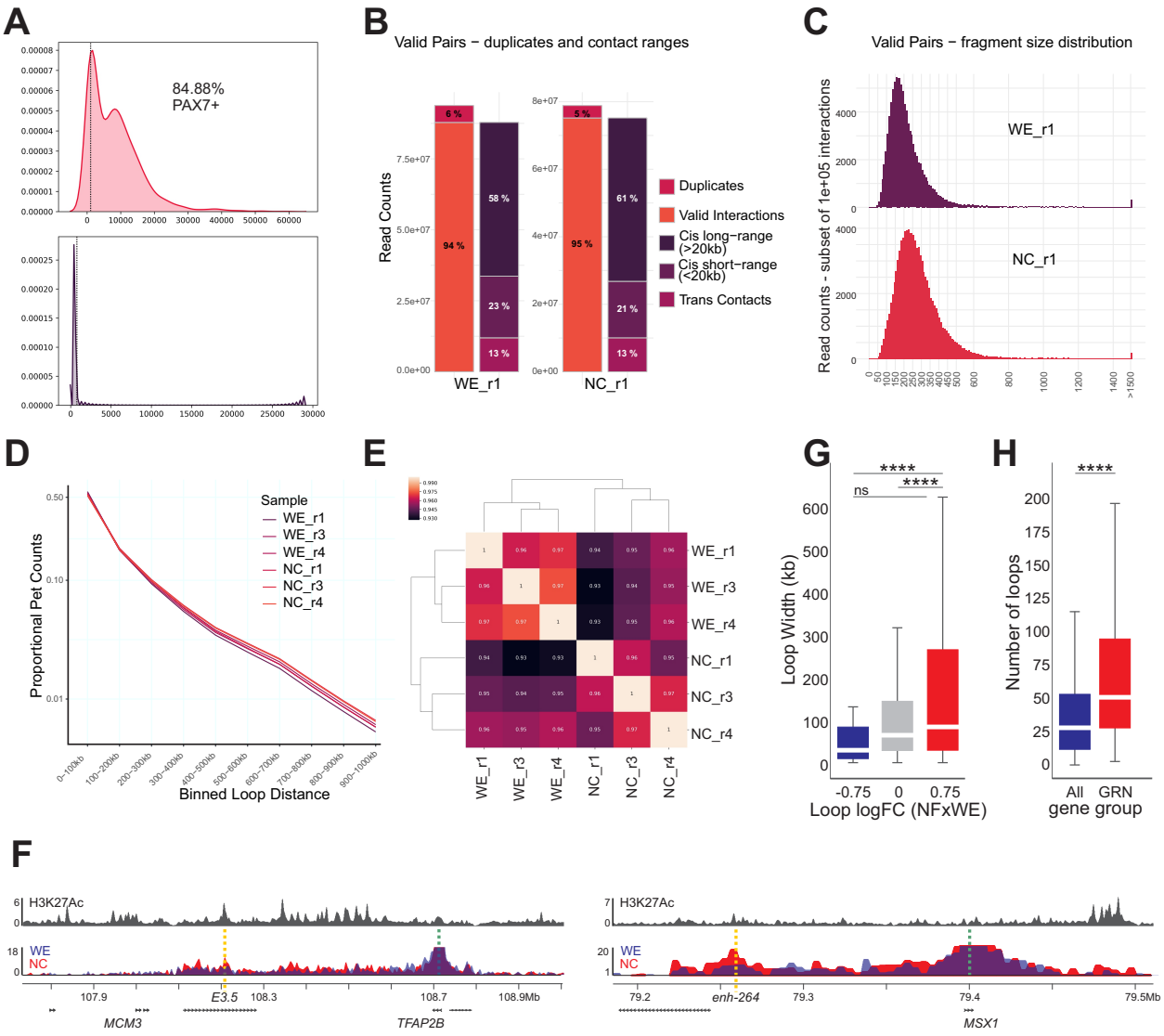

Figure S1. Quality metrics for assembly of a high-resolution map of active enhancers in avian neural crest cells. Related to Figure 1.

(A) Histograms displaying fluorescence quantification of microdissected neural crest cells after PAX7 immunostaining. Neural crest dissections contained 84.88% Pax7+ cells (upper panel). Lower panel shows immunostaining control (with suppression of primary antibody).

(B) Bar plots depicting percentages of duplicates and contact types of valid chromatin pairs for one pair of replicates. Y-axis represents read counts.

