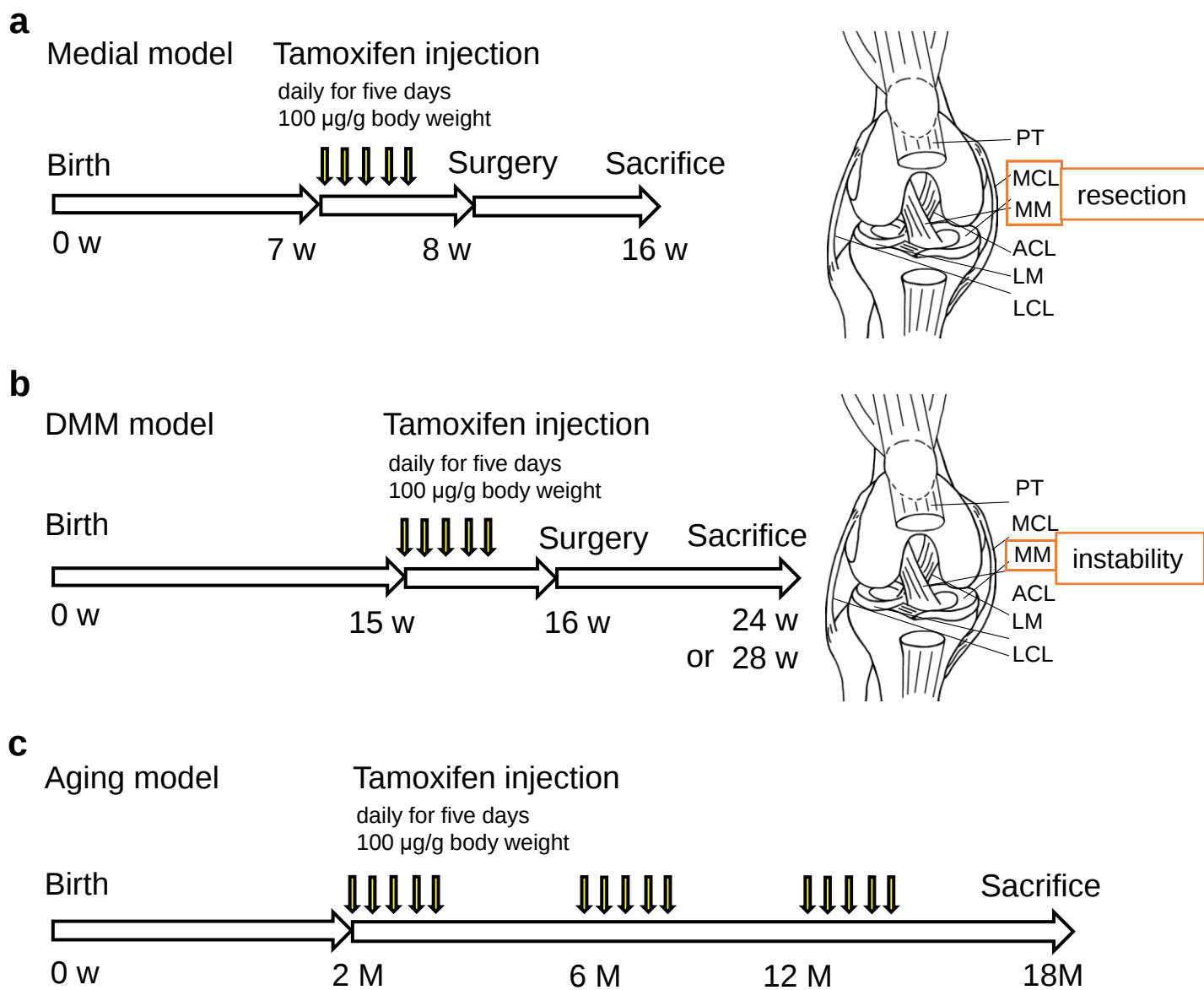
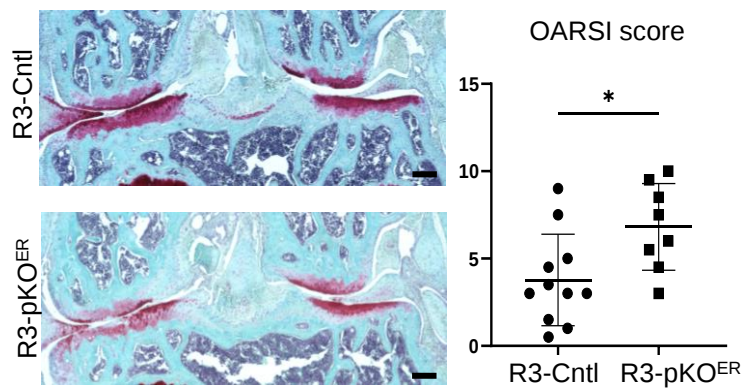


Runx2 and Runx3 differentially regulate articular chondrocytes during osteoarthritis development

