Promethazine downregulates Wnt/β-catenin signaling and increases biomechanical forces of injured Achilles tendon in early stage of healing

Competing Interests:
The authors declare that no competing interests exist.

Running title: Promethazine for tendon injury

Supplementary Table S1. Primer sequences for rat genes

Gene	Forward primer	Reverse primer
Axin2	CTGGCTATGTCTTTGCACCA	AGGAGGGATTCCATCTACGC
Scx	TCATCCCGACCGAGCCAGCA	CCGCAGGCTTCACCCACCAG
Mkx	TTTACAAGCACCGTGACAACCC	ACAGTGTTCTTCAGCCGTCGTC
Tnmd	TGGAAATGGCACCGATGAAAC	GCAGGAACCCAAATCACTGACTG
Wnt1	GGGTTTCTGCTACGTTGCTACT	GGAGGTGATTGCGAAGATAAAC
Wnt3	AGTCATTTCCAACCTCAAGTGG	CAGGCTGTCATCTATGGTGGTA
Wnt4	ATTGAGGAGTGCCAATACCAGT	CTCTCGGACGTCTACAAAGGAC
Wnt7a	CGAACCCTCATGAACTTACACA	GGGTCCTCTTCACAGTAATTGG
Wls	TGGGATTTCCATGACCTTTATC	ATGATGAAAGCCATAGCCAGTT
Dkk1	TTGCCGAAAGCGCAGGAAC	CCTCGAGGTAAATGGCTGTG
Col1a1	CGGCAGAAGTCTCAAGATGGTGGCCG	CTCTCCGCTCTTCCAGTCAGA
Col3a1	TGATGGGATCCAATGAGGGAGA	GAGTCTCATGGCCTTGCGTGTTT
Il1b	AAAAATGCCTCGTGCTGTCT	TCGTTGCTTGTCTCTCCTTG
Il6	GCCCTTCAGGAACAGCTATGA	TGTCAACAACATCAGTCCCAAAGA
Mmp1	GGCTACCAGCTCATACAGTTTCC	CCTCATAGCACTCAGGGTTTCAG
Mmp2	CACCACCGAGGATTATGACC	GTTGCCCAGAAAAGTGAAGG
Mmp3	CAGGCATTGGCACAAAGGTG	CTGAAACACACGACGCCTTC
Tnfa	AGAGCCCCCAATCTGTGTC	TTCAGCGTCTCGTGTGTTTC
Fn1	GGGCTTTGGCAGTGGTCATTT	CTCATCCGCTGGCCATTTTCTC
Ctgf	CGGAGCGTGATCCCTGCGAC	GGTGCACCATCTTTGGCAGTGC
Dcn	GACAACAACAAACTCCTCA	AGAAGTCATGCTCCCAAA
Blvrb	GTATGACTAGGACCCTGGCTG	AAATTGATTGATCCCTCCATGTGTG
Dpyd	GAATACAAGCTCATGCAACTCTC	GCTTCTCACAGGTAAAGCAGT
Ca1	GTGCAGTTGGTTATTCCAAATATCA	TGGTTGTTTCCGTTGGCAAT
Gapdh	GGGTGTGAACCACGAGAAAT	ACTGTGGTCATGAGCCCTTC

Legends for Supplementary Figures

Fig. S1. A rat model of injured Achilles tendon.

(A) The right Achilles tendon was injured with a dermal punch (1.5 mm in diameter) at the midpoint between the calcaneus and the gastrocnemius muscle. A 15-ml Falcon tube is placed beneath the leg. Temporal profiles of our analyses are indicated on the right side. **(B)** Relative expressions of *Scx*, *Mkx*, and *Tnmd* (n = 3 tendons each) on postoperative day 14. Each mRNA expression was normalized for *Gapdh* mRNA, and also for the mean of sham-operated tendons. No statistical difference by Student's t-test. **(C)** Representative immunostaining for β-catenin, Scx or Tnmd (green) with DAPI (blue) in the serial sections of the injured site. The upper left injured site, where the DAPI-positive cells are accumulated and expression of β-catenin is low, is indicated by a dotted line. The lower right area where Tnmd expression is high is indicated by a solid line. **(D)** Representative images of double immunostaining for Scx (green) and β-catenin (red) with DAPI (blue) in a section of the injured site. Percentages of Scx- and/or β-catenin-positive cells per DAPI-stained cells are indicated in a pie chart (n = 311 DAPI-stained cells in 3 tendons). Scale bar = 10 mm in **A**, 200 μm in **C**, and 10 μm in **D**.

