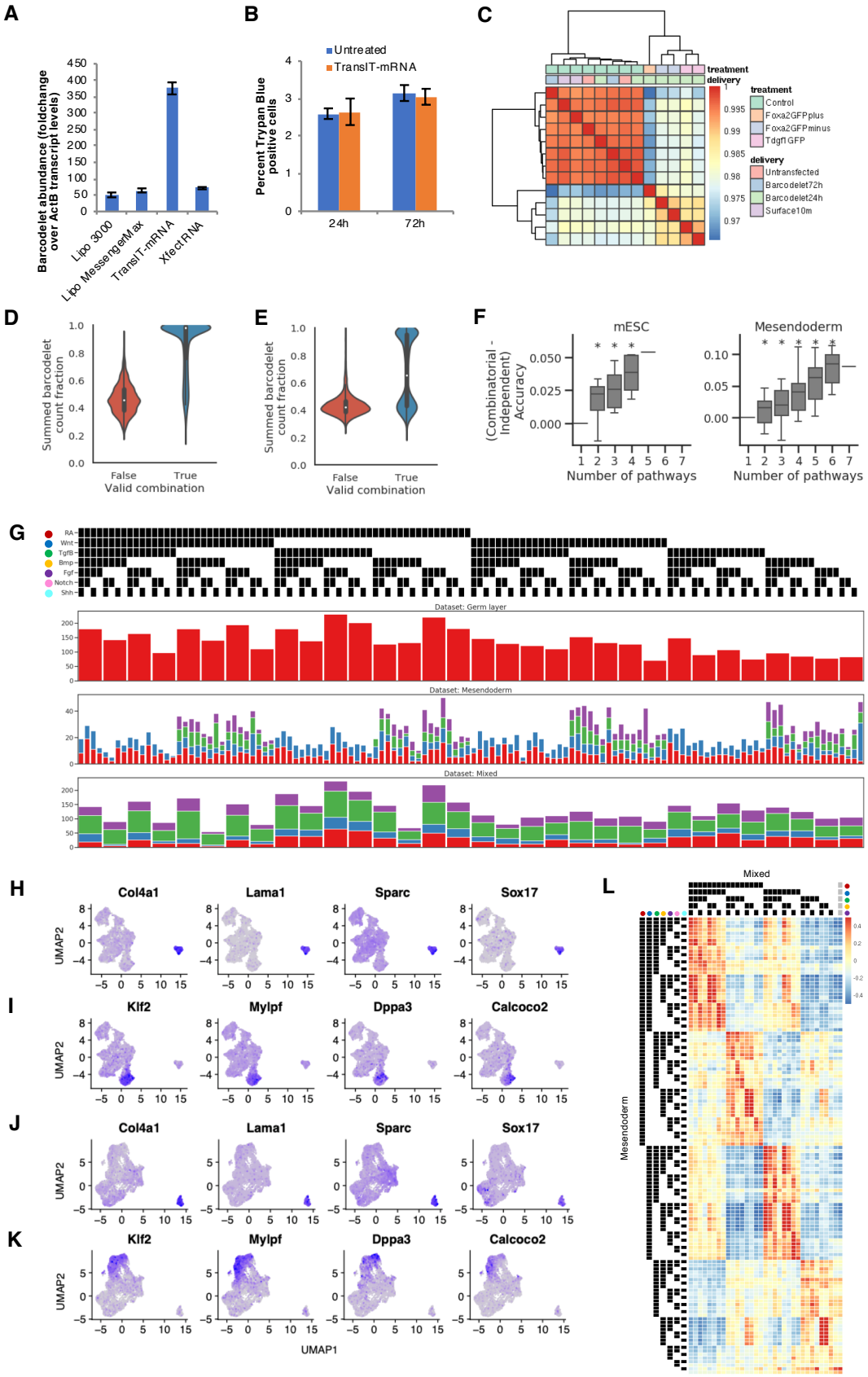

A
Barcodelet abundance (foldchange over ActB transcript levels)
Lipo 3000
Lipo MessengerMax
TransIT-mRNA
XfectRNA
B
Untreated
TransIT-mRNA
Percent Trypan Blue positive cells
24h
72h
C
treatment
delivery
Control
Foxa2GFPplus
Foxa2GFPminus
TdgfGFP
Untransfected
Barcodelet72h
Barcodelet24h
Surface10m
D
Summed barcodelet count fraction
False
True
Valid combination
E
Summed barcodelet count fraction
False
True
Valid combination
F
mESC
Mesendoderm
(Combinatorial - Independent) Accuracy
Number of pathways
Number of pathways
G
RA
Wnt
TgfB
Bmp
Fgf
Notch
Shh
Dataset: Germ layer
Dataset: Mesendoderm
Dataset: Mixed
H
Col4a1
Lama1
Sparc
Sox17
UMAP2
I
Klf2
Mylpf
Dppa3
Calcoco2
UMAP2
J
Col4a1
Lama1
Sparc
Sox17
UMAP2
K
Klf2
Mylpf
Dppa3
Calcoco2
UMAP2
UMAP1
L
Mixed
Mesendoderm

