Supplementary Information
Nature Materials
NM17061887

Parenchymal and Stromal Tissue Regeneration of Tooth Organ by Pivotal Signals Reinstated in Decellularized Matrix

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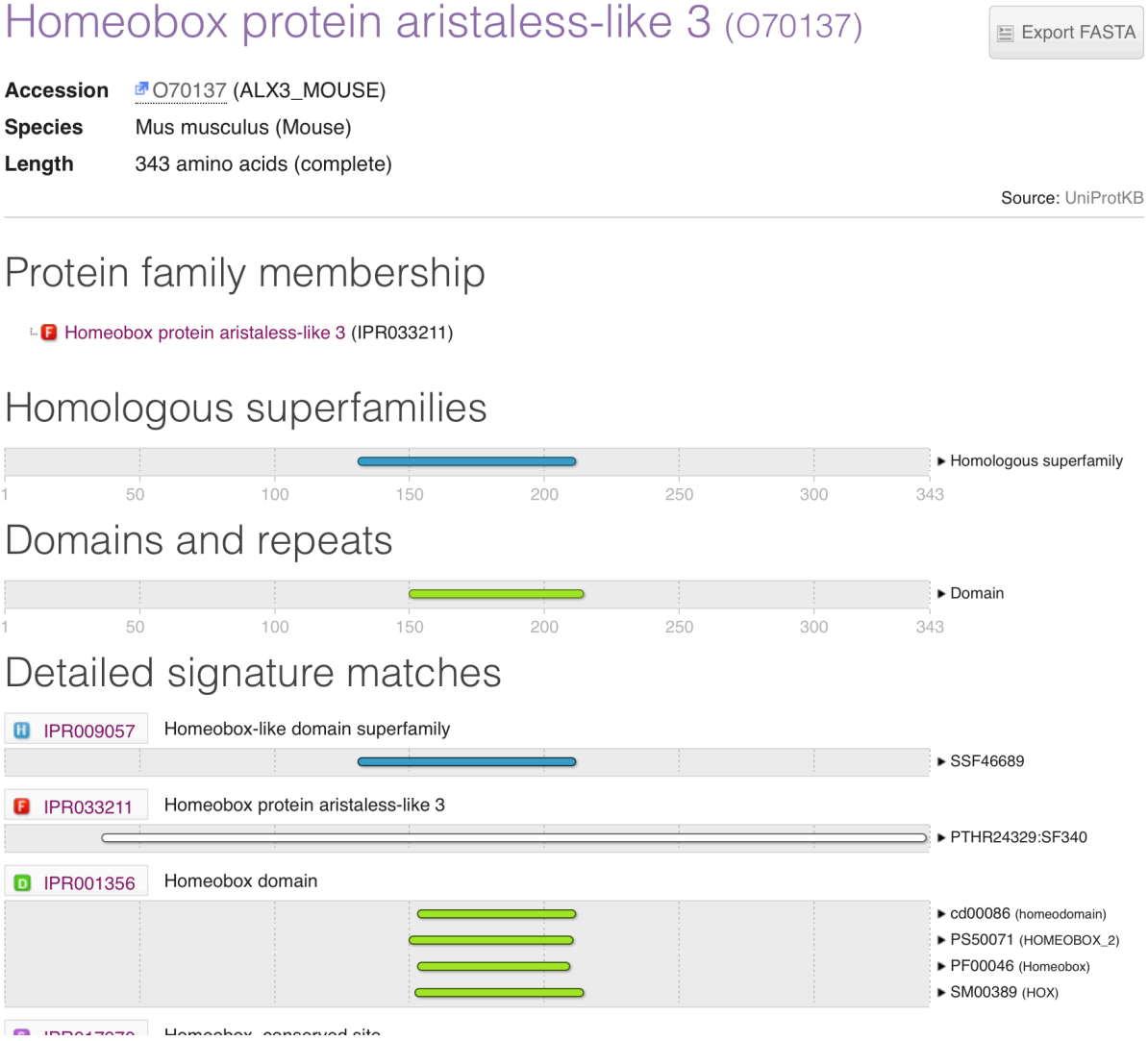
Contents:

Supplementary notes

CRISPR/Cas9 - Alx3 KO

Design:

- Homeobox domain resides between aa125 and aa210.
- Locate the exon harboring this homeobox domain.
- Design two guides that may cut in front of this HB domain.
- Design two guides that may cut after this HB domain.
- Generate the sgRNAs and inject these sgRNAs + nlsCas9 (RNP) into B6CBAF1 eggs to generate mice with the HB domain deletion.



Alx3-5-203R: catttcggggaagtgctctg
TTCTAATACGACTCACTATAGGcatttcggggaagtgctctgGTTTTAGAGCTAGA

Alx3-5-34F: tagcagaggcatgagcctga
TTCTAATACGACTCACTATAGGtagcagaggcatgagcctgaGTTTTAGAGCTAGA

Alx3-3-49R: acagatatttggactccaag

TTCTAATACGACTCACTATAGGacagatatttggactccaagGTTTTAGAGCTAGA

Alx3-3-121R: ggcaacagaaatccattgag
TTCTAATACGACTCACTATAGggcaacagaaatccattgagGTTTTAGAGCTAGA

0.05 umole PAGE oligos were ordered from Sigma on 2/23/2018 ($169.06)

- performed CRISPR EZ with combinational guides in B6CBAF1 eggs.
- eggs recovered in aKSOM and electroporation in aKSOM X 4 PULSES.

Assay the editing (deletion) by Alx3delat1F/1R PCR

(1) 1649bp-836bp deletion=813bp amplicon (didn't test this set)

sgRNA-Alx3-5-203R (1368ng/ul)	2.9ul	
sgRNA-Alx3-3-49R (1848ng/ul)	2.2ul	
1N NaCl		2ul
40uM Cas9(QB3, Berkeley)		5ul

(2) 1649bp-908bp deletion=741bp amplicon

sgRNA-Alx3-5-203R (1368ng/ul)	2.9ul	
sgRNA-Alx3-3-121R (2344ng/ul)	1.7ul	
1N NaCl		2ul
40uM Cas9(QB3, Berkeley)		5ul

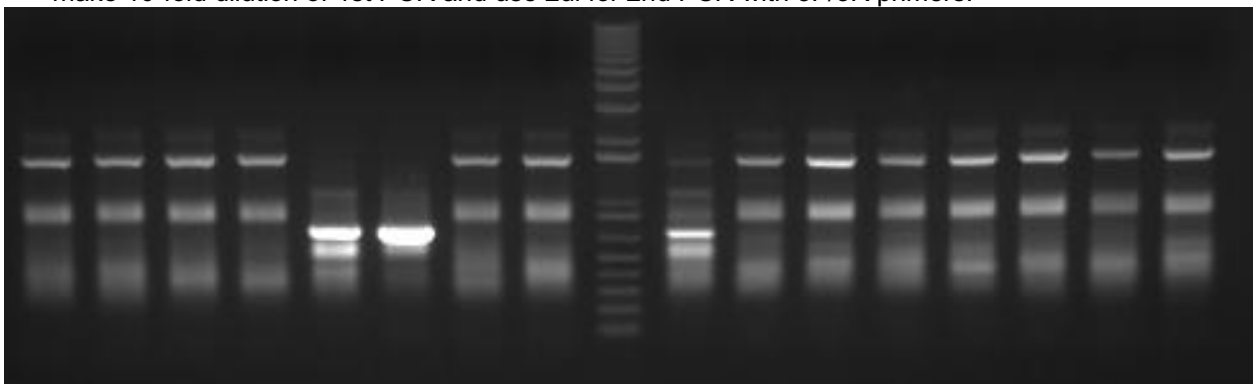
(3) 1649bp-1006bp deletion=643bp amplicon

sgRNA-Alx3-5-34F (820ng/ul)		4.9ul
sgRNA-Alx3-3-49R (1848ng/ul)	2.2ul	
1N NaCl		2ul
40uM Cas9(QB3, Berkeley)		5ul

