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Supplemental Information

Genomic Knockout of Two Presumed Forelimb *Tbx5* Enhancers Reveals They Are Nonessential for Limb Development

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Supplemental Figures and Legends

Mouse *Tbx5* intron 2 gene edited allele (357 bp deletion):

KEY: **EXONS (BOLD)**
INTRONS (NORMAL TYPE)
N_{20}(sgRNA)**NGG(PAM)**
REPORTED: HOX BINDING SITES, RARE, Tcf/Lef (Wnt/β-catenin)
GENOTYPING PRIMERS
DELETED REGION (357 bp)

mm10: chr5:119836798-119838792 Tbx5-intron2-Δ357

CTTCAGCTTGGAGTGAAGGGTCAAACAGCTTGTCCCCTATGTCGCTAGACACTCTCCAACTCCCTCTCTTGTCCTCAG**AATAGAACCTCGCGCGGGCACAGGAAC**
CCCTGCGCACCATGGCCGATACAGATGAGGGCTTTGGCCTGGCGCGCACGCCTCTGGAGCCTGATTCCAAAGACAGGTCTTGCGATTCGAAACCTGAGAGTGCTC
TGGGGGCTCCCAGCAAGTCTCCATCATCCCCGCAGGCTGCCTTCACCCAGCAGGTAAGAAAAGCCGGCCCAGCGGCAGGCGTAGACCGAGGACTGAGCCCAGCTC
TCTGCTAACGGTGCAGGGGGGTGGGCAGGGCCAGCTATTCCCTCTATTTCCTGGCTAGTCTCACCAAATCTCCCCCAATTCCACTCTATCCCAATCCACTTTATC
GAGGCGTTTATCTTTATGGCCTGCGTATTTTAGAAA**CCT**ATTTACATTATTGGAGAGCTCTCAATTATCCGACTATTTGGGGTCAGCTAACAACGGGCCAGATAA
TTCTCTCCCAGCTGCCCTGGGTATGCCTTATTAAGGGTCGGGTCAGCGGCCGCTTCAGGCCGGCATGAGCGATGACTTGACCCTCCTTAGATCTTTCTTAGCCTT
CGCCTCGGCTCGCCTGCTCTGCGCAACCCGCCTCGCCCCTCATACAGTACCATTTTATCTTGCTCCGGGGCCTGGAGGAGCTCCCTGACCCGCCGAGGAGAGCACA
CAGGGGCTGGAACCAAGGCACCCAGAGCCCGGCTGACTGGAAGTTGGTCCGGAGTGGAGGGAGAGGTCTTGTGGTCTGTTATTTGAG**TGG**CCATCGTTTTTTGGG
GGCGCTAAGTAGGTTTAAAACTAGGTGTCACTGGGAAGGCTTGACCAGAGAGATGAGTTTGATCTAGAAATCTTGTCCAATTGATGTGTGTATAGCTTTTAATAA
AAGCAAAGAGGCTTAAGAATAGTTTGGGGAAATTTACATAACATTTTGAAAAGGGAAAACAAGTTCACTTTTTAGTCGGTGGTTGATAATTGTTTCACAATGAAG
AGTAGTGTCTGACTGTTTTGGTATTGGTTTTTAGAAATAACTTCCAGTGGAAAGAGAAAATGTTAAAAGGCTTAATTGGTTGCCCCAAGTCAAAGAAAGGCTGGG
CGGTGGTGCTAGATAAGGCAAGAGTCTGGAAAGATTGTTAATATAGACACTGAGGGGGAAAACTACTTTTTTTGGTCCTTGAGAAGAAAAACGATTTTAAAATTG
AAAATTTCTACATAGTGGCTCACATGTGTTGGAATCTTAGCACTTAGGAGGGTGAGGCAGGAGGGTTGCCAAGAGTTTGAGACCACTTTTATGCGCTGCATAGTG
AATTCCAGGCCTGTCTGGGCTGCAGAGCAAGACTTTGTCTCCAATATTAATAACAACAATAATATTGAAAATTTTTGTATGTTCATATTTAGACCCAGTGATGAC
GGACAGCGATGTATTTGCATAGCTGTGTGTTTTGCATCTGGGTGACAATACAAAAATATCCTAGTGGGGTTTTCTAGATGGATCCCCTCCCAATCATGTTTCAGC
TAAAAATGGATCCAAATTAGATGGCCTGGTCCTCTAGGGGGGAGCTCTGTGGAGGGGAATAATTCCCAGGCCTCAATCTGGTGGAAGAGACAGCAGGTACTAAGG
ACTGAGATGCTGCTGGACGCAAGCTCCAACTCACCTAGGGAGCTGTAGGCAGATGCCATGGGTCGCCCTAGACTTTCCATCACTCCCCAGAAGTCAGAGCGCTCT
TTCTTTAACCGCACAGACTTTGGTAGGTACTTGGCCTCTGCATATAGGGGGGAGTTTGAGAAAGGATTCC**CCGCTAAGACCCATCACTGA**AAATTTTCTCTTTGT
CTATCAAG**GGCATGGAAGGAATCAAGGTGTTTCTTCATGAACGTGAACTGTGGCTGAAGTTCCACGAAGTGGGCACAGAGATGATCATCACCAAGGCAGGGAG**GT

