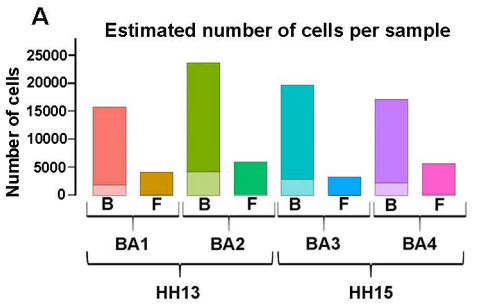

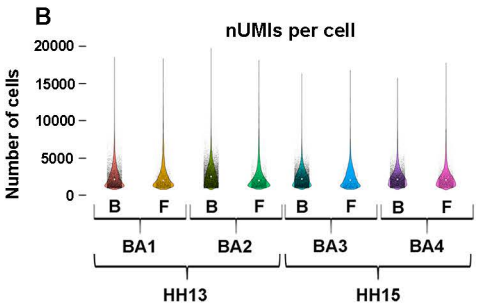

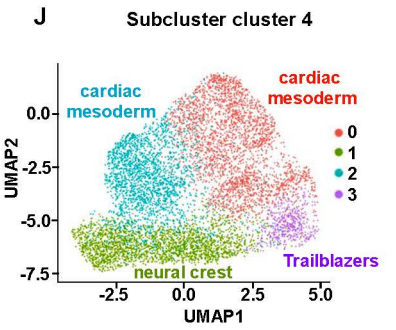

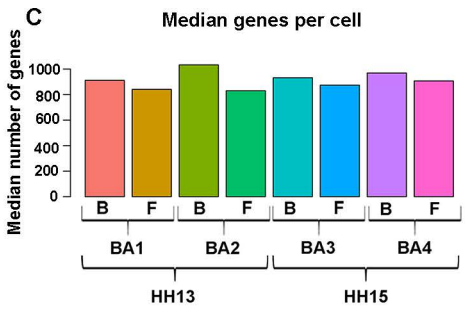

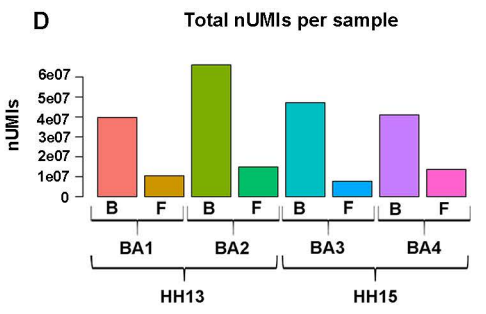

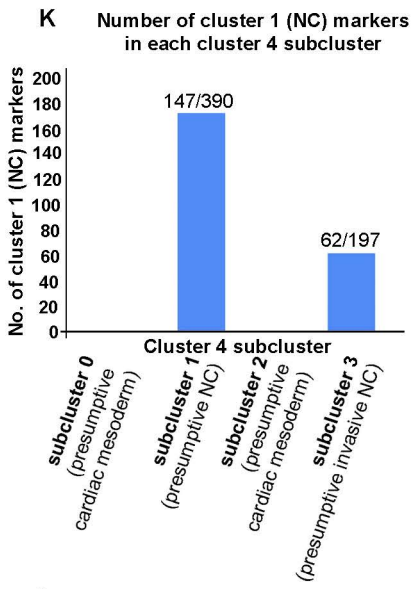

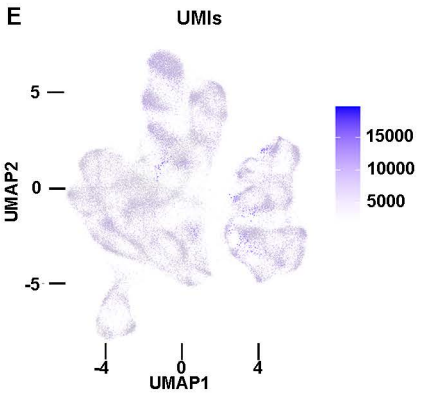

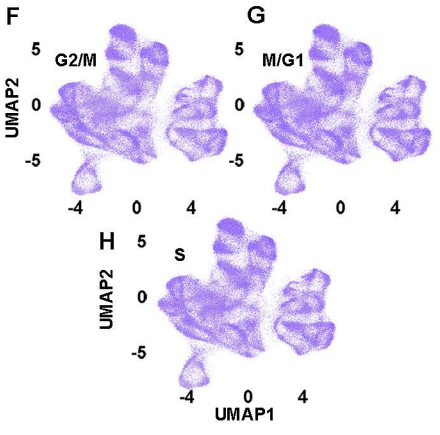

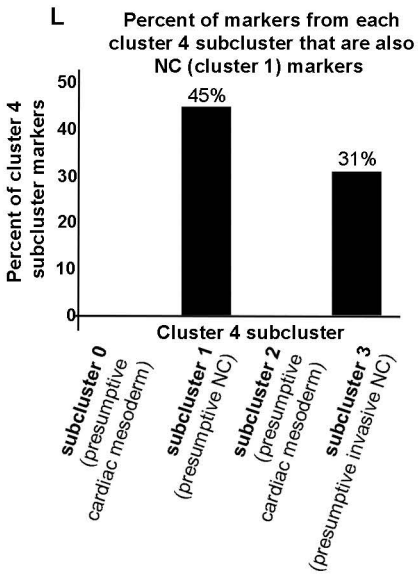

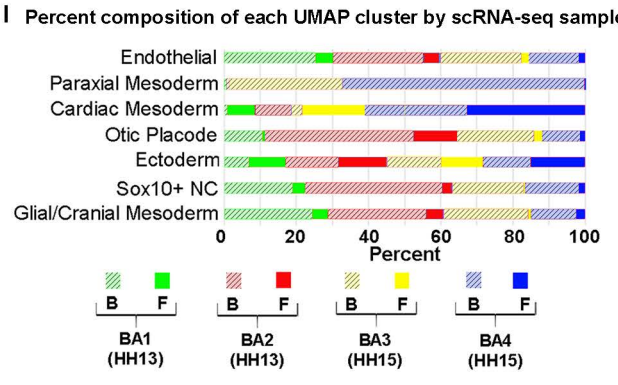

Fig. S1. Quality control and analytics of chick BA1-4 scRNA-seq. (A) Estimated number of cells per sample. Lighter-shaded areas within each bar represent the estimated number of multiplets within each sample. (B) nUMIs per cell. Gray dots identify the median nUMIs for each sample type. (C) Median genes per cell for each sample. (D) Total nUMIs per sample. Back (B) and Front (F) subpopulations are labeled according to each of the branchial arches (BA) 1 through 4 at HH13 and HH15 in each graph. (E) Read depth does not significantly affect scRNA-seq clustering. UMAP feature plot colored by the number of reads per cell shows consistent profiling across all clusters analyzed. (F-H) Cell cycle does not significantly affect scRNA-seq