(4) 1649-1078bp deletion=571bp amplicon

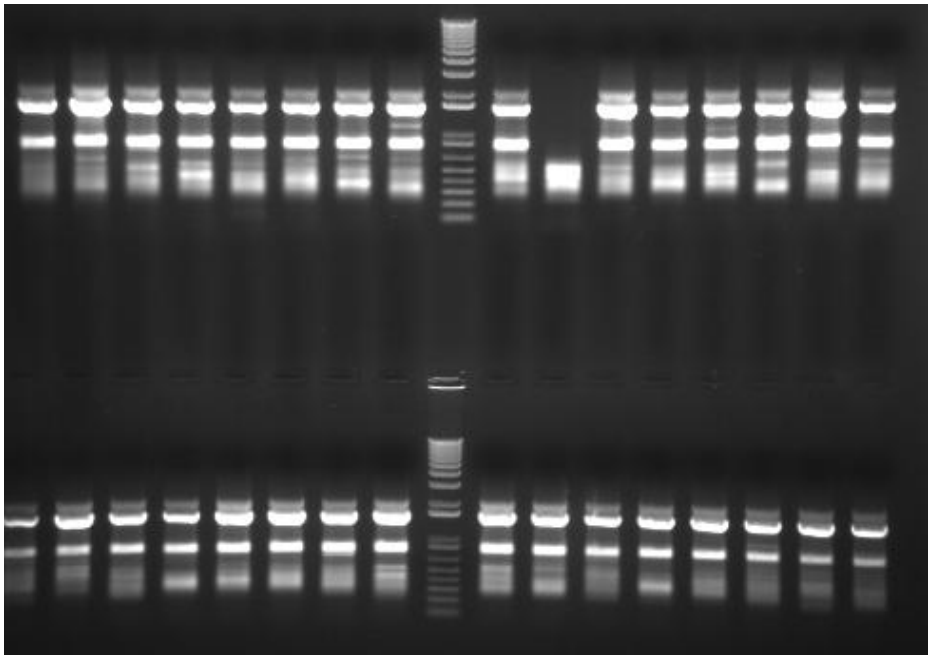
sgRNA-Alx3-5-34F (820ng/ul)		4.9ul
sgRNA-Alx3-3-121R (2344ng/ul)	1.7ul	
1N NaCl		2ul
40uM Cas9(QB3, Berkeley)		5ul

- mix 14ul RNP with 10ul aKSOM/30 embryos.
- 4 pulses, 8% droop.
- culture t blastocyst stage for genotyping
- over 90% of the embryos became blastocysts when checked at 4pm Sunday.
- lyse blastocysts in 10ul lysis buffer.
- perform 34 cycle PCR with Alx3Delta1F/1R.
- make 10-fold dilution of 1st PCR and use 2ul for 2nd PCR with 3F/3R primers.



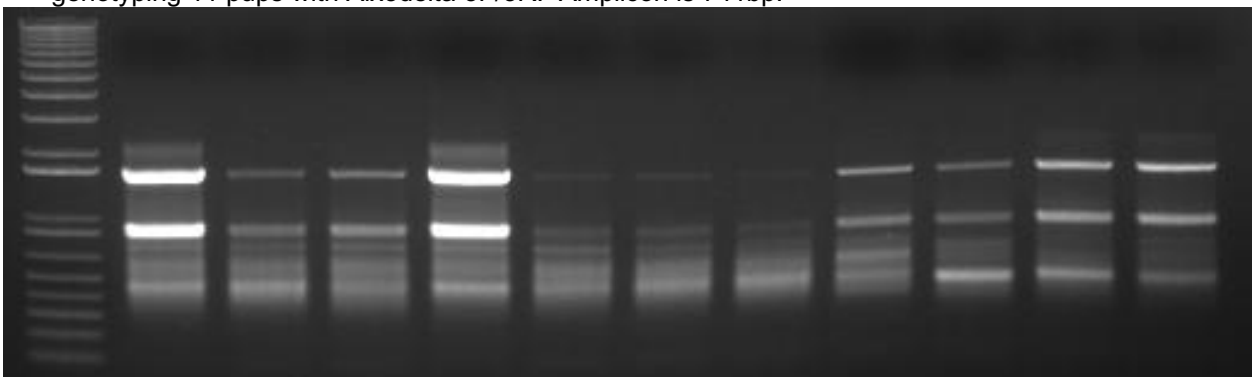
1649bp-908bp deletion=741bp amplicon

#5, #6, and #9 have the edited allele. The 1.6kb amplicon is the WT and the 0.8kb amplicon is non-specific and appears in all samples. Don't say #5 and #6 are homozygotes as edited allele will compete with the WT allele in this PCR. The result just confirms that we can generate this deletion in blastocysts with CRISPR technique. Mice with the same deletion should come once we transfer the embryos into the foster moms.

