Table S1. List of primers.

Purpose	Primer	
	Name	Sequence (5' – 3')
Cloning of *col10a1* knock-in donor plasmid	Mbait9bp_FP	TTACGCCAGTCGACCACCTCTG
	Cre_pA_RP	CCCCACTTTGTACAAGATCAAGCTGTGGCAGGG AAAC
	Cre_pA_FP	TCCCTGCCACAGCTTGATCTTGTACAAAGTGGGGGAT
	Cmlc_HA2_RP	GATGCGTGATCAACCTAAAAGCTTAAATCAGTTGTGT
	Cmlc_HA2_FP	CAACTGATTTAAGCTTTTAGGTTGATCACGCATCAGG
	Mbait2bp_RP	AGCGCGCAATTAACCCTCACTCC
	P2A_Cre_FP1	CCCTGGACCTATGTCCAATTTACTAACCGTACACCA
	stopHA2_Cre_RP1	GTGATCAACCTATCAAGCTGTGGCAGGGAAACCCTCT
	P2a_HA1_RP1	AGTTTGTAGCCGTTGAAGCGATGAGAAAACCG
	Cre_stop_HA2_FP1	GCCACAGCTTGATAGGTTGATCACGCATCAGGTTC
	HA1_p2a_FP1	CTCATCGCTTCAACGGCTACAAACTTCAGCCTGCTG
	Mbait_cre_FP	CCCCCCCACCTCTGGAACCGCAGCAGCCATGTC CAATTTACTAACCGTA
	PolyA_mbait_RP	CCCCCCCACCTCTGGAACCGCAGCAGCCAAAA AACCTCCCACACCTCC
	HR_cre_mbait_RP	GTGGCGTCGACTGTATCTCCCTATGGGGTGAAA CAGCAGA
	HR_polyA_mbait_FP	GTGTGGGAGGTTTTTTGGTGGGAGCACTTTTTT GCCGTTCC
Cloning of *col2a1a* knock-in donor plasmid	c2o1_mbait_FP1	TTACGCCAGTCGACCACCTC
	mbait_c2HA1_RP1	GGTTTCCTGTGGCCGGGCTATGGGGTGAAACAGC
	mbait_c2HA1_FP1	GTTTCACCCCATAGCCCGGCCACAGGAAACCTGAAGA
	c2HA1_p2a_RP1	GGCTGAAGTTTGTAGCCAAAAAGCAGACGGGGCCTATGT
	c2HA1_p2a_FP1	GCCCCGTCTGCTTTTTGGCTACAAACTTCAGCCTGCTGA
	cmlc2_c2HA2_RP1	GTTGCAATACTCAACTAAAGCTTAAATCAGTTGTGTT
	cmlc2_c2HA2_FP1	AACTGATTTAAGCTTTAGTTGAGTATTGCAACGGCCC
	c2HA2_mbait_RP1	ACGGCAAAAAAGTGCTCTATTGCCGGTGCCCTTAAGT
	c2HA2_mbait_FP1	AGGGCACCGGCAATAGAGCACTTTTTTGCCGTTCC
	c2o1_mbait_RP1	CAAGCTATGCATCAAGCTTGGTACCCTC
Cloning of *osr1* knock-in donor plasmid	c2o1_mbait_FP1	TTACGCCAGTCGACCACCTC
	mbait_o1HA1_RP1	AAAAATGGCTGAGGGCTATGGGGTGAAACAGC
	mbait_o1HA1_FP1	TTTCACCCCATAGCCCTCAGCCATTTTTAGCGG
	o1HA1_p2a_RP1	GCTGAAGTTTGTAGCCTTGACCTTGGCTGGCTT
	o1HA1_p2a_FP1	CCAGCCAAGGTCAAGGCTACAAACTTCAGCCT
	cmlc2_o1HA2_RP1	TGTCCCGGTCGCTTGACAAAGCTTAAATCAGTTGTG
	cmlc2_o1HA2_FP1	AACTGATTTAAGCTTTGTCAAGCGACCGGGACACT

