Figures and figure supplements

FoxP2 isoforms delineate spatiotemporal transcriptional networks for vocal learning in the zebra finch

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Figure 1. Overexpression of FoxP2 isoforms. (A) Schematics show full-length (FoxP2.FL) and 10+ (FoxP2.10+) isoforms. Regions whose transcripts were targeted by the complementary riboprobes are shown in red. (B) Left panel depicts experimental design to test for isoform-specific expression in vivo. Middle and right images depict two sections from the same female brain. For purposes of validation only, the bird’s right hemisphere (shown on left) was injected with an AAV expressing FoxP2.FL while the left hemisphere was injected with the FoxP2.10+ construct. Two weeks post-injection, robust signals were observed in the striatopallidum of both hemispheres using the mid probe but only in the hemisphere injected with the FoxP2.FL construct using the 3’ probe. Signals reflect both the endogenous FoxP2 expression pattern (Teramitsu and White, 2006; Teramitsu et al., 2004; Teramitsu et al., 2010) as well as enhanced levels due to viral-driven expression. (C) FoxP2 expression quantified by qRT-PCR in juvenile males that were bilaterally injected with one of the constructs at 35d using primers that identify both isoforms (left graph) or only the FoxP2.10+ isoform (right graph). Using the Figure 1 continued on next page.
former primers, enhanced expression is observed in the FoxP2.FL (grey: 126.5 ± 13.53%; n = 6) and FoxP2.10+ (red: 162.4 ± 26.77%; n = 6) groups relative to levels of birds that received the GFP control construct (green: 100 ± 7.54%; n = 7). Using the ‘FoxP2.10+ Only’ primers, enhanced expression is only observed in the FoxP2.10+ group (red: 279 ± 52.69%; n = 6) vs. the FoxP2.FL (grey: 126.16 ± 24.61%; n = 6) and GFP (green: 100 ± 22.95%; n = 7). Values represent percentage relative to GFP ± SEM. * and # denote p=0.031 and p=0.084, respectively, of an unpaired two-tailed bootstrap test. (D) A cell in the zebra finch striatopallidum expressing GFP (indicating viral transduction; green), endogenous FoxP2 as revealed by an antibody directed to the C-terminus (red), and Xpress-FoxP2.10+ revealed by an antibody to the Xpress tag (cyan). The Xpress signal is reminiscent of FoxP2.10+ aggresomes observed by Vernes et al. (Vernes et al., 2006). Orthogonal views of the cell are presented below. Scale bar = 5 μM.
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Figure 1—figure supplement 1. FoxP2 isoform protein expression. Immunoblot loaded with 20 and 10 micrograms of zebra finch whole brain homogenate. Incubation with an antibody (Proteintech, Rosemont, IL, USA, Cat. No. 20529–1-AP) against the N-terminus of FoxP2 reveals bands at the predicted molecular weights in zebra finch (FL, ~77 and 79 kDa, 10+, ~47 kDa). GAPDH signals (~32 kDa) were used as a loading control.

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Figure 1—figure supplement 2. FoxP2 qRT-PCR in VSP samples. In contrast to Area X, FoxP2 overexpression is not observed in VSP samples.