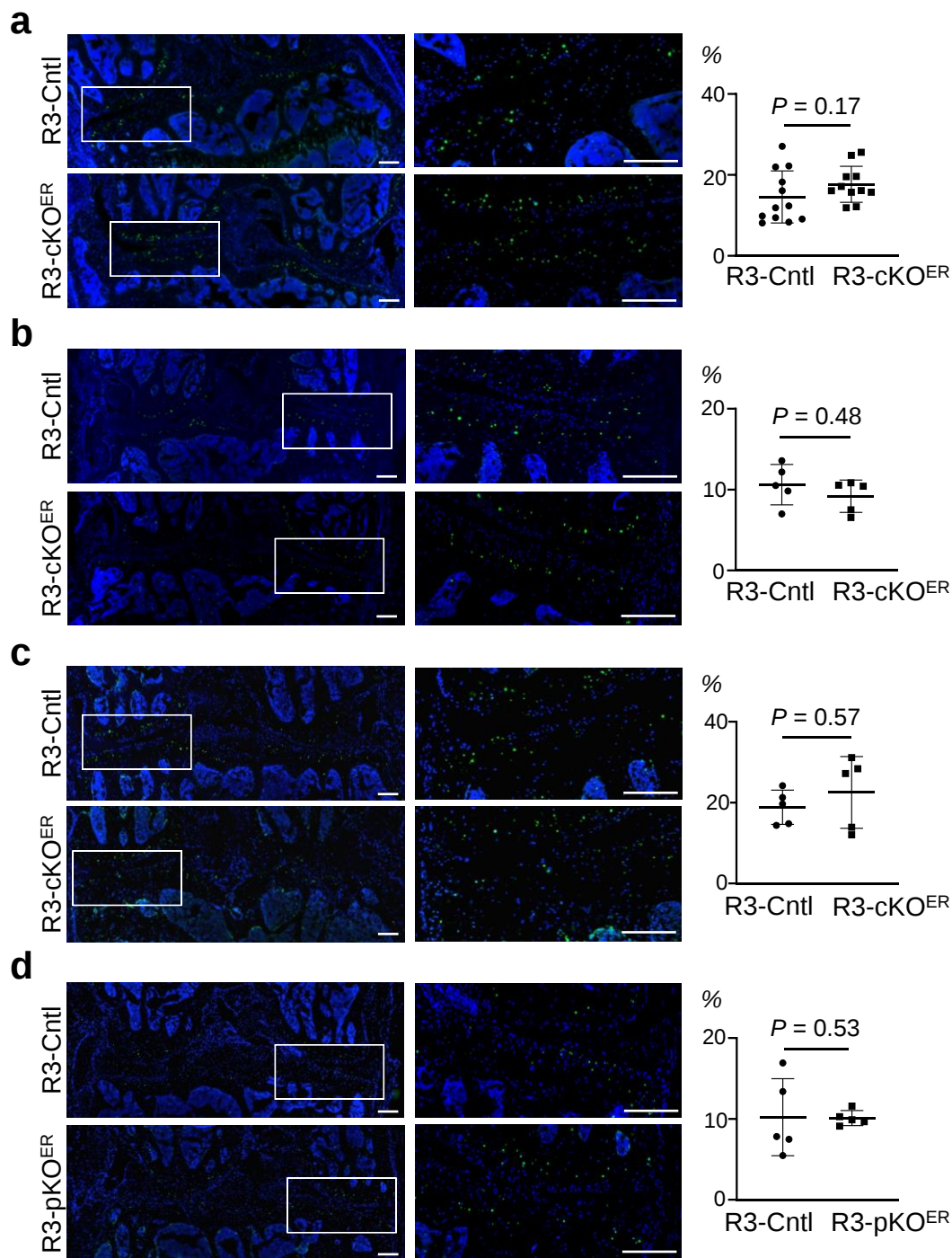
Supplementary information



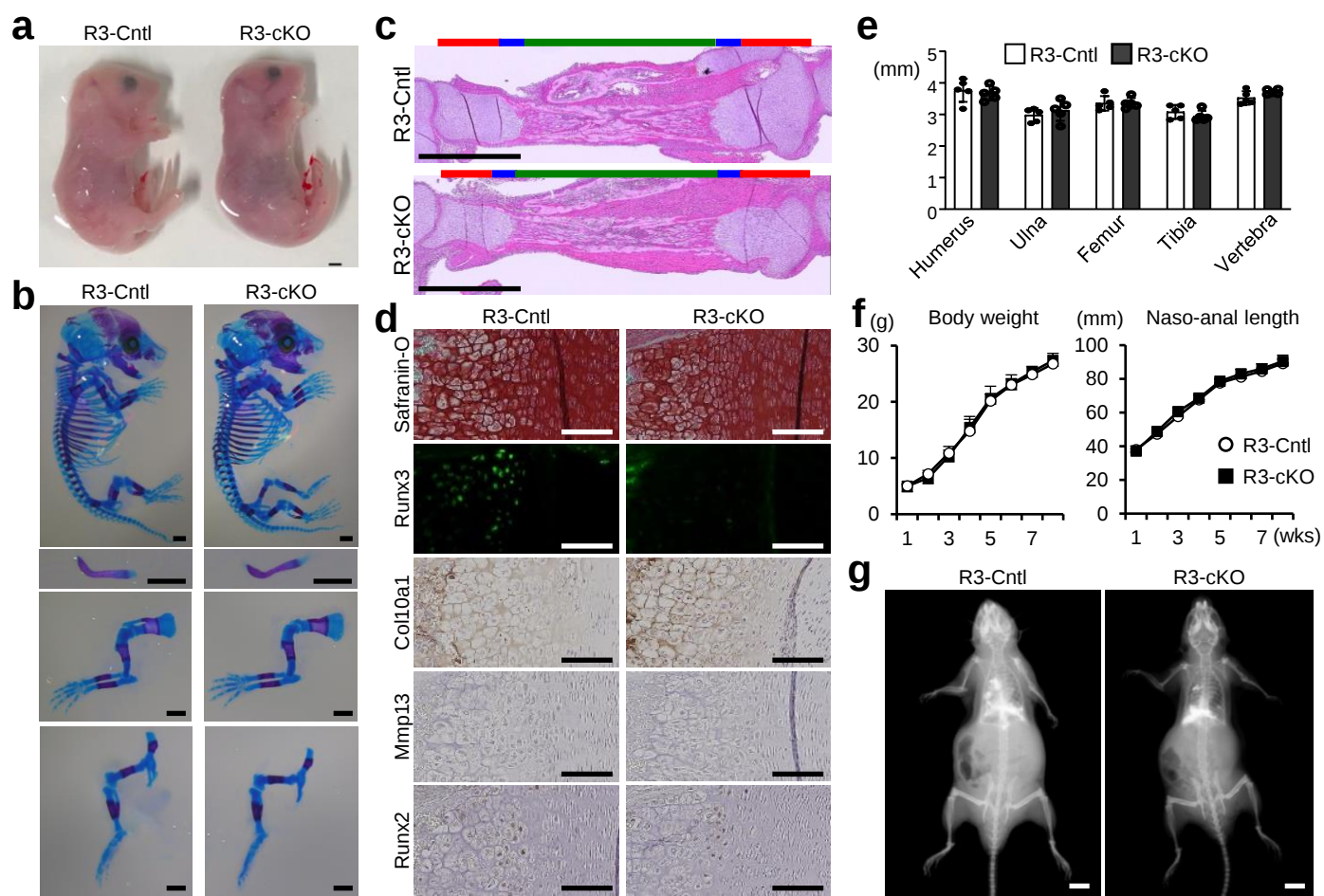
Supplementary Fig. 1 Schematic time course of osteoarthritis (OA) models. **a** Surgical model induced by resecting the medial collateral ligament and medial meniscus (medial model). **b** Surgical model induced by destabilizing the medial meniscus (DMM model). *Runx3* and *Runx2* knockout mice were sacrificed eight weeks and twelve weeks after surgery, respectively. **c** Natural course with aging (aging model). PT, patellar tendon; MCL, medial collateral ligament; MM, medial meniscus; ACL, anterior cruciate ligament; LM, lateral meniscus; LCL, lateral collateral ligament.



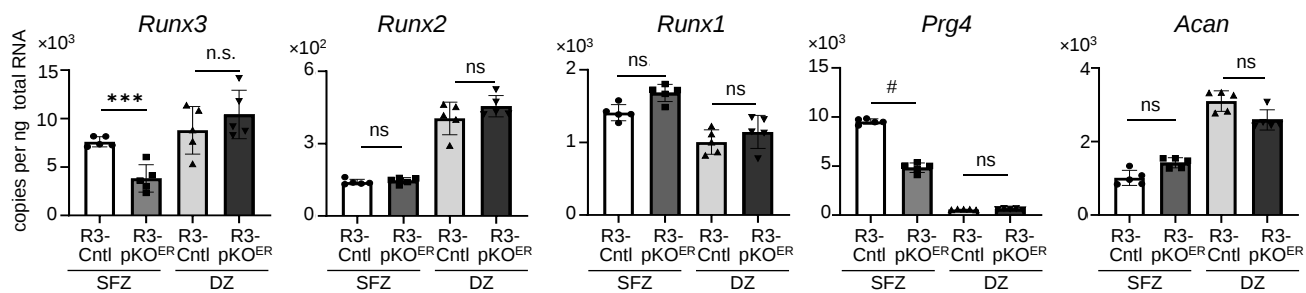
Supplementary Fig. 2 Development of OA in *Runx3^{fl/fl}* (R3-Cntl) and *Prg4-Cre^{ERT2};Runx3^{fl/fl}* (R3-pKO^{ER}) mice by destabilization of the medial meniscus (DMM model) at the age of 16 weeks. Tamoxifen injection was performed one week before surgery. The mice were sacrificed 8 weeks after the surgery. Scale bars indicate 200 μ m. Experimental groups consisted of $n = 11$ and 8, respectively. OARSI scores are expressed as dot plots and means $\pm$ standard deviation (S.D.). * $P < 0.05$, two-tailed Mann-Whitney U test.