Fig. S2. The effects of IWR-1 and promethazine (PH) on the sham-operated rat Achilles tendon.

(A, B) Mean and SEM (n = 3 rats each) of Bonar scores of sham-operated Achilles tendons treated with IWR-1 **(A)** and PH **(B)** on postoperative weeks 2 are indicated. **(C, D)** Mean and SEM (n = 3 rats each) of peak force, stiffness, peak stress, elastic modulus, and cross-sectional area of sham-operated Achilles tendons treated with IWR-1 **(C)** and PH **(D)** on postoperative weeks 2 are indicated. **(E, F)** Mean and SEM (n = 3 rats each) of peak force, stiffness, peak stress, elastic modulus, and cross-sectional area of sham-operated **(E)** and injured **(F)** Achilles tendons treated with PH on postoperative weeks 8. No statistical difference by Student's t-test.

Fig. S3. Promethazine (PH) did not change the expressions of genes for Wnt ligands (*Wnt1*, *Wnt3*, *Wnt5*, and *Wnt7a*), a Wnt secretion mediator (*Wls*), and a Wnt inhibitor (*Dkk1*) in rat tendon-derived cells (TDCs).

Relative expressions of *Wnt1*, *Wnt3*, *Wnt5*, *Wnt7a*, *Wls*, and *Dkk1* in TDCs treated with the indicated concentration of PH for 48 h. Each mRNA expression was normalized for *Gapdh* mRNA, and then for the mean of untreated cells. The gene expressions are indicated by mean and SEM (n = 6 wells each). P-values by the Jonckheere-Terpstra trend test to evaluate dose responsiveness are indicated at the top of each graph.

Fig. S4. Promethazine (PH) suppressed *Col3a1*, *Il1b*, *Mmp2*, and *Fn1* in the injured rat Achilles tendon.

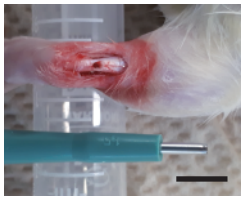
(A) Staining of vehicle- and PH-treated injured tendons with DAPI (blue). PH treatment did not change the number of DAPI-stained cells. Scale bar = 10 μm. **(B-E)** Relative expressions of tendon-related genes **(B)**, inflammatory genes **(C)**, fibrosis-related genes **(D)**, and genes that were previously reported to be upregulated in tendon injury[1] **(E)** on postoperative day 14 in vehicle- and PH-treated injured tendons. Each mRNA expression was normalized for *Gapdh* mRNA, and then for the mean of vehicle-treated tendons. Mean and SEM are indicated (n = 3 tendons each). *$p < 0.05$ and **$p < 0.01$ by Student's t-test.

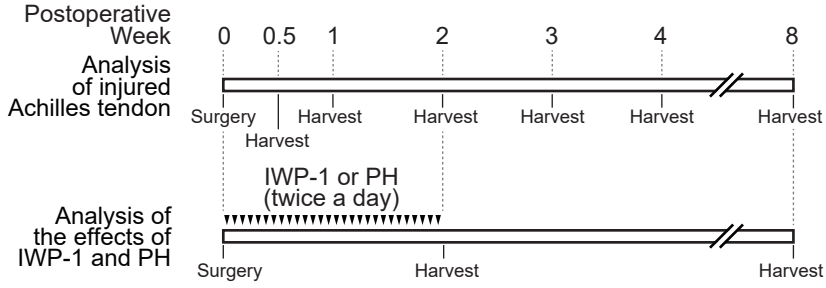
Reference

1. Shen H, Yoneda S, Sakiyama-Elbert SE, et al. Flexor Tendon Injury and Repair. The Influence of Synovial Environment on the Early Healing Response in a Canine Model. *J Bone Joint Surg Am.* 2021;103(9):e36.