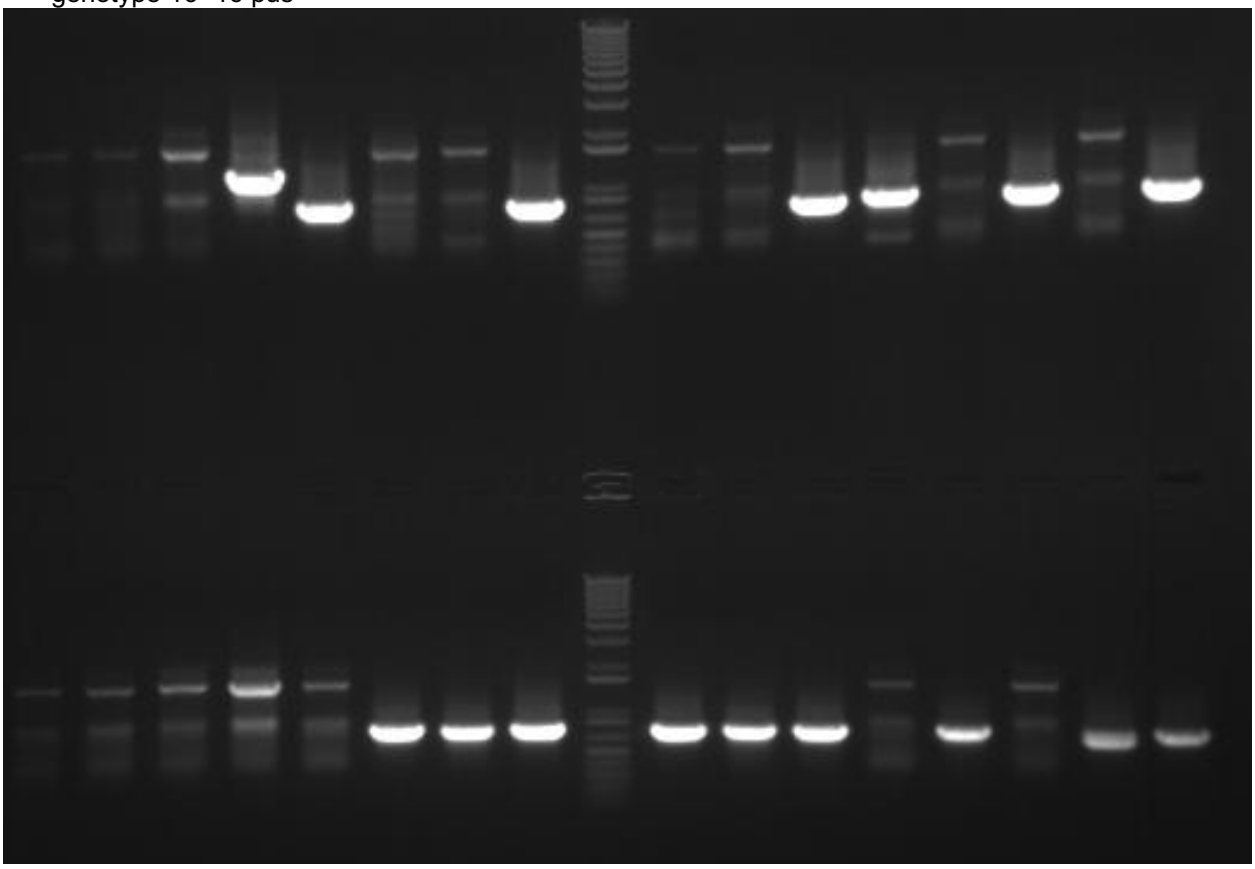


upper:(3) 1649bp-1006bp deletion=643bp amplicon
lower:(4) 1649-1078bp deletion=571bp amplicon

- genotyping 11 pups with Alx3delta 3F/3R. Amplicon is 741bp.



- genotype 16+16 pus



- 15 out of 32 have Alx3 deletion.
- A nested PCR was used for this genotyping. PCR-A was performed according to the following parameters and the PCR product was diluted 10 times before running the 2nd PCR-B.
- DNA ladder used in this gel is Invitrogen's 1kb plus DNA ladder.

PCR-A

	PCR Reaction (20ul)	
		X10
2ul	Tail lysate	
15.3ul	water	153ul
2ul	10X NEB ThermoPol PCR buffer	20ul
0.4ul	10 mM dNTP	4ul
0.05ul	Forward primer (100 pmol/ul)	0.5ul
0.05ul	Reverse primer (100 pmol/ul)	0.5ul
0.2ul	Taq (5 units/ul)	2ul
20ul	total	

PCR parameters

94º	60s	X1
94º 60º 72º	10s 10s 30s	X34
72º	2m	X1
4º	HOLD	X1
PCR product: WT:1649		

Alx3delta 1F: **acagcactgagggtaatgagggaag**
Alx3delta 1R: **acacaaagtggtgaccttaggcaaa**

PCR-B

PCR Reaction (20ul)

		X10
2ul	10-fold dilution of the PCR-A	
15.3ul	water	153ul
2ul	10X NEB ThermoPol PCR buffer	20ul
0.4ul	10 mM dNTP	4ul
0.05ul	Forward primer (100 pmol/ul)	0.5ul
0.05ul	Reverse primer (100 pmol/ul)	0.5ul
0.2ul	Taq (5 units/ul)	2ul
20ul	total	

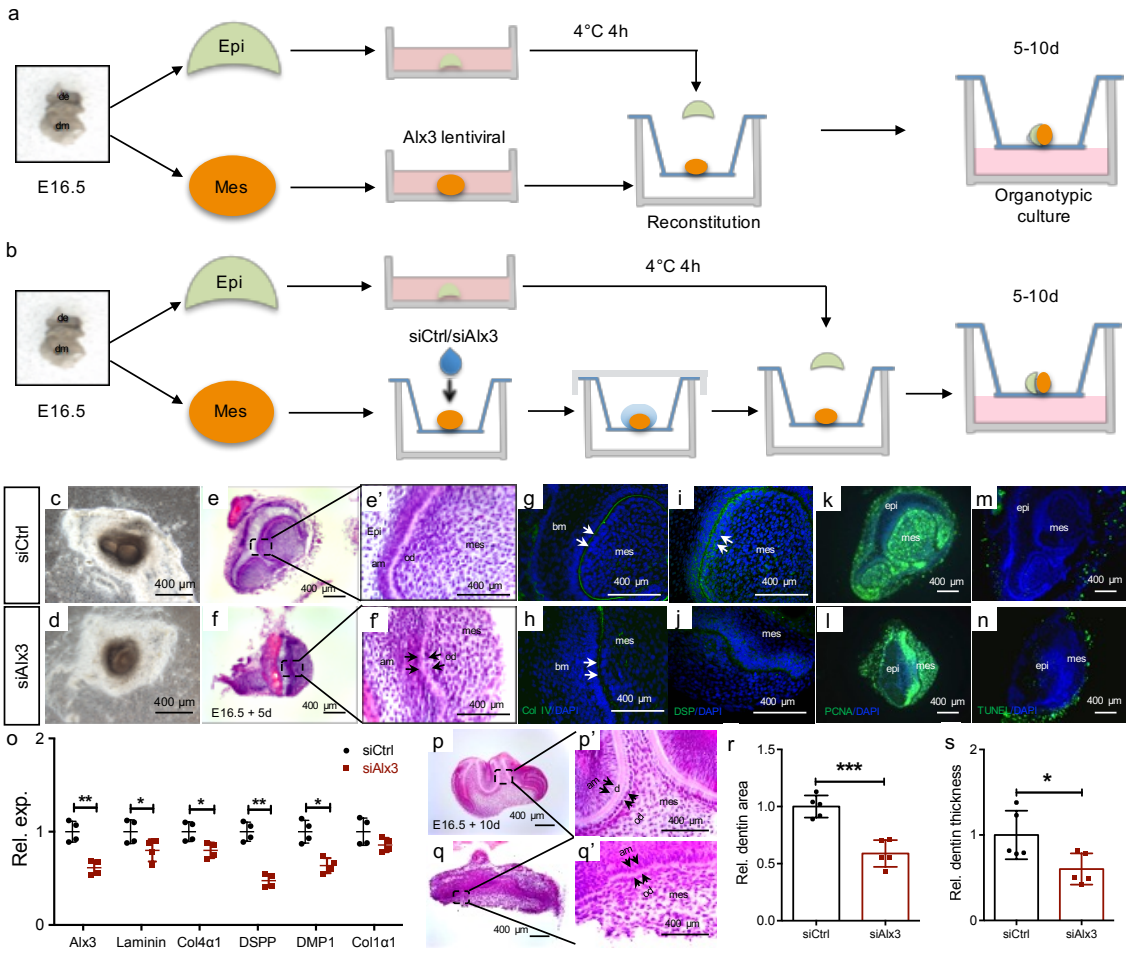
0.5kb PCR parameters

94º	60s	X1
94º 60º 72º	10s 10s 30s	X34
72º	2m	X1
4º	HOLD	X1
PCR product: WT:1616bp Delta: 741bp		

Alx3delta 3F: **acagcactgagggtaatgagggaag**
Alx3delta 3R: **gggtttttctctgtgcagaaggact**