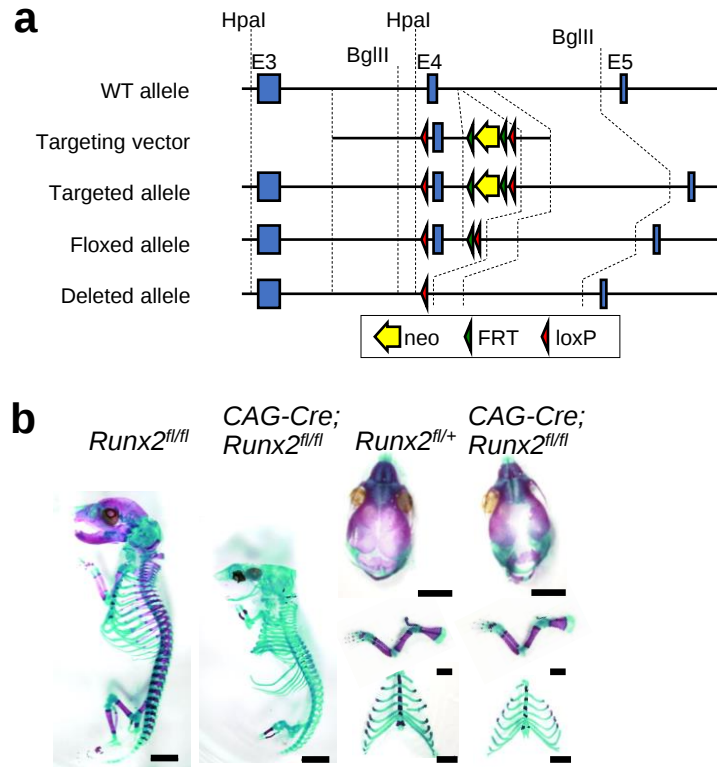
Supplementary Fig. 3 Chondrocyte apoptosis in articular cartilage of *Runx3^{fl/fl}* (R3-Cntl) and *Col2a1-Cre^{ERT2};Runx3^{fl/fl}* (R3-cKO^{ER}) or *Prg4-Cre^{ERT2};Runx3^{fl/fl}* (R3-pKO^{ER}) mice. TUNEL staining of operated (**a**) and sham-operated (**b**) knee joints of 16-week-old R3-Cntl and R3-cKO^{ER} mice. Tamoxifen was injected at seven weeks, and the medial model surgery was performed at eight weeks. **c** TUNEL staining of knee joints of 18-month-old R3-Cntl and R3-cKO^{ER} mice, into which Tamoxifen was injected at 8 weeks, 6, and 12 months. **d** TUNEL staining of sham-operated knee joints of 16-week-old R3-Cntl and R3-pKO^{ER} mice, into which Tamoxifen was injected at seven weeks. Inset boxes in the low magnification images indicate regions of the high magnification images. Scale bars, 200 μ m. Experimental groups consisted of $n = 12$ vs. 11 (**a**), or 5 (**b**, **c**, **d**), respectively. Rates of TUNEL-positive cells in the Safranin O-positive area are shown in the right panels. All data are expressed as dot plots and means $\pm$ S.D. All P values were over 0.05 in two-tailed Mann-Whitney U test.



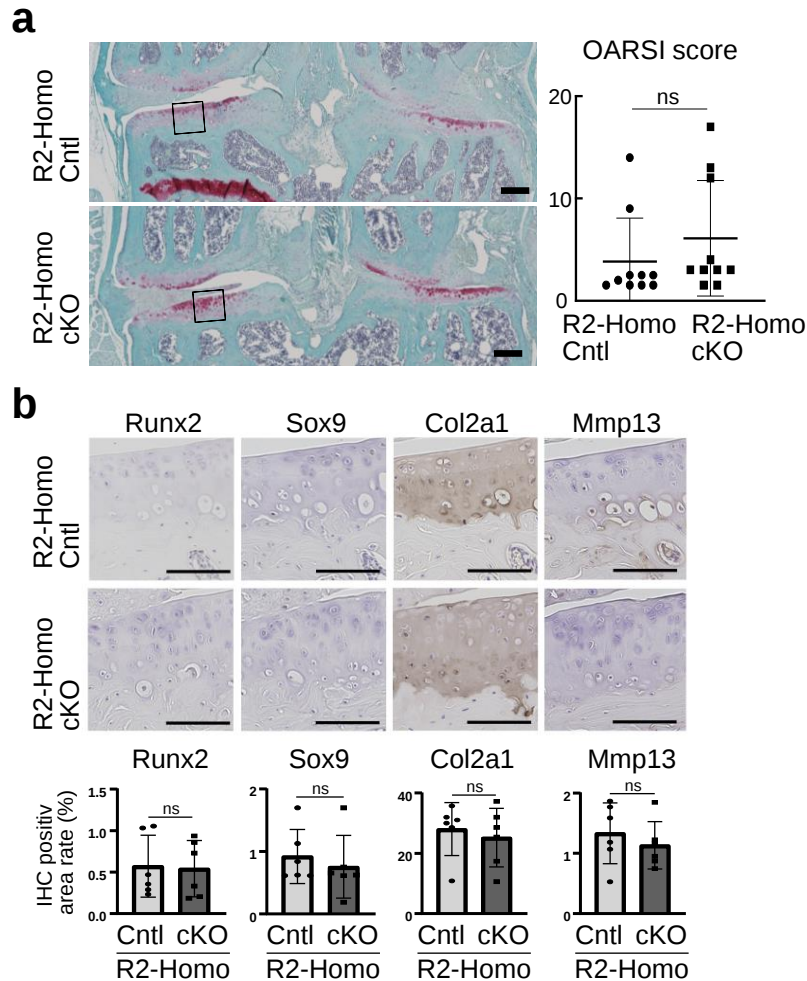
Supplementary Fig. 4 Skeletal formation in *Col2a1-Cre;Runx3^{fl/fl}* mice. **a** Gross appearance of *Runx3^{fl/fl}* (R3-Cntl) and *Col2a1-Cre;Runx3^{fl/fl}* (R3-cKO) littermate E18.5 embryos. Scale bars indicate 1 mm. **b** Double staining with Alizarin red and Alcian blue of the whole skeleton, clavicles, upper extremities, and lower extremities of R3-Cntl and R3-cKO littermate E18.5 embryos. Scale bars indicate 1 mm. **c** H&E staining of whole tibias of R3-Cntl and R3-cKO littermate E18.5 embryos. Scale bars indicate 1 mm. Upper bars indicate lengths of the proliferative zone (red), hypertrophic zone (blue), and bone area (green). **d** Safranin O staining and immunohistochemistry of Runx3, Col10a1, Mmp13, and Runx2 in proximal tibias of R3-Cntl and R3-cKO littermate E18.5 embryos. Scale bars indicate 200 μ m. Representative images among five mice in each genotype were shown. **e** Length of long bones and vertebra (first to fifth lumbar spines) of R3-Cntl and R3-cKO littermate E18.5 embryos. Data are expressed as means $\pm$ S.D. of five mice per group. **f** Body weight and naso-anal length of R3-Cntl and R3-cKO littermates from 1- to 8-week-old. Data are expressed as means $\pm$ S.D. of R3-Cntl (n = 5) and R3-cKO mice (n = 7). **g** Plain radiographs of the entire bodies of R3-Cntl and R3-cKO littermates at 16 weeks. Scale bars indicate 1 cm.