	o1HA2_mbait_RP1	GGCAAAAAAGTGCTCCACAGGGGTTTTTCAAGGTCC
	o1HA2_mbait_FP1	CTTGAAAAACCCCTGTGGAGCACTTTTTTGCCGT
	c2o1_mbait_RP1	CAAGCTATGCATCAAGCTTGGTACCCTC
Incorporation of *col10a1* wobble bases	pA_Col10a1_e2wob_FP	GGGAGGTGTGGGAGGTTTTTTCTGCACTTTCTCTGGGTTC CTGATTGC
	Col10a1e2endwob_RP	AAAACCGGAAAACGTACAATGTACATTCTCAGCAGCAAA GACACCATTGG
Incorporation of *col2a1a* wobble bases	c2wob_FP1	AAAACCTCACGCTTGCCCATAGTCGAC
	c2wob_RP1	GTCGACTATGGGCAAGCGTGAGGTTTT
Incorporation of *osr1* wobble bases	o1wob_FP1	TTTGCTGCGATCAGCACAGGTGGGTCA
	o1wob_RP1	TGACCCACCTGTGCTGATCGCAGCAAA
Genotyping of *col10a1*$^{p2a\text{-}CreERT2}$ medaka	Col10a1_e2_1481_FP (FP1)	GGCAGCCCCATTAAGTTCGACC
	CreERT2_81_RP (RP1)	GGCGATCCCTGAACATGTCCAT
	Col10a1_i2_244_RP (RP2)	TGTCTCCGTTACAAAAGGTCACCG
Sequencing of *col10a1* knock-in at 3' and 5' homology regions	Col10a1_e2_1481_FP	GGCAGCCCCATTAAGTTCGAC
	CreERT2_81_RP	GGCGATCCCTGAACATGTCCAT
	Col10a1_i2_244_RP	TGTCTCCGTTACAAAAGGTCACCG
	GFP_66_RP	GTCGCCGTCCAGCTCGACCA
Sequencing of *col2a1a* knock-in at 3' and 5' homology regions	c2a1a_i53_FP (FP1)	AAGGGCCATCTTCTGAGTGGA
	CreERT2_81_RP (RP1)	GGCGATCCCTGAACATGTCCAT
	col2a1_end_RP1 (RP2)	AACTTGGTTTGCTTGGTCCCT
	GFP_66_RP (FP2)	GTCGCCGTCCAGCTCGACCA
Sequencing of *osr1* knock-in at 3' and 5' homology regions	osr1_i1_1296_FP (FP1)	TTGCACTGGAGAAGGGGTTTAG
	CreERT2_81_RP (RP1)	GGCGATCCCTGAACATGTCCAT
	osr1_end_RP1 (RP2)	TGGCTTTGATCATTCTGGGGC
	GFP_66_RP (FP2)	GTCGCCGTCCAGCTCGACCA

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Table S2. Sequence of guide RNAs (gRNAs).

Name	Sequence (5' – 3')
mbait gRNA	CCACCTCTGGAACCGCAGCAGCC
col10a1 gRNA	CCACTGCACTTTCTCTGGGTTCC
osr1 gRNA	TGACACACGTTTGCTGAGCGCGG
col2a1a gRNA	AACATCCCGCCTGCCAATCGTGG

