A direct fate exclusion mechanism by Sonic Hedgehog-regulated transcriptional repressors

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Abstract

Sonic hedgehog (Shh) signaling patterns the vertebrate spinal cord by activating a group of transcriptional repressors in distinct neural progenitors of somatic motor neuron and interneuron subtypes. To identify the action of this network, we performed a genome-wide analysis of the regulatory actions of three key ventral determinants in mammalian neural tube patterning: Nkx2.2, Nkx6.1 and Olig2. Previous studies have demonstrated that each factor acts predominantly as a transcriptional repressor, at least in part, to inhibit alternative progenitor fate choices. Here, we reveal broad and direct repression of multiple alternative fates as a general mechanism of repressor action. Additionally, the repressor network targets multiple Shh signaling components providing negative feedback to ongoing Shh signaling. Analysis of chromatin organization around Nkx2.2, Nkx6.1 and Olig2 bound regions, together with co-analysis of engagement of the transcriptional activator Sox2, indicate that repressors bind to, and likely modulate the action of, neural enhancers. Together, the data suggest a model for neural progenitor specification downstream of Shh signaling wherein Nkx2.2 and Olig2 direct repression of alternative neural progenitor fate determinants, an action augmented by the overlapping activity of Nkx6.1 in each cell type. Integration of repressor and activator inputs, notably activator inputs mediated by Sox2, is likely a key mechanism in achieving cell type-specific transcriptional outcomes in mammalian neural progenitor fate specification.

Introduction

Sonic hedgehog (Shh) signaling is critical for the specification of ventral neural progenitor types that give rise to molecularly and functionally distinct classes of ventral neurons in the developing vertebrate central nervous system (Dessaud et al., 2008). Several lines of evidence, including the direct binding of Gli transcription factors to their cis-regulatory modules, have identified Nkx2.2, Nkx6.1 and Olig2 as direct transcriptional targets of Shh's ventral neural patterning activity (Lei et al., 2006; Oosterveen et al., 2012; Peterson et al., 2012; Wang et al., 2011). Each of these factors has been shown to function as a transcriptional repressor in neural patterning: Olig2 is required for specification of somatic motor neuron progenitors, Nkx2.2 for the specification of V3 interneuron progenitors, while Nkx6.1 expression overlaps V2 and V3 interneurons and somatic motor neuron progenitors and is essential for normal specification of both populations (Briscoe et al., 2000; Briscoe et al., 1999; Lu et al., 2002; Mizuguchi et al., 2001; Muhr et al., 2001; Novitch et al., 2001; Sander et al., 2000; Vallstedt et al., 2001; Zhou and Anderson, 2002; Zhou et al., 2001) (Fig.1A). Although their general roles in specifying respective neural progenitor types downstream of Shh pathway have been documented through mis-expression studies (Briscoe et al., 2000; Mizuguchi et al., 2001; Novitch et al., 2001), their direct DNA associated regulatory actions and target specificity is not understood.

In this report, we undertook an integrative, functional genomic approach to identify genomic binding regions and target genes of Nkx2.2, Nkx6.1, and Olig2 in embryonic stem cell derived neural progenitors. Our data highlight previously unappreciated breadth of direct fate exclusion, modulation of ongoing upstream Shh signaling input through multiple signaling nodes (Lek et al., 2010), and Sox2 input into available enhancers with a resulting cell-type specific output directing a specific neural progenitor type (Oosterveen et al., 2012; Peterson et al., 2012).

Materials and Methods

Chromatin immunoprecipitation analysis

Neural progenitors were derived from mouse ESCs in serum-free suspension culture in all-*trans* retinoic acid (RA, 500nM) (Sigma) and SAG (Calbiochem; 100-800nM) as described previously (Peterson et al., 2012; Wichterle et al., 2002). ChIP-seq was performed as described in (Peterson et al., 2012) and analyzed using CisGenome 2-sample conditional binomial algorithm in an mm9 genome assembly; data is accessible through GEO (GSE65462). In bioinformatics studies, the nearest genes 5' and 3' of the transcription factor binding region where considered potential transcriptional targets. Conformational and potential isoform bias in ChIP-seq was minimized by employing a cocktail of factor specific antibodies: Millipore [AB9610] for Olig2, the cocktail of (DSHB [74.5A5], Sigma [HPA003468], and custom rabbit polyclonal [gift from T. Jessell]) for Nkx2.2, and the cocktail of (DSHB [F55A10, F55A12, F64A6B4, and F65A2], RD Systems [AF5857], and custom rabbit polyclonal [gift from T. Jessell]) for Nkx6.1. Gli3-FLAG ChIP was performed with anti-FLAG (M2, Sigma) on a mouse ESC line with a 3xFLAG-Avi tag inserted immediately upstream of the Gli3 start codon. Motif analysis was performed on the top 2,000 peaks with CisGenome or DREME combined with TOMTOM (Bailey, 2011). Aggregate plot and heatmap clustering were performed with HOMER (Heinz et al., 2010). Gene ontology annotation was performed through the DAVID program (http://david.abcc.ncifcrf.gov/). Sox2 [ESC] (GSE11724), Gli1, Sox2, H3K4me2, and H3K27ac [all NPC] (GSE42132), H3K4me2 [ESC] (GSE11172), H3K27ac [ESC] (GSE24164), and DNaseI-seq [E14.5 brain] (GSM1014197) were used in this study. A crude

neural tube preparation for ChIP-qPCR was performed on limbless, decapitated E10.5 mouse trunk samples, in biological and technical duplicates. See Table S3 for primer sequences.

Ectopic expression study

Open reading frames (ORF) were targeted to the engineered *HPRT* locus in the mA2.lox.Cre mESC line to express the ORFs under the control of a tetracycline response element (Iacovino et al., 2011). Neural progenitors were induced with 500nM RA as described in the previous section and the transgene was activated with 1µg/ml doxycycline (Dox) after two days of RA treatment. RNA samples were collected up to 24hr post Dox induction and subjected to RNA-seq or BioMark (Fluidigm) RT-qPCR assay. RNA-seq was performed at 12hr post induction in biological duplicates and technical triplicates and analyzed with STAR aligner and DEseq2 (GEO accession number: GSE65462). The following DNA binding deficient mutant forms of each repressor factor were employed in the study: WFQ**N**HRY [Nkx2.2] (Pradhan et al., 2012), WFQ**N**RRT [Nkx6.1](Lee et al., 2008), NSR**E**RKR [Olig2](Longo et al., 2008)). See Table S3 for primer sequences.

Protein binding microarray (PBM) experiments

Full-length mouse Olig2 was purified from *E. coli* as a GST fusion. PBM experiments were conducted using 200 nM Olig2 in the PBM binding reactions essentially as described previously, with the addition of 0.023% Triton X-100 to the binding buffer, using two custom "all-10mer" array designs (AMADID #015681 and #016060) (Berger and Bulyk, 2009). PBM data were quantified and normalized, and data from the two arrays were combined as described previously (Berger and Bulyk, 2009) to determine the *in vitro* DNA binding specificity of Olig2. The resulting PWMs were trimmed as described previously (Gordan et al., 2011) to remove flanking sequence of low information content.

Results and Discussion

To examine the direct regulatory actions of the Shh-initiated transcriptional network (Lei et al., 2006; Oosterveen et al., 2012; Peterson et al., 2012), we performed ChIP-seq for Nkx2.2, Nkx6.1 and Olig2 on neural progenitors derived *in vitro* from mouse embryonic stem cells (mESCs); a model system that recapitulates *in vivo* patterning processes (Peterson et al., 2012; Wichterle et al., 2002) (Table S1). The binding events were reproducibly detected in biological replicates (Fig. S1A); moreover, binding was confirmed in neural tube preparations from E10.5 embryos at 28 out of 36 loci tested (Nkx2.2: 7/11, Nkx6.1: 11/11, Olig2: 10/14) (Fig. S1B). DNA regions bound by each factor showed considerable overlap (Fig. 1B); an even greater overlap was observed in the potential target genes: assigned as the nearest 5' and 3' neighboring gene to the bound region (Fig. 1C). These data suggest that the three factors engage a common set of target genes though cis-regulatory elements many of which bind all three factors, as well as discrete regulatory elements engaging specific members of the regulatory trio.

To assess the significance of the predicted target gene overlap, we performed Gene Ontology (GO) term analysis. "Neural Differentiation" and "Transcription Regulator Activity" GO terms were strongly enriched in the gene sets targeted by all three repressors (3.1 fold and 2.0 fold) when compared to single or pair-wise targeted gene sets. This data suggests that co-targeting defines the most relevant neural targets within the repressor network in neural fate specification. Detailed analyses showed that a number of known neural fate determinants as well as components of the Hedgehog pathway were co-targeted (Fig. 1D-F, S1-4). Targeted neural fate regulators included both progenitor expressed transcription factors (eg. *Pax6*, *Irx3*) as well as transcriptional regulators active in post-mitotic neurons (eg.

Isl1, *En1*) (Fig. S1). These results are consistent with at least two regulatory strategies for the ventral repressor program: the repression of alternative neural subtype fates at both progenitor and post-mitotic levels and feedback modulation of the Hedgehog pathway. Interestingly, though Nkx2.2, Nkx6.1 and Olig2 mediate tissue patterning as Shh primary targets (Lei et al., 2006; Oosterveen et al., 2012; Peterson et al., 2012; Wang et al., 2011), intersectional analysis showed a limited overlap with Gli1 and Gli3 bound regions overall (Fig. 1G, S6D), with the exception of putative cis-regulatory regions around ventral neural progenitor sub-type specifiers including Nkx2.2, Olig2 and Nkx6.1 (Fig. S2) (Lei et al., 2006; Oosterveen et al., 2012; Peterson et al., 2012; Wang et al., 2011). At the target gene-level, Gli factors showed extensive overlap, particularly with genes targeted by all three repressors (Fig. 1H, S6E). Thus, the initial Shh/Gli input, and downstream Nkx2.2, Nkx6.1 and Olig2 repressor programs, share common target genes but act through distinct cis-regulatory modules.