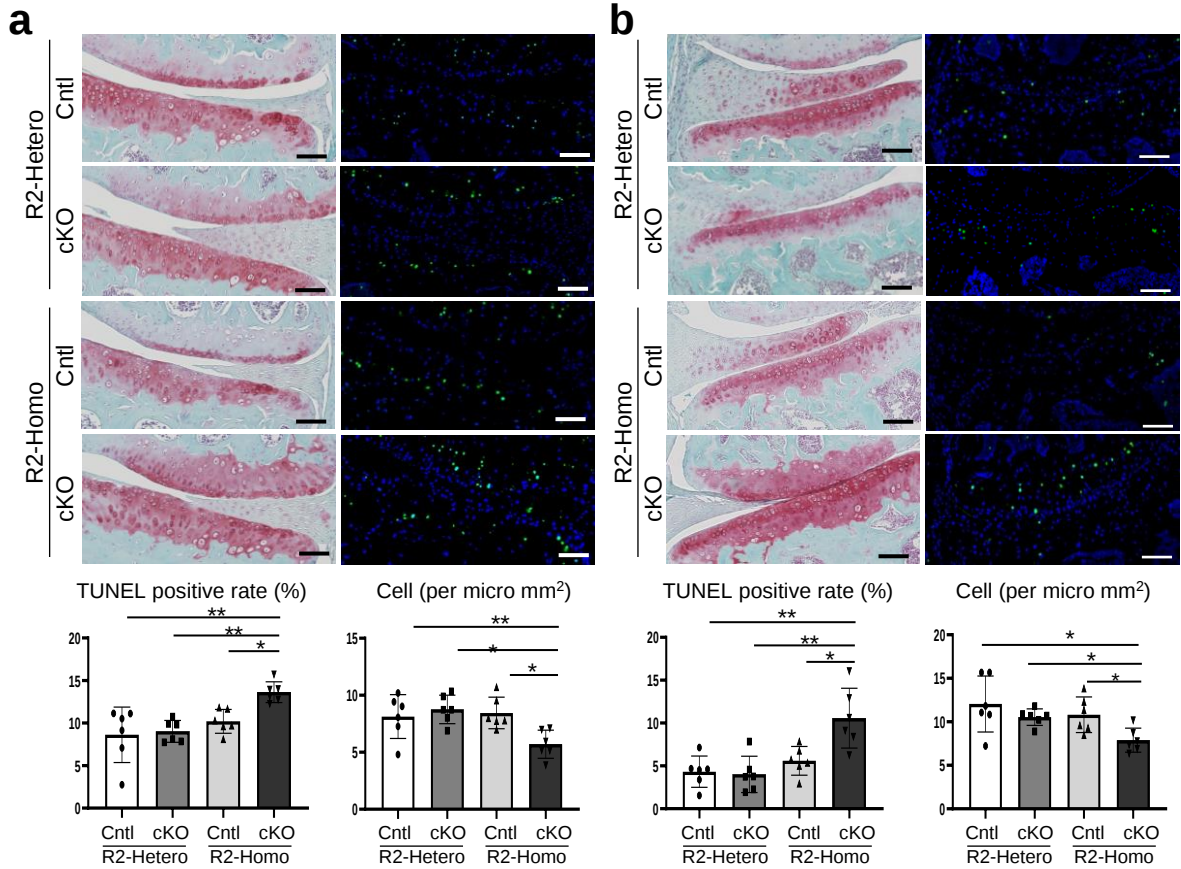
Supplementary Fig. 5 mRNA levels of Runx family members, *Prg4*, and *Acan* in primary SFZ and DZ chondrocytes obtained from *Runx3^{fl/fl}* (R3-Cntl) and *Prg4-Cre^{ERT2};Runx3^{fl/fl}* (R3-pKO^{ER}) mice. Data are expressed as means $\pm$ S.D. of five mice per group. *** $P < 0.001$, # $P < 0.0001$, n.s., not significant; ordinary one-way ANOVA test with Turkey's post hoc test.



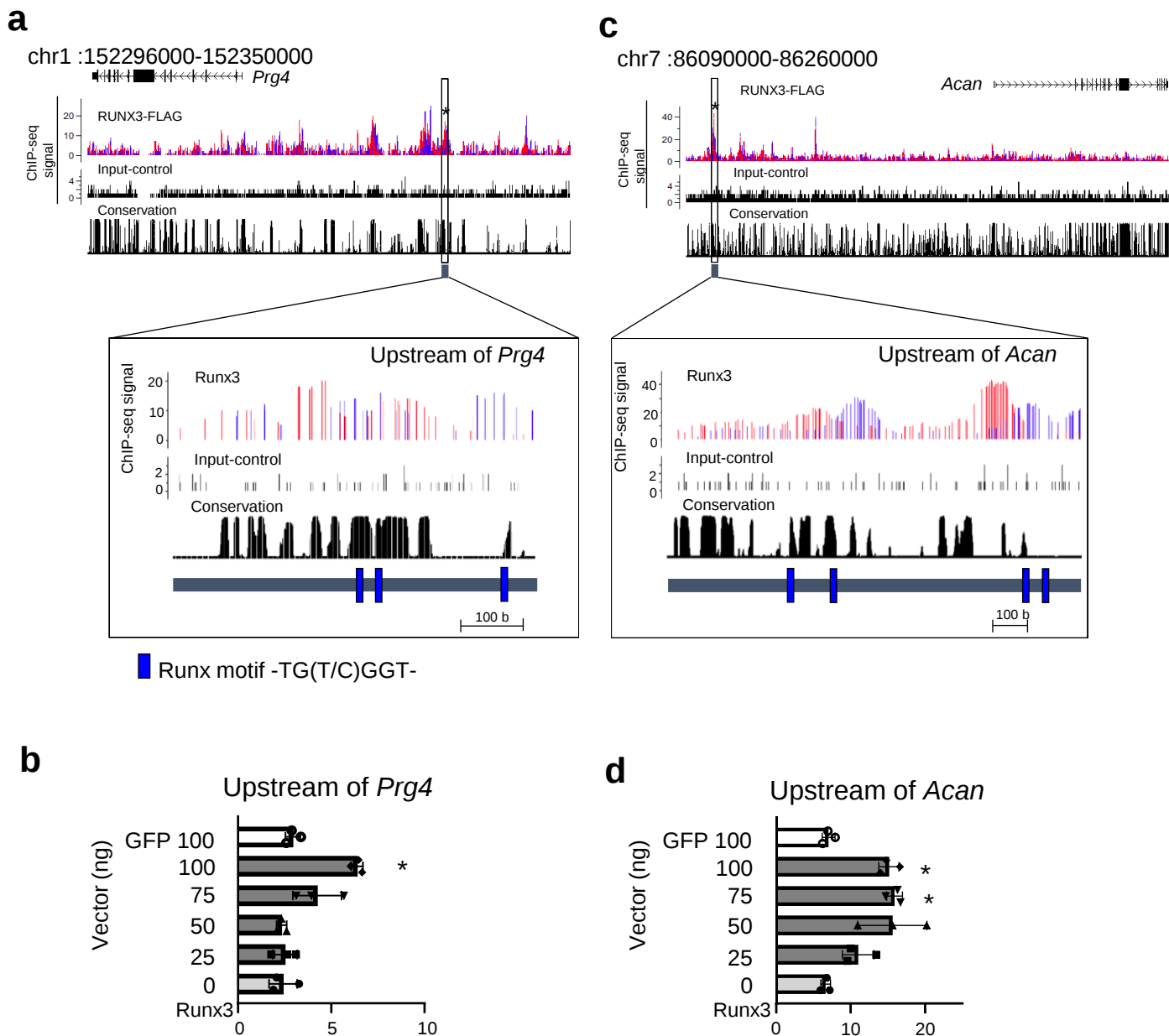
Supplementary Fig. 6 Generation and validation of *Runx2-flox* mice. **a** Generation of *Runx2-flox* mice. Diagram showing the *Runx2* genomic locus with exons 4 and 5, *Runx2*-targeting vector including the neo-cassette (Targeting Vector), and recombination between homologous regions of the targeting vector and *Runx2* locus, leading to generation of the *Runx2*-targeted allele (Targeted Allele). The floxed *Runx2* allele was generated by flippase recombinase target (FRT)-mediated recombination of the *Runx2*-targeted allele (Floxed allele), and the *Runx2*-deleted allele was generated by Cre-mediated recombination of the floxed *Runx2* allele (Deleted allele). **b** Skeletal elements of E18.5 embryos of stained with Alcian blue (cartilage) and Alizarin red (mineralized tissue). Scale bars, 2 mm.