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Figure 2. Overexpression of FoxP2 isoforms affect song learning and/or song variability. (A) Timeline of experimental procedures relative to critical periods in song development. (B) Schematic illustrates NS-UD or UD-UD experiments performed on adjacent days. (C) The effect size of two hours of song duration, amplitude, pitch, and other features. (D) % Similarity to tutor over time for different conditions. (E) Spectrograms showing differences in tutor and experimental groups. (F) Summary table of learning and variability outcomes.
UD singing on syllable CV was calculated using the formula \((\text{NS-UD})/(\text{NS} + \text{UD})\) after an NS-UD, UD-UD experiment performed at ~60d and 61d as in (B). Overexpression of FoxP2.0FL (grey bars; \(n = 16\) syllables; Duration = \(-0.059 \pm 0.029\); AM = \(-0.010 \pm 0.028\); Entropy = \(-0.038 \pm 0.04\)) diminishes singing induced variability relative to that seen in GFP-expressing controls (green bars; \(n = 9\) syllables; Duration = \(-0.128 \pm 0.071\); AM = \(-0.065 \pm 0.035\); Entropy = \(-0.091 \pm 0.034\)). In contrast, overexpression of FoxP2.10+ (red bars; \(n = 13\) syllables; Duration = \(0.070 \pm 0.054\); AM = \(0.088 \pm 0.047\); Entropy = \(0.048 \pm 0.029\)) leads to a singing-induced state of relative invariability. Values and bar heights represent the average effect size for all syllables within the virus construct group ±SEM. * denotes significant result in one-way ANOVA (Duration: \(F(2,35) = 3.95, p=0.028\); AM: \(F(2,35) = 3.96, p=0.028\); Entropy: \(F(2,35) = 3.63, p=0.037\)) and Tukey’s HSD post-hoc test (\(p<0.05\)). (D) Learning curves plot the relationship between percentage similarity to tutor as a function of time. Animals overexpressing GFP (green, letter ‘B’; \(n = 7\) birds; ~65 d similarity = 67.2 ± 6.64%) or FoxP2.10+ (red, letter ‘A’; \(n = 5\) birds; ~65 d similarity = 75.8 ± 2%) learn significantly better than those overexpressing FoxP2.0FL (grey, letter ‘C’; \(n = 5\) birds; ~65 d similarity = 44.3 ± 10.1%). Values are mean ±SEM. Data are binned by day (top panel; bold points represent group mean and shifted smaller points are individual birds) or by individuals (bottom panel). Significantly different groups tested by one-way ANOVA (Bin 1: ~40d \(F(2,11) = 6.06, p=0.016\); Bin 3: ~55d \(F(2,13) = 6.04, p=0.014\); Bin 4: ~60d \(F(2,14) = 9.94, p=0.002\); Bin 5: ~65d \(F(2,14) = 4.76, p=0.026\)) and Tukey HSD post-hoc test (\(p<0.05\)) are denoted by capital and lowercase lettering. (E) Exemplar motifs of a tutor and three of his 65d pupils, each of which was injected with a different viral construct at 30d. These examples illustrate the percent similarity depicted in panel D. (F) Summary of the learning and variability phenotypes observed after virus injection.

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Figure 2—figure supplement 1. Raw acoustic feature variability in the NS-UD and UD-UD conditions by virus group. The raw acoustic feature CVs transformed by the calculation in Figure 2C show the variability relationship between NS-UD and UD-UD paradigms for all measured acoustic features. For most song features, UD singing drives increases in CV in the GFP group. This effect is blocked or reversed in the FoxP2.FL and FoxP2.10+ groups, respectively. Notably, the songs of FoxP2.10+ animals following 2 hr of UD song were significantly less variable than those after 2 hr of non-singing. Asterisks indicate a significant difference (p<0.05) in a paired resampling test within virus construct. See Materials and methods for more information regarding the transformation of raw data to effect size.

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Figure 3. WGCNA yields behaviorally relevant modules. (A) Dendrogram (top) illustrates the topological overlap between genes in the juvenile Area X overall network. Modules delineated by automated tree trimming are shown below and are depicted by arbitrary colors. Beneath the color bar, gene

Figure 3 continued on next page
significances to the quantified behaviors (number of motifs sung, tutor similarity, acute variability changes, and overall variability; see Results) are indicated by a heatmap wherein red indicates a positive correlation and blue indicates a negative correlation (see B for scale). (B) Correlations between module eigengenes and each behavior are presented as a heatmap. The Pearson’s $r$ and, in parentheses, Student's asymptotic $p$-values for modules where $p \leq 0.05$ are displayed. P-values are uncorrected for multiple hypothesis testing but those that pass FDR correction at $p \leq 0.05$ are denoted by * (See ‘Correlation of behavior to gene expression’ in Materials and methods). (C) For all significant module-trait correlations, the relationship between gene significance and module membership is plotted for each gene in the module. Dashed lines represent the linear regression and the Pearson’s $r$ (‘cor’) and p-value as determined by Fisher’s z-transformation are indicated above each plot. (D) The average whole network connectivity ($k_{Total}$) within each module reveals that the purple, green, and pink modules are composed of the most strongly connected genes in the network. (E) Term significances for the black, darkred, and green modules are indicated for disease, gene ontology biological process and molecular function, as well as for pathways for categories annotated as ‘neuronal’ in the GeneCards GeneAnalytics software. (F) Network plots of the modules presented in panel E where nodes represent genes scaled by the node’s intramodular connectivity and edge width displays the topological overlap between genes.