To functionally address predicted repressor program, we used a doxycycline-inducible transgenic mESC system (Iacovino et al., 2011; Mazzoni et al., 2011) to ectopically express Nkx2.2, Nkx6.1 and Olig2 singly, or in pairwise combinations: Nkx2.2 and Nkx6.1 (Nkx6.1-2A-Nkx2.2) and Olig2 and Nkx6.1 (Nkx6.1-2A-Olig2). Samples were subjected to global analysis of transcriptional activity by RNA-seq 12 h post-Dox induction, and targeted analysis of a subset of genes by microfluidic-based RT-qPCR (Fluidigm) (Fig. 2A, B). At the global level, we observed an extensive set of targets displaying reduced mRNA levels on individual or pairwise activation of Nkx2.2, Nkx6.1, and Olig2, as well as their pairwise combinations (Fig. 2C, Table S2), in good agreement with predictions of the direct DNA interaction data (Fig. 1B-E, S1). Factor specific effects were only evident for a small set of genes (clustered toward the left in Fig. 2C). Overall, 76-96 genes were downregulated by individual factor expression, and 172 to 192 in pairwise combinations, setting a 2-fold cutoff

in target gene repression (Table S2). Of these down-regulated genes, 57-71% were associated with binding of the respective factors, a 1.9 - 4.7 fold enrichment over random expectation ($p<<0.01$) (Table S2). Consistent with the ChIP-seq GO profile, the RNA-seq GO profile showed enrichment for neural differentiation and transcription factor terms (Table S2). Importantly, alternative fate determinants and Shh pathway components were strongly represented in the highly down-regulated gene set (Fig. 2C).

Select genes representing ventral and dorsal neural progenitor fate determinants and Shh pathway components were subjected to a more extensive temporal analysis of regulation by RT-qPCR (Fluidigm). Consistent with the RNA-seq data, Nkx2.2, Nkx6.1 and Olig2 reduced mRNA levels for all tested ventral fate determinants individually (Fig. 2D); their effects were evident within 6 hrs of Dox-mediated induction. Interestingly, Nkx6.1 enhanced the repressive phenotype observed with Olig2 and Nkx2.2 (Fig. 2D). Together, the results suggest a direct repressive action of the ventral patterning factors on the expression of other transcriptional determinants of neural patterning that is likely through independent regulatory mechanisms given the additive effects observed in the co-expression analysis. However, genes encoding dorsal neural progenitor fate determinants showed a marked weighting in their specific responses to individual factors: some genes showed little response (e.g. *Msx2* and *Zic1*) while expression of others was reduced on activation of a specific factor, or combination of factors (e.g. *Pax3*, *Pax7*, *Msx1*) (Moore et al., 2013) (Fig. 2E, Table S2). Such differential sensitivities to repressor input suggest the regulatory systems restricting dorsal and ventral progenitor fates are largely distinct programs (Briscoe et al., 2000). The targets of the repressor network revealed here are notably broader than previously appreciated (Briscoe et al., 2000; Moore et al., 2013; Oosterveen et al., 2012). This likely reflects redundancies in the regulatory circuitry that obscure de-repression effects in mutant analysis

and an emphasis on a restricted set of local cross-repressive interactions from ectopic expression studies.

Repression was also highly selective for Hedgehog signaling components with *Nkx2.2* displaying a stronger inhibitory activity on gene expression than Olig2. Though expression of genes enhancing (e.g. *Gli2*, *Gas1*, and *Boc*) and inhibiting (e.g. *Gli3*) Shh signaling showed reduced expression, the strongest effect was observed on key genes that promote Shh signaling including *Gli2*, the predominant transcriptional activator in the Hedgehog pathway, and *Gas1*, a co-receptor in Shh signaling (Fig. 2F). These results are consistent with the notion that Nkx2.2 exerts its patterning action in part by negative feedback regulation of Hedgehog pathway components (Lek et al., 2010).

To determine whether the observed repressive effects were dependent on DNA binding, point mutations predicted to abolish direct DNA binding (see Methods) were introduced into the DNA-binding domains of Nkx2.2, Nkx6.1 and Olig2 producing transgenes. Each mutant form showed a loss of repression in the assay (Fig. S5). Thus direct binding to target DNA binding sites within cis-regulatory elements is likely the primary mode whereby each regulatory factor controls gene activity. This conclusion is supported by motif analysis that recovered centrally positioned DNA recognition motifs for each of the factors in factor specific ChIP-seq (Fig. 3A-C).

To gain additional mechanistic insights into regulatory control processes within the regions identified by Nkx2.2, Nkx6.1 and Olig2 interactions, we analyzed bound regions for overrepresented motifs. The clear similarity of the primary ChIP motif to motifs determined by *in vitro* binding of factors supports the argument of direct DNA engagement by each factor (Fig. 3A-C). Moreover, the data revealed additional features of DNA engagement modes: the Nkx6.1 primary motifs appear to contain the Nkx6.1 *in vitro* binding motif and an

additional motif separated by a spacer, consistent with complex formation, possibly with Pbx (Fig. 3B, compare Nkx6.1 (c-2), Nkx6.1 (P), and Pbx (c)). bHLH factors like Olig2 bind an E-box motif (CAXXTG). Comparison between the unambiguous *in vitro* Olig2 homodimer motif (CA**TA**TG) and the more flexible *in vivo* motifs (CA **T/G** **A/G** TG), as well as inspection of E-box sequences at ChIP peaks (data not shown) suggest that Olig2 binds as both homo- and hetero-dimers (Fig. 3B). Interestingly, centered Fox and nuclear hormone receptor (NHR) motif predictions in Nkx2.2 bound regions, and a Pbx motif recovered from Nkx6.1 bound regions suggests a direct regulatory interplay (Fig.3A, B). SoxB1 transcription factors (Sox1, 2, and 3) play key roles in the active maintenance and fate determination of neural progenitors (Bergsland et al., 2011; Bylund et al., 2003; Graham et al., 2003; Oosterveen et al., 2012; Peterson et al., 2012). Examination of the Nkx2.2, Nkx6.1 and Olig2 data sets showed a consistent enrichment of a Sox motif in bound regions (Fig. 3A-C).

We explored a potential Sox factor association at repressor bound regions by intersecting Sox2 binding data in neural progenitors (Peterson et al., 2012). Sox2 is best known in the neural lineage for its role in progenitor state maintenance, a general property shared by all progenitors independent of progenitor specificity (Bylund et al., 2003; Graham et al., 2003). In our data, we uncovered extensive overlap of Sox2 binding and DNA regions targeted by all three repressors: 57% of Sox2 associated regions intersected with DNA domains bound by Nkx2.2, Nkx6.1, or Olig2 (Fig. S6A). Collectively, these data suggest that both repressor and activator inputs governing progenitor programs are mediated through a common set of enhancers (Fig. 4A). Similarly, Sox3 DNA target interactions in neural progenitors (Bergsland et al., 2011) showed an extensive overlap with the repressor trio (46%) (Fig. S6B). As only a small percentage of these Sox2/repressor trio bound regions can be identified in Sox2's ESC regulatory profile (Marson et al., 2008) (9%: Fig. S6C) the data reveal a distinct Sox2 engagement with the neural regulatory genome.

To address the regulatory role of the repressor and activator bound enhancers, we analyzed the presence of histone modifications associated with active cis-regulatory elements accompanying neural progenitor specification *in vitro* (Fig. 4B) (Creyghton et al., 2010; Heintzman et al., 2007; Rada-Iglesias et al., 2011). Regions bound by Nkx2.2, Nkx6.1 and Olig2 associated overall with acetylation of lysine 27 on histone 3 (H3K27ac) in the mixed populations of dorsal and ventral neural progenitors (Fig. 4C-E, S7G) suggesting that repressors likely engage at active transcriptional enhancers (Creyghton et al., 2010; Rada-Iglesias et al., 2011). Interestingly, Nkx2.2, Nkx6.1, or Olig2 binding regions that do not overlap with Sox2 binding showed only low levels of H3K27ac modification in neural progenitors, or in ESC-derivatives prior to neural specification (Fig. 4H), while those overlapping with Sox2 showed markedly elevated H3K27ac levels in a primarily neural progenitor specific manner (Fig. 4G). Thus, Sox2 engagement correlates with an active enhancer signature at this subset of the repressor targeted genome. Importantly, Sox2 binding regions that do not overlap with Nkx2.2, Nkx6.1, or Olig2 bound regions showed similar enrichment of H3K27ac suggesting these are also active enhancers (Fig. 4F). Nkx2.2, Nkx6.1, and Olig2 bound regions that do not show significant H3K27ac signal could act as transcriptional silencer domains, a possibility that requires further study. Overall, we observed similar observations and correlations to those with H3K27ac analyzing H3K4me2, a second chromatin modification linked to enhancer signatures (He et al., 2010) (Fig. S7A-F, H). Whether the set of enhancers identified here is engaged by distinct repressor networks in more dorsally located neural progenitors, or acts independent of repressor networks, remains to be determined.

In sum, our data support a model wherein neural progenitor diversity in the developing mammalian central nervous system follows from the suppression of alternative neural pathway choices by the action of Shh-dependent transcriptional repressors coupled with Sox-

family-mediated transcriptional activation of available cis-regulatory modules within a given progenitor type. The core dorso-ventral neural patterning network is ancient: the spatial arrangements and actions of several key transcriptional components including *vnd*/*Nkx2*, *ind/Gsh* and *msh/Msx* are conserved from insects to mammals (Cornell and Ohlen, 2000). In the mammalian pancreas, transcriptional networks involving Nkx2.2 and Nkx6.1 also play central roles in islet cell specification together with a number of other factors linked to neural fate determination including Foxa2, Mnx1 and Isl1 (Arda et al., 2013). Exploring the mechanisms at play in neural systems in invertebrate organisms and patterning in mammalian pancreatic development may prove useful for further defining the underlying operating principles of these repressor networks in cell fate specification in animal development.