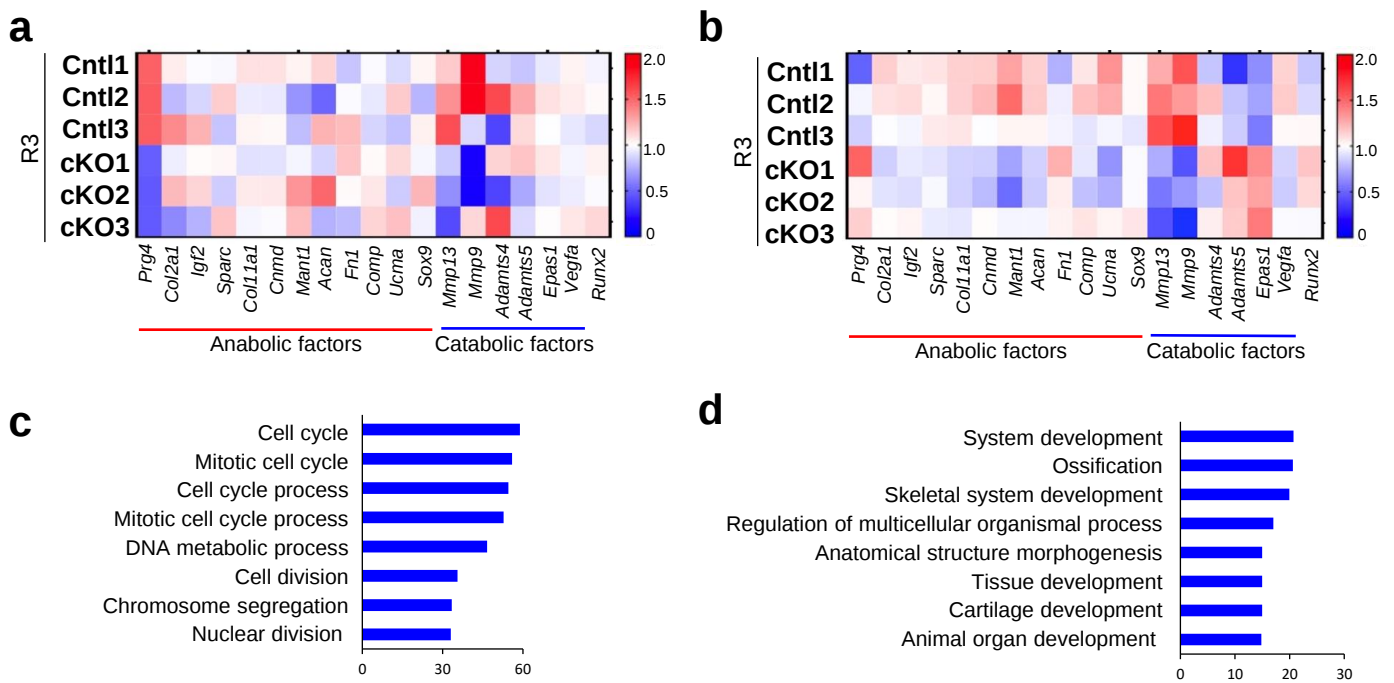
Supplementary Fig. 7 OA development of chondrocyte-specific Runx2-knockout mice with aging. **a** Representative Safranin O staining of 18-month-old homozygous *Runx2^{fl/fl}* (R2-Homo Cntl) and *Col2a1-Cre^{ERT2};Runx2^{fl/fl}* (R2-Homo cKO) knee joints (aging model). OARS scores are shown in the right panel. Data are expressed as mean $\pm$ S.D., $n = 10$ biologically independent animals. Scale bars, 200 μ m. **b** Immunohistochemistry of Runx2, Sox9, Col2a1, and Mmp13 in 18-month-old R2-Homo Cntl and R2-Homo cKO knee joints. Six biologically independent animals which displayed OARS scores close to the median for each group were analyzed. Scale bars, 100 μ m. Inset boxes in Safranin O staining indicate regions of immunohistochemistry. All data are expressed as dot plots and mean $\pm$ S.D. ns, not significant; two-tailed Mann-Whitney U test.



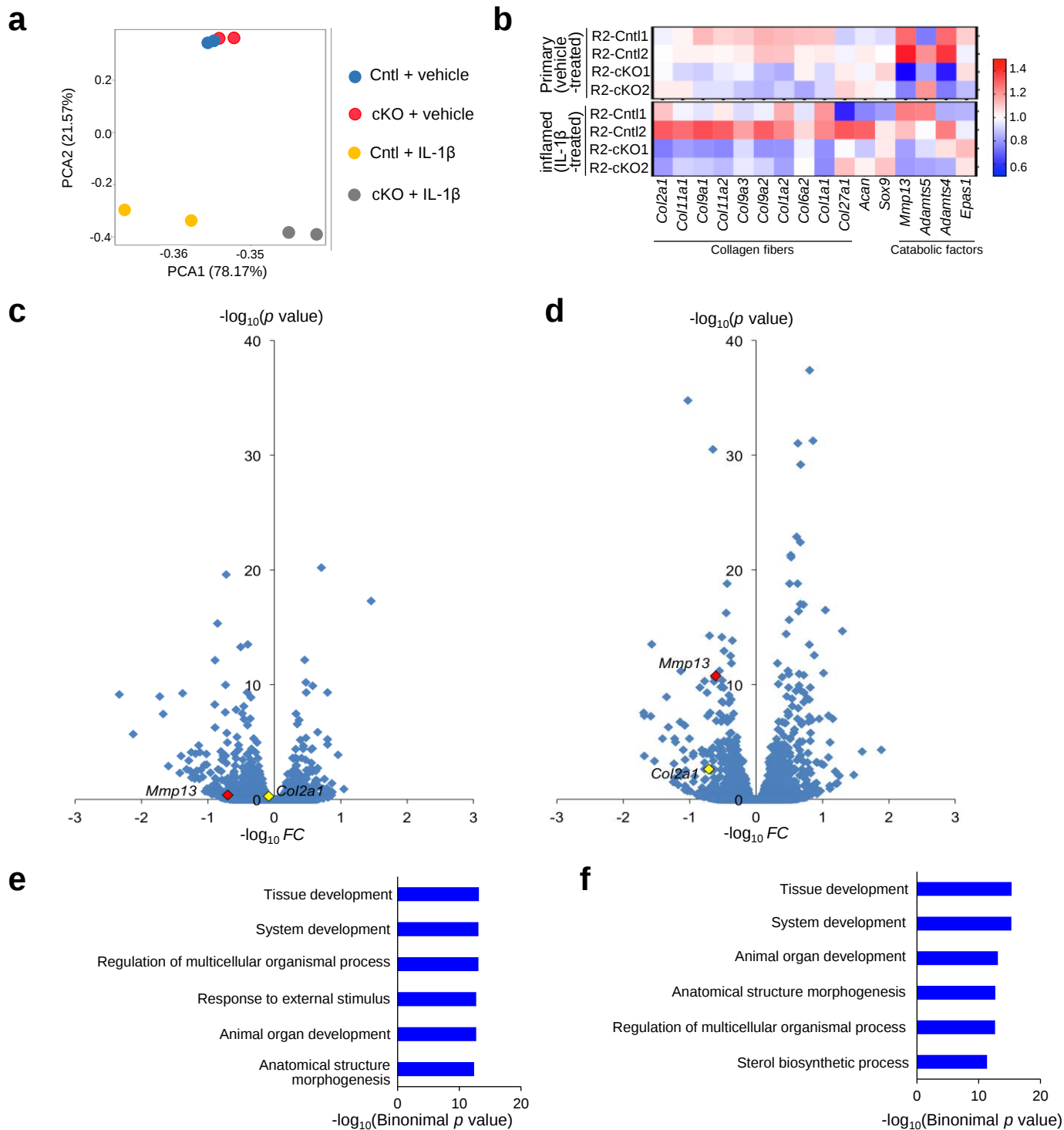
Supplementary Fig. 8 Chondrocyte apoptosis in articular cartilage of *Runx2^{fl/+}* (R2-Hetero Cntl), *Col2a1-Cre^{ERT2};Runx2^{fl/+}* (R2-Hetero cKO), *Runx2^{fl/fl}* (R2-Homo Cntl), and *Col2a1-Cre^{ERT2};Runx2^{fl/fl}* (R2-Homo cKO) mice. Safranin O and TUNEL staining of sham knee joints of medial model mice (**a**) and 8-week-old mice into which tamoxifen was injected at 7 weeks (**b**). Scale bars, 100 μ m. Percentages of TUNEL-positive and DAPI-positive cells in the Safranin O-positive area are shown below. $n = 6$ biologically independent animals. All data are expressed as dot plots and mean $\pm$ S.D. * $P < 0.05$; ** $P < 0.01$; one-way ANOVA test with Turkey's post hoc test.