(C) Fragment size distribution of valid chromatin contacts of representative H3K27Ac-HiChIP replicates. Y-axis represents read counts.

(D) Distribution of PET (Paired-end tags) per sample as a function of the distance separating the anchors. All samples present similar behavior, an indicative of comparable sequencing depths among samples.

(E) Pairwise Pearson correlation of all three replicates for each condition.

(F) Genomic loci of *TFAP2B* and *MSX1* genes depicting WE (blue) and NC (red) HiChIP 1D tracks and H3K27ac CUT&RUN profile. Green dotted lines mark promoter regions. The active enhancers *E3.5 (TFAP2B),* and *enh-264* (*MSX1)* (Williams et al., 2019) are located in neural crest enriched putative enhancer-promoter contacts (yellow dotted lines).

(G) Boxplot depicting loop width of the strongest contact per gene at putative enhancer-promoter contacts binned by logFC (NCxWE). Genes with neural crest-enriched contacts present longer loops. Statistical significance was determined via Welch's unpaired two-tailed t-test.

(H) Boxplot displaying number of loops contacting promoters of neural crest GRN genes. Statistical significance was determined via Welch's unpaired two-tailed t-test.

NC, neural crest; WE, whole embryo; Kb, kilobase; GRN, gene regulatory network; ****p<0.0001.