Supplementary figures and figure legends

Supplementary Figure 1

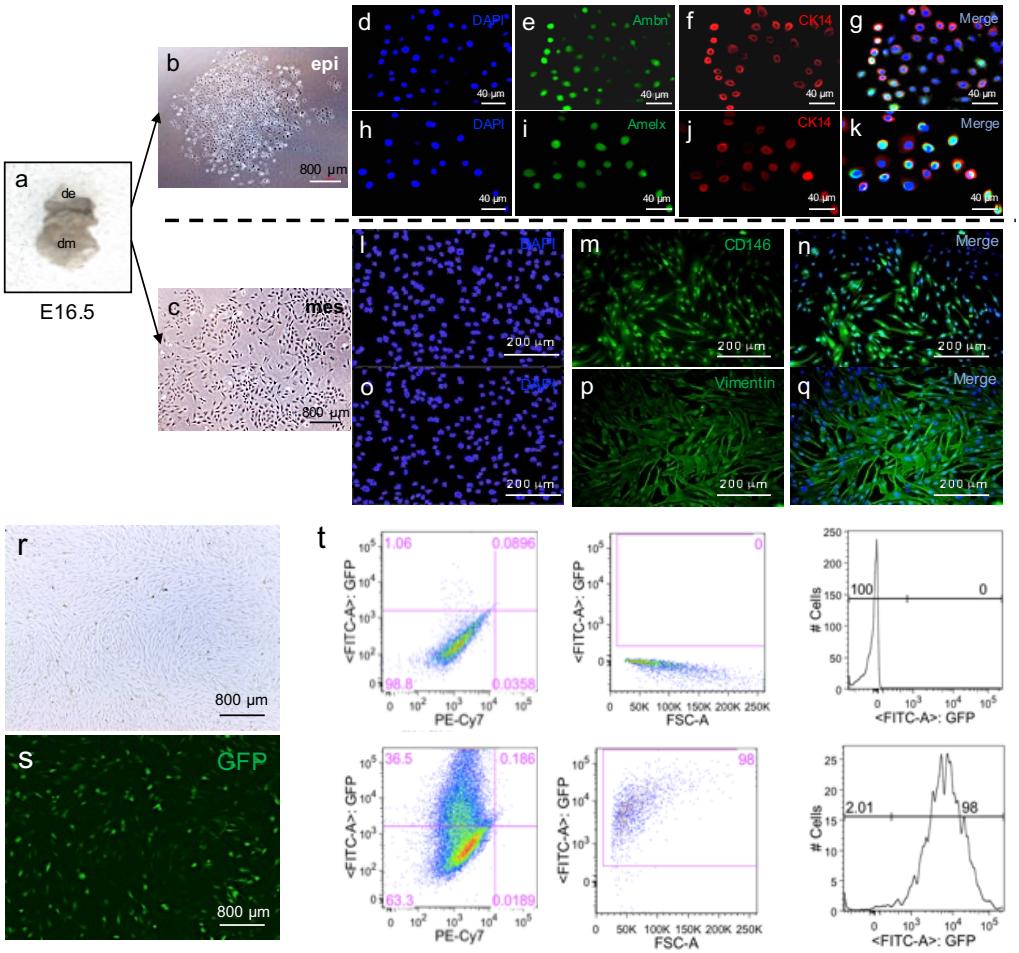


Supplementary Figure 1

Epithelium and mesenchyme reconstitution and Alx3 manipulation. **a:** Epithelium (Epi) and mesenchyme (Mes) tissues were microdissected. Mes was upregulated with Alx3 lentiviral constructs, whereas Epi was immersed in organ culture medium. The isolated Epi and Mes were reconstituted and cultured for 5 to 10 days. **b:** Epi and Mes isolation and reconstitution as in **a**, except for Alx3 knockdown by siRNA in Mes. **c-f':** Reconstituted E16.5 tooth organs with Alx3 knockdown followed by 5-day culture. Scale bar, 400 μm.

g,h: Collagen IV immunofluorescence; basement membrane (bm) formation (white arrows). **i,j:** Dentin sialoprotein (DSP) immunofluorescence (white arrows). **k,l:** Proliferating cell nuclear antigen (PCNA) immunofluorescence. **m,n:** TUNEL staining. Scale bar, 400 μm. **c-n:** n=4 biological independent samples. **o:** Real-time PCR of Alx3, laminin, collagen4α1, DSPP (dentin sialophosphoprotein), DMP1 (dentin matrix protein-1) and Col1α1 upon Alx3 knockdown by 5-day culture. (n=4 biological independent samples. Presented with mean ± SD; *P* values calculated by multiple two-sided Student's t-test. *: $p<0.05$; **: $p<0.01$). **p-q':** HE images of E16.5 reconstituted tooth organs following 10-day culture. Black arrows: ameloblasts (am) and odontoblasts (od); d: dentin. Scale bar, 400 μm. **p-q':** n=5 biological independent samples. **r,s:** Quantified dentin area and thickness. (n=5 biological independent samples. Presented with mean ± SD; *P* values calculated by two-sided Student's t-test. *: $p<0.05$; ***: $p<0.001$).