Figure S1. Related to Figure 1B, 1E, 1G, 2B

(A) Levels of barcodelet transcripts detected by RT-qPCR 24 hours after transfection with Lipofectamine 3000 (Lipo 3000), Lipofectamine MessengerMax (Lipo MessengerMax), TransIT-mRNA, and Xfect RNA, shown as fold difference compared to Actb levels. Error bars represent standard deviation from two biological replicates.

(B) Percent Trypan Blue positive dead cells 24 and 72 hours after transfection with barcodelet and TransIT-mRNA (orange) or untreated control (blue). Error bars represent standard deviation from two biological replicates.

(C) Correlation of bulk RNA-sequencing of cell populations which vary according to the treatment they received and barcodelet delivery method. Barcodelets were delivered via transfection and the populations assessed after 24h and 72h in the Barcodelet24h and Barcodelet72h respectively. Control cells did not observe any treatments, while untransfected cells did not receive any barcodelets. Foxa2GFPplus, Foxa2GFPminus, and Tdgf1GFP conditions refer to cell lines which received some combination of treatments as well as barcodelets via transfection

(D, E) Distribution of summed barcodelet count fraction of top 3 most abundant barcodelets in mesendoderm dataset (D) and mixed dataset (E), grouped by whether or not the top 3 most abundant barcodelets from a valid combination

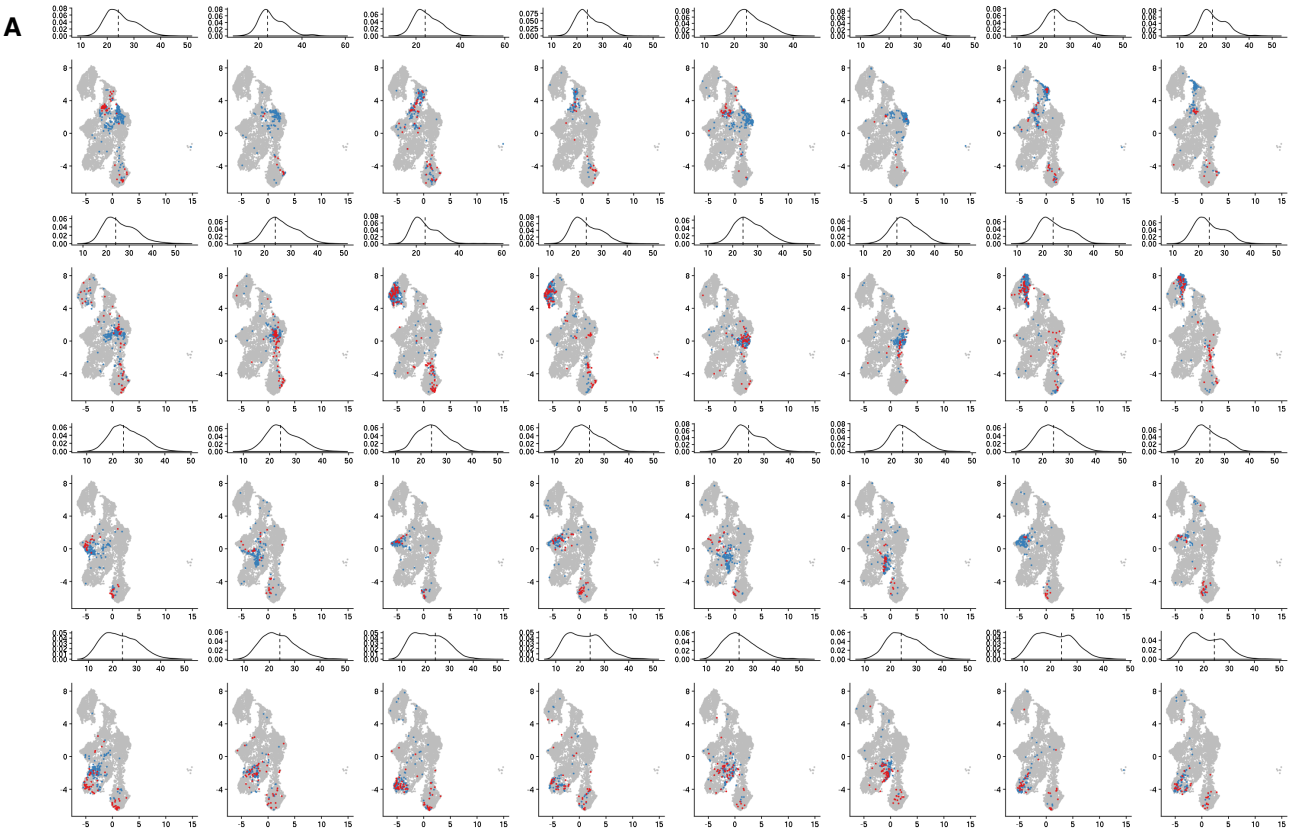
(F) Difference in model accuracy on randomly-held out test set with increasing number of pathways when predicting combinatorial pathway status vs. each pathway independently. * indicates bonferonni-corrected p-value for paired t-test < 0.05