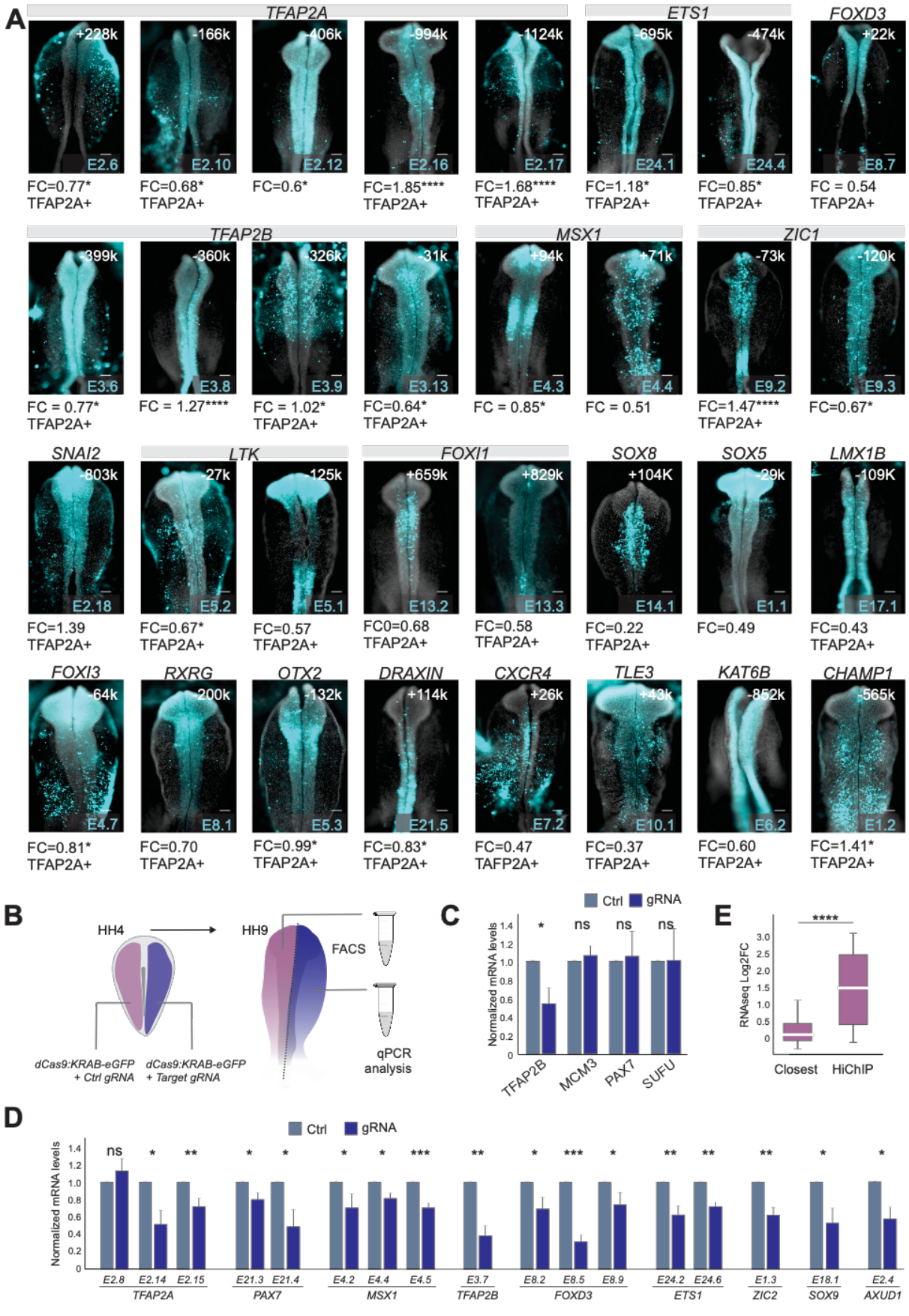
A
TFAP2A
+228k
E2.6
FC=0.77*
TFAP2A+
-166k
E2.10
FC=0.68*
TFAP2A+
-406k
E2.12
FC=0.6*
-994k
E2.16
FC=1.85****
TFAP2A+
-1124k
E2.17
FC=1.68****
TFAP2A+
ETS1
-695k
E24.1
FC=1.18*
TFAP2A+
-474k
E24.4
FC=0.85*
TFAP2A+
FOXD3
+22k
E8.7
FC = 0.54
TFAP2A+
TFAP2B
399k
E3.6
FC = 0.77*
TFAP2A+
-360k
E3.8
FC = 1.27****
-326k
E3.9
FC = 1.02*
TFAP2A+
-31k
E3.13
FC=0.64*
TFAP2A+
MSX1
+94k
E4.3
FC = 0.85*
+71k
E4.4
FC = 0.51
ZIC1
-73k
E9.2
FC=1.47****
TFAP2A+
-120k
E9.3
FC=0.67*
SNAI2
-803k
E2.18
FC=1.39
TFAP2A+
LTK
-27k
E5.2
FC=0.67*
TFAP2A+
-125k
E5.1
FC=0.57
TFAP2A+
FOXI1
+659k
E13.2
FC0=0.68
TFAP2A+
+829k
E13.3
FC=0.58
TFAP2A+
SOX8
+104K
E14.1
FC=0.22
TFAP2A+
SOX5
-29k
E1.1
FC=0.49
LMX1B
-109K
E17.1
FC=0.43
TFAP2A+
FOXI3
-64k
E4.7
FC=0.81*
TFAP2A+
RXRG
-200k
E8.1
FC=0.70
TFAP2A+
OTX2
-132k
E5.3
FC=0.99*
TFAP2A+
DRAXIN
+114k
E21.5
FC=0.83*
TFAP2A+
CXCR4
+26k
E7.2
FC=0.47
TAFP2A+
TLE3
+43k
E10.1
FC=0.37
TFAP2A+
KAT6B
-852k
E6.2
FC=0.60
TFAP2A+
CHAMP1
-565k
E1.2
FC=1.41*
TFAP2A+
B
HH4
HH9
FACS
qPCR
analysis
dCas9:KRAB-eGFP
+ Ctrl gRNA
dCas9:KRAB-eGFP
+ Target gRNA
C
Ctrl
gRNA
Normalized mRNA levels
TFAP2B
MCM3
PAX7
SUFU
E
RNAseq Log2FC
****
Closest
HiChIP
D
Ctrl
gRNA
Normalized mRNA levels
E2.8
E2.14
E2.15
TFAP2A
E21.3
E21.4
PAX7
E4.2
E4.4
E4.5
MSX1
E3.7
TFAP2B
E8.2
E8.5
E8.9
FOXD3
E24.2
E24.6
ETS1
E1.3
ZIC2
E18.1
SOX9
E2.4
AXUD1

Figure S2. Additional enhancers tested with reporter assays in transgenic embryos. Related to Figure 2.

(A) Whole-mount view of transient transgenic embryos expressing novel distal elements identified by enhancer-promoter contact enrichment in neural crest cells. Contact elements are identified by loop width (+, upstream or -, downstream of a gene's promoter). The enhancer-promoter contact logFC and the presence of TFAP2A peaks is presented below each image.

(B) Schematic representation of the CRISPRi assay for enhancer targeting. Control (left) and target (right) guide RNAs were bilaterally electroporated with the vector dCas:KRAB-eGFP at HH4. Embryos were incubated until HH9 stage, when half heads were dissected and GFP+ cells FACS sorted. Samples were then processed for qPCR. Gene expression levels were normalized to the reference gene *HPRT1* and presented as fold change over control side (control gRNA).