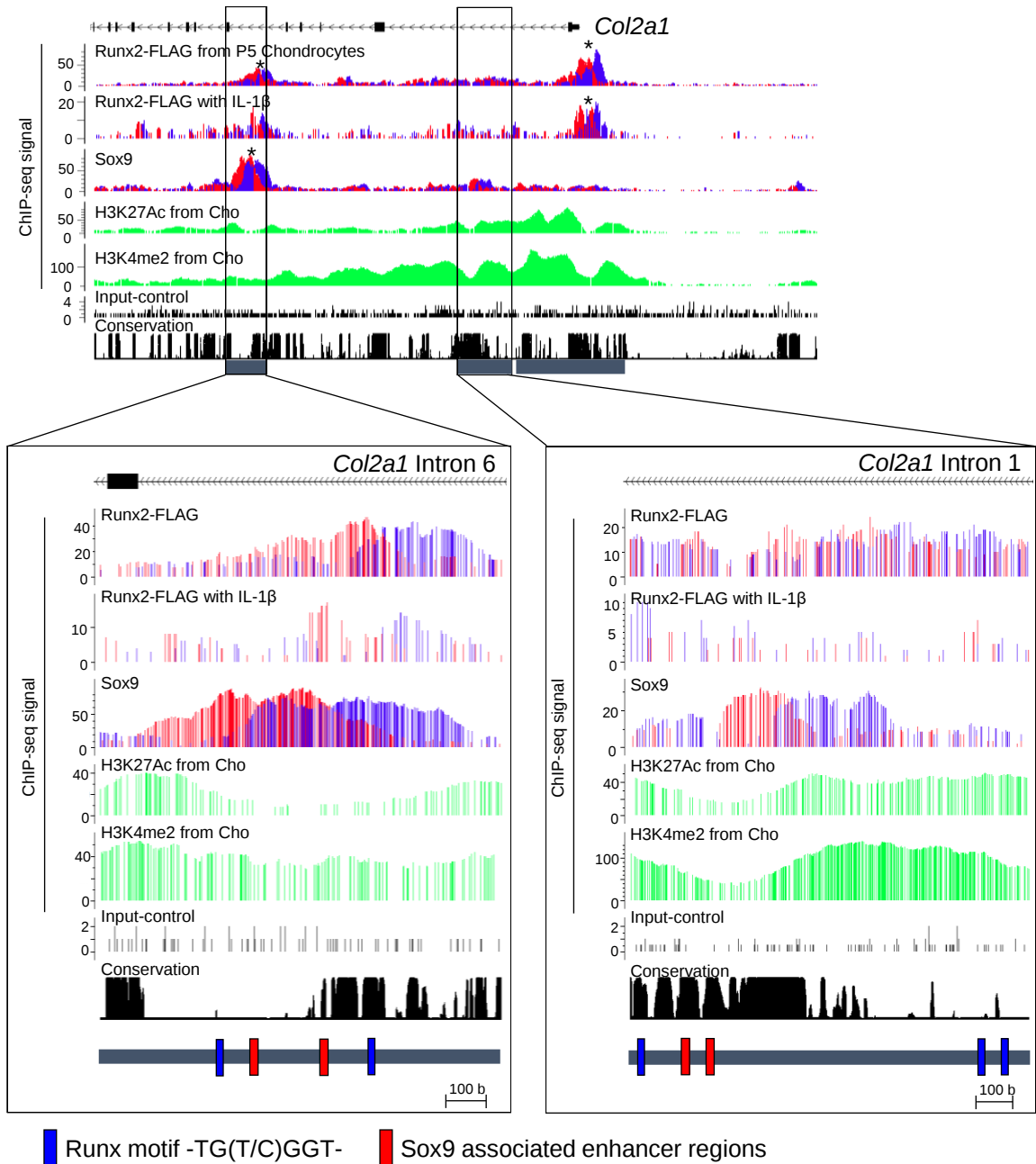
Supplementary Fig. 9 CisGenome browser screenshots showing Runx3 engagement, input control, and a conservation index around *Prg4* (a) and *Acan* (c) genes. Lower left and right panels indicate Chr1: 152337736–152338319 and Chr7: 86098728–86099908 in the mm9 database, respectively. Luciferase activities of *Prg4* (b) and *Acan* (d) enhancer regions in mouse chondrocytes transfected with various amounts of Runx3 or GFP control. $n = 3$ biologically independent experiments. Data shown as mean $\pm$ S.D. * indicates increased values, respectively, compared with control (white bars) with $P < 0.05$ in one-way ANOVA test with Turkey's post hoc test.



Supplementary Fig. 10 RNA-seq analyses of SFZ and DZ cells obtained from three individual *Runx3^{fl/fl}* (R3-Cntl) and *Col2a1-Cre;Runx3^{fl/fl}* (R3-cKO) littermate mice ($n = 3$). Heat maps showing extracellular matrix related genes, and articular cartilage catabolic factor related genes for SFZ (**a**) and DZ cells (**b**). Gene Ontology (GO) analyses in the top 2,000 downregulated genes in R3-cKO samples for SFZ (**c**) and DZ cells (**d**) showing top seven enriched GO terms.