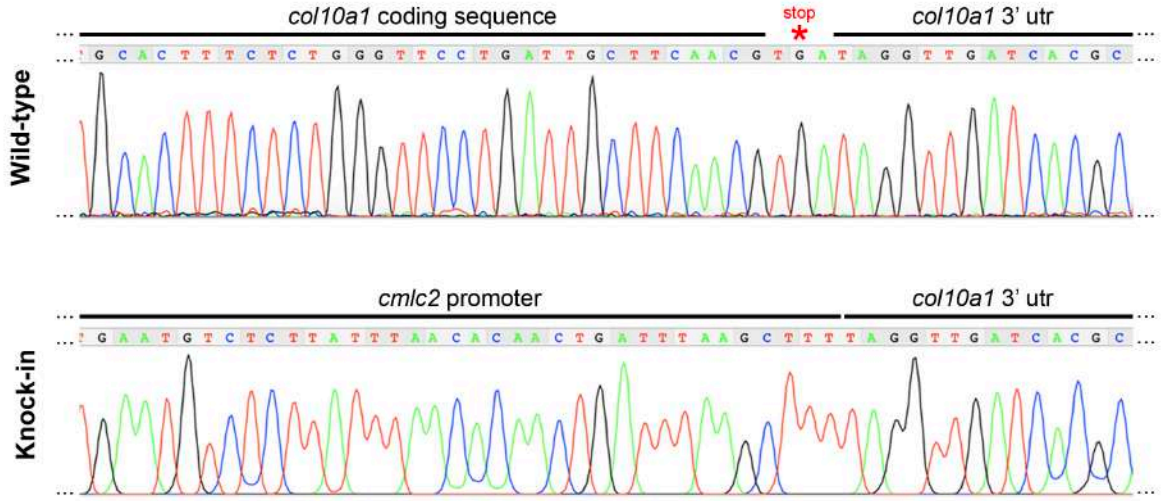


Fig. S1. DNA sequencing confirms precise knock-in at the 3' homology region in *col10a1*$^{p2a\text{-}CreERT2}$ medaka. In *col10a1*$^{p2a\text{-}CreERT2}$ medaka, *cmlc2* promoter sequence begins immediately upstream of the *col10a1* 3' untranslated region (utr).