(C) Quantification of fold change in TFAP2B transcripts level in neural crest cells when the endogenous *E3.5* enhancer is targeted with a specific gRNA. The effect of *E3.5* disruption is specific to *TFAP2B* since no significant change in expression level was observed for *MCM3*, the closest gene with active expression in neural crest cells, the neural crest marker *PAX7* or the neural marker *SUFU*. Error bars represent ± SEM. Statistical significance determined via an unpaired t-test.

(D) Quantification of fold change in expression levels of neural crest genes when assigned enhancers were targeted with specific gRNA. Error bars represent ± SEM. Statistical significance determined via an unpaired t-test.

(E) HiChIP contact map gene assignment of neural crest enhancers led to a stronger gene enrichment in neural crest cells when compared to the closest gene assignment. Statistical significance was determined via Welch's unpaired two-tailed t-test.

FC, loop fold change; Scale bar, 100um. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

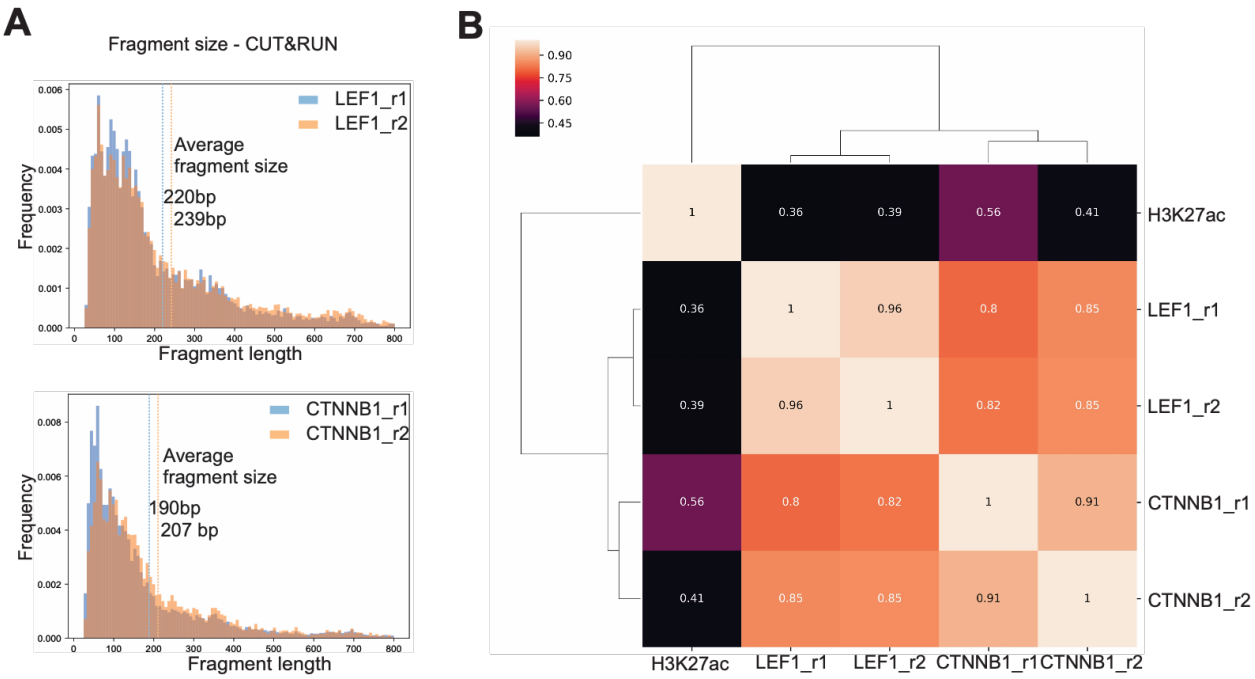

Figure S3. Controls for genome-wide mapping of Wnt nuclear effectors with CUT&RUN. Related to Figure 3.

(A) Fragment size distribution of the two replicates of LEF1 and CTNNB1 CUT&RUN read pairs.

(B) Pairwise Pearson correlation of LEF1 and CTNNB1 CUT&RUN replicates.