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Author Contributions

Y.N. performed the ChIPseq and ectopic expression experiments, K.A.P purified Olig2 protein, A.V. performed the Olig2 protein binding microarray experiments and analysis, and Y.N., X.Z., J.J., and K.A.P. performed the computational analysis. Y.N. and A.P.M. conceived and designed the experiments and wrote the manuscript. M.L.B., W.A.H. and A.P.M. supervised research. All authors contributed to and approved the final manuscript.

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Figures

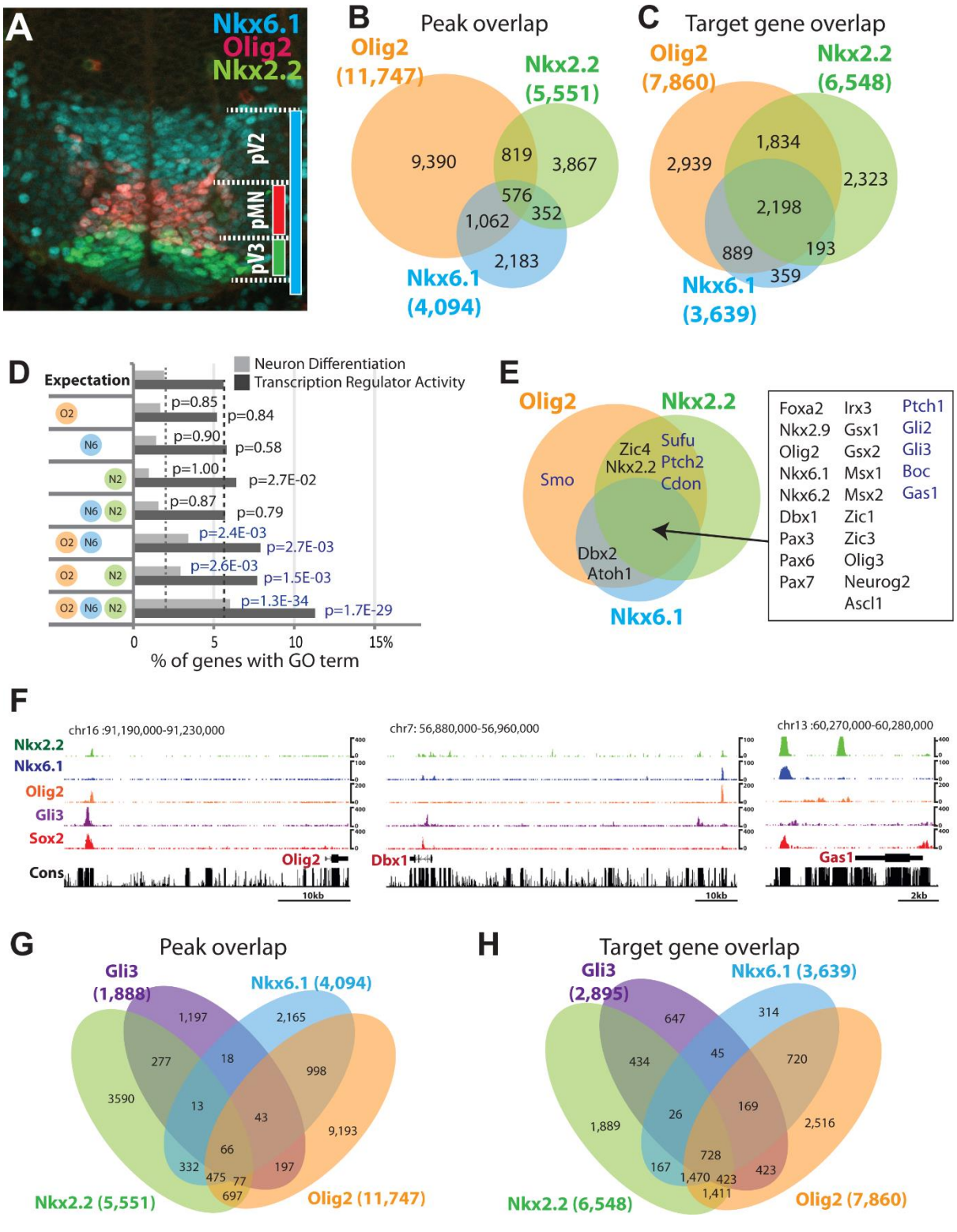


Figure 1. Characteristics of Nkx2.2, Nkx6.1 and Olig2 target genes

(A) Immunofluorescence assay on transverse E10.5 neural tube section at forelimb level with indicated antibodies. (B) Venn diagram intersection of Nkx2.2, Nkx6.1, and Olig2 binding regions. (C) Venn diagram intersection of Nkx2.2, Nkx6.1, and Olig2 target genes. (D) Gene Ontology analysis summary for genes targeted by different combinations of factors. (E) Target gene Venn diagram highlighting neural progenitor fate determinants and Sonic Hedgehog pathway components. (F) genome browser snapshots showing indicated ChIP-seq signal. Cons: Phastcon 30 conservation score. (G, H) Venn diagram for binding region overlap (G) and target gene overlap (H) between Nkx2.2, Nkx6.1, Olig2, and Gli3.

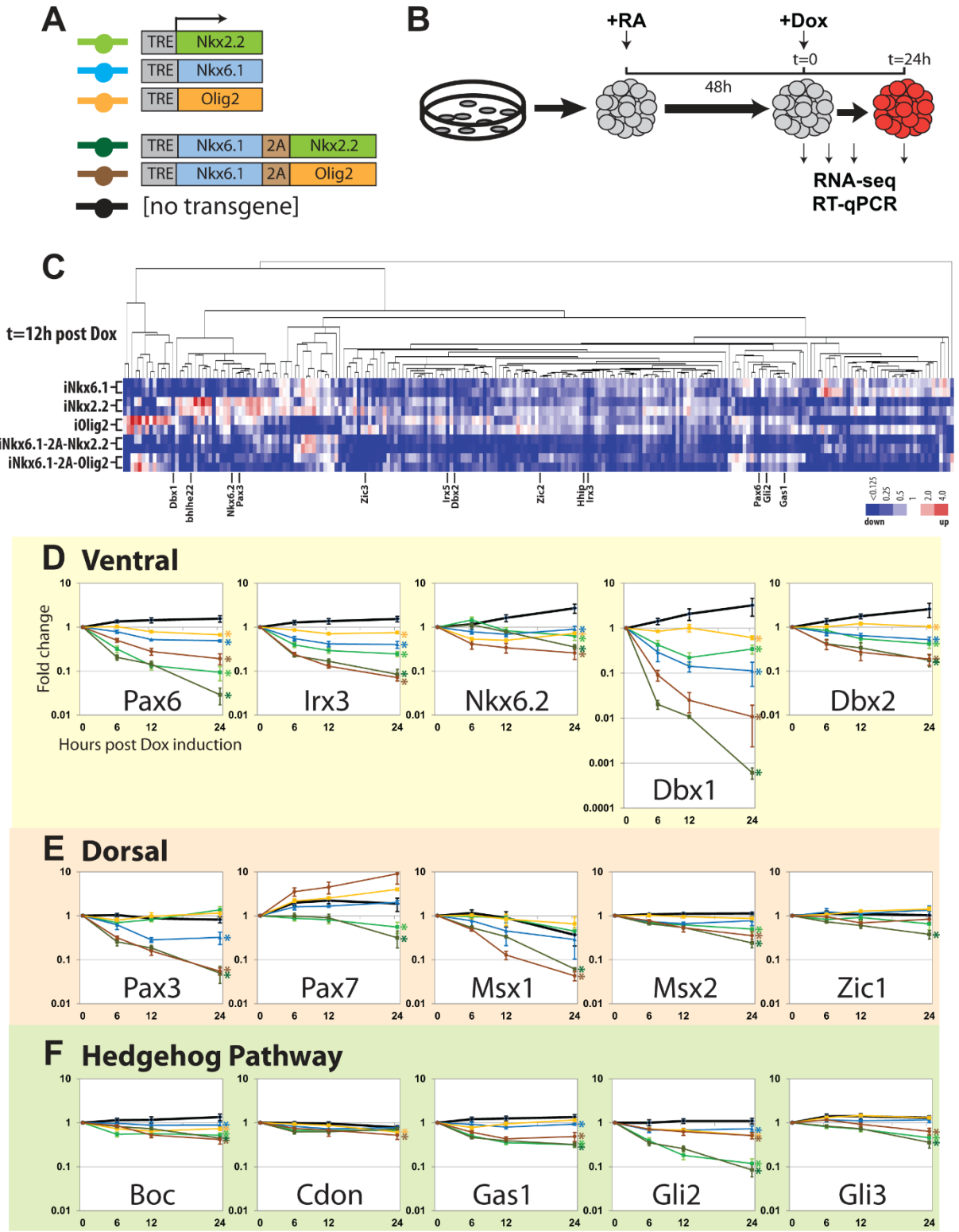


Figure 2. Nkx2.2, Nkx6.1, and Olig2 overexpression assay in neural progenitors

(A) A schematic describing transgene structures. (B) A schematic of overexpresssion experiment design. Cell aggregates were generated from mESC and subjected to neural differentiation and transgene activation. RA: all-trans retinoic acid. Dox: doxycycline. (C) Hierarchical clustering of genes displaying a two-fold or greater change in mRNA-seq data relative to the parental reference population 12 hours following Dox-mediated activation of transcriptional repressors. Fold change to the parental cell line is shown. (D-F) RT-qPCR time course repression assay. See panel A for color designations. X-axis: hours post Dox induction, y-axis: fold change from Dox induction (t=0). Error bars: standard error based on 3 biological replicates. Asterisks indicate significant difference from non-transgenic control based on the standard errors.