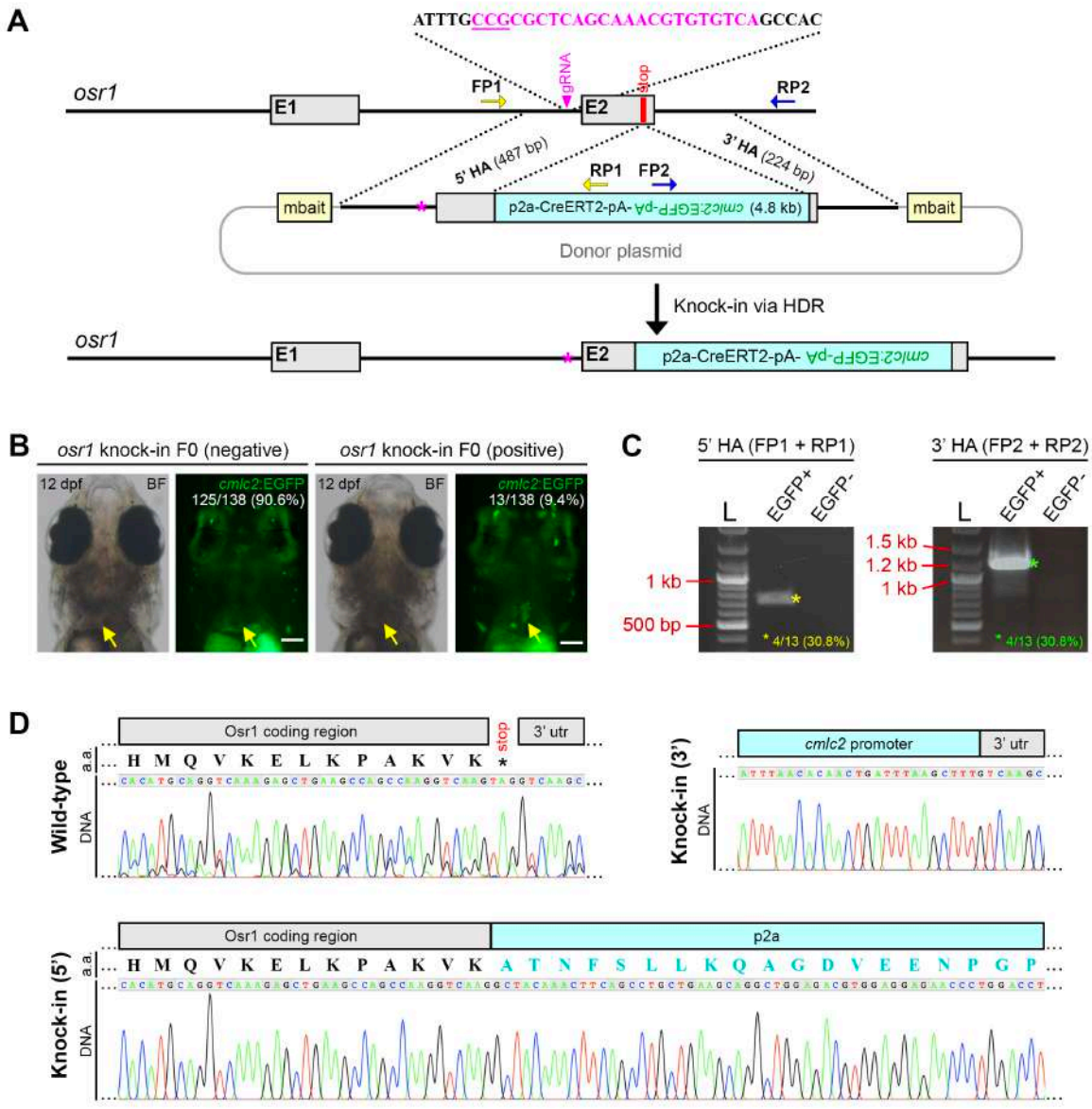


Fig. S2. Knock-in of p2a-CreERT2;*cmlc2*:EGFP into the medaka *osr1* locus using CRISPR/Cas9 and homology-directed repair (HDR).

A. Strategy for knock-in of p2a-CreERT2;*cmlc2*:EGFP into the *osr1* locus. A gRNA targeting *osr1* (indicated in magenta; PAM sequence underlined) was designed. A donor plasmid containing two mbait gRNA sites (yellow boxes) and two *osr1* homology arms (5' HA and 3' HA, 487 bp and 224 bp, respectively) flanking the p2a-CreERT2;*cmlc2*:EGFP sequence was used. Magenta asterisks indicate wobble bases incorporated at the *osr1* gRNA site. The endogenous *osr1* stop codon is indicated in red. Yellow arrows (FP1, RP1) and blue arrows (FP2, RP2) indicate primer pairs used for identification of precise knock-in at the 5' HA and 3' HA, respectively. **B.** Stereomicroscope fluorescent images showing *osr1* knock-in injected embryos (F0) with no

cmlc2:EGFP expression (left panel, yellow arrows; 125 out of 138 injected embryos) or mosaic *cmlc2*:EGFP expression (right panel, yellow arrows; 13 out of 138 injected embryos) in the heart. Scale bars: 100 µm. **C.** DNA agarose gel images showing PCR-genotyping of *cmlc2*:EGFP-positive (EGFP+) and *cmlc2*:EGFP-negative (EGFP-) larvae (F0) using the primer pairs as indicated in A. Yellow and green asterisks indicate the expected 712 bp and 1.2 kb, respectively. L, DNA ladder. Expected bands at the 5' and 3' homology regions were observed in 4 out of 13 EGFP+ larvae (30.8%). **D.** DNA sequencing of the 5' and 3' bands as shown in C (yellow and green asterisks, respectively) revealed precise knock-in at the *osr1* locus. Black asterisk indicates the endogenous *osr1* stop codon. a.a., amino acid.