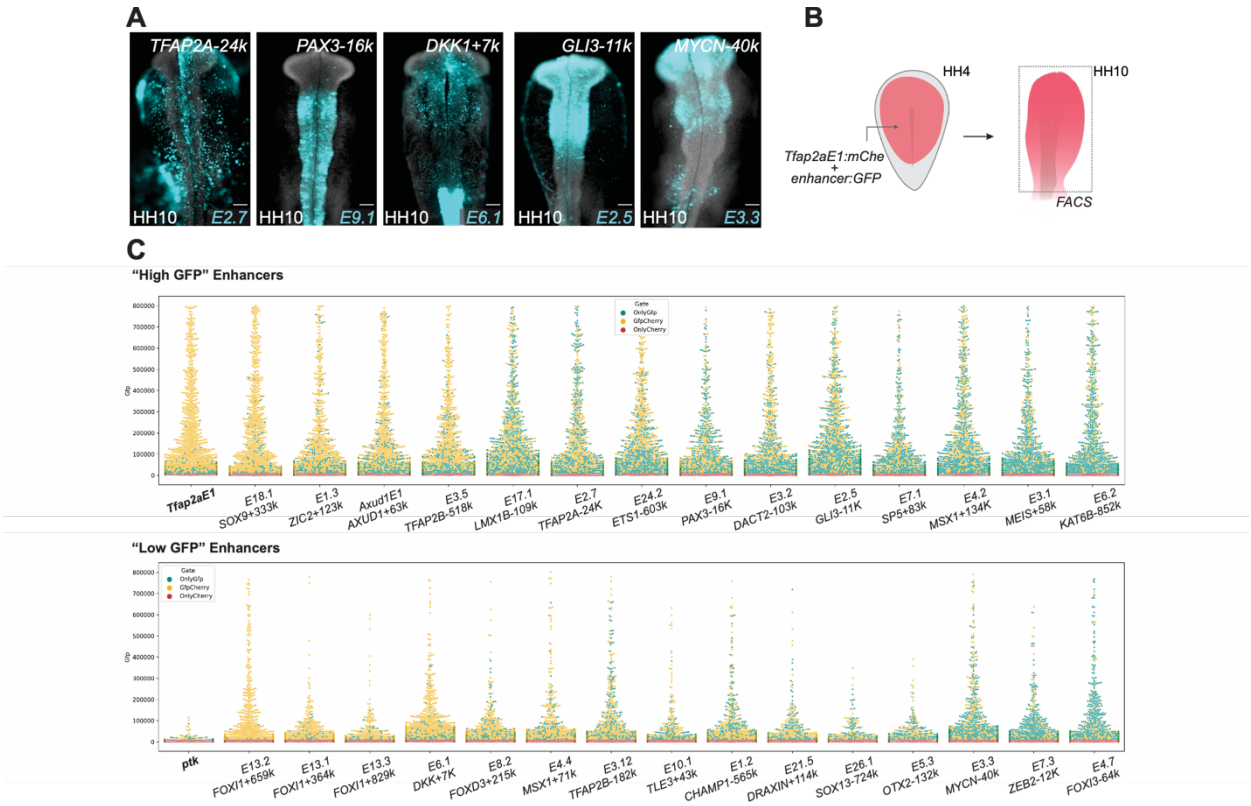

Figure S4. Flow cytometry analysis of strength and specificity of Wnt-associated enhancers. Related to Figure 4.

(A) Reporter assay and whole-mount view of embryos electroporated with putative enhancers of neural crest expressing genes driving GFP expression. All regions present peaks co-occupied by LEF1/CTNNB1. *E2.7 (TFAP2A-24k), E9.1 (PAX3-16k), E6.1 (DKK+7k), E2.5 (GLI3-11k)* and *E3.3 (MYCN-40k)* elements are found in the same PET (Paired-end tags) as the indicated gene promoter.

(B) Schematic representation of the enhancer-reporter assay quantification. Each putative enhancer, cloned into pTK-eGFP, was co-electroporated with *Tfap2aE1:mChe* in gastrula stage embryos (HH4). Embryos were incubated until HH10 stage, when the cranial region was dissected and processed for single-cell analysis with flow cytometry.

(C) Scatter plots displaying enhancer-reporter assays for all the LEF1/CTNNB1-bound putative enhancers displayed in (Figure 4B) cloned into pTK-eGFP. As described above, all enhancers were co-electroporated with *Tfap2aE1:mChe*. Y-axis represents the eGFP intensity of HH10 cranial single cells. Green dots represent cells expressing only eGFP. Red dots represent cells expressing only the neural crest marker (mCherry). Yellow dots represent double-positive cells (eGFP+/mCherry+). Upper plot depicts highly active enhancers, while lower depicts enhancers with lower activity (Figure 4B). Enhancers are ordered from left to right following the specificity score (Figure 4B).