Supplementary Fig. 12 Downstream targets of Runx2 in chondrocytes under normal and inflammatory conditions determined by RNA-seq. **a** Principal component analysis plot. **b** Heat maps of collagen fiber-associated genes, *Acan*, *Sox9*, and catabolic factor-associated genes. **c**, **d** Volcano plots from RNA sequencing data comparing the positions of *Col2a1* and *Mmp13* in primary chondrocytes (**c**) and inflamed chondrocytes (**d**). **e**, **f** Genomic Regions Enrichment of Annotations Tool gene ontology analyses of the top 2,000 expressed genes in primary chondrocytes treated with vehicle (**e**) and inflamed chondrocytes exposed to 1 ng per mL IL-1 β (**f**).



Supplementary Fig. 13 CisGenome browser views of Runx2 and reference engagement ChIP-seq around *Col2a1* (Chr15: 97825000–97840000). Lower panels show magnified views. Lower left and right panels indicate Chr15: 97827723–97828731 and Chr15: 97832509–97833631 in the mm9 database, respectively. ChIP-seq of Runx2-FLAG and Sox9 was performed by a paired-end method. Peak center positions are represented as red (data from forward reads) and blue (reverse reads) bars. Asterisks indicate peaks in ChIP-seq following peak calling criteria.

Supplementary Table 1. Top 10 down-regulated genes by Runx3 knockout in the SFZ cells, in order of FC value, evaluated by DESeq2 based on a model using the negative binomial distribution by two-tailed. FC, Fold Change.

Rank	Gene Name	GeneID	Log2FC	Average expression		P value
				R3-Cntl	R3-cKO	
1	<i>Prn</i>	111368	-2.273	15.897	3.289	< 0.001
2	<i>Mmp9</i>	17395	-1.523	158.309	55.094	0.001
3	<i>Tnnt2</i>	21956	-1.294	75.494	30.792	0.004
4	<i>Ccl2</i>	20296	-1.235	277.869	118.034	0.003
5	<i>Prg4</i>	96875	-1.153	1297.231	583.379	0.008
6	<i>Chrna1</i>	11435	-1.129	130.824	59.812	0.014
7	<i>Mylpf</i>	17907	-1.099	208.527	97.375	0.013
8	<i>Klhl30</i>	70788	-1.090	82.500	38.752	0.011
9	<i>Ein</i>	13717	-1.089	7882.319	3705.051	0.018
10	<i>Ibsp</i>	15891	-1.081	2259.332	1068.033	0.016

Supplementary Table 2. Top 10 down-regulated genes by Runx3 knockout in the DZ cells, in order of FC value, evaluated by DESeq2 based on a model using the negative binomial distribution by two-tailed. FC, Fold Change.

Rank	Gene Name	GeneID	Log2FC	Average expression		P value
				R3-Cntl	R3-cKO	
1	<i>C1qc</i>	12262	-1.885	12.788	3.462	< 0.001
2	<i>Zfp648</i>	100503355	-1.837	121.701	34.055	< 0.001
3	<i>Pthlh</i>	19227	-1.824	44.674	12.615	< 0.001
4	<i>Apoe</i>	11816	-1.772	320.277	93.774	< 0.001
5	<i>Tnfrsf19</i>	29820	-1.717	111.573	33.947	0.001
6	<i>Myo1f</i>	17916	-1.703	9.620	2.956	0.001
7	<i>Mpeg1</i>	17476	-1.701	31.592	9.715	0.001
8	<i>Alpl</i>	11647	-1.645	2480.344	793.254	< 0.001
9	<i>Col15a1</i>	12819	-1.614	1068.835	349.253	< 0.001
10	<i>Tnfrsf9</i>	21942	-1.592	27.470	9.112	< 0.001

Supplementary Table 3. Top 10 up-regulated genes by Runx3 knockout in the SFZ cells, , in order of FC value, evaluated by DESeq2 based on a model using the negative binomial distribution by two-tailed. FC, Fold Change.

Rank	Gene Name	GeneID	Log2FC	Average expression		P value
				R3-Cntl	R3-cKO	
1	<i>Fbxo15</i>	50764	1.131	4.764	10.437	0.013
2	<i>Lrrtm1</i>	74342	0.962	83.678	163.059	0.026
3	<i>Jph3</i>	57340	0.944	9.205	17.713	0.040
4	<i>Gm13083</i>	279185	0.921	2.690	5.092	0.044
5	<i>Penk</i>	18619	0.918	39282.223	74239.531	0.046
6	NA	NM_001305677	0.903	39.147	73.203	0.017
7	<i>Gsta4</i>	14860	0.890	218.037	404.136	0.040
8	<i>Cdkl4</i>	381113	0.876	4.695	8.619	0.056
9	<i>Cyp4f14</i>	64385	0.876	39.558	72.613	0.057
10	<i>Pm20d1</i>	212933	0.872	48.034	87.911	0.027

Supplementary Table 4. Top 10 up-regulated genes by Runx3 knockout in the DZ cells, in order of FC value, evaluated by DESeq2 based on a model using the negative binomial distribution by two-tailed. FC, Fold Change.

Rank	Gene Name	GeneID	Log2FC	Average expression		P value
				R3-Cntl	R3-cKO	
1	<i>Hoxc13</i>	15422	2.194	30.384	139.067	4.E-07
2	<i>Dsp</i>	109620	2.020	60.358	244.778	1.E-07
3	<i>Lce3c</i>	94060	1.885	2.154	7.957	2.E-04
4	<i>Car3</i>	12350	1.879	98.052	360.598	1.E-04
5	<i>Rab3b</i>	69908	1.758	205.651	695.508	3.E-04
6	<i>Crct1</i>	74175	1.726	3.783	12.517	0.001
7	<i>Serpine1</i>	18787	1.706	1017.046	3318.122	3.E-04
8	<i>Dlx2</i>	13392	1.699	48.422	157.176	1.E-05
9	<i>Slurp1</i>	57277	1.680	22.486	72.039	0.001
10	<i>Serpib9b</i>	20706	1.675	10.446	33.347	0.001

Supplementary Table 5. Different expression gene lists of RNA sequencing in primary chondrocytes from *Runx2^{fl/fl}* (R2-Homo Cntl) and *Runx2^{fl/f};Col2a1-Cre^{ERT2}* (R2-Homo cKO) mice, in order of FC value, evaluated by DESeq2 based on a model using the negative binomial distribution by two-tailed. FPKM, Fragments Per Kilobase of transcript per Million fragments Mapped; FC, Fold Change.

Gene Symbol	Expression level (FPKM)		Log2 FC	P value	Gene Symbol	Expression level (FPKM)		Log2 FC	P value
	R2-Homo Cntl	R2-Homo cKO				R2-Homo Cntl	R2-Homo cKO		
Tmem254b	1234.051	244.214	-2.337	7.280E-10	Ddx3y	21.352	1482.747	6.118	6.230E-79
Mup18	29.671	6.787	-2.128	2.020E-06	Eif2s3y	11.411	605.925	5.731	5.450E-83
Aoc3	222.588	67.298	-1.726	1.080E-09	Kdm5d	9.557	424.667	5.474	3.230E-67
Plin1	179.383	56.118	-1.677	3.650E-08	Uty	11.551	372.215	5.01	6.440E-59
Gm3500	18.725	6.197	-1.595	1.266E-03	NA	8.394	74.941	3.158	1.100E-15
B3galt2	45.484	16.951	-1.424	4.828E-03	Tcerg1l	5.633	16.355	1.538	2.317E-03
Adipoq	129.996	49.087	-1.405	1.629E-04	Esr1	229.908	632.026	1.459	4.980E-18
Bglap2	327.966	126.067	-1.379	5.640E-10	Pnp2	5.059	11.898	1.234	2.547E-02
Car3	84.085	33.674	-1.32	2.366E-03					
Il17b	116.841	47.88	-1.287	4.156E-04					
Fabp4	733.023	304.746	-1.266	3.363E-03					
Hhip	187.607	78.935	-1.249	7.640E-05					
Hsd11b2	60.391	25.484	-1.245	1.256E-02					
Wnt10b	67.869	29.581	-1.198	1.544E-02					
Spink5	85.563	37.372	-1.195	8.184E-03					
Gpd1	238.102	104.658	-1.186	1.022E-04					
Olfml2a	113.961	50.739	-1.167	3.185E-03					
Cd36	258.085	117.241	-1.138	1.940E-05					
Lamc3	75.381	34.489	-1.128	1.664E-02					
Hp	202.173	92.87	-1.122	7.590E-05					
Ihh	128.499	59.727	-1.105	2.592E-03					
Gper1	97.937	46.428	-1.077	1.119E-02					
C3	201.53	97.416	-1.049	9.630E-04					
Gli1	253.036	124.343	-1.025	1.118E-04					
Podnl1	186.699	92.096	-1.02	9.524E-04					