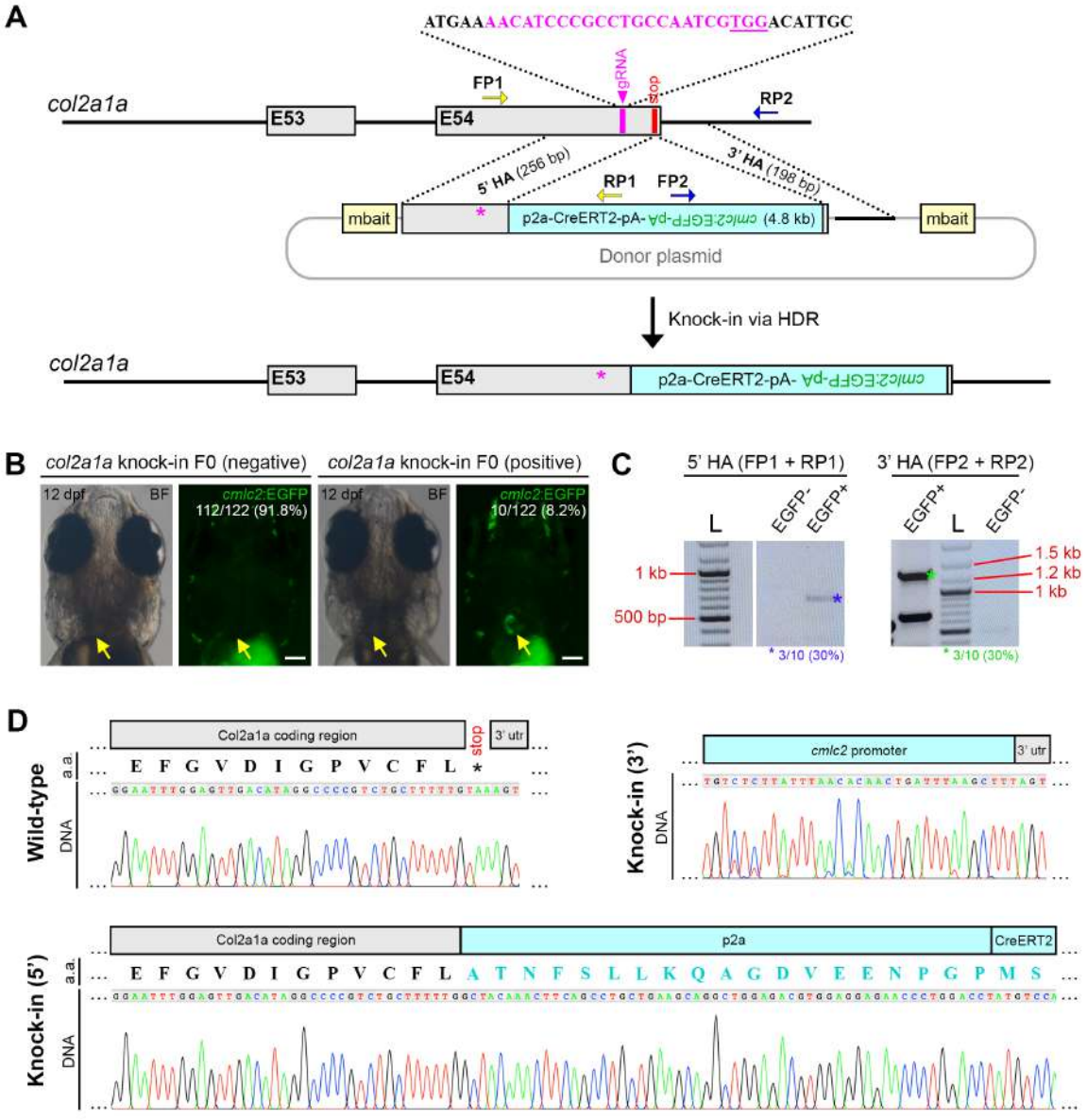


Fig. S3. Knock-in of p2a-CreERT2;*cmlc2*:EGFP into the medaka *col2a1a* locus using CRISPR/Cas9 and homology-directed repair (HDR).

