{bc}	0.0008	0.0002	NS
Tb.Th (μm)	29.0±1.65	32.2±2.07	28.8±2.34	32.6±2.47	NS	NS	NS
Tb.N (/mm)	1.71±0.11	2.4±0.01 ^{ac}	0.9±0.1 ^{ab}	1.43±0.12 ^{bc}	<0.0001	<0.0001	NS
Tb.Sp (μm)	586.1±30.9	390.4±17.9 ^c	1202±154 ^{ab}	701.7±61.1 ^{bc}	<0.0001	<0.0001	0.0082
MAR (μm/day)	1.63±0.2	2.12±0.27	1.12±0.13 ^b	1.97±0.18 ^c	0.007	NS	NS
MS/BS (%)	35.19±2.06	37.7±2.37	30.8±4.8	42.9±2.04 ^c	0.0194	NS	NS
BFR/BS(μm³/μm²/year)	212.8±34	296±45.8 ^c	127.8±27.2 ^b	307.4±38.3 ^c	0.0064	NS	NS
Ob.S/B.Pm (%)	18.75±1.63	25.5±1.1 ^{ac}	8.9±1.16 ^{ab}	20.0±2.35 ^{bc}	<0.0001	0.0004	NS
N.Ob/B.Pm (/mm)	14.6±0.92	19.3±0.41 ^{ac}	7.2±0.82 ^{ab}	15.2±1.65 ^{bc}	<0.0001	0.0001	NS
OS/BS (%)	13.05±1.53	16.9±1.84 ^c	7.32±1.9 ^b	13.6±2.6 ^c	0.0261	0.0435	NS
O.Th (μm)	4.35±0.30	4.80±0.32 ^c	2.68±0.51 ^{ab}	4±0.25 ^c	0.025	0.0025	NS
N.Oc/B.Pm (/mm)	4.22±0.37	4.56±0.27 ^c	6.31±0.46 ^{ab}	5.11±0.38 ^c	NS	0.0064	NS
Oc.S/B.Pm (%)	12.58±0.6	12.77±0.51 ^c	16.9±1.18 ^{ab}	14.1±1.31 ^c	NS	0.0087	NS
ES/BS (%)	4.51±0.7	4.46±0.47	5.14±0.48	5.8±0.74	NS	NS	NS

Data are expressed as Mean±SEM. Two-Way ANOVA followed by Fisher's LSD post-hoc test.

a=p<0.05 compared to *control* mice, b= p<0.05 compared to *Rspo3^{+/-}* mice, c= p<0.05 compared to *Dkk1-Tg* mice.