HH, Hamburger and Hamilton. Scale bar, 100um.

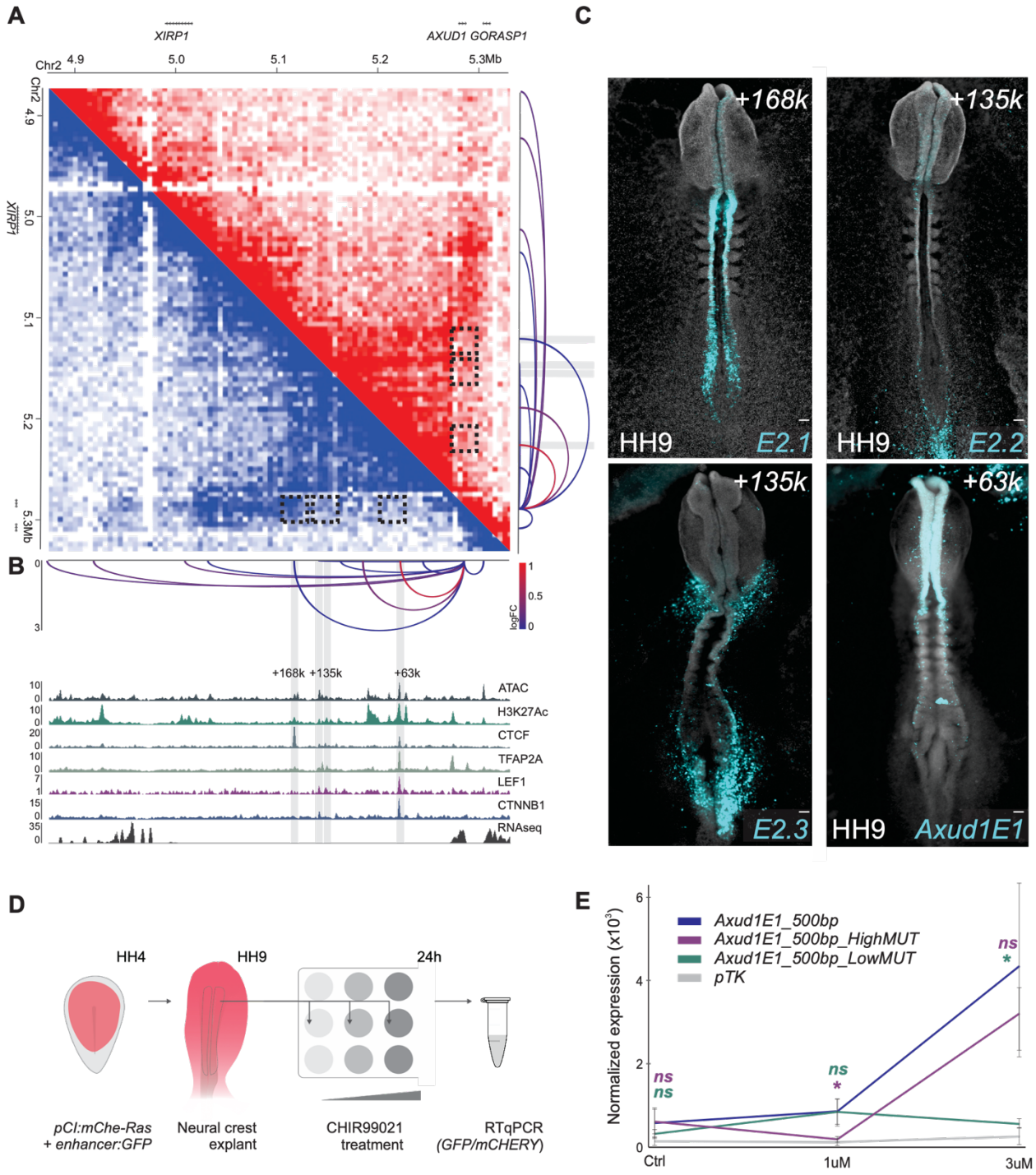

Figure S5. Identification of AXUD1 enhancers by HiChIP. Related to Figure 5.

(A) HiChIP contact matrix depicting normalized contact frequencies for dissected neural crest cells (NC, red, top right) and whole embryo (WE, blue, bottom left) of valid contacts at the *AXUD1* locus.