Supplementary Figure 2

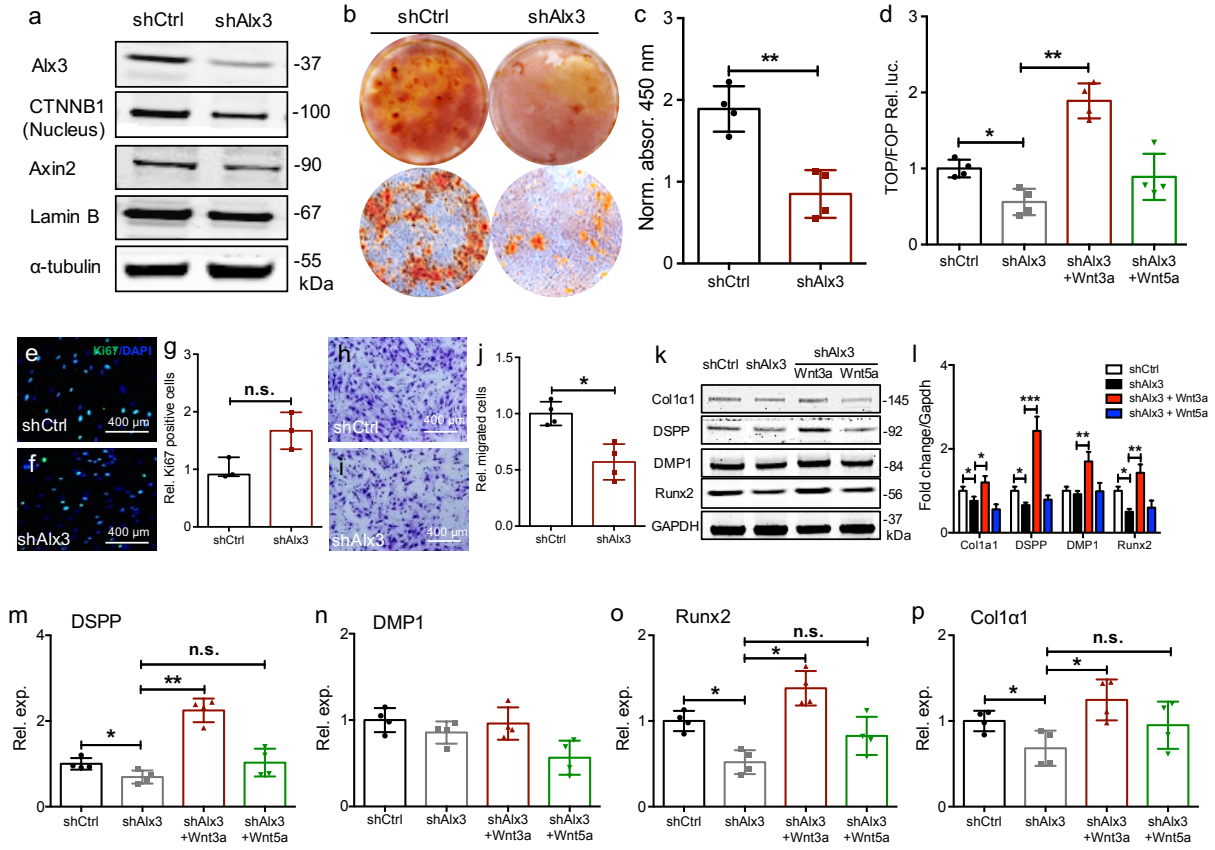


Supplementary Figure 2

Cell isolation, characterization and Alx3 overexpression. **a:** E16.5 tooth organ. **b,c:** E16.5 dental epithelium cells (Epi) and mesenchyme cells (Mes) isolated and plated. Scale bar, 800 µm. **d-k:** Co-expression of ameloblastin (Ambn), amelogenin (Amelx) and cytokeratin 14 (CK14) by isolated Epi cells. Scale bar, 40 µm. **l-q:** CD146 and vimentin expression by isolated Mes cells. Scale bar, 200 µm. **a-q:** n=5 biological independent samples. **r,s:** Phase contrast and immunofluorescence image of lentiviral transfected adult,

human dental-pulp mesenchyme stem/progenitor cells and lentiviral Alx3-GFP transfection. Scale bar, 800 μm. **t:** FACS sorted Alx3-GFP cells following three passages showing 98% Alx3-GFP positivity. **r-t:** n=3 biological independent samples.

Supplementary Figure 3

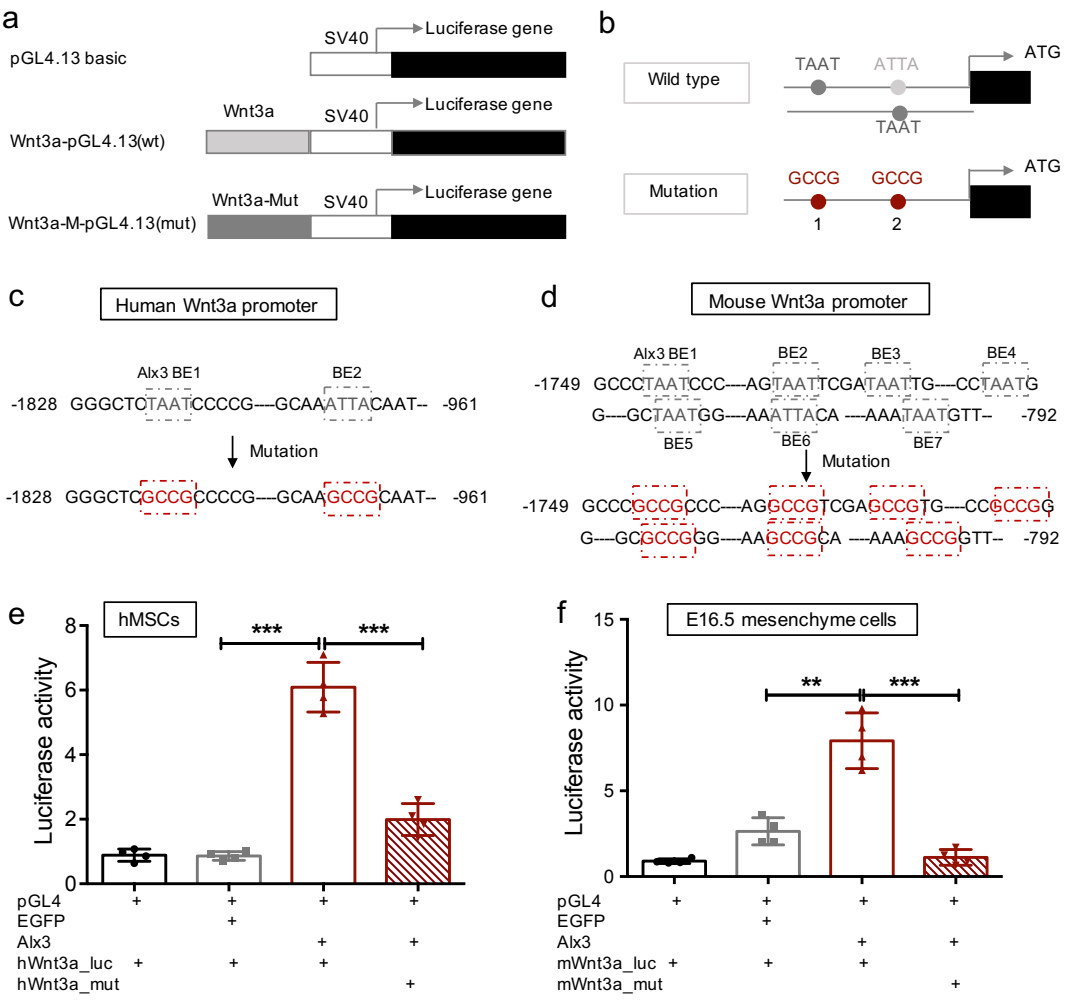


Supplementary Figure 3

Attenuated cell migration and differentiation upon Alx3 knockdown. **a:** Alx3 knockdown by shRNA (short-hairpin RNA) with nuclear CTNNB1 and Axin2 expression. n=3 biological independent samples. **b,c:** Alizarin Red-S staining and quantification (n=4 biological independent samples. Presented with mean ± SD; *P* value calculated by two-sided Student's t-test. **: $p<0.01$). **d:** Top/Fop Flash of Wnt activity following Alx3 knockdown by shRNA with or without exogenous Wnt3a (100 ng/ml) (n=4 biological independent samples. Presented with mean ± SD; *P* values calculated by one-way ANOVA with Bonferroni. *: $p<0.05$; **: $p<0.01$).

e-g: Ki67 immunofluorescence and quantification indicative of cell proliferation. (n=3 biological independent samples. Presented by median with range; *P* value calculated by Mann-Whitney test. n.s.: not significant). Scale bar, 400 μm. **h-j:** Transwell migration assay of Alx3 knockdown and shRNA controls, with relative migrated cell number quantification (n=4 biological independent samples. Presented with mean ± SD; *P* value calculated by two-sided Student's t-test. *: $p<0.05$). Scale bar, 400 μm. **k,l:** Western blot of collagen1α1, DSPP, DMP1 and Runx2 of shAlx3 cells with Wnt3a (100 ng/ml) or Wnt5a (200 ng/ml) supplement and quantification (n=4 biological independent samples. Presented with mean ± SD; *P* values calculated by one-way ANOVA with Bonferroni. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$). **m-p:** Real-time PCR of DSPP, DMP1, Runx2 and Col1α1 of shRNA control and shAlx3 cells with Wnt3a (100 ng/ml) or Wnt5a (200 ng/ml) supplement (n=4 biological independent samples. Presented with mean ± SD; *P* values calculated by one-way ANOVA with Bonferroni. *: $p<0.05$; **: $p<0.01$; n.s.: not significant).