Mouse *Tbx5* intron 2 gene edited allele (548 bp deletion):

KEY: **EXONS (BOLD)**
INTRONS (NORMAL TYPE)
N_{20}(sgRNA)**NGG(PAM)**
REPORTED: HOX BINDING SITES, RARE, Tcf/Lef (Wnt/β-catenin)
GENOTYPING PRIMERS
DELETED REGION (548 bp)

mm10: chr5:119836798-119838792 Tbx5-intron2-Δ548

CTTCAGCTTGGAGTGAAGGGTCAAACAGCTTGTCCCCTATGTCGCTAGACACTCTCCAACTCCCTCTCTTGTCCTCAG**AATAGAACCTCGCGCGGGCACAGGAAC**
CCCTGCGCACCATGGCCGATACAGATGAGGGCTTTGGCCTGGCGCGCACGCCTCTGGAGCCTGATTCCAAAGACAGGTCTTGCGATTCGAAACCTGAGAGTGCTC
TGGGGGCTCCCAGCAAGTCTCCATCATCCCCGCAGGCTGCCTTCACCCAGCAGGTAAGAAAAGCCGGCCCAGCGGCAGGCGTAGACCGAGGACTGAGCCCAGCTC
TCTGCTAACGGTGCAGGGGGGTGGGCAGGGCCAGCTATTCCCTCTATTTCCTGGCTAGTCTCACCAAATCTCCCCCAATTCCACTCTATCCCAATCCACTTTATC
GAGGCGTTTATCTTTATGGCCTGCGTATTTTAGAAA**CCT**ATTTACATTATTGGAGAGCTCTCAATTATCCGACTATTTGGGGTCAGCTAACAACGGGCCAGATAA
TTCTCTCCCAGCTGCCCTGGGTATGCCTTATTAAGGGTCGGGTCAGCGGCCGCTTCAGGCCGGCATGAGCGATGACTTGACCCTCCTTAGATCTTTCTTAGCCTT
CGCCTCGGCTCGCCTGCTCTGCGCAACCCGCCTCGCCCCTCATACAGTACCATTTTATCTTGCTCCGGGGCCTGGAGGAGCTCCCTGACCCGCCGAGGAGAGCACA
CAGGGGCTGGAACCAAGGCACCCAGAGCCCGGCTGACTGGAAGTTGGTCCGGAGTGGAGGGAGAGGTCTTGTGGTCTGTTATTTGAG**TGG**CCATCGTTTTTTGGG
GGCGCTAAGTAGGTTTAAAACTAGGTGTCACTGGGAAGGCTTGACCAGAGAGATGAGTTTGATCTAGAAATCTTGTCCAATTGATGTGTGTATAGCTTTTAATAA
AAGCAAAGAGGCTTAAGAATAGTTTGGGGAAATTTACATAACATTTTGAAAAGGGAAAACAAGTTCACTTTTTAGTCGGTGGTTGATAATTGTTTCACAATGAAG
AGTAGTGTCTGACTGTTTTGGTATTGGTTTTTAGAAATAACTTCCAGTGGAAAGAGAAAATGTTAAAAGGCTTAATTGGTTGCCCCAAGTCAAAGAAAGGCTGGG