clustering. UMAP feature plot colored by the mean normalized expression of G2/M (F) M/G1 (G) S (H) phase cell cycle genes identified by Whitfield et al. (Whitfield et al., 2002). (I) Cell type composition of each of the spatially segregated scRNA-seq samples. (J) Subcluster of cluster 4 (cardiac mesoderm + invasive/trailblazer NC) into 4 subpopulations (k means = 4). The subclusters are labeled as subpop 0 (red)-cardiac mesoderm; subpop 1 (green)-neural crest; subpop 2 (blue)-cardiac mesoderm; subpop 3 (purple)-invasive/trailblazer neural crest cells. Markers distinguishing the 4 subclusters are listed in Supplemental Table 4. (K-L) Subclusters 1 & 3 within cluster 4 are comprised of NC cells. NC markers (cluster 1 markers from Supplemental Table S1) are also markers of each of the 4, cluster 4 subclusters (J; Supplemental Table S4). None of the 64 or 147 markers of subclusters 0 or 2 (presumptive cardiac mesoderm) contain markers of NC cells. 147 of the 390 markers (45%) of subcluster 1 (presumptive NC) also mark cluster 1 NC, and 62 of the 197 (31%) markers of subcluster 3 (presumptive invasive NC) overlap with the markers of cluster 1 NC.