(G) Number of cells collected per treatment condition across datasets. Different colors correspond to different replicates multiplexed within the same dataset

(H, I) Expression of known ExEN gene markers for mESC (B) and mesendoderm (D) starting states

(J, K) Expression of known mESC gene markers for mESC (C) and mesendoderm (E) starting states

(L) Heatmap depicting correlation of mean expression profiles for cells starting at the mesendoderm stage assigned to each treatment combination. The last column (indicated by grey circles) corresponds to control cells. Legend colors are as in (A)


A

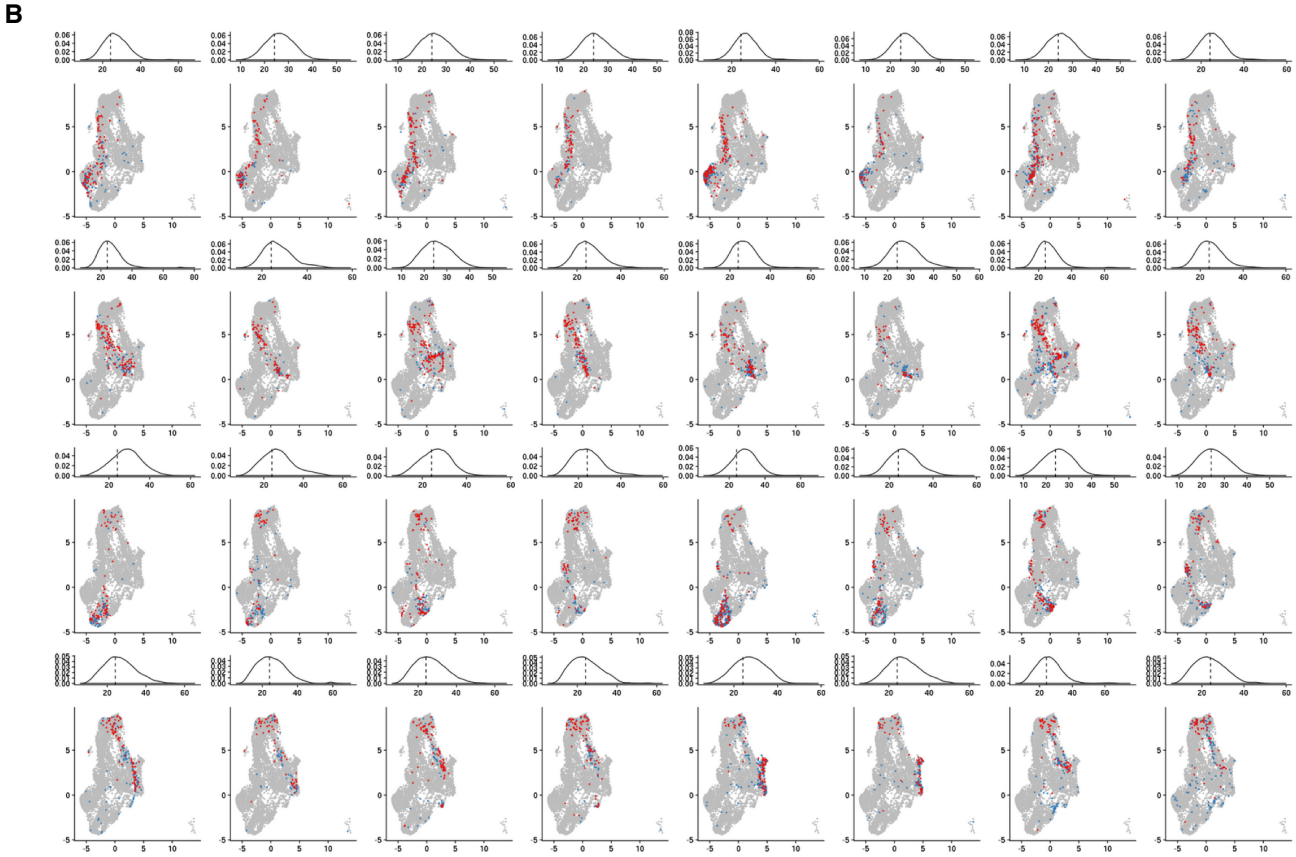

B