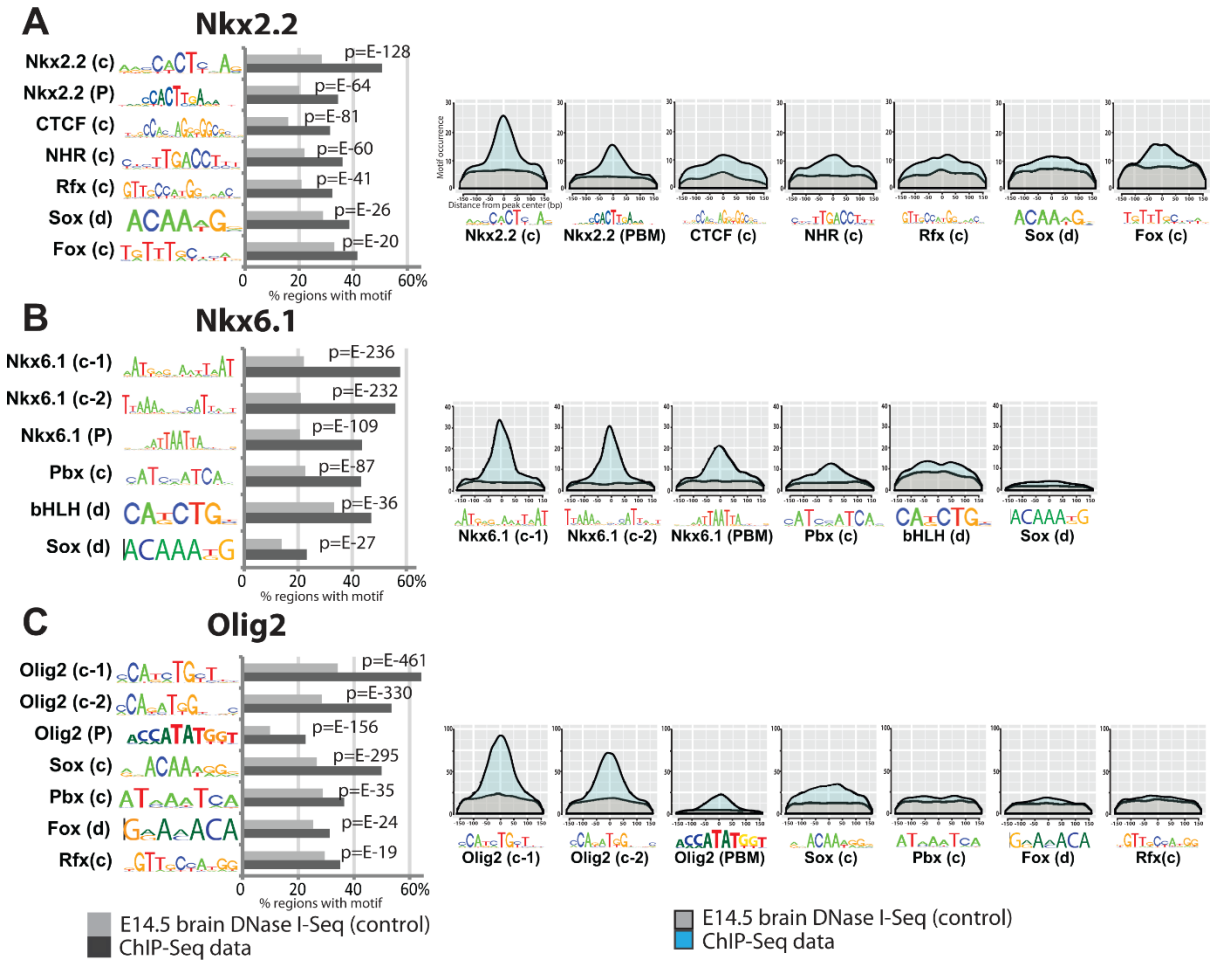


Figure 3. Analysis of enriched binding motifs

(A-C) Occurrence of ChIP-recovered and in vitro-determined motifs. c: CisGenome motif recovery, d: DREME motif recovery, P: protein binding microarray. Grey: E14.5 brain DNaseI-seq control data set. Black: ChIPseq data. (left) Motif distribution histogram relative to binding peak center. X-axis: cumulative motif occurrence, y-axis: bp from peak center. Grey: E14.5 brain DNaseI-seq control, light blue: ChIPseq data.

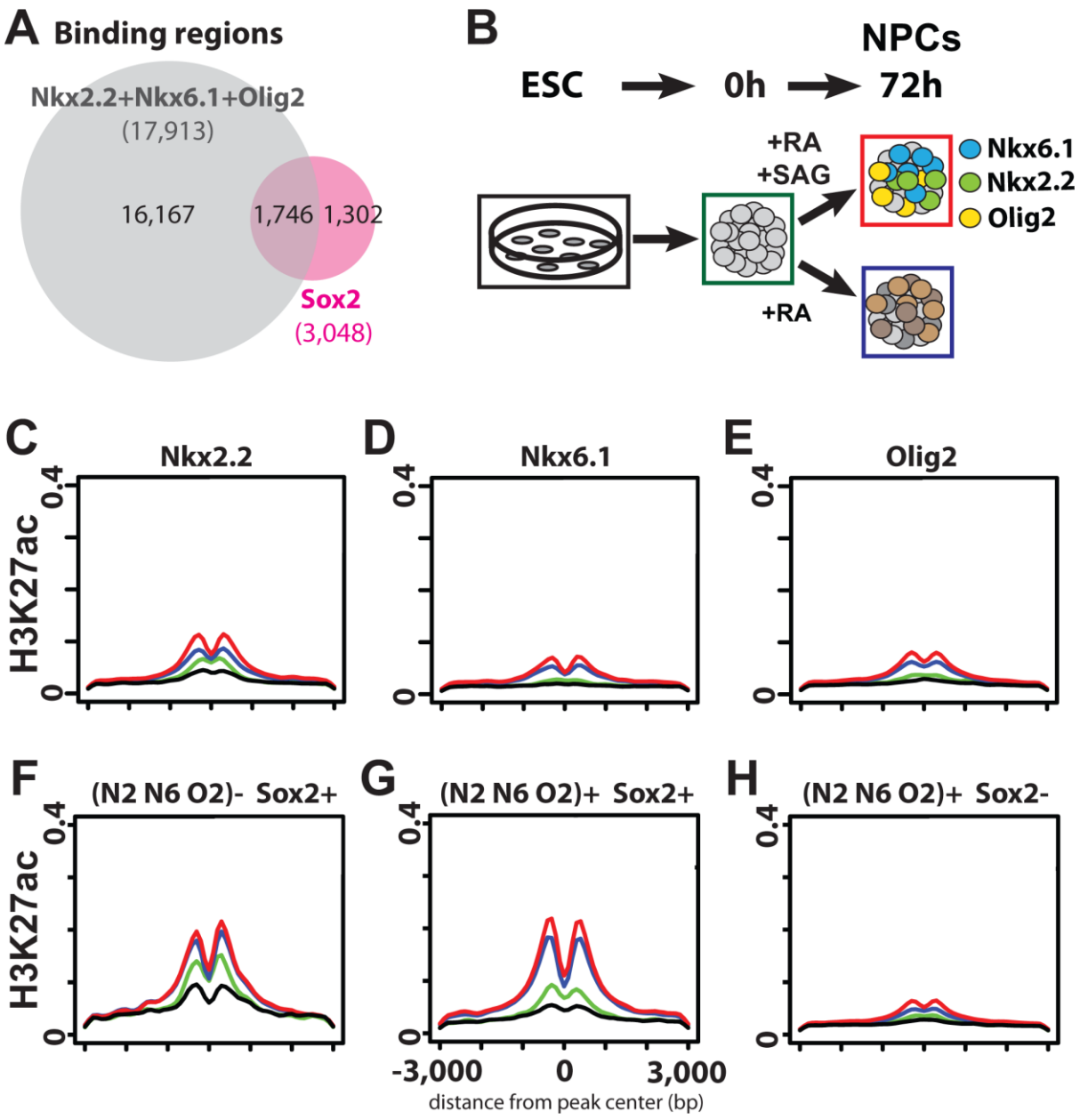


Figure 4. Analysis of Sox2 inputs into ventral repressor-bound regions

(A) Venn diagram intersection between Sox2 binding regions and the union of Nkx2.2, Nkx6.1, and Olig2 binding regions. (B) A schematic describing neural progenitor differentiation protocol. Each condition analyzed in (C-H) is annotated with a solid box with the corresponding color. (C-H) Aggregate analysis of H3K27ac modification status along neural progenitor differentiation paths. Black: ESCs, green: pre-neural induction, blue: dorsal

neural progenitors, red: ventral neural progenitors. Also see (B) for color coding. (C-E) Individual plot for Nkx2.2, Nkx6.1, and Olig2 binding regions. (F) Sox2 binding regions that do not overlap with Nkx2.2, Nkx6.1, or Olig2. (G) Sox2 binding regions that overlap with Nkx2.2, Nkx6.1, or Olig2. (H) Nkx2.2, Nkx6.1, or Olig2 binding region that do not overlap with Sox2 binding.