Supplementary Table 6. Different expression gene list of RNA sequencing in inflamed chondrocytes (treated with 1 ng per mL IL-1 β for 24 hours) from *Runx2^{fl/fl}* (R2-Homo Cntl) and *Runx2^{fl/fl};Col2a1-Cre^{ERT2}* (R2-Homo cKO) mice, in order of FC value, evaluated by DESeq2 based on a model using the negative binomial distribution by two-tailed. FPKM, Fragments Per Kilobase of transcript per Million fragments Mapped; FC, Fold Change.

Gene Symbol	Expression level (FPKM)		Log2 FC	P value	Gene Symbol	Expression level (FPKM)		Log2 FC	P value
	R2-Homo Cntl	R2-Homo cKO				R2-Homo Cntl	R2-Homo cKO		
Mylpf	160.668	49.620	-1.695	2.840E-08	NM_001305819	7.208	51.933	2.849	8.93E-11
Myh1	56.546	17.597	-1.684	1.654E-04	Raet1b	6.099	25.708	2.076	1.54E-05
Myh8	145.108	45.163	-1.684	5.030E-08	Ifit1	12.508	46.196	1.885	4.83E-05
Actc1	165.294	54.993	-1.588	5.580E-08	Ces2e	7.871	25.444	1.693	1.13E-03
Ibsp	367.421	123.671	-1.571	3.070E-14	Ano3	28.708	86.801	1.596	6.95E-05
Ttn	66.201	22.936	-1.529	4.641E-04	Gm14430	88.864	246.747	1.473	7.63E-03
Sp7	154.509	58.208	-1.408	5.260E-06	Capn11	6.223	16.153	1.376	1.76E-02
Podnl1	291.177	114.184	-1.351	1.180E-09	Mrgprf	222.209	546.158	1.297	2.20E-15
Bglap	205.567	82.190	-1.323	5.180E-07	Stfa3	26.378	62.782	1.251	8.69E-03
Atp2a1	46.853	19.733	-1.248	3.180E-02	Itga2	28.302	66.838	1.240	7.50E-03
Des	108.582	46.054	-1.237	7.607E-04	S100a8	15.676	36.606	1.224	3.99E-02
Myog	42.461	18.123	-1.228	3.025E-02	H60a	68.454	157.252	1.200	4.47E-02
Acta1	231.711	99.026	-1.226	5.090E-06	Sncg	40.474	92.550	1.193	3.67E-03
Neb	57.484	24.605	-1.224	1.478E-02	Chdh	138.242	307.787	1.155	9.84E-08
Myh3	202.111	87.078	-1.215	1.060E-05	Serpina3n	2012.897	4354.166	1.113	1.12E-05
Smpd3	1754.054	789.284	-1.152	4.429E-03	Mgmt	84.358	179.775	1.092	2.14E-04
Tmem119	298.302	134.934	-1.145	1.840E-07	Esr1	168.661	359.174	1.091	5.10E-08
Alpl	518.483	236.375	-1.133	6.690E-12	Ptgs1	474.049	975.514	1.041	3.21E-17
Mfap5	82.824	37.776	-1.133	9.406E-03	Notch3	81.955	165.771	1.016	1.00E-03
Wnt10b	202.773	94.877	-1.096	5.020E-05	Dglucy	319.079	644.004	1.013	9.77E-12
Bglap2	362.647	172.052	-1.076	3.670E-07					
Thy1	181.539	86.608	-1.068	3.491E-04					
Myl1	132.910	63.682	-1.061	2.953E-03					
Gpx3	4298.849	2110.731	-1.026	1.700E-35					
Ddx3y	1857.833	925.398	-1.005	5.180E-06					

Supplementary Table 7. Primers used for genotyping.

<i>Runx2-flox</i>	F	aacatacaaactgtcttcctcaac
	R	caggtcttgagctaaaagcagaagg
<i>Cre</i>	F	gcatttctggggattgctta
	R	cccggcaaacacaggtagtta
<i>Prg4-Cre^{ERT2}</i>	Common F	tgactatctcaggaattcaagctg
	Wild type R	cgcttttgacgctgaagttac
	Mutant R	gaacttcagggtcagcttgc
<i>Runx3-flox</i>	F	tatccctctctgggccttct
	R	ggaaactgagtcagccaag
<i>Runx2-FLAG</i>	tag-F	gcttgatcatcgatccttgtagtc
	F	ttgcaagatcatgactagggatt
	R	ccccaactgtttgaattctagc

Supplementary Table 8. Primers used for qRT-PCR.

Gene Symbol	Species		Sequence
<i>Gapdh</i>	mouse	F	tgaccaccaactgcttagc
		R	ggatgcagggatgatgttct
<i>Runx3</i>	mouse	F	tcacaatcaccgtgttcacc
		R	ctgggtgtgcttggtgttac
<i>Runx2</i>	mouse	F	gactgtggttaccgtcatggc
		R	actgggttttcataacagcgga
<i>Runx1</i>	mouse	F	ccagcctctctgcagaactt
		R	caggtaggtgtggtagcgaga
<i>Prg4</i>	mouse	F	tccattcagaccaccatctcc
		R	gagcagccactatcttcctgt
<i>Acan</i>	mouse	F	ccaaaccagcctgacaactt
		R	tctagcatgctccaccactg
<i>Col2a1</i>	mouse	F	gccaagacctgaactctgc
		R	gccatagctgaagtggaagc
<i>Sox9</i>	mouse	F	cgactacgctgaccatcaga
		R	agactgggtgttcccagtc
<i>Mmp9</i>	mouse	F	tgaatcagctggcttttg
		R	gtggatagctcgggtggtgtt
<i>Mmp13</i>	mouse	F	aggccttcagaaaagccttc
		R	tccttgagtgatccagacc
<i>Vegfa</i>	mouse	F	caggctgctgtaacgatgaa
		R	tttcttgcgcttcggtttt
<i>Hif2a</i>	mouse	F	tgagtggctcatgagttgc
		R	ctcacggatctcctcatggt

Supplementary Table 9. Locations of the cloned sequences for luciferase reporter vectors.

Reporter	Colned sequence (MGSCv37/mm9)	Length
Col2a1 Intron6	Chr15:97827723-97828731	1,009 bp
Col2a1 Intron1	Chr15:97832509-97833631	1,123 bp
Col2a1 Around TSS	Chr15:97833508-97835758	2,251 bp
Mmp13 Upstream of TSS	Chr9:7271588-7272547	960 bp
Prg4 Upstream	Chr1:152337736-152338319	584 bp
Acan Upstream	Chr7:86098728-86099908	1,181 bp