A. For knock-in of p2a-CreERT2;*cmlc2*:EGFP into the *col2a1a* locus, a gRNA targeting *col2a1a* (indicated in magenta; PAM sequence underlined) was designed. A donor plasmid containing two mbait gRNA sites (yellow boxes) and two *col2a1a* homology arms (5' HA and 3' HA, 256 bp and 198 bp, respectively) flanking the p2a-CreERT2;*cmlc2*:EGFP sequence was used. Magenta asterisks indicate wobble bases incorporated at the *col2a1a* gRNA site. The endogenous *col2a1a* stop codon is indicated in red. Yellow arrows (FP1, RP1) and blue arrows (FP2, RP2) indicate primer pairs used for identification of precise knock-in at the 5' HA and 3' HA, respectively. **B.** Stereomicroscope fluorescent images showing *col2a1a* knock-in injected embryos with no *cmlc2*:EGFP expression (left panel, yellow arrows; 112 out of 122 injected embryos) or mosaic *cmlc2*:EGFP expression (right panel, yellow arrows; 10 out of 122 injected embryos) in the heart. Scale bars: 100 µm. **C.** DNA agarose gel images showing PCR-genotyping of *cmlc2*:EGFP-positive (EGFP+) and *cmlc2*:EGFP-negative (EGFP-) larvae (F0) using the primer pairs as indicated in A. Blue and green asterisks indicate the expected 612 bp and 1279 bp, respectively. L, DNA ladder. Expected bands at the 5' and 3' homology regions were observed in 3 out of 10 EGFP+ larvae (30%). **D.** DNA sequencing of the 5' and 3' bands as shown in C (blue and green asterisks, respectively) revealed precise knock-in at the *col2a1a* locus. Black asterisk indicates the endogenous *osr1* stop codon. a.a., amino acid.

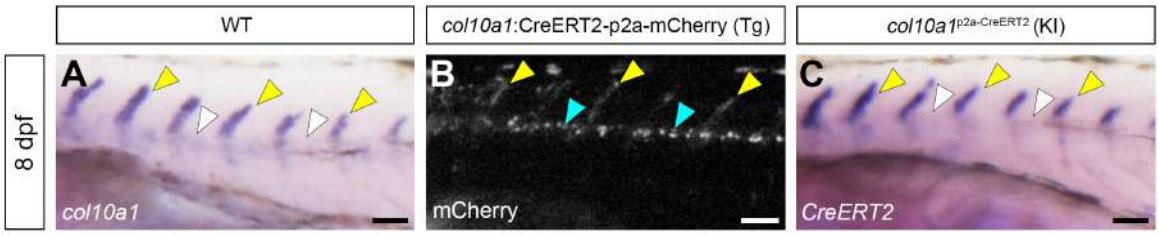


Fig. S4. Expression of endogenous *col10a1*, transgenic *col10a1*:CreERT2-p2a-mCherry and *CreERT2* in the *col10a1*$^{p2a\text{-}CreERT2}$ knock-in line at the medaka vertebral column. Lateral view of the 8 dpf medaka trunk showing whole-mount RNA *in situ* hybridization (WISH) of *col10a1* in wild-type (WT) medaka (A), mCherry fluorescence in *col10a1*:CreERT2-p2a-mCherry transgenic (Tg) medaka (B) and WISH of *CreERT2* in *col10a1*$^{p2a\text{-}CreERT2}$ knock-in (KI) medaka (C). Yellow arrows in A-C indicate expression in neural arches. Cyan arrows in B indicate expression in spinal cord neurons. White arrows in A, C indicate absence of expression in spinal cord neurons. Scale bars: 50 μm.