(B) Arc plot depicting putative enhancer-promoter contacts, ATAC-seq, RNA-seq and CUT&RUN profiles for H3K27ac, CTCF, TFAP2A, LEF1 and CTNNB1 at the *AXUD1* locus. Arc plot y-axis represents Zscores calculated from the mean counts of NC HiChIP replicates and the heatmap represents loop logFC (NCxWE). Gray bars highlight distal elements enriched for TFAP2A binding.

(C) Whole-mount view of embryos electroporated with the distal elements highlighted in (**b**), *E2.1 (AXUD1+168k), E2.2 and E2.3 (AXUD1+135k),* and *E2.4 (Axud1E1 – AXUD1+63k).* The element cloned from the loop *AXUD1+63k* (*Axud1E1*) presents robust and specific activity in the dorsal neural tube.

(D) Schematic representation of the enhancer-reporter assay quantification under Wnt agonist treatment. Each *Axud1E1* wild type and mutant variants, cloned into pTK-eGFP, were co-electroporated with *PCI:mCherry-Ras* in gastrula stage embryos (HH4). Embryos were incubated until HH9 stage, when the dorsal neural folds were dissected and cultured for 24h in the presence of the Wnt agonist CHIR99021. Treated explants were then processed for qPCR and the *GFP* levels (enhancer activity) were normalized to *mCHERRY*.

(E) GFP normalized expression of *Axud1E1-500bp*, *Axud1E1_500bp_HighMUT*, *Axud1E1_500bp_LowMUT* and pTK under CHIR99021 treatment (1uM and 3uM). Error bars represent ± SEM. Statistical significance determined via an unpaired t-test.

HH, Hamburger and Hamilton; Mb, megabase. Scale bar, 100um. *p<0.05.

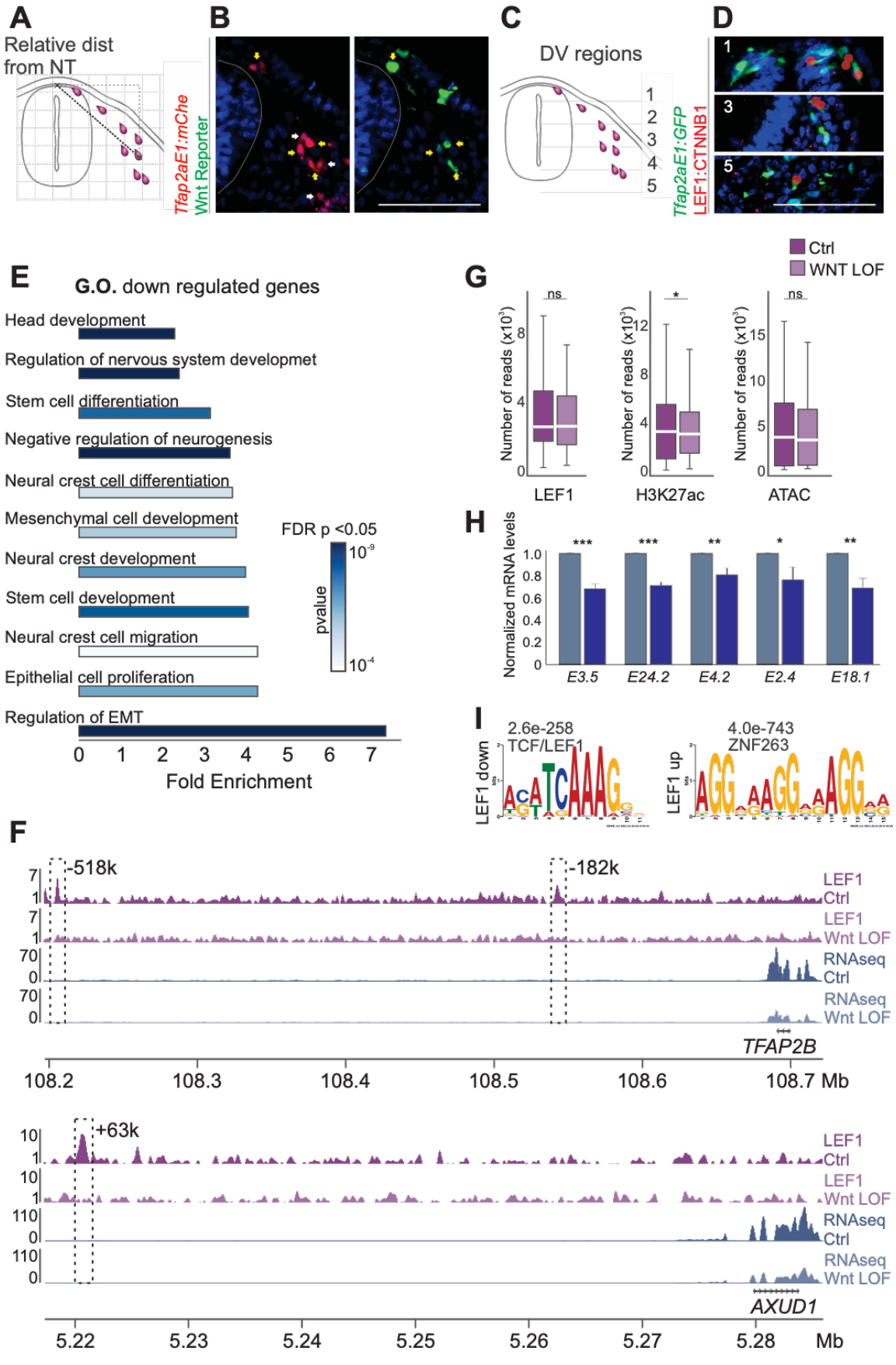
A
Relative dist from NT
Tfap2aE1:mChe
Wnt Reporter
B
C
DV regions
1
2
3
4
5
D
Tfap2aE1:GFP
LEF1:CTNNB1
E
G.O. down regulated genes
Head development
Regulation of nervous system developmet
Stem cell differentiation
Negative regulation of neurogenesis
Neural crest cell differentiation
Mesenchymal cell development
Neural crest development
Stem cell development
Neural crest cell migration
Epithelial cell proliferation
Regulation of EMT
FDR p <0.05
pvalue
Fold Enrichment
G
Ctrl
WNT LOF
ns
*
Number of reads (x10³)
LEF1
H3K27ac
ATAC
H
Normalized mRNA levels
E3.5
E24.2
E4.2
E2.4
E18.1
I
2.6e-258
TCF/LEF1
LEF1 down
4.0e-743
ZNF263
LEF1 up
F
-518k
-182k
LEF1 Ctrl
LEF1 Wnt LOF
RNAseq Ctrl
RNAseq Wnt LOF
TFAP2B
108.2
108.3
108.4
108.5
108.6
108.7 Mb
+63k
AXUD1
5.22
5.23
5.24
5.25
5.26
5.27
5.28
Mb