CGGTGGTGCTAGATAAGGCAAGAGTCTGGAAAGATTGTTAATATAGACACTGAGGGGGAAAACTACTTTTTTTGGTCCTTGAGAAGAAAAACGATTTTAAAATTG
AAAATTTCTACATAGTGGCTCACATGTGTTGGAATCTTAGCACTTAGGAGGGTGAGGCAGGAGGGTTGCCAAGAGTTTGAGACCACTTTTATGCGCTGCATAGTG
AATTCCAGGCCTGTCTGGGCTGCAGAGCAAGACTTTGTCTCCAATATTAATAACAACAATAATATTGAAAATTTTTGTATGTTCATATTTAGACCCAGTGATGAC
GGACAGCGATGTATTTGCATAGCTGTGTGTTTTGCATCTGGGTGACAATACAAAAATATCCTAGTGGGGTTTTCTAGATGGATCCCCTCCCAATCATGTTTCAGC
TAAAAATGGATCCAAATTAGATGGCCTGGTCCTCTAGGGGGGAGCTCTGTGGAGGGGAATAATTCCCAGGCCTCAATCTGGTGGAAGAGACAGCAGGTACTAAGG
ACTGAGATGCTGCTGGACGCAAGCTCCAACTCACCTAGGGAGCTGTAGGCAGATGCCATGGGTCGCCCTAGACTTTCCATCACTCCCCAGAAGTCAGAGCGCTCT
TTCTTTAACCGCACAGACTTTGGTAGGTACTTGGCCTCTGCATATAGGGGGGAGTTTGAGAAAGGATTCC**CCGCTAAGACCCATCACTGA**AAATTTTCTCTTTGT
CTATCAAG**GGCATGGAAGGAATCAAGGTGTTTCTTCATGAACGTGAACTGTGGCTGAAGTTCCACGAAGTGGGCACAGAGATGATCATCACCAAGGCAGGGAG**GT

Figure S1. CRISPR/Cas9 gene editing of mouse *Tbx5* intron 2 element. Related to Figure 1. Shown is the DNA sequence in the vicinity of the mouse *Tbx5* intron 2 DNA element conserved in mammals (Minguillon et al., 2012). Within the intron 2 DNA element are shown regions that were reported to bind HOX4 or HOX5 proteins (orange), a retinoic acid response element (red), and a Tcf/Lef (Wnt/□-catenin) element (green) (Minguillon et al., 2012; Nishimoto et al., 2014; Nishimoto et al., 2015). The locations of sgRNAs used for CRISPR/Cas9 gene editing are shown, resulting in mice carrying the deletions shown by gray shading.