Supplementary Figure 4

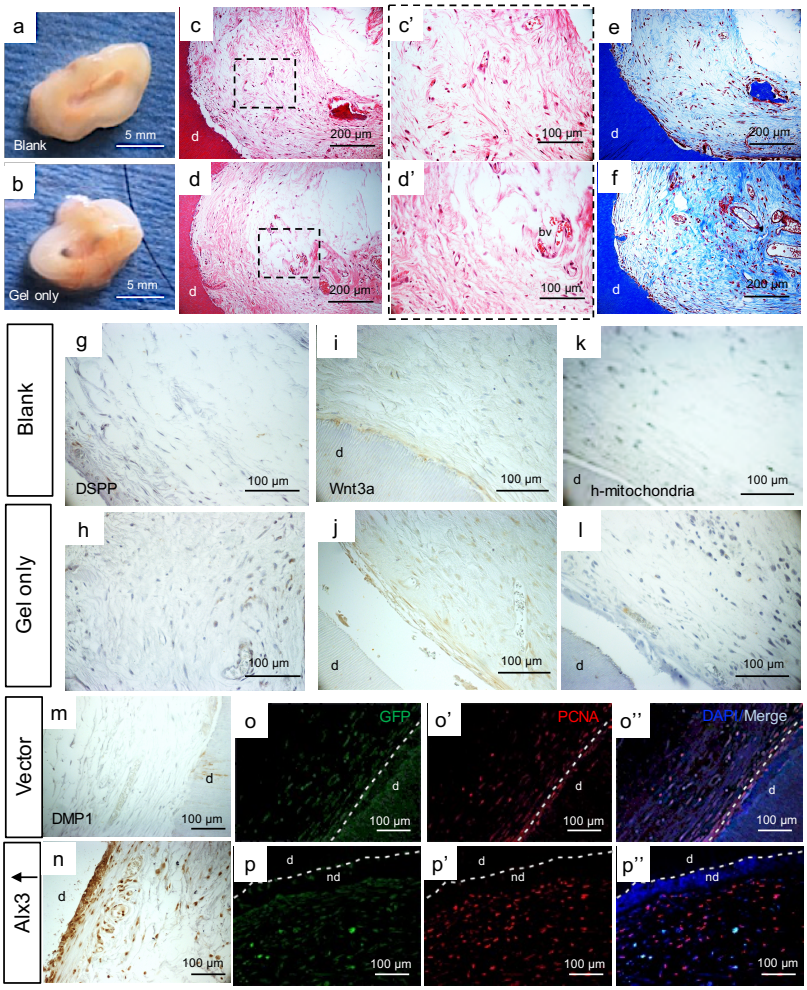


Supplementary Figure 4

Alx3 transactivates Wnt3a expression at transcriptional level. **a,b:** Wnt3a promoter luciferase reporter constructs. wt: wild type; mut: mutation. Mutation from Alx3 binding element TAAT or reverse chain ATTA to GCCG in Wnt3a promoter reporters. **c:** Human Wnt3a promoter reporter with two Alx3-binding elements (BE). **d:** Mouse Wnt3a promoter reporter with seven Alx3-binding elements. **e,f:** Wnt3a luciferase assay upon Alx3 transfection in both human dental-pulp mesenchyme stem/progenitor cells (**e**) and mouse E16.5

mesenchyme cells (**f**). (n=4 biological independent samples. Presented with mean ± SD; *P* values calculated by one-way ANOVA with Bonferroni. **: $p<0.01$; ***: $p<0.001$).

Supplementary Figure 5

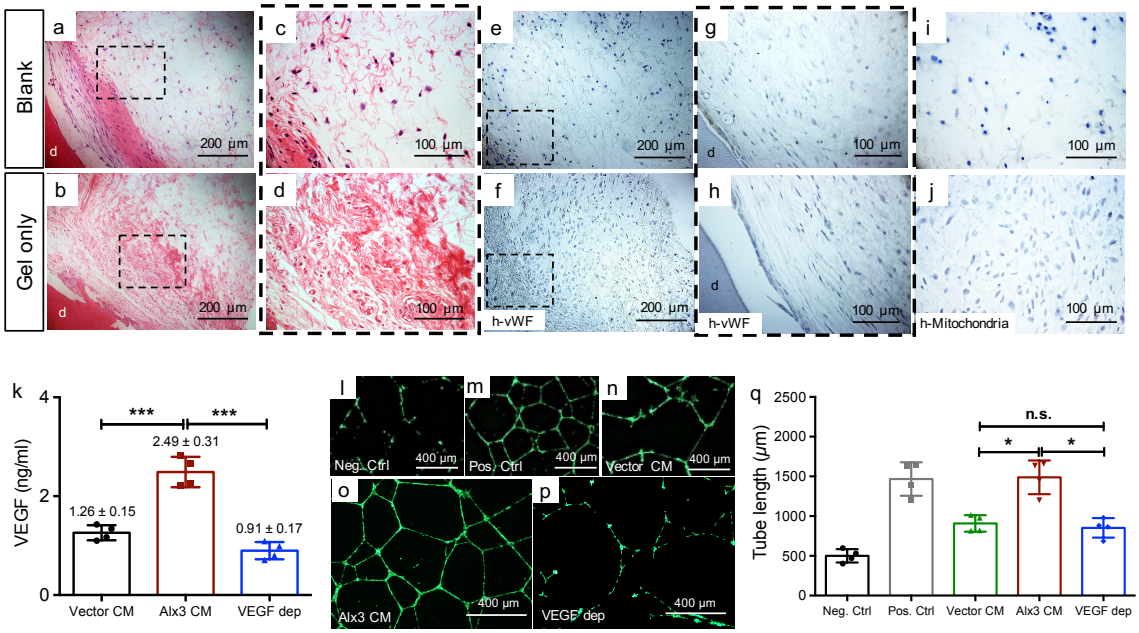


Supplementary Figure 5

Retrieved *in vivo* 8-wk implantation samples. **a,b:** Retrieved decellularized scaffolds only (Blank) and decellularized scaffolds with collagen gel (Gel only). Scale bar, 5 mm. **c-d':** HE staining. **c,d:** scale bar, 200 μm; **c',d':** scale bar, 100 μm. **e,f:** Masson's Trichrome staining. Scale bar, 200 μm. **a-f:** n=5 biological independent samples. **g,h:** DSPP (dentin sialophosphoprotein) immunohistochemistry of Blank and Gel only controls. **i,j:** Wnt3a immunohistochemistry of Blank and Gel only controls. **k-l:** Human mitochondria immunohistochemistry of Blank and Gel only controls. **m,n:** DMP1 (dentin matrix protein 1)

immunohistochemistry of vector control (**m**) and Alx3 overexpression (**n**) samples. **o-p":** Proliferating cell nuclear antigen (PCNA) immunofluorescence of vector control (**o-o"**) and Alx3 overexpression (**p-p"**) samples. **g-p":** n=3 biological independent samples. d: dentin; bv: blood vessel; nd: newly-formed dentin. White dash lines: boundary between native dentin and newly-formed dentin. Scale bar, 100 μm.