Figure S6. Shifts in activity of Wnt signaling affect regulation of neural crest genes by LEF1 and CTNNB1. Related to Figure 6.

(A) Diagram outlining the parameters for single neural crest cell measurement of Wnt reporter fluorescence.

(B) Section of an embryo displaying *Tfap2aE1:mChe* positive (white arrows) and double positive (Tfap2aE1:mChe/Wnt reporter:GFP) cells (yellow arrows).

(C) Diagram outlining the dorsal-ventral (DV) regions employed for LEF1:CTNNB1 PLA puncta quantification in single neural crest cells.

(D) Proximity ligation assays (PLA) visualized in transverse sections of DV regions 1, 3 and 5. Positive interactions, indicated by red puncta, are enriched in dorsal most regions of the embryo.

(E) Bar plot showing significantly enriched GO terms from Gene Ontology analysis of downregulated genes upon Wnt knockdown. Heatmap displays pValue range.

F, LEF1 signal and RNA-seq profiles at control and Wnt loss-of-function conditions at the *TFAP2B* and *AXUD1* loci. Dotted boxes highlight the putative enhancers *E3.5* (*TFAP2B-518k*), *E3.12* (*TFAP2B-182k*) and *Axud1E1* (*AXUD1+63k*) tested in (Figure 4B).

(G) Box plots depicting total LEF1 and H3K27ac binding and ATAC signal at LEF1/CTNNB1 co-occupied peaks in control and Wnt loss-of-function conditions. Statistical significance was determined via Welch's unpaired two-tailed t-test.

(H) Quantification of fold change in eRNA levels in control and Wnt loss-of-function conditions. Error bars represent ± SEM. Statistical significance determined via an unpaired t-test.

(I) Motif enrichment analysis via MEME-ChIP shows a strong enrichment for the TCF/LEF1 binding motif at peaks that lost LEF1 binding upon Wnt knockdown while peaks presenting increased LEF1 binding are enriched for the ZNF263 motif. Values indicate significance of motif occurrence as reported by MEME.

NT, neural tube; DV, dorsal-ventral; LOF, loss-of-function; Mb, megabase. Scale bar, 100um. *p<0.05, **p<0.01.