Figure S2. UMAP visualization of cells assigned to different treatment groups. Related to Figure 2C-D
(A, B) Visualization of cells assigned to a few treatment groups for cells starting at mESC (A) and mesendoderm (B) states. Order of treatment conditions left to right, top to bottom is as in Figure S2A. For each subplot; Top: Distribution of distances between cells assigned to that treatment condition and control cells. Bottom: UMAP visualization of cells assigned to that treatment condition, colored by dataset

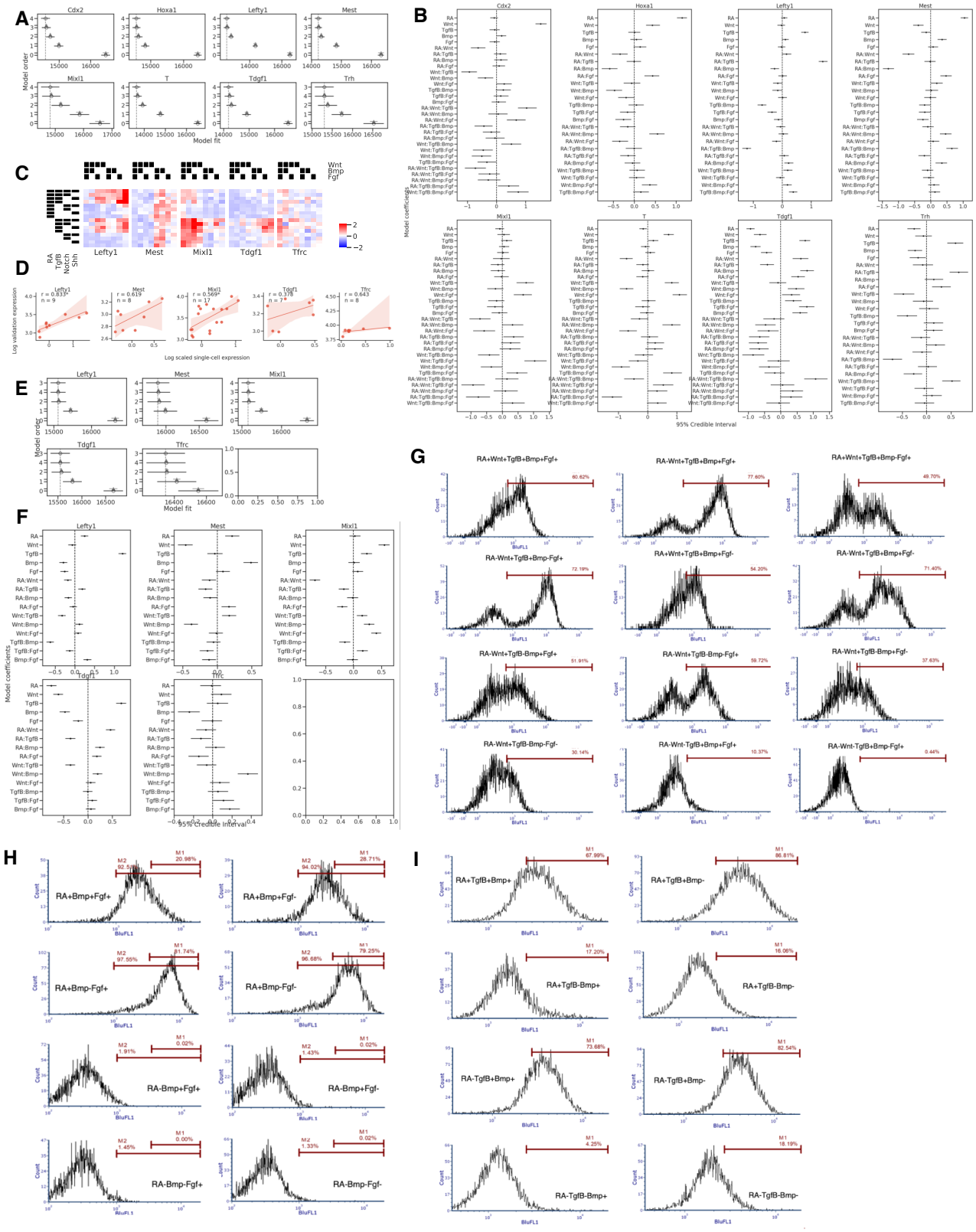


Figure S3. Related to Figure 3

(A-B) Bayesian model regression analysis of genes in cells starting at the mESC stage.