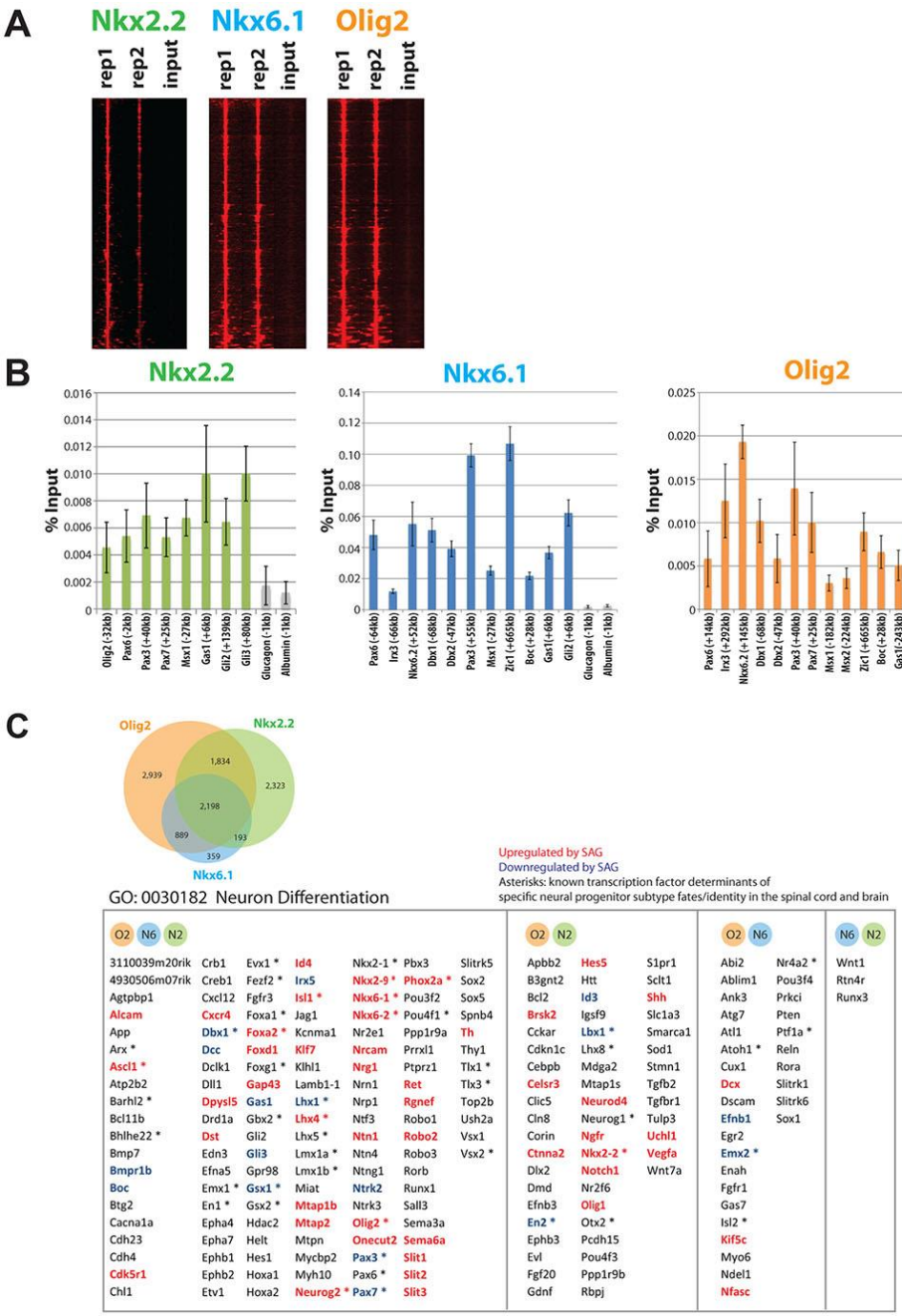
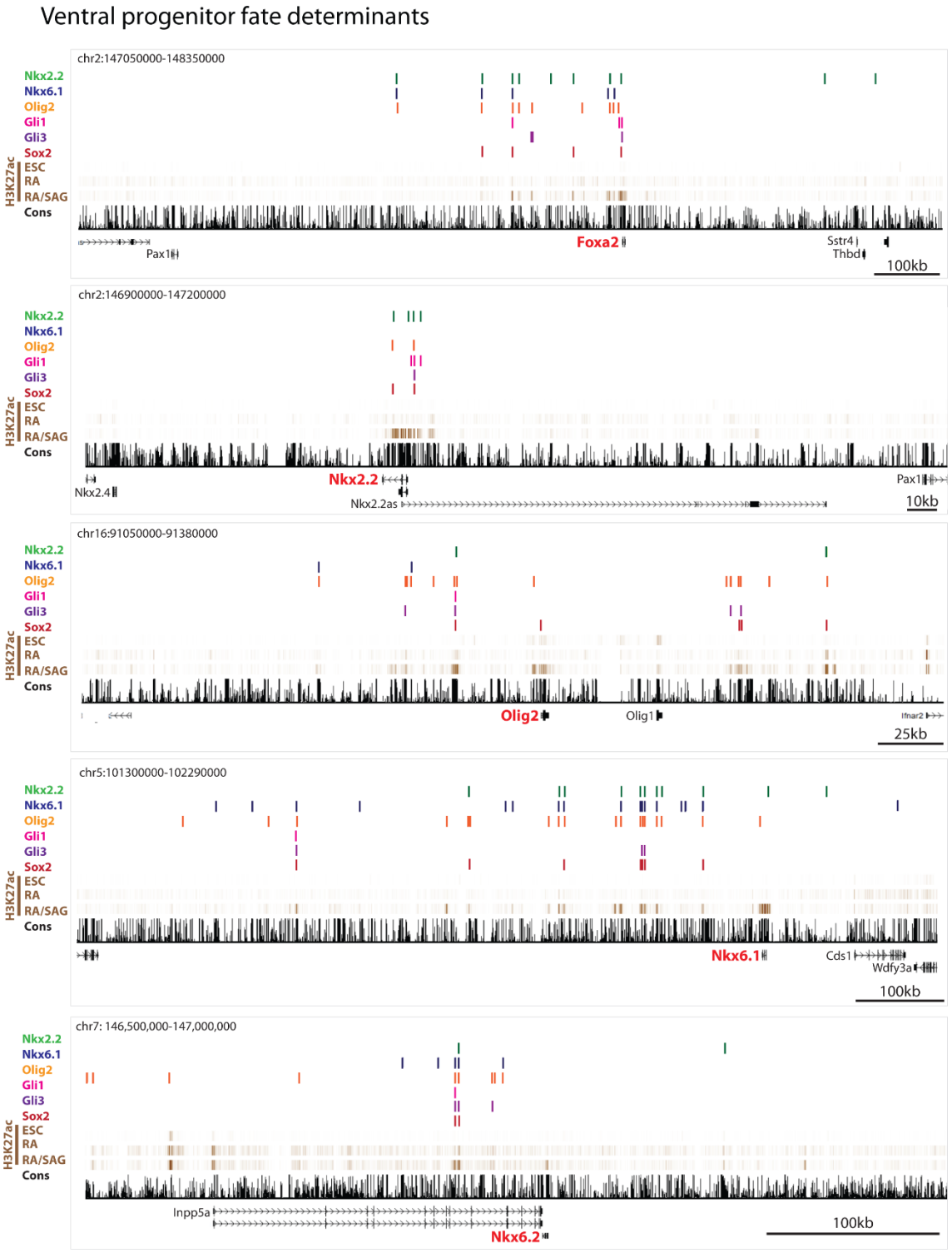


Figure S1. Characterization of Nkx2.2, Nkx6.1, and Olig2 binding and their target genes

(A) Heatmap analysis of two ChIP-seq biological replicates. (B) ChIP-qPCR analysis on E10.5 embryonic trunk preparation. Error bars are standard error based on 2 biological replicates. (C) Genes co-targeted by Nkx2.2, Nkx6.1, and Olig2 that fall into Neuron Differentiation GO term are shown. SAG: agonist for Shh pathway. Genes upregulated and downregulated by SAG are associated with ventral and dorsal progenitor identities, respectively.