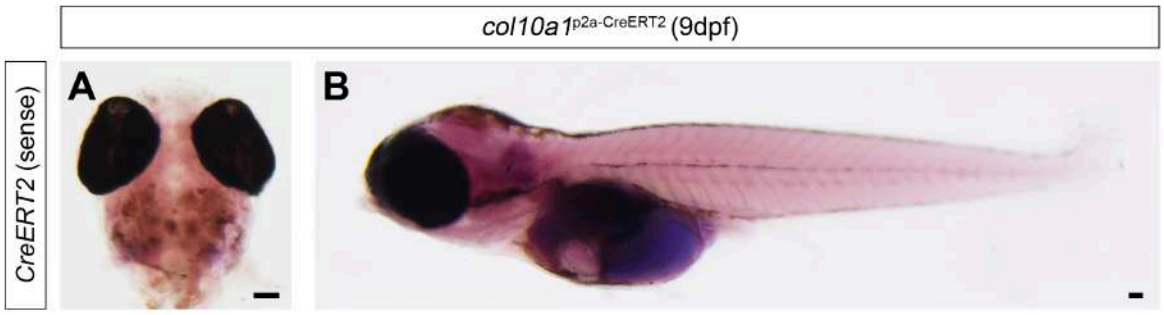


Fig. S5. Whole-mount RNA *in situ* hybridization using *CreERT2* sense riboprobes on 9 dpf *col10a1*$^{p2a\text{-}CreERT2}$ larvae. No specific staining was observed in the head (A, ventral view), trunk and caudal fin (B, lateral view). Scale bars: 100 µm.

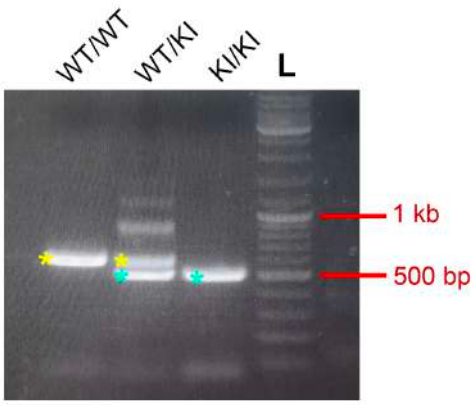


Fig. S6. DNA agarose gel showing genotyping of heterozygous *col10a1*$^{p2a\text{-}CreERT2}$ (WT/KI), homozygous *col10a1*$^{p2a\text{-}CreERT2}$ (KI/KI) and a wild-type sibling (WT/WT). Genotyping was performed via PCR using the primers indicated in Fig. 1 (FP1, RP1 and RP2). For WT/WT, a single 596 bp band was observed (yellow asterisk on the first lane). For KI/KI, a single 505 bp band was observed (cyan asterisk on the third lane). For WT/KI, both the 596 and 505 bp bands were observed (yellow and cyan asterisks, respectively). L, DNA ladder.

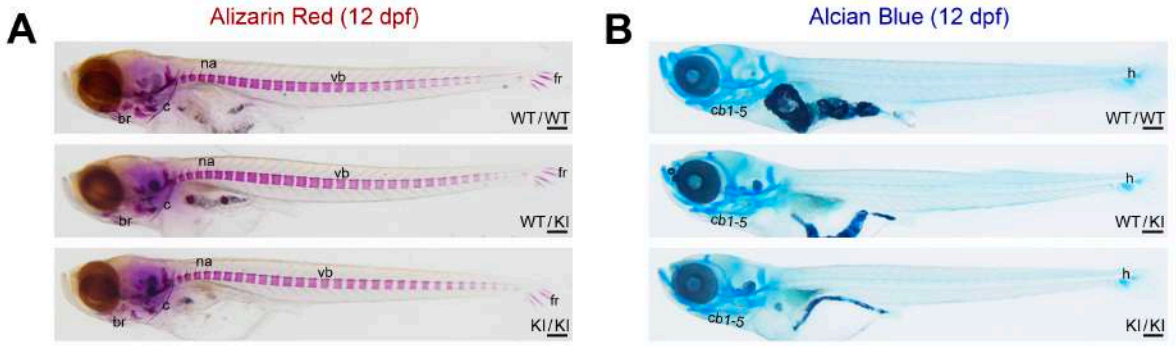


Fig. S7. Alizarin Red bone staining and Alcian Blue cartilage staining on 12 dpf heterozygous *col10a1*$^{p2a\text{-}CreERT2}$ (WT/KI), homozygous *col10a1*$^{p2a\text{-}CreERT2}$ (KI/KI) and WT siblings (WT/WT). Bone (A) and cartilage (B) formation were normal in WT/KI and KI/KI larvae compared to WT/WT controls. Images show the lateral view. br, branchiostegal ray. cb1-5, ceratobranchials 1-5. c, cleithrum. fr, fin ray. h, hypural. na, neural arch. vb, vertebral body. Scale bars: 200 µm.