(A) Model comparisons for validation genes. Empty circles indicate the mean score of that model, black horizontal lines indicate the standard deviation of the score, and grey horizontal lines indicates the standard deviation of the difference between the score of that model and the best model. Lower scores imply a better model fit.

(B) 95% credible intervals on coefficients for optimal models

(C-F) Gene-level analysis of cells starting at the mesendoderm stage
(C) Mean scaled gene expression of genes chosen for validation of cells that observed different treatment conditions
(D) Correlation of average scRNA-seq expression with GFP expression for reporter cell lines. Spearman rank correlation coefficient is reported for each gene and is significant at $p < 0.05$ for Lefty1 and Mixl1. 95% confidence intervals are estimated via bootstrap ($n = 1000$).
(E) Model comparisons for validation genes. Empty circles indicate the mean score of that model, black horizontal lines indicate the standard deviation of the score, and grey horizontal lines indicates the standard deviation of the difference between the score of that model and the best model. Lower scores imply a better model fit.
(F) 95% credible intervals on coefficients for optimal models
(G-I) GFP expression of cells in T (G), Hoxa2 (H) and Lefty1-GFP(I) cell lines in response to combinatorial treatments

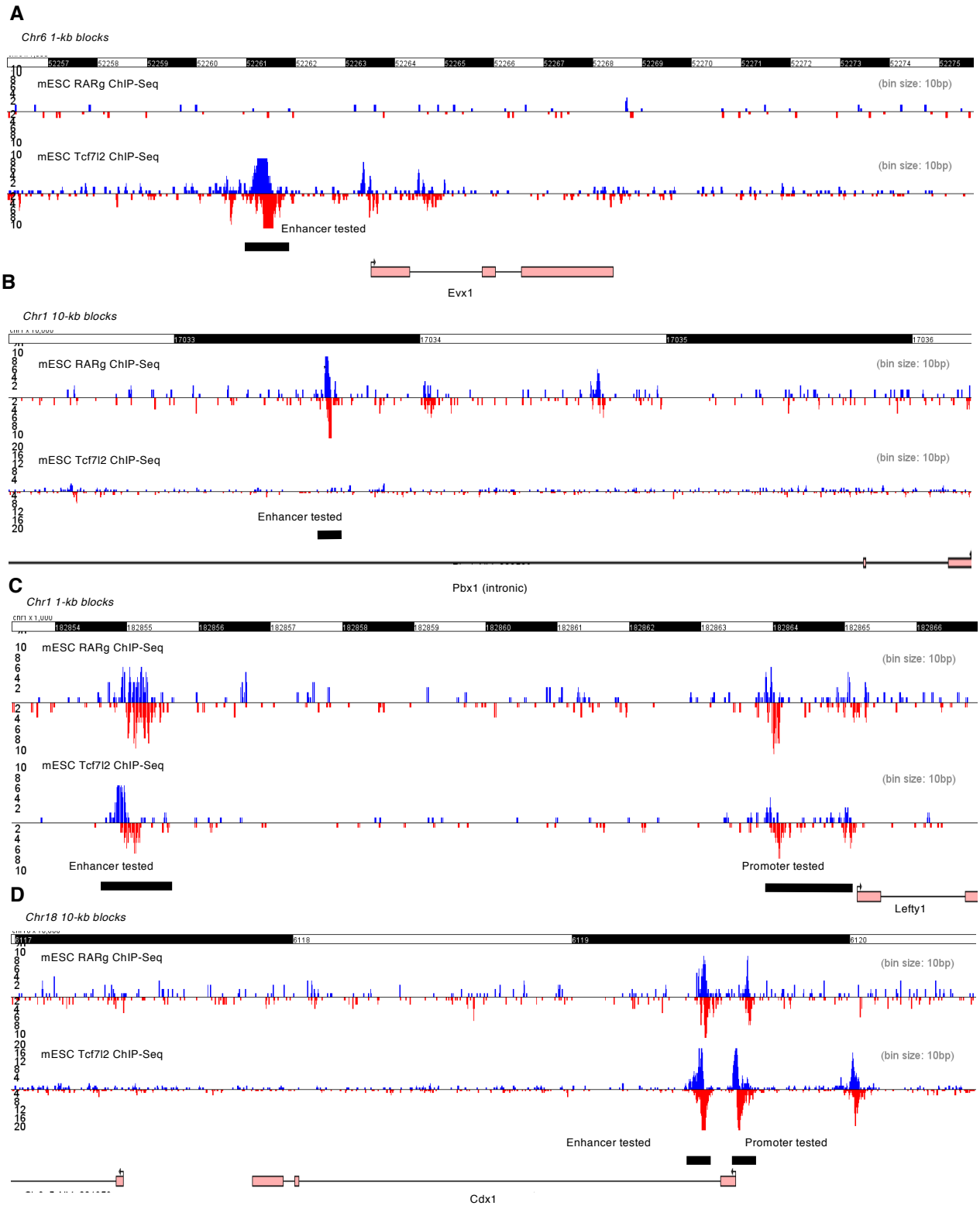


Figure S4. ChIP-seq tracks for the RA effector Rarg and Wnt effector Tcf7l2 for Evx1, Pbx1, Lefty and Cdx1 (A-D). Related to Figure 4.

Enhancers and promoters tested in Figure 4 are annotated in black.