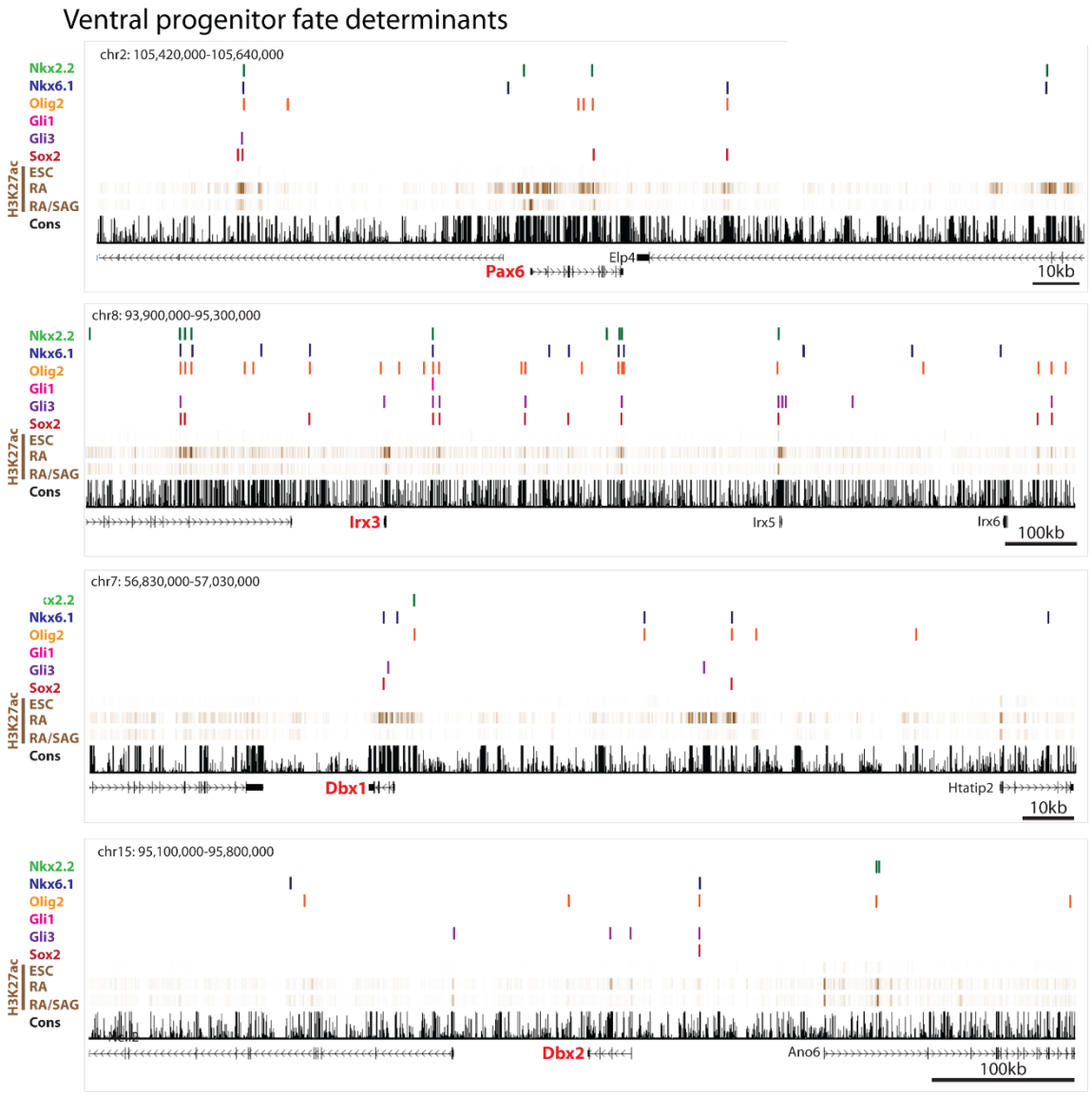


Figure S2. Transcription factor binding and H3K27ac status at ventral neural fate determinants

Indicated transcription factor binding is shown as ticks and H3K27ac signal and phastcon conservation score as heatmap at ventral neural fate determinant loci. RA: neural progenitor culture treated with RA for 72hrs. RA/SAG: neural progenitor culture treated with RA and SAG for 72hrs.

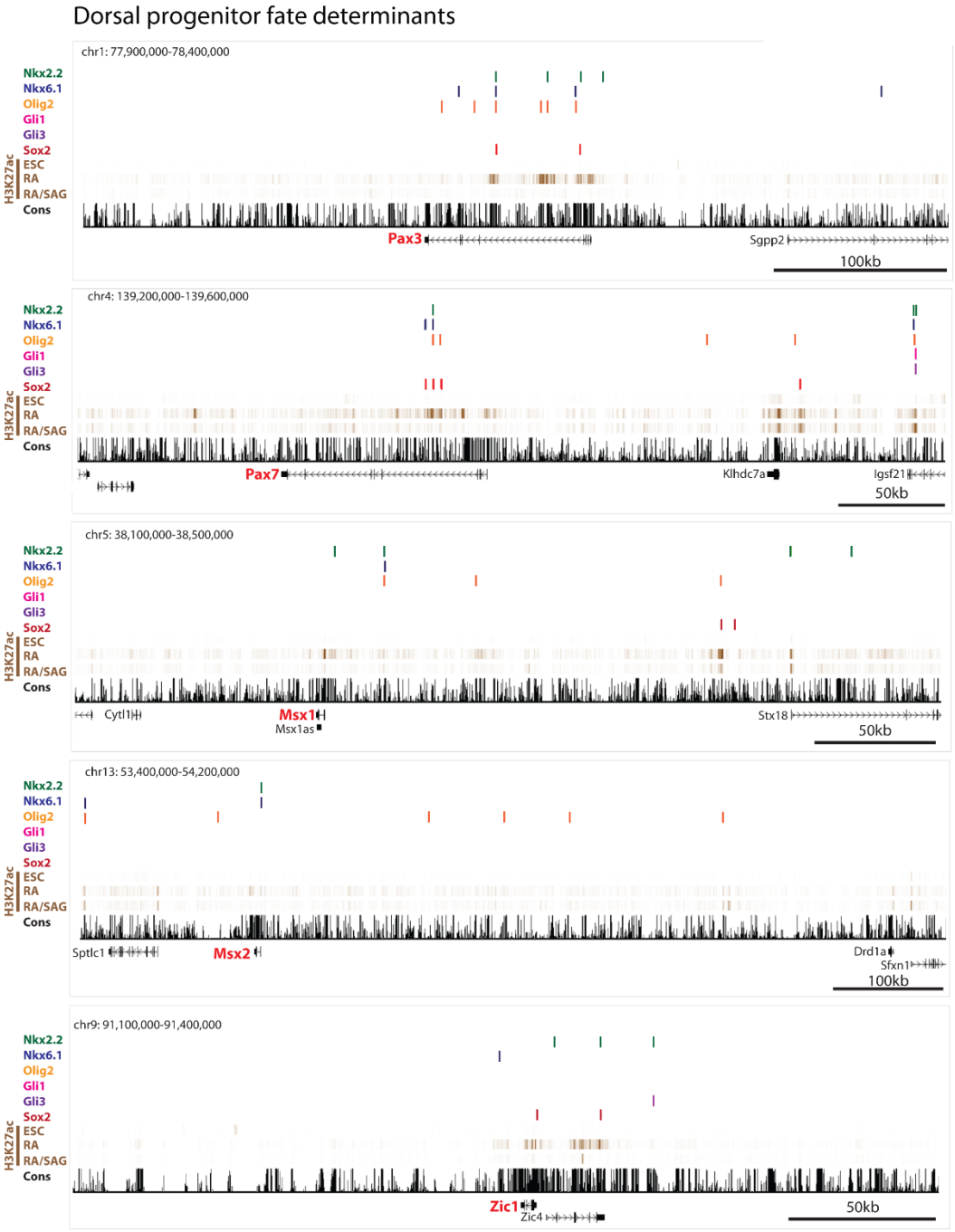
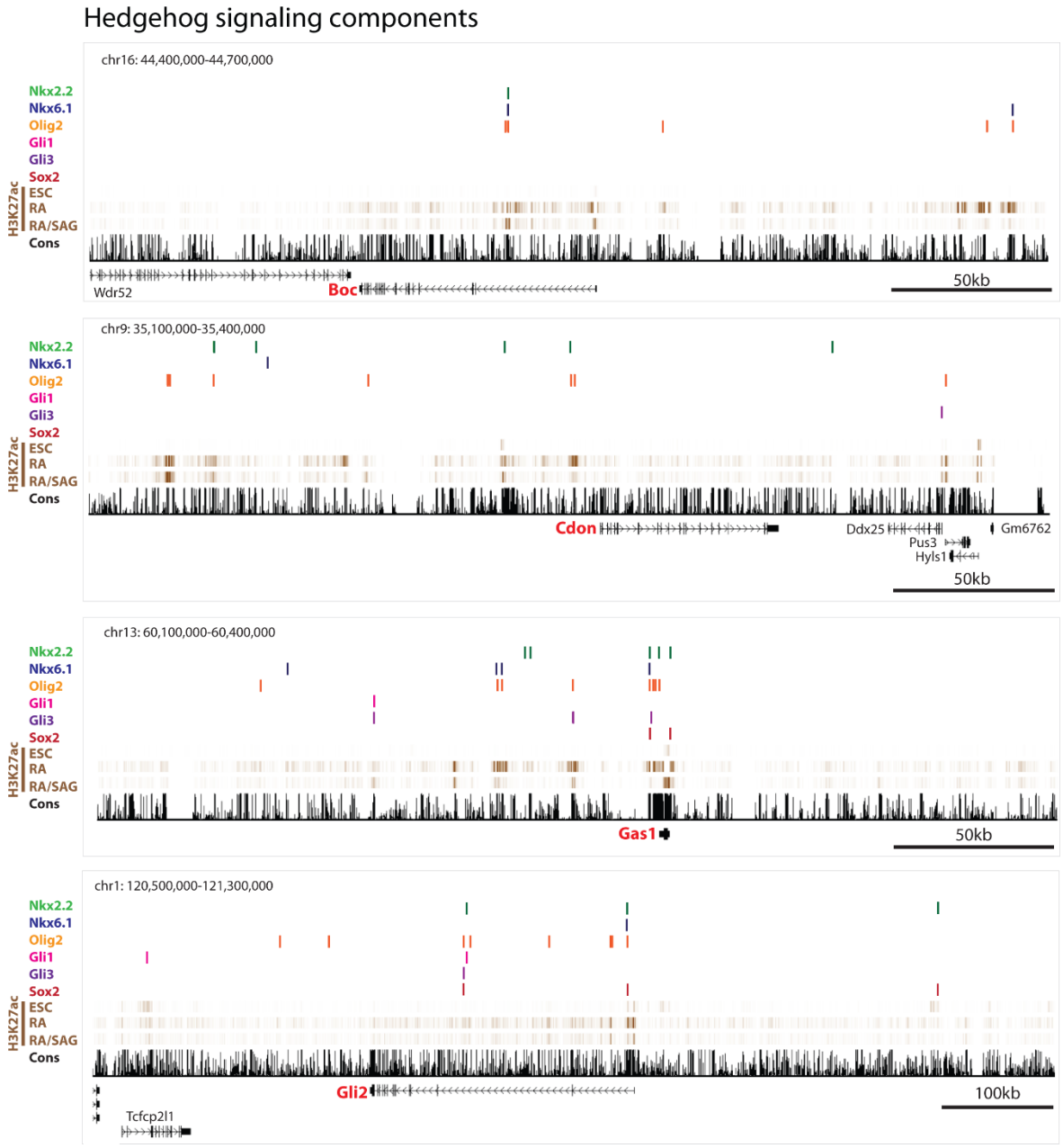


Figure S3. Transcription factor binding and H3K27ac status at dorsal neural fate determinants

Indicated transcription factor binding is shown as ticks and H3K27ac signal and phastcon conservation score as heatmap at dorsal neural fate determinant loci. RA: neural progenitor culture treated with RA for 72hrs. RA/SAG: neural progenitor culture treated with RA and SAG for 72hrs.