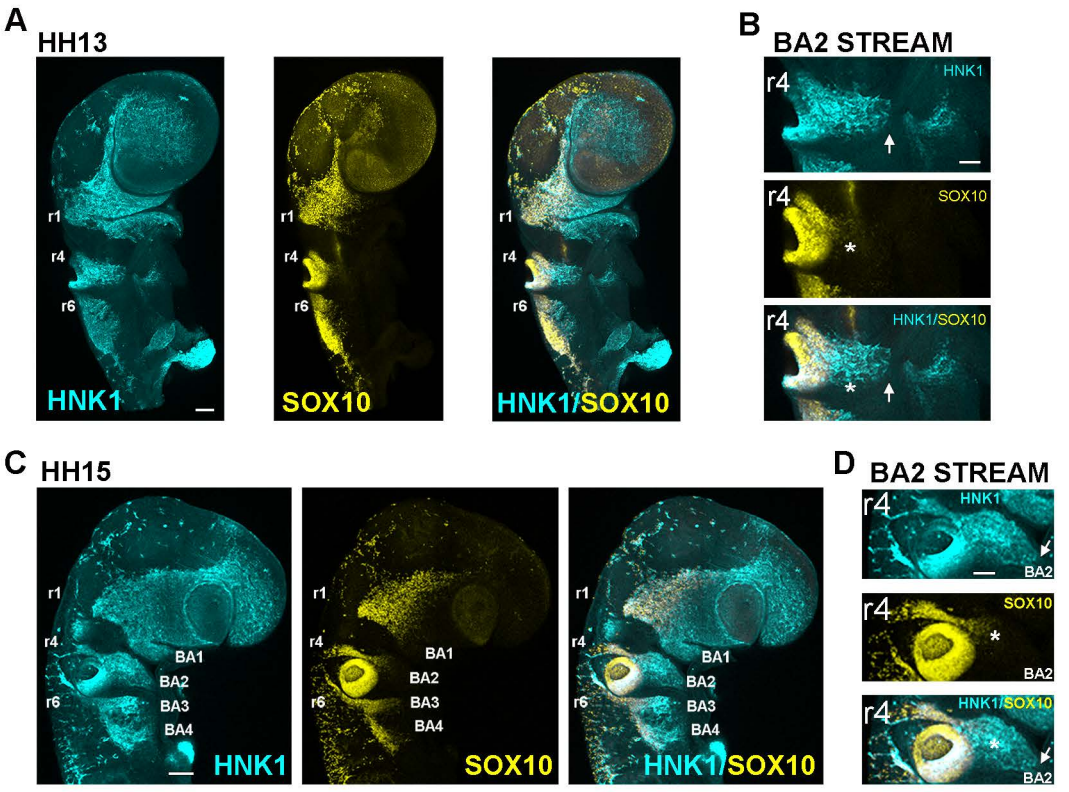

Fig. S2. *SOX10/HNK-1* expression analysis with respect to BA1-4 streams and REVIGO analysis.

(A-D) At HH13 and 15, migrating all cranial-to-cardiac neural crest cells are strongly labeled by HNK-1 (arrows), while *SOX10* expression using RNAscope (asterisks) inefficiently labels neural crest cells at the migratory front. (E) Reduce and Visualize Gene Ontology (REVIGO) analysis of 45 gene invasion signature. Branchial Arch 2 (BA2); otic vesicle (OV). Scalebars are 100um (A,C) and 50um (B,D).