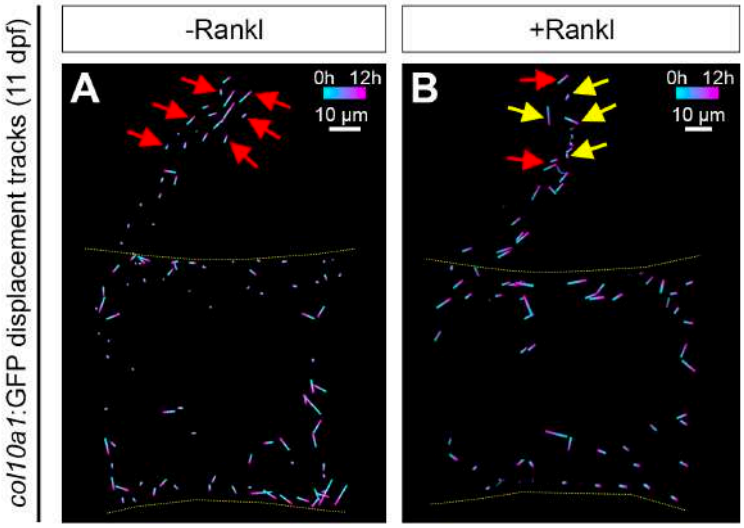
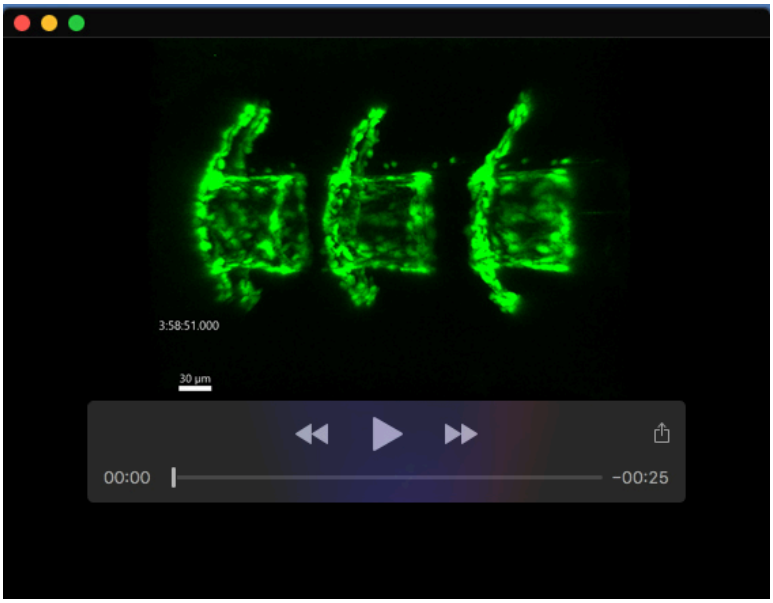


Fig. S8. Displacement tracks of *col10a*1:GFP cells in a vertebra of the vertebral column in non-Rankl and Rankl-induced conditions at 11 dpf. Tracks were generated from a 12 h time-lapse videos at 11 dpf in the absence of Rankl induction (A) or 2 days after Rankl induction (B). Yellow dotted lines demarcate the vertebral body. Arrows indicate *col10a1*:GFP cells at the neural arch. Yellow arrows indicate cells migrating ventrally (towards the vertebral body). Red arrows indicate cells migrating dorsally (away from the vertebral body). Scale bar: 10 µm.



Movie 1. Time-lapse video showing *col10a*1:GFP osteoblast progenitors in medaka vertebral column beginning at 11 dpf. Time-lapse duration: 12 hours. Scale bar: 30 µm.