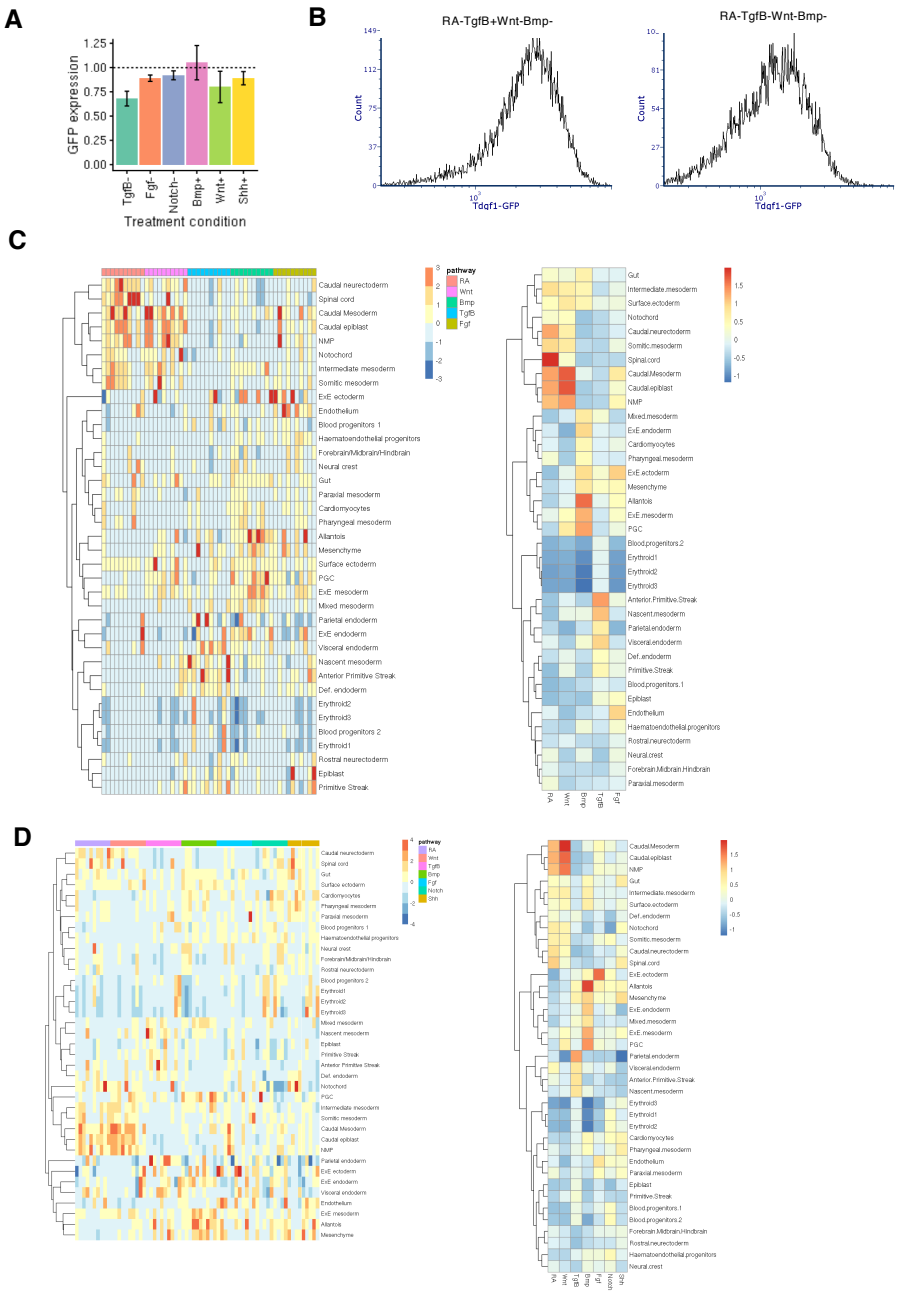


Figure S5. Related to Figure 6, 2.
(A) Average and standard deviation of GFP expression normalized to the TgfB+Fgf+Bmp-Notch+Wnt-Shh- baseline in TgfB-GFP cell line
(B) GFP expression in TgfB-GFP cell line comparing TgfB+ vs. TgfB- treatments
(C-D) Average expression of top ten genes most predictive of each pathway's activity from Figure 2E (left) and aggregated average expression of these genes by pathway (right) for mESC (C) and mesendoderm (D).