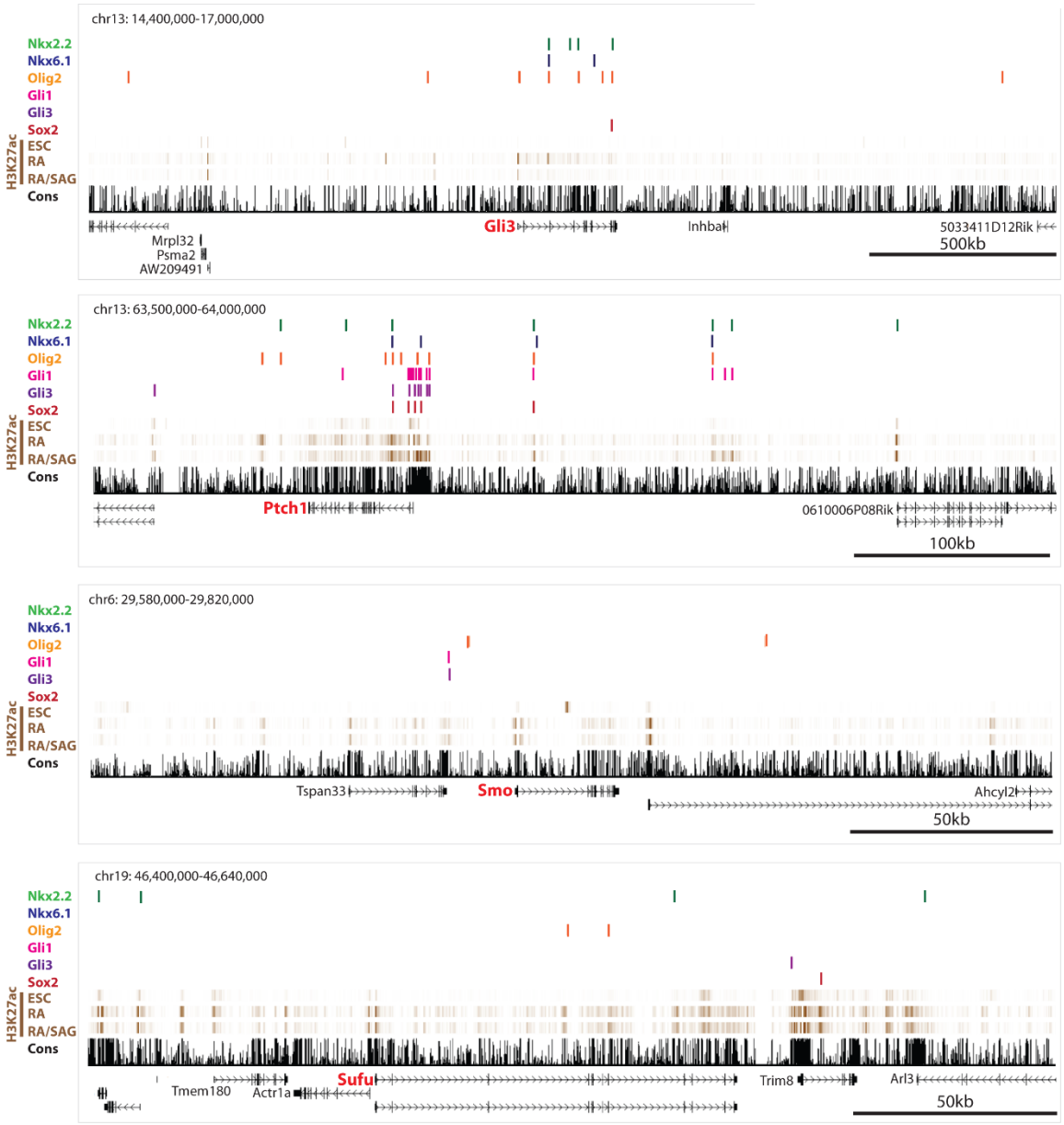


Figure S4. Transcription factor binding and H3K27ac status at Hedgehog pathway components

Indicated transcription factor binding is shown as ticks and H3K27ac signal and phastcon conservation score as heatmap at Hedgehog pathway component loci. RA: neural progenitor culture treated with RA for 72hrs. RA/SAG: neural progenitor culture treated with RA and SAG for 72hrs.

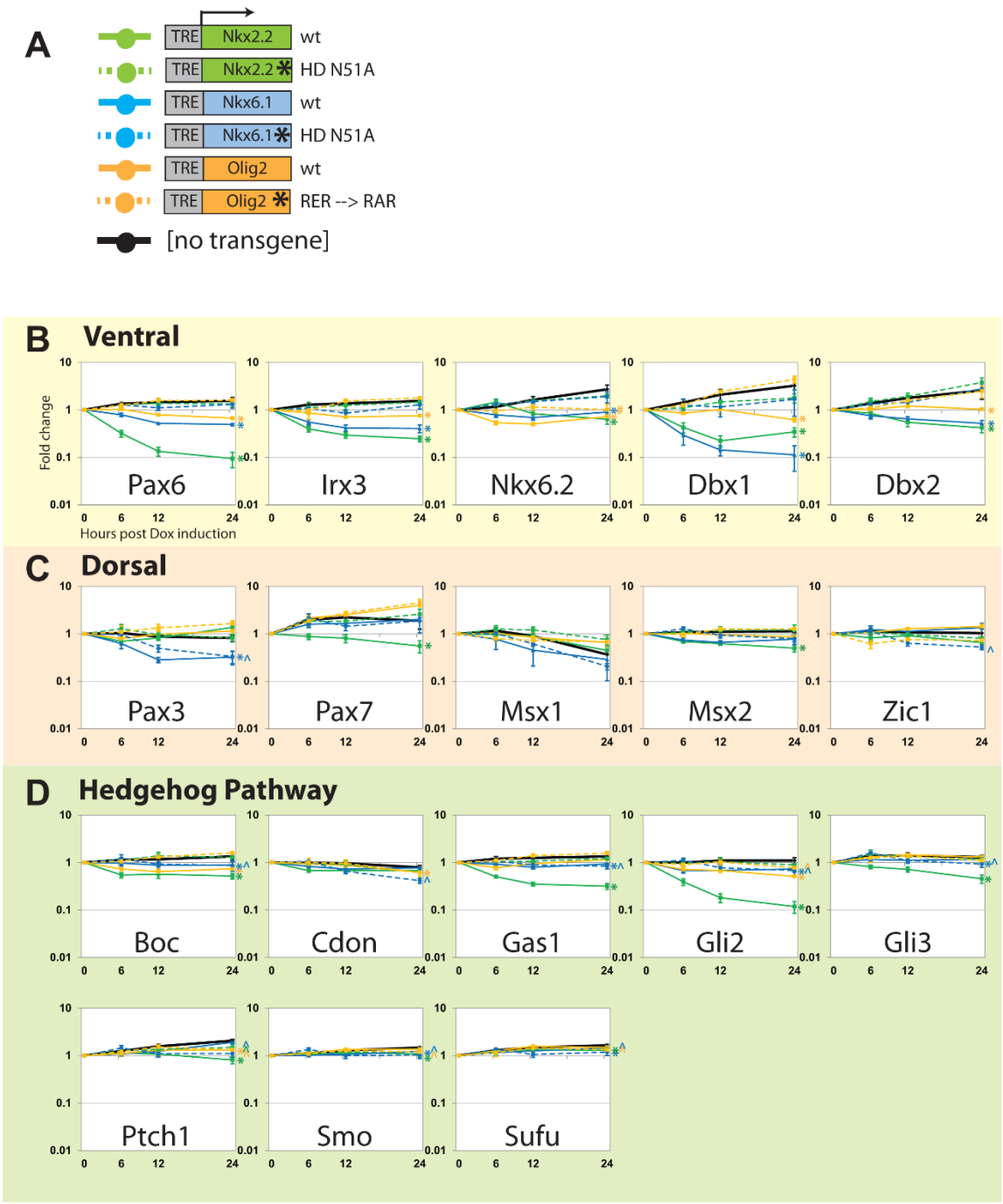


Figure S5. Overexpression assays with DNA binding deficient Nkx2.2, Nkx6.1, Olig2 mutants

(A) A schematic describing transgene structures. (B-D) Expression change plot. See panel A for color and stroke designations. X-axis: hours post Dox induction, y-axis: fold change from Dox induction (t=0). Error bars: standard error based on 3 biological replicates.