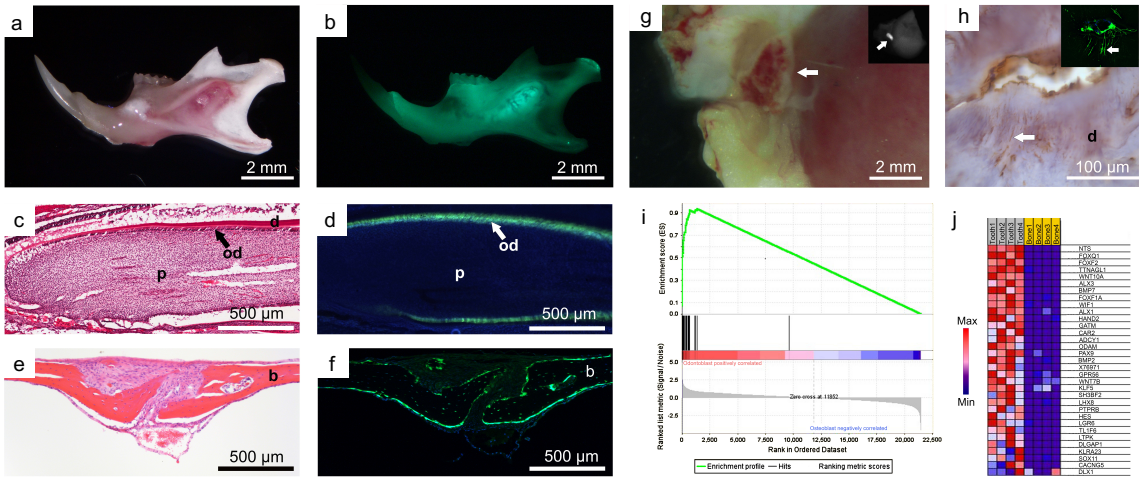
Supplementary Figure 6



Supplementary Figure 6

Retrieved *in vivo* 4-wk implantation samples and HUVEC angiogenesis assays. **a-d:** HE staining of decellularized scaffolds only (Blank) and decellularized scaffolds with collagen gel (Gel only). d: dentin. **a,b:** scale bar, 200 μm; **c,d:** scale bar, 100 μm. **e-h:** human von Willebrand factor (h-vWF) immunohistochemistry. **e,f:** scale bar, 200 μm; **g,h**: scale bar, 100 μm. **i,j:** human mitochondria immunohistochemistry. Scale bar, 100 μm. **a-j:** n=5 biological independent samples. **k:** ELISA of VEGF content in vector conditioned medium (CM), or Alx3 CM with or without VEGF depletion (VEGF dep). (n=4 biological independent samples. Presented with mean ± SD; *P* values calculated by one-way ANOVA with Bonferroni. ***: $p<0.001$). **l-p:** Tube formation (calcein staining) by HUVECs treated by vector CMs, Alx3 CM and VEGF depletion. n=4 biological independent samples. Scale bar, 400 μm. **q:** Tube length quantification (n=4 biological independent samples. Presented with mean ± SD; *P* values calculated by one-way ANOVA with Bonferroni. *: $p<0.05$; n.s.: not significant).

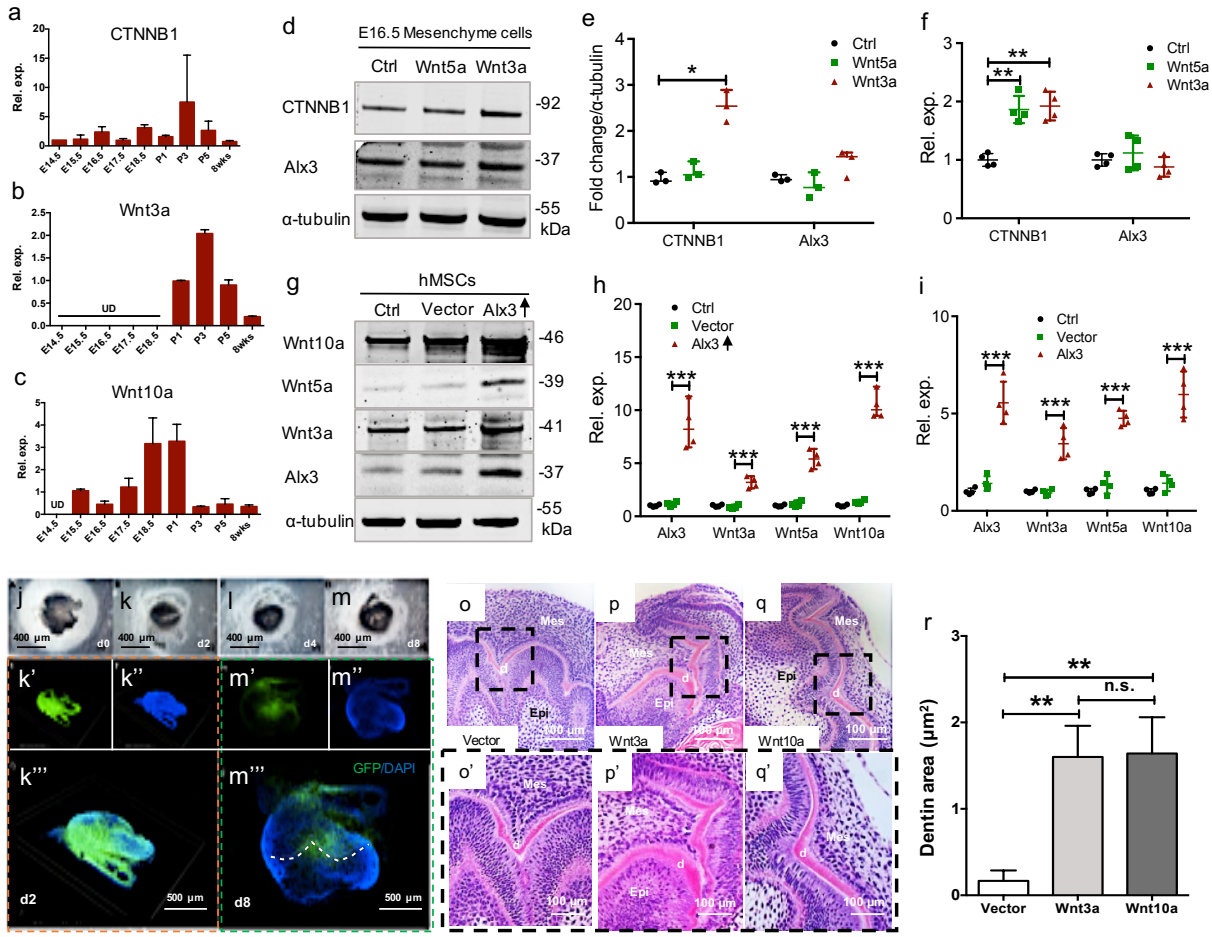
Supplementary Figure 7



Supplementary Figure 7

Identification of pivotal molecules in dentin development. **a:** Mandible of a transgenic 2.3 kb collagen type Iα1 chain (col1α1)-EGFP mouse. **b:** GFP expressed in mandibular teeth and bone. Scale bar, 2 mm. **c:** Dental pulp (p) of the mandibular incisor with odontoblasts (od) aligned immediately adjacent to mineralized dentin (d). **d:** GFP expressed in odontoblasts (od) but not the rest of dental pulp (p) cells. **e:** Calvarial bone (b) with marrow and cranial suture. **f:** GFP expressed in osteoblasts and osteocytes of the calvarial bone (b). Scale bar, 500 µm. **g:** GFP+ odontoblasts infused ectopically into the subrenal capsule of athymic mice in a collagen gel carrier (arrow). A radiographically visible mineral nodule formed in 6 wks (arrow in insert). Scale bar, 2 mm. **h:** Mineral nodule formed by infused GFP+ odontoblasts with GFP+ processes (arrow in insert) extending into DSPP (d) positive structures (arrow). Scale bar, 100 µm. **i,j:** Donor-matched odontoblasts and osteoblasts profiled by genome microarray followed by GSEA and pathway analyses. (n=4 biological independent samples. *P* values calculated by one-way ANOVA with Bonferroni).