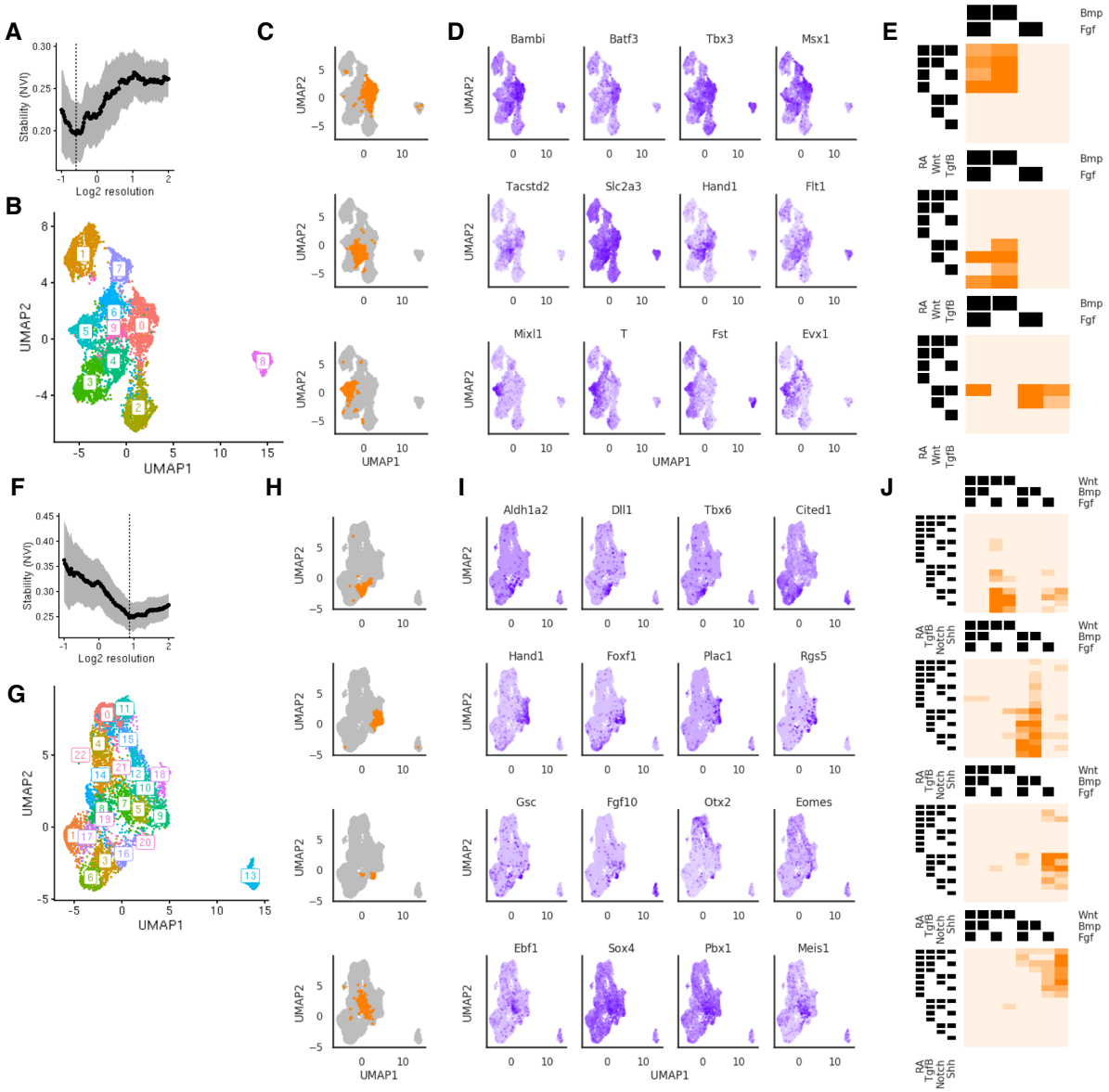


Figure S6. Related to Figure 5-7.
(A-J) Unsupervised clustering analysis for cells starting at the mESC stage (A-E) and mesendoderm stage (F-J) identifies cell subpopulations also discovered via comparison with cell atlas.
(A, F) Stability estimated as normalized variation of information with respect to small perturbations in the resolution parameter. Grey ribbon indicates standard deviation in stability. Dashed line corresponds to resolution chosen for final clustering results
(B, G) Clustering results given by resolution in (A, F)
(C, H) Stable clusters of cells identified by unsupervised clustering analysis