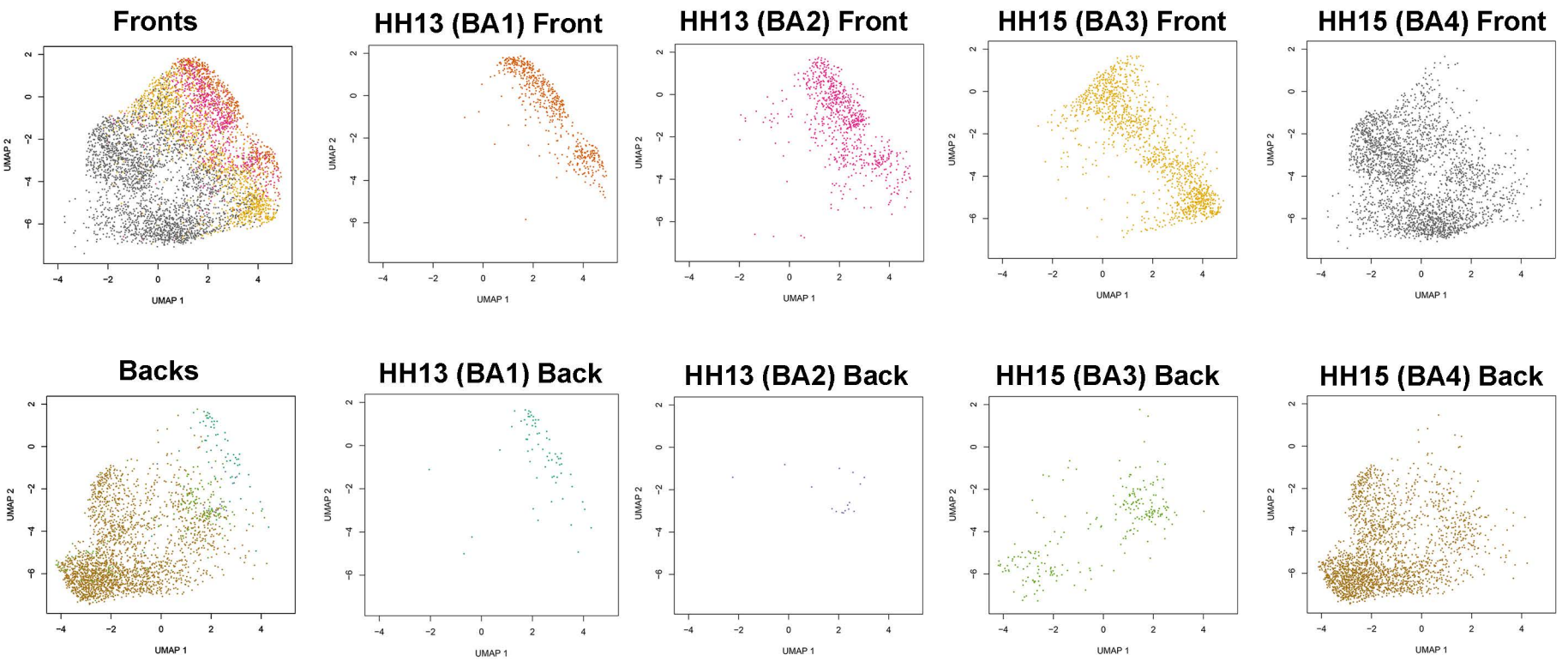

Fig. S3. Clustering of only cardiac mesoderm cells by sample. UMAP of all cardiac mesoderm cluster (cluster 4) cells color-coded by sample type: HH13 BA1 Front (orange), HH13 BA1 Back (teal), HH13 BA2 Front (pink), HH13 BA2 Back (purple), HH15 BA3 Front (yellow), HH15 BA3 Back (green), HH15 BA4 Front (gray) and HH15 BA4 Back (brown).

Table S1. Markers of the 7 UMAP clusters of cells isolated from branchial arches 1-4

Click here to download Table S1

Table S2. Contribution of each spatially distinct sample to each UMAP cluster

Click here to download Table S2

Table S3. Markers of the most invasive/trailblazer neural crest cells from branchial arches 1-4

Click here to download Table S3

Table S4. Markers of the 4 subclusters from cluster 4 (cardiac mesoderm + invasive/trailblazer NC).

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Table S5. 34 published non-neural crest related cell invasion signatures

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