Supplementary Fig. 1

A





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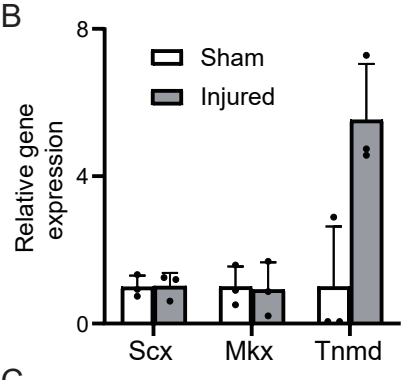

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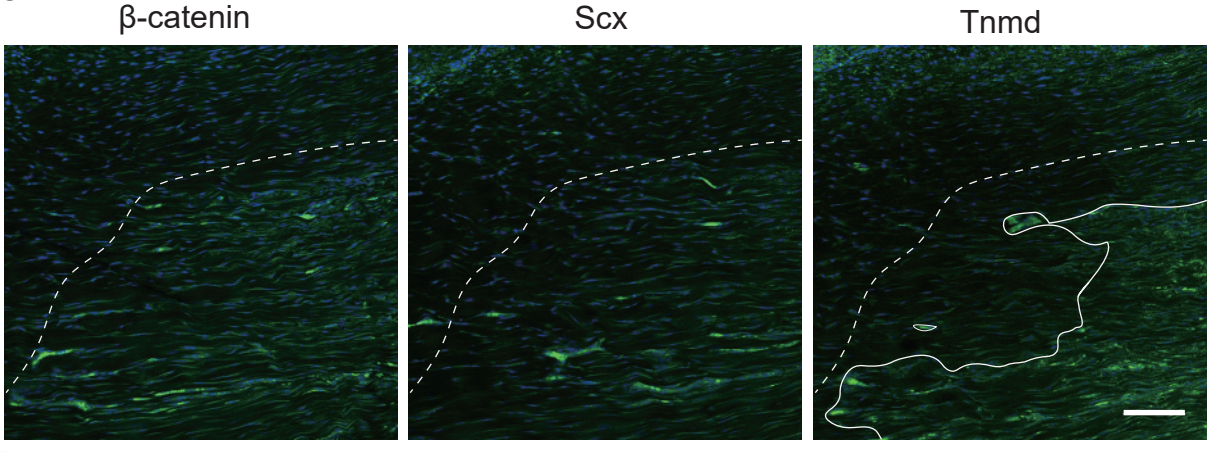