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Figure 3—figure supplement 1. GFP-only Area X network. A gene coexpression network built only from GFP birds is presented. Significant module-trait correlations are shown in a heatmap, right. Colors are consistent with Figure 3A.
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Figure 3—figure supplement 2. FoxP2.FL-only Area X network. A gene coexpression network built only from FoxP2.FL birds is presented. Significant module-trait correlations are shown in a heatmap, right. Colors are consistent with Figure 3A.

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Figure 3—figure supplement 3. FoxP2.10+-only Area X network. A gene coexpression network built only from FoxP2.10+ birds is presented. Significant module-trait correlations are shown in a heatmap, right. Colors are consistent with Figure 3A.

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Figure 3—figure supplement 4. Module preservation between GFP vs FoxP2 FL and FoxP2.10+ combined, GFP vs. FoxP2-FL, and GFP vs. FoxP2.10+ networks. Plots depict module preservation vs. module size for each virus construct pair and allow for visual assessment of whether a specific module exists in two conditions. Middle and upper dashed horizontal lines indicate thresholds for low and high preservation, respectively.
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Figure 3—figure supplement 5. Juvenile Area X gene coexpression network. The gene coexpression network is displayed with genes represented as nodes and colors indicating the module assignment of each gene. Nodes are scaled by their degree and edge color is the combination of the module colors of nodes connected by the edge. Poorly connected nodes are excluded (see Methods).
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Figure 3—figure supplement 6. Intersample correlation for Area X samples. The intersample correlation for the Area X samples does not clearly delineate clusters by virus construct. Virus construct is indicated in green (GFP), black (FoxP2.FL), and red (FoxP2.10+). Below, time singing is indicated on a blue-red color scale where the samples from birds that sang the most are colored the deepest red.

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Figure 4. Juvenile Area X singing related gene coexpression patterns are not preserved in juvenile VSP. (A) Dendrogram (top) displays the topological overlap in Area X between genes common to both juvenile Area X and VSP networks. Beneath, the module assignments and the gene significances for Figure 4 continued on next page.
each gene as calculated using expression from VSP ('V') or Area X ('X') for all behaviors are quantified as in Figure 3A. Module colors are consistent with those presented in Figure 3. (B) Module preservation (Zsummary) for all modules that were present in both Area X and VSP displayed as a function of module eigengene correlation to motifs. Lower and upper dashed horizontal lines indicate thresholds for low and high preservation, respectively. (C) Circle plots display the adjacencies between the 20 most well-connected genes in the Area X black, cyan, green, royalblue, and blue modules. The adjacency between genes is indicated by edge thickness. Genes grouped together in the black, cyan, royalblue, and blue song modules in Area X have numerous and strong connections. Those connections are weakened or nonexistent in VSP such that genes sort into different modules in VSP. In contrast, the green learning-related module genes maintain their common grouping and connections in VSP. (D) Raw gene expression is tightly correlated between Area X and VSP for the genes in the black, cyan, green, royalblue, and blue modules (top). Only the intramodal connectivity of the genes in the green learning-related module is correlated between Area X and VSP (bottom). Dashed lines represent the linear regression.

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Figure 5. Area X song production but not learning-related modules are preserved into adulthood. (A) Dendrogram (top) displays the topological overlap in juvenile Area X between genes common to both juvenile and adult Area X networks. The module assignments and the gene significances to Figure 5 continued on next page.
motifs in juveniles and adults are presented below. Module colors are consistent with those presented in Figure 3. (B) Module preservation (Zsummary) for all modules that were present in both juvenile and adult Area X displayed as a function of ME correlation to motifs. Lower and upper dashed horizontal lines indicate thresholds for low and high preservation, respectively. (C) Circle plots display the adjacencies between the 20 most well-connected genes in the juvenile Area X black, cyan, green, royalblue, and blue modules. The adjacency between genes are indicated by edge thickness. Genes grouped together in the black, cyan, royalblue, and blue song modules in Area X have numerous and strong connections that are mostly maintained in adulthood. The densely interconnected green learning-related module genes found in juveniles do not maintain these relationships in adulthood. (D) Strong positive correlations between gene significance to motifs exist for all modules (top row). Ranked expression values for the genes in each module also show positive correlation (middle row). Intramodular connectivity is more positively correlated between ages for the black, cyan, royalblue, and blue song production modules than for the green learning-related module (bottom row).