Mouse *Tbx5* cns12sh DNA element gene edited allele (779 bp deletion):

KEY: NON-CODING (all)
N_{20}(sgRNA)**NGG(PAM)**
CNS12sh DNA element
GENOTYPING PRIMERS
DELETED REGION (779 bp)

mm10: chr5:119977426-119979000 Tbx5-CNS12-enhancer-region-Δ779

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CCAGGGGATGCAGCTGATGGTGGAGCAAGCGTGAGAACGCTGGCCTAGAATGTTTAAGGCCCTCAGCTTTGTCCTCGGCACTACATACGAGAAAAACCAACTTTA
TCAAGACCCCCAAACTCTGTATGTACATGAGGTTTCTTAGCTTCCAGGGTGGGATGGCACAGGCAGAGGTAAGGGTTTCTTTTCTTAATTTATTTTTTAAGCATG
TGTGTGTGTGTGTGTGTGTGTGTGTATGTTTGTGTGTGTTTGTGCGTGTTGTACTAATTTTCTGAACTTTACCCCCCTGATACACAGACACACTTCTCATCTAGT
GTTTCCCTGGCTTCTCCACTGTCATATGTGGCCATCCCCATCCTGTGATATCCTTCAATTGACCTGTTTGGTTGGTGACCCACTAGAGGACATGAGGGAGTTCCC
ACAGGCAGCCCTGGACCAGGCCTGACCCAGCCATACCTAGGTTCCCTCCTGGCTTGTCAGGATGTTTATGGATTTGTCAGGGGCAAAGGAGATGGAGTTTGTACA
CATGGGAAAGGAGCAGTAAGCCTATACTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGCGTGTGTGTATGTGTGCCCGCATCTCCCCTTTC
CATTCTACCTCATGTTCTGCCTCCTCTTCTCCCCCTCTTACCTTAGACCTGCCCCTGACATGGGTGAATAGAAGCCCAGAGCCCAACGCTCAGGAAAGCCAGGCA
CCTGGGGTCGGAGGCTCAAGAGCCCACCAAGAGTTAATCCCTGAGGAACAAGGAGCTGAGTGAATCCTGCCCTGCCCAACACCCTCATCATCATTAGTCATGCTC
GCTGGACAGGAGGCTCCCCACTGGCGCACCTCTTCCTGGCTTCTGTCCTGCGAGCAGGCAGCCATATTTCATGCTCTGAAAACAGATGACCCCGGGGGTCACAGA
AATGAAAGACCCGTTGAACGGGCTGGTCAGGGGCTCCACGGAAATGGAGGACAAGGAGAAGGTGGCAGACCAGTGTCTCCAGAAGCAGCCCTGGGCTGTGTTTCC
TGGCAGCCCGCTCTGCTTCCTTAGGCCAGGTAGGGAGACAGGGCTACTGGGGCTGTTCCTTTAAGGAAGCACAAGGCAGGGGTGGGGCTGGGAAGCCATATTTGC
ATAGAGACTCTTCCCATGATGCCCTGGAACAGACTGGATCAGATTGGGTCACAATACAGTGAGGACAAGACCAGCCTACCCACTTGGCTTTCAGTTTACCAGACT
ATTCCAGATTCTTCACAGTGAACAGGGAAGAATTAAATGTCATAGTTATAGGTATGTTGTGGGTTTTTTCCCCTTGTATTGAGGGAAAAATAAATGAAACACATA
GTGCCTATGAAAATCCATAAATATTTCCTAAAGAAGAAGACACGATAAAATGCTTATTTAAAACTATAATGGTTAGGTCTGGGTACATACCTTTAATCCCGGGCA
GAGAGGCAAGTGAATCTCTGTGAGTTCAAGACCAGCCTGGTACACACAGTTTCAGACCAACCAAGGCTACAGAGTGAAAGCCAGTCTAAAAAACGAAACAAAACA
```

Mouse *Tbx5* cns12sh DNA element gene edited allele (772 bp deletion):

KEY: NON-CODING (all)
N_{20}(sgRNA)**NGG(PAM)**
CNS12sh DNA element
GENOTYPING PRIMERS
DELETED REGION (772 bp)

mm10: chr5:119977426-119979000 Tbx5-CNS12-enhancer-region-Δ772

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CCAGGGGATGCAGCTGATGGTGGAGCAAGCGTGAGAACGCTGGCCTAGAATGTTTAAGGCCCTCAGCTTTGTCCTCGGCACTACATACGAGAAAAACCAACTTTA