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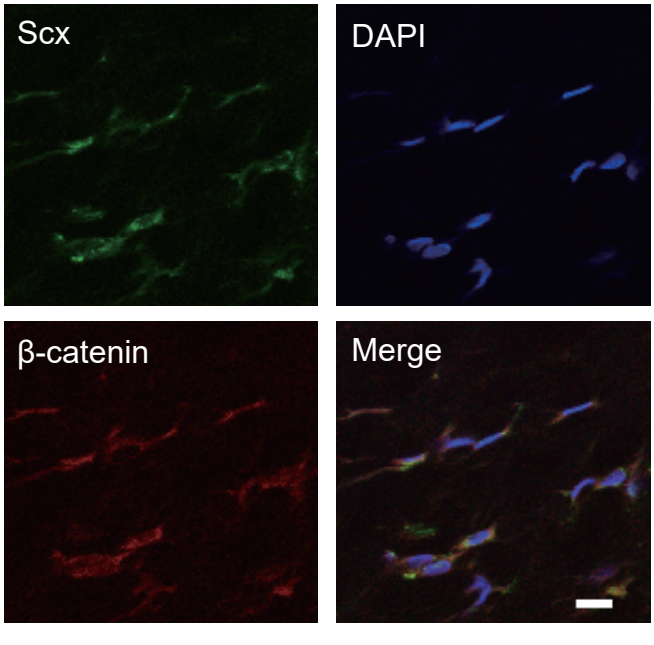
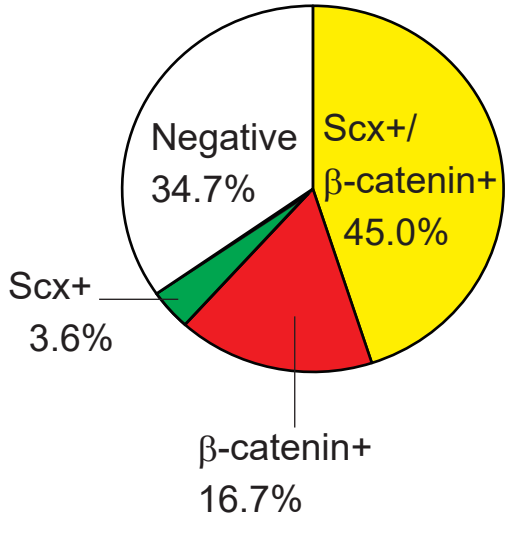