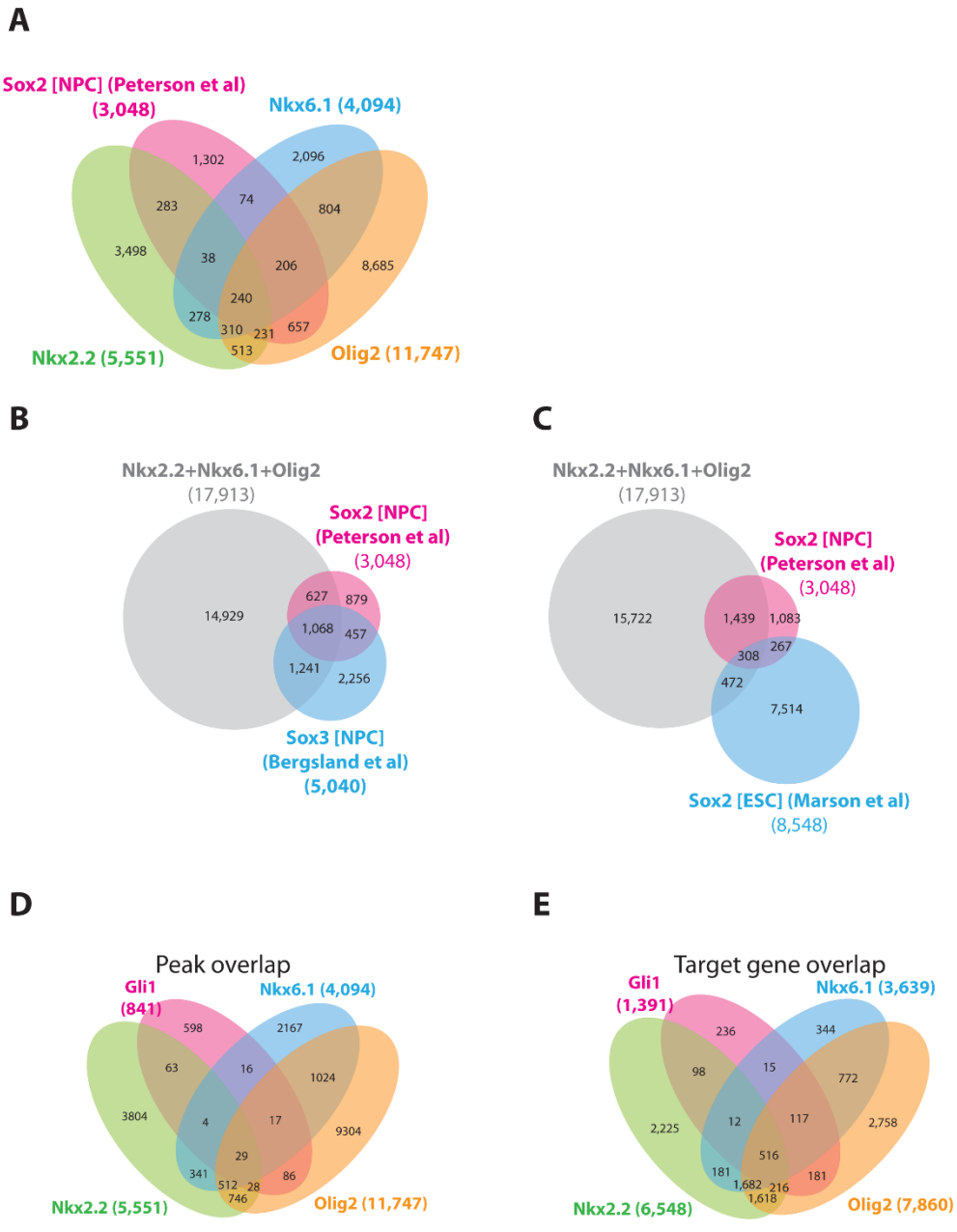


Figure S6. Sox2 and Gli1 binding region intersectional analysis

(A) 4-way intersectional analysis between Nkx2.2, Nkx6.1, Olig2, and Sox2. (B) Venn diagram intersection between the union of Nkx2.2, Nkx6.1, and Olig2 binding regions, Sox2, and Sox3. (C) Venn diagram intersection between the union of Nkx2.2, Nkx6.1, and Olig2 binding regions, Sox2 binding regions in neural progenitors, and Sox2 binding regions in ESCs. (D, E) Venn diagram for binding region overlap (D) and target gene overlap (E) between Nkx2.2, Nkx6.1, Olig2, and Gli1.

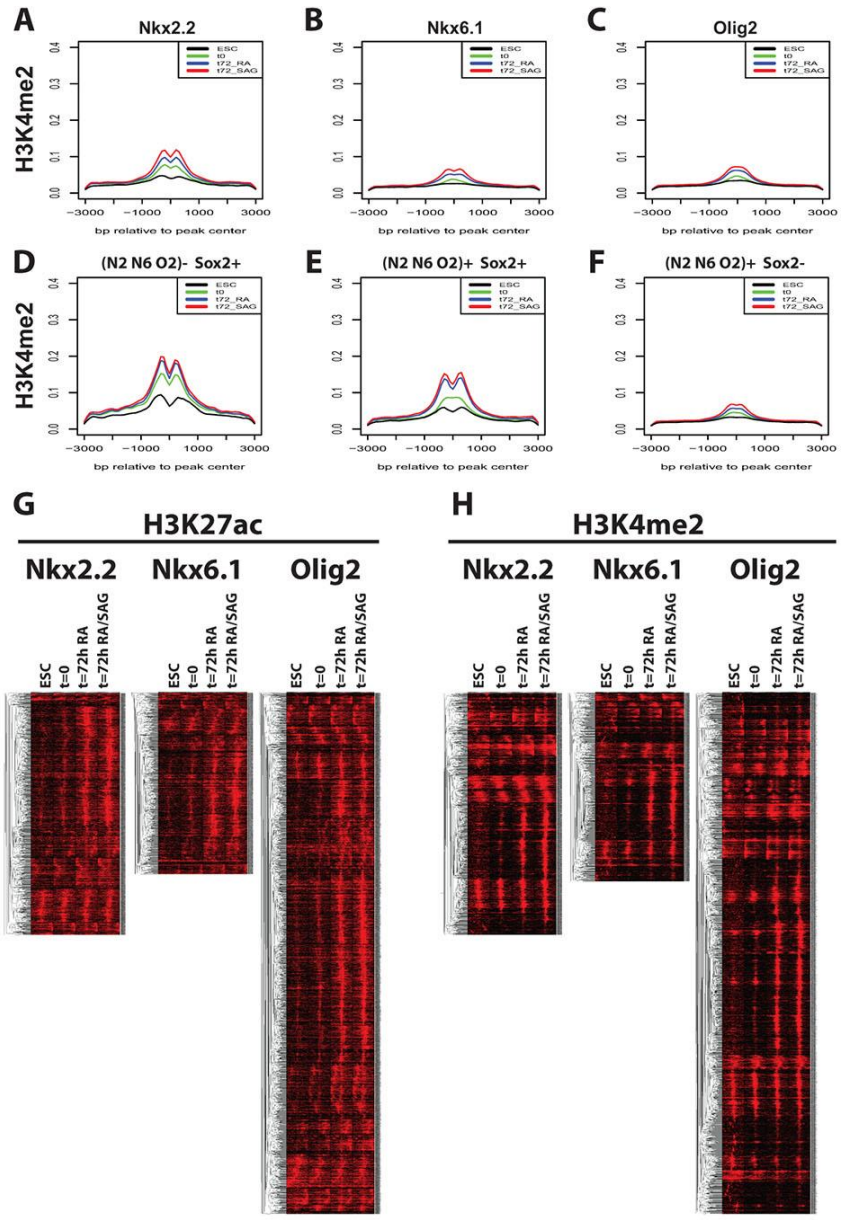


Figure S7. Analysis of active chromatin signatures at Nkx2.2, Nkx6.1, Olig2 and Sox2 binding regions

(A-F) Aggregate plot for H3K4me2 at Nkx2.2, Nkx6.1, Olig2, and Sox2 binding regions. (A-C) Individual binding regions. (D) Sox2 binding regions that do not overlap with Nkx2.2, Nkx6.1, or Olig2. (E) Sox2 binding regions that overlap with Nkx2.2, Nkx6.1, or Olig2. (F) Nkx2.2, Nkx6.1, or Olig2 binding region that do not overlap with Sox2 binding. (G, H) Heatmap clustering analysis of H3K4me2 and H3K27ac modifications at Nkx2.2, Nkx6.1, and Olig2 binding regions.

Table S1. Nkx2.2, Nkx6.1, and Olig2 binding regions

(Tab1-3) Primary peak call and annotation. Column A: peak rank according to peak score. Column B-D: binding peak coordinates. Column E-U: genes associated with the peak. Nearest gene: the gene whose transcriptional start site is the closest to the peak. Nearest left gene: the closest gene located left to the peak, Nearest right gene: the closest gene located right to the peak. 5x Nearest left/right genes: five closest genes to the left and to the right from the peak. Gene symbol: official gene name. refseq: Refseq ID. Relative2peak: relative location of the peak to the gene. TSS_upstream: the binding peak is upstream of the transcriptional start site of the gene. TES_downstream: the binding peak is downstream of the transcriptional end site of the gene. Dist2TSS: relative distance of the peak to the transcriptional start site of the gene in bp. (Tab4) Integrated peak list and annotation for Nkx2.2, Nkx6.1, Olig2, Sox2, and Gli1. See above for designations for column B-L. Column M-Q: binding peak count for indicated transcription factors. Column R-AK: transcription factor binding motif count in the binding region. See Fig. 3A-C for motif details. Column AL-AP: mouse pronuclear injection enhancer assay test results from http://enhancer.lbl.gov/.

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Table S2. Repression assay RNAseq data

(Tab1) rpkm values for all samples and all analyzed genes. (Tab2) Expression value ratio between indicated overexpression line and the parental line for genes repressed by 2-fold or greater in at least one overexpression group. This data was used for hierarchical clustering shown in Fig 3C. (Tab3) List of genes down-regulated in each transgene expression experiment. Down-regulation cutoff was 2-fold relative to parental cell line using replicate average. Genes associated with binding of respective factor(s) are indicated in red. (Tab4-9) DAVID GO term analysis on down-regulated genes. (Tab4) Genes down-regulated at least in one cell line. (Tab5-9) Genes down-regulated in each cell line from Tab3.

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Table S3. Primers used in this study

(Tab1) Primer sequences for the RT-qPCR assays in this study. Column A: target gene. Column B,C: left primer. Column D,E: right primer. (Tab2) Primers used in the embryonic ChIP-qPCR assay. Column A: target gene. Column B: distance from the assay region to the TSS of the target gene. Column C: transcription factor bound to the assay region. Column D, E: left primer. Column F, G: right primer.

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