(D, I) Expression of gene markers identified for each cell type classifier visualized on the UMAP. Gene markers shown are differentially expressed with respect to the rest of the cell population at q-value < 0.05
(E, J) Fraction of cells assigned to each treatment combination that belong to the corresponding cluster in (A)

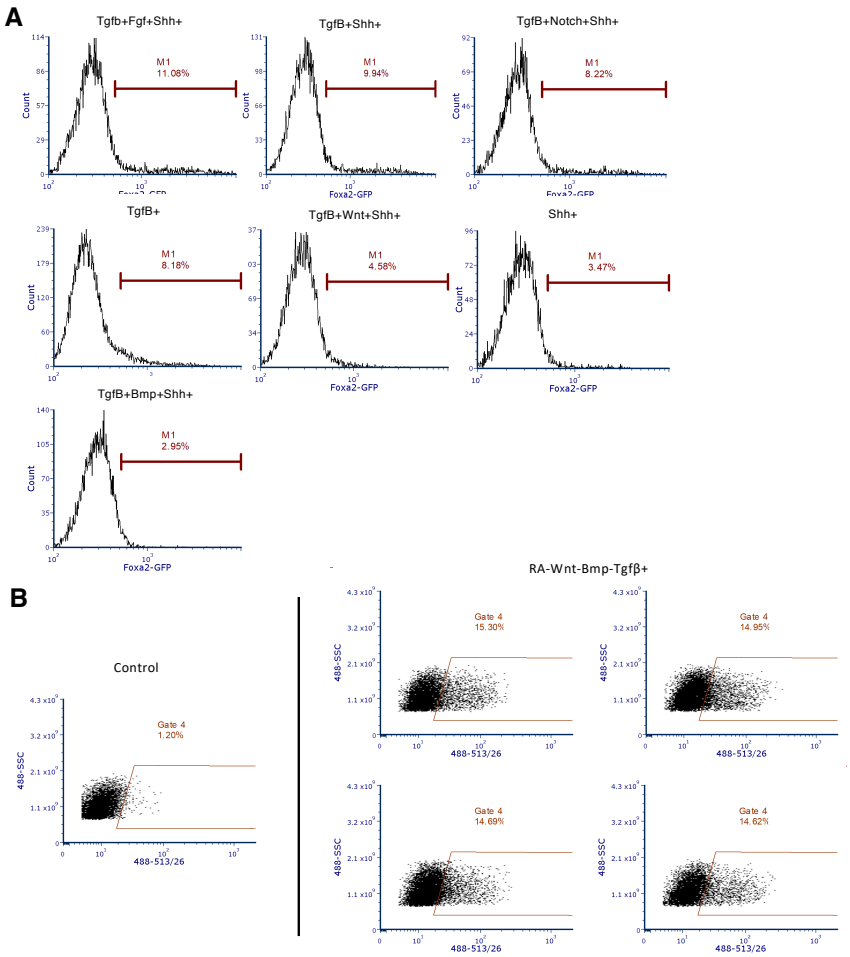


Figure S7. Cells expressing notochord markers are selected for by activating TgfB+. Related to Figure 7.
(A) GFP expression in Foxa2-GFP cell line in response to combinatorial treatments
(B) Flow cytometry plots showing Foxa2-GFP expression in the presence of RA-Wnt-Bmp-Tgfβ+. On average, 14.9% of cells show Foxa2-GFP expression.