TCAAGACCCCCAAACTCTGTATGTACATGAGGTTTCTTAGCTTCCAGGGTGGGATGGCACAGGCAGAGGTAAGGGTTTCTTTTCTTAATTTATTTTTTAAGCATG
TGTGTGTGTGTGTGTGTGTGTGTGTATGTTTGTGTGTGTTTGTGCGTGTTGTACTAATTTTCTGAACTTTACCCCCCTGATACACAGACACACTTCTCATCTAGT
GTTTCCCTGGCTTCTCCACTGTCATATGTGGCCATCCCCATCCTGTGATATCCTTCAATTGACCTGTTTGGTTGGTGACCCACTAGAGGACATGAGGGAGTTCCC
ACAGGCAGCCCTGGACCAGGCCTGACCCAGCCATACCTAGGTTCCCTCCTGGCTTGTCAGGATGTTTATGGATTTGTCAGGGGCAAAGGAGATGGAGTTTGTACA
CATGGGAAAGGAGCAGTAAGCCTATACTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGCGTGTGTGTATGTGTGCCCGCATCTCCCCTTTC
CATTCTACCTCATGTTCTGCCTCCTCTTCTCCCCCTCTTACCTTAGACCTGCCCCTGACATGGGTGAATAGAAGCCCAGAGCCCAACGCTCAGGAAAGCCAGGCA
CCTGGGGTCGGAGGCTCAAGAGCCCACCAAGAGTTAATCCCTGAGGAACAAGGAGCTGAGTGAATCCTGCCCTGCCCAACACCCTCATCATCATTAGTCATGCTC
GCTGGACAGGAGGCTCCCCACTGGCGCACCTCTTCCTGGCTTCTGTCCTGCGAGCAGGCAGCCATATTTCATGCTCTGAAAACAGATGACCCCGGGGGTCACAGA
AATGAAAGACCCGTTGAACGGGCTGGTCAGGGGCTCCACGGAAATGGAGGACAAGGAGAAGGTGGCAGACCAGTGTCTCCAGAAGCAGCCCTGGGCTGTGTTTCC
TGGCAGCCCGCTCTGCTTCCTTAGGCCAGGTAGGGAGACAGGGCTACTGGGGCTGTTCCTTTAAGGAAGCACAAGGCAGGGGTGGGGCTGGGAAGCCATATTTGC
ATAGAGACTCTTCCCATGATGCCCTGGAACAGACTGGATCAGATTGGGTCACAATACAGTGAGGACAAGACCAGCCTACCCACTTGGCTTTCAGTTTACCAGACT
ATTCCAGATTCTTCACAGTGAACAGGGAAGAATTAAATGTCATAGTTATAGGTATGTTGTGGGTTTTTTCCCCTTGTATTGAGGGAAAAATAAATGAAACACATA
GTGCCTATGAAAATCCATAAATATTTCCTAAAGAAGAAGACACGATAAAATGCTTATTTAAAACTATAATGGTTAGGTCTGGGTACATACCTTTAATCCCGGGCA
GAGAGGCAAGTGAATCTCTGTGAGTTCAAGACCAGCCTGGTACACACAGTTTCAGACCAACCAAGGCTACAGAGTGAAAGCCAGTCTAAAAAACGAAACAAAACA
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Figure S2. CRISPR/Cas9 gene editing of mouse *Tbx5* cns12sh element. Related to Figure 1. Shown is the DNA sequence in the vicinity of the mouse *Tbx5* cns12sh DNA element located ~100 kb downstream of the mouse *Tbx5* coding region (Adachi et al., 2016). Blue sequence refers to the region highly conserved in jawed vertebrates. The locations of sgRNAs used for CRISPR/Cas9 gene editing are shown, resulting in mice carrying the deletions shown by gray shading. Single cns12sh mutants contained the Δ779 bp deletion allele, whereas double mutants contained the cns12sh Δ772 bp deletion allele along with the intron 2 Δ548 deletion allele.

Zebrafish *tbx5a* cns12sh DNA element gene edited allele (248 bp deletion):