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Figure 5—figure supplement 1. Differential connectivity as a function of green module membership. Differential connectivity (kTotal.Juvenile - kTotal.Adult) is plotted against green module membership. Points are colored by correlation to tutor percent similarity from low to high on a blue-white-red scale. The genes most differentially connected are among the most strongly learning related and are well-correlated to the green module eigengene. Data points representing genes in the green module have a green stroke.

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Figure 6. Gene significance and network position implicate MAPK11 as a molecular entry point to vocal learning mechanisms. (A) The 20 genes with the highest to lowest gene significances to tutor similarity (sorted from top to bottom) are shown. Each column represents a bird and columns are sorted in order of increasing tutor similarity from left to right. Gene expression is scaled such the highest and lowest expression across samples have the brightest shade of red or blue, respectively. (B) Expression of MAPK11 is replotted, here separated by virus group and then sorted by increasing tutor percentage similarity. (C) The FoxP2 binding sequence as annotated by the JASPAR database (top) and a potential binding site found in the MAPK11 ‘promoter’. (D) Amplification of genomic DNA (‘Genomic’) with primers for a region of the MAPK11 ‘promoter’ that contains a putative FoxP2 binding site enrich a fragment of predicted size (red arrowhead) in the pull-down lane (FoxP2) but not the control (IgG) lane. (E) MAPK11 and its 10 closest network neighbors, including green learning-related module members and hub gene ATF2, as defined by topological overlap.

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Figure 6—figure supplement 1. MAPK11 PCR Product Sequencing. The MAPK11 NCBI RefSeq sequence (highlighted in grey) contains a potential FoxP2 binding motif (red). Genomic DNA was isolated and subjected to PCR with primers (highlighted in yellow) amplifying the region of interest. The product of each primer was sequenced showing our primers amplify a fragment containing the proposed FoxP2 binding motif.

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Figure 7. Protein-level interactions between song production and learning-related module genes in juvenile Area X. A protein interaction network plot using the STRING database between genes in learning-related (darkred, green, greenyellow) and song production (black, blue, darkgreen, orange, royalblue) modules. Nodes are scaled by number of connections. Edge width is determined by scaling the STRING protein interaction confidence score for the two nodes by the product of each node’s intramodular connectivity. Interactions within learning or song production modules are omitted for clarity.

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Figure 8. Changes in vocal plasticity state between juvenile and adult birds. (A) Schematics depict the juvenile straitopallidum (left) in a 'plastic' state in which genes in learning-related modules (green) are densely interconnected and of high importance in the network. Simultaneously, singing driven...
gene coexpression patterns (blue) occur in Area X. In the adult striatopallidum (right), song production modules (blue) exist as they do in juveniles, but the learning-related modules do not and are replaced by coexpression patterns that presumably underlie the maintenance of song (red). (B) Area X modules in the juvenile brain are plotted to emphasize their preservation in adult Area X (x-axis) and juvenile VSP (y-axis). Points representing the module colors are scaled by the module’s absolute correlation to learning (left) or the absolute correlation to singing (right), emphasizing the preservation of singing coexpression patterns into adulthood and learning coexpression patterns in the juvenile striatopallidum. (C) Genes in song production or learning-related modules that are within two steps of ATF2 in the high-confidence protein interaction network are shown. Nodes are scaled by intramodular connectivity in juveniles (left) or adults (right) with edge width indicative of adjacency between genes in the coexpression network. The change in coexpression patterns across age groups causes decreased connectivity of many learning-related genes, driving an alteration in the network’s landscape which may underlie the transition from song learning to song maintenance.

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