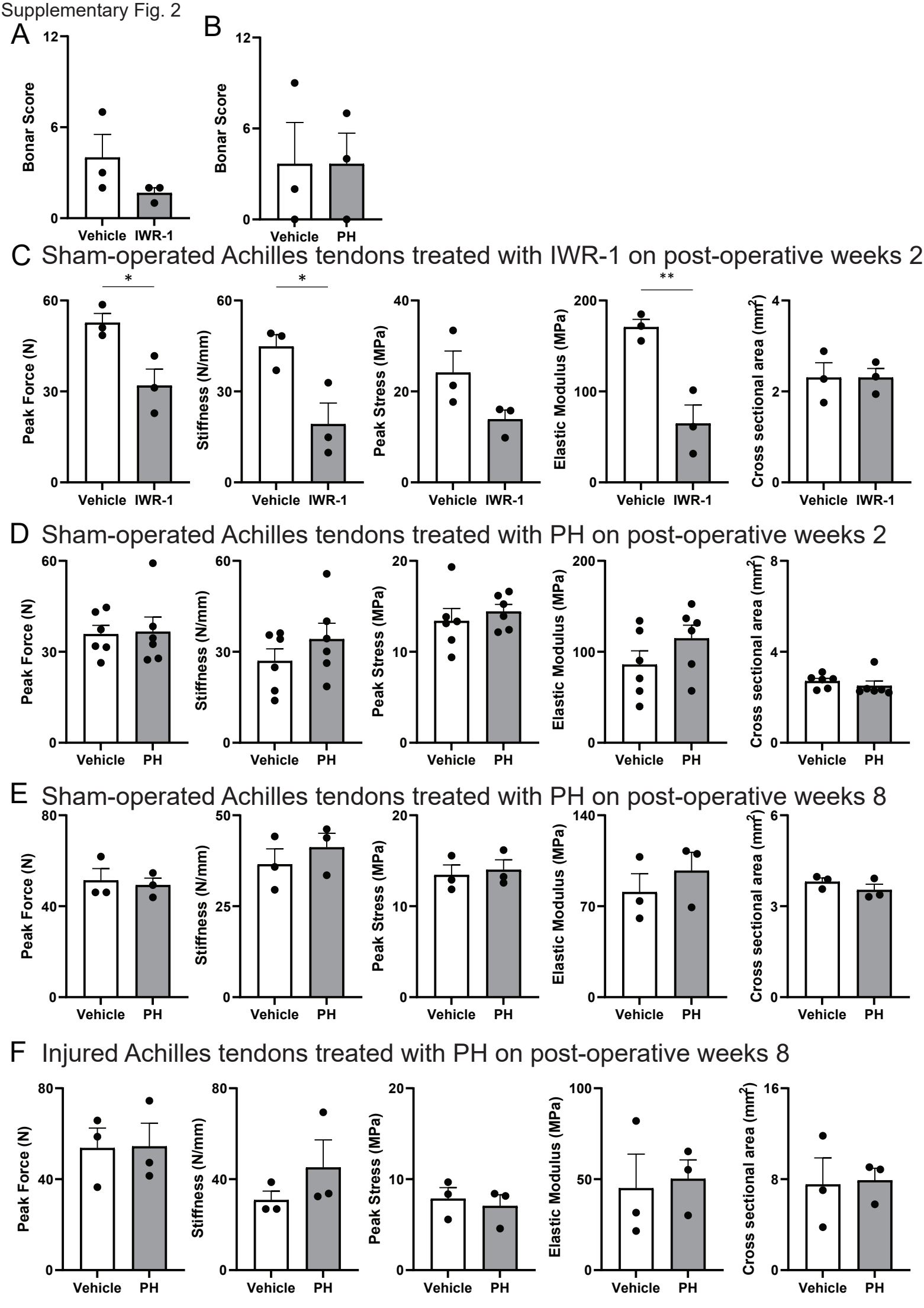
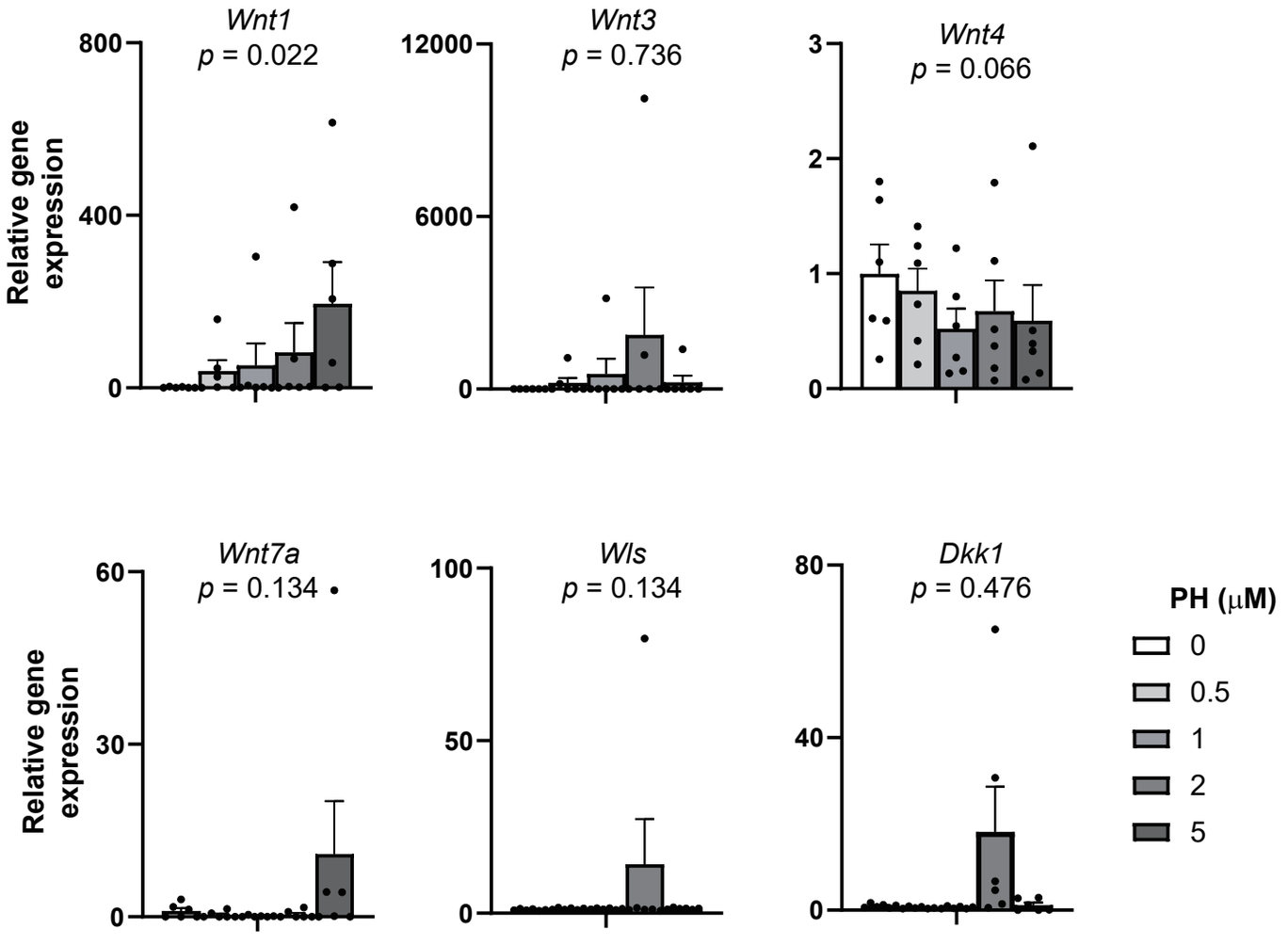




Supplementary Fig. 2



Supplementary Fig. 3



Supplementary Fig. 4