KEY: NON-CODING (all)

N_{20}(sgRNA) **NGG (PAM)**

CNS12sh DNA element

GENOTYPING PRIMERS

DELETED REGION (248 bp)

CCTCAGACAAATTCCAAGCGTGGCGGCAGGATAAGGCCATGGAAAGATCTCTTTTATCTCCTCGGCACTTTTGCCTTTTG
TTGATGATACACTGACCTTCCTCATCGATCTCCAGCTCTAAGCTCCTGTGCGCTCTGATAATGAGACTCCGCTGATAATG
AGGAAAACTAATTGTCCCGGCGGTGTGTTGTGGCTGTCCGGCGGCATCTGACCCGCATTGTCTGCACACATCTTGATTT
CATTAACATCTCTTCCTTCTACTGGCTGTCACAAAGACTCTTGTTCTGCTGGAGTGATCCACCATGACTGTCTAGTGGC
CTTCTAAAGGGGATCTACTATGCAGGAACCGCTTTATAAGGGGTGGCATGGTGGCGCAGTGGGTAGCACAATCATCT
TACAGCGAGAAGGTCACCCCGGCTGGGTCAGGTGACATTTCTGTGTGGAGTTTGCATGTTCTCCTAGTGTTGTCATGG
GTTTCCTCCATCAAAAAACATGTGGTATAGGGGAATTGGGTAAGCTAAATTGTTGGTAGTGTGTATGGATGTTTCCCAG
TGATGGGTTGCAGCTGGAAGGGCATTCGCTGCATTGAAAAAATATGCTGGATAAGTTGGCGGTTCATTCCACTGTGGA
AACCCCAGATTAATAAAGGAACTAAGCTGAAAATTAATGAATGAATGATCAGACTTGATGAATGAAACACTTTGCTTGA
CATTCCCTCTTTGAACGTGCCACGCCAACACGAGGAGAACATGCAAACTCCACAGAAACACCAAATGACCCAGCCGGG
ACTCAAACCAGCAACCTTCTTGCTGTGAGGTGATCGTGCTACCCACTGCACCACCGTGACGCTCTTCACGAGATGTTTTT
CAACCGAAAACTTTGTTCACA

Figure S3. CRISPR/Cas9 gene editing of zebrafish *tbx5a* DNA element. Related to Figure 4. Shown is the DNA sequence in the vicinity of the zebrafish cns12sh DNA element located ~30 kb downstream of the *tbx5a* coding region, showing a 248 bp region deleted using CRISPR/Cas9 gene editing. Bold sequence refers to the region highly conserved in jawed vertebrates (Adachi et al., 2016).

Supplemental References

Adachi, N., Robinson, M., Goolsbee, A., and Shubin, N.H. (2016). Regulatory evolution of Tbx5 and the origin of paired appendages. Proc Natl Acad Sci USA *113*, 10115-10120.

Minguillon, C., Nishimoto, S., Wood, S., Vendrell, E., Gibson-Brown, J.J., and Logan, M.P. (2012). Hox genes regulate the onset of Tbx5 expression in the forelimb. Development *139*, 3180-3188.

Nishimoto, S., Minguillon, C., Wood, S., and Logan, M.P. (2014). A combination of activation and repression by a colinear Hox code controls forelimb-restricted expression of Tbx5 and reveals Hox protein specificity. PLoS Genetics *10*, e1004245.

Nishimoto, S., Wilde, S.M., Wood, S., and Logan, M.P. (2015). RA Acts in a Coherent Feed-Forward Mechanism with Tbx5 to Control Limb Bud Induction and Initiation. Cell Reports *12*, 879-891.