Supplementary Figure 8



Supplementary Figure 8

Wnt ligand expression patterns in tooth development and upon Alx3 transfection in reconstituted E16.5 tooth organ. **a-c**: Real-time PCR of CTNNB1, Wnt3a and Wnt10a from E14.5 to postnatal 8 wks. UD: undetectable. n=4 biological independent samples. **d:** Western blot of CTNNB1 and Alx3 upon exogenous Wnt5a (200 ng/ml) or Wnt3a (100 ng/ml) treatment in isolated E16.5 mesenchyme cells, and quantification in **e**. (n=3 biological independent samples. Presented by median with range; *P* values calculated by Kruskal-Wallis test. *: $p<0.05$). **f:** Real-time PCR of CTNNB1 and Alx3 upon exogenous Wnt5a (200 ng/ml) or

Wnt3a (100 ng/ml) treatment in isolated E16.5 mesenchyme cells (n=4 biological independent samples. Presented with mean ± SD; *P* values calculated by one-way ANOVA with Bonferroni. **: $p<0.01$). **g:** Western blot of Wnt10a, Wnt5a, Wnt3a and Alx3 upon Alx3 overexpression in adult human dental-pulp mesenchyme stem/progenitor cells and quantification (**h**) (n=4 biological independent samples. Presented with mean ± SD; *P* values calculated by one-way ANOVA with Bonferroni. ***: $p<0.001$). **i:** Real-time PCR of Wnt5a, Wnt3a and Alx3 upon Alx3 overexpression (n=4 biological independent samples. Presented with mean ± SD; *P* values calculated by one-way ANOVA with Bonferroni. ***: $p<0.001$). **j-m**: Reconstituted tooth organ from day 0 to day 8. Scale bar, 400 μm. **k'-m''':** Confocal images of GFP-lentiviral transfected dental mesenchyme reconstituted with dental epithelium in organotypic culture. White dash line: boundary between epithelium and mesenchyme. Scale bar, 500 μm. **o-q':** Basement membrane and dentin formation in vector control, Wnt3a- and Wnt10a-overexpressed samples following 8-day organotypic culture. **j-q':** n=5 biological independent samples. Epi: epithelium; Mes: mesenchyme; d: dentin. Scale bar, 100 μm. **r:** Quantified dentin area (n=5 biological independent samples; Presented with mean ± SD; *P* values calculated by one-way ANOVA with Bonferroni; **: $p<0.01$; n.s.: not significant).

Supplementary tables

Supplementary Table 1. ChIP-PCR primer sequences.

Name	Primer	Sequence (5' --> 3')
DSPP-1	Forward	ACCTTTGGACATCTTGCTACTT
	Reverse	CTCTGTCACCCTGACATTTCTT
DSPP-2	Forward	TGAATGTGTGTGTGGGTAAGG
	Reverse	CAAGATGTCCAAAGGTGCATAAAG
DSPP-3	Forward	CACCAACCTCCACTGATGAA
	Reverse	CACCATTCTCTTGAACCCTAGAT
DSPP-4	Forward	GCCTTCCATTGTGGTCAGTA
	Reverse	GGCAGATTAAAGGGCCTAAGA
DSPP-5	Forward	GGGCTCAGAATACGTTCTATCTT
	Reverse	CCCTTACCCACACACACATT
Dmp1	Forward	ATAACTTGTCATAAGGGGTGG
	Reverse	CCATATTCTGTTGGTCAATCAA
Col1a1	Forward	ATGAAGGTCCATCCCTCCATTG
	Reverse	CTGGTTTTGTGCAACGAAGACG
Msx1	Forward	CGCCCAGTTAAAAGTCGAGGGA
	Reverse	CCATTGATGACTGCGGGGTTC
Msx2	Forward	CCTGTGTGCCTTATAGCTGAC
	Reverse	ACAAGGCAAGGTGCAGGCTATA
SFRP	Forward	TAGGCAACAAGAGCGAAACTG
	Reverse	CGGGGAGCCTGGATCATACTT
Wnt3a	Forward	AGTGCCTCTTAACCATCCCAGT
	Reverse	AGTGACCAGTCAACTCTCCAGCT
Wnt5a	Forward	ATCATACCTTGAGCACGACGAA
	Reverse	AAAGGGGTGAGGCAGAAAAGA
Lef1	Forward	AAAACCCACTGGAGACCTAG

	Reverse	TGGAGACAAGGGAACACACT
SMURF2	Forward	CGGGTGAGTGGTGAGAAAGA T
	Reverse	AGCCAGTGGACTGGGA TGTGA
BMP1	Forward	CCGAGACACCAGAGGCTAATT
	Reverse	GCAGGAGTGATGGGGTTACAT
BMPR2	Forward	ACCTGGGAGGTGGAGGTTGCA
	Reverse	GGCGGAGTCTTGCTCTGTCGA
Shh	Forward	ACGTGTTGTCTGGAGTAGCCTCT
	Reverse	ATATGCTGCGATGGTAGACAGG
GAPDH	Forward	CAATTCCCCATATCAGTCGT
	Reverse	TAGTAGCCGGGCCCTACTTT

Supplementary Table 2. Quantitative RT-PCR primer sequences.

Species	Name	Primer	Sequence (5' --> 3')
Human	Alx3	Forward	GCTCAGGGTAAAGCCCAAGGAG
		Reverse	TGGGAGCGACTGGGAATGGAAA
	DSPP	Forward	GTGGGGTTGCTACACATGAAAC
		Reverse	CCATCACCAGAGCCTGTATCTTC
	DMP1	Forward	TGTGAACTACGGAGGGTAGAGG
		Reverse	ACTGGGAGAGCACAGGATAATCC
	Runx2	Forward	ATGAAATGCTGGAGTGATGTGG
		Reverse	ATGAAGCCTGGCGATTTAGAGT
	Col1a1	Forward	GAGGGCCAAGACGAAGACATC
		Reverse	CAGATCACGTCATCGCACAAC
	GAPDH	Forward	TCAGCAATGCCTCCTGCAC
		Reverse	TCTGGGTGGCAGTGATGGC
Mouse	Alx3	Forward	AAGCCCCTCTCCTGCCCCCCACCCCCCGCG
		Reverse	TTTTATTTTGATCGATGTCCAATATGGTTTTCA
	Laminin1	Forward	ACTATGCCGTCAGCGATACAG
		Reverse	GGCACCAGCTTTGAATAATACGA
	Col IV	Forward	GGCCCCAAAGGTGTTGATG
		Reverse	CAGGTAAGCCGTTAAATCCAGG
	DSPP	Forward	AACTCTGTGGCTGTGCCTCT
		Reverse	TATTGACTCGGAGCCATTCC
	DMP1	Forward	CAGTGAGGATGAGGCAGACA
		Reverse	TCGATCGCTCCTGGTACTCT
	Col1a1	Forward	CCTGACGCATGGCCAAGAAGA
		Reverse	GCATTGCACGTCATCGCACA
	Msx1	Forward	AAGTTCCGCCAGAAGCAGTA
		Reverse	TCAGGTGGTACATGCTGTAG
	BMP4	Forward	CTGTTGTGTCCCCACTGAACT

	Reverse	ACCCCTCTACCACCATCTCC
Pax9	Forward	GCATCCGCTCCATCACCGACC
	Reverse	TGGACGCTCCCATCAGAGTGC
Shh	Forward	CTGGCCAGATGTTTTCTGGT
	Reverse	TAAAGGGGTCAGCTTTTTGG
Wnt3a	Forward	TTTGGAGGAATGGTCTCTCG
	Reverse	GACCCCTCCAAGTAGGAACC
Wnt5a	Forward	CTGGCAGGACTTTCTCAAGG
	Reverse	CTCTAGCGTCCACGAACTCC
GAPDH	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	TGTAGACCATGTAGTTGAGGTCA