Intellectual disability risk gene RFX4 regulates cortical neurogenesis by restraining neuronal differentiation

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Abstract

Despite the recent identification of RFX4 as a neurodevelopmental disorder risk gene, its role in cortical development remained unclear. Here, we identified both shared and lineage-specific RFX4 requirements for human cortical development using new human stem cell models of deficiency and pathogenic mutation. We found that RFX4 restrains neurogenesis by acting cooperatively with NOTCH signaling, specifically repressing pro-neuronal and synaptic gene expression in neural progenitors. We also determined that genome-wide binding of RFX3, another neurodevelopmental disorder risk gene, depends upon RFX4 to regulate synaptic gene expression. Furthermore, we identified lineage-specific functions for RFX4 in regulating proliferation during cortical inhibitory neuron development. Ultimately, we demonstrated that RFX4 deficiency persistently dysregulates neuronal gene expression through neuronal differentiation and disrupts cortical neuron stratification in organoid models. These consequences were absent in neurons generated by direct differentiation, confirming that neuronal phenotypes resulted from unconstrained neurogenesis. Finally, we modeled pathogenic missense mutation of the RFX4 DNA-binding domain. While this mutation strongly reduced DNA binding, it dysregulated synaptic gene expression distinctly from our deficiency models, supporting pathogenic mechanisms distinct from haploinsufficiency. Together, this work identified both shared and lineage-specific requirements for RFX4 during cortical development, building a necessary foundation for elucidating the etiology of RFX4-associated disorders.

Introduction

Development of the human cerebral cortex is a highly regulated process, with disruptions contributing to the etiology of neurodevelopmental disorders (NDDs) including intellectual disability (ID) and autism spectrum disorder (ASD) (Lui et al. 2011; Albert et al. 2017; Cadwell et al. 2019). A central hypothesis for the neuronal dysfunction underlying these NDDs involves imbalanced excitatory and inhibitory neuronal activity in the cerebral cortex (E/I imbalance) (Siegel-Ramsay et al. 2021; Hollestein et al. 2023) resulting from altered development and/or function of glutamatergic excitatory neurons (cEXs) and/or GABAergic inhibitory neurons (cINs). A major contributor to E/I imbalance is changes in the highly regulated process of cortical neurogenesis, a process by which proliferative neural progenitors differentiate into post-mitotic cortical neurons. While transcription factors (TFs) are major regulators of cortical neurogenesis (Cholfin and Rubenstein 2007; Villalba et al. 2021; Chapman et al. 2024; Vasan et al. 2025), with pathogenic mutations in these identified as frequent NDD contributors, it remains unclear how NDD-associated TF dysfunction differentially affects cEX versus cIN development to cause NDDs in humans. Thus, identifying both cell type-specific and -agnostic roles for NDD-associated TFs in regulating cortical neurogenesis is critical to understanding NDD etiology.

Towards this end, we modeled epigenetic regulation of human cIN development in prior work, deriving an atlas describing histone modification state changes accompanying cIN specification and differentiation from human pluripotent stem cells (hPSCs) (Chapman et al. 2024). This work identified Regulatory Factor X (RFX) 4 and RFX3 as key TFs regulating this process. As both *RFX3* and *RFX4* were also recently identified as NDD risk genes, with pathogenic mutations associated with global developmental delay, epilepsy, and ASD (Harris et al. 2021), these observations merited further study. Furthermore, while RFX3 is broadly expressed during human cortical development and included in multi-gene deletions that contribute to 9p deletion syndrome (Banerjee et al. 2019; Ajami et al. 2023; Wang et al. 2025), RFX4

65 expression is largely restricted to neural progenitors (Wang et al. 2025), suggesting a distinct role for
66 RFX4 in regulating cortical neurogenesis.

67 RFX4 was initially identified as a gene involved in ciliogenesis (Ashique et al. 2009), while work in
68 murine Rfx4 knockout (KO) models demonstrated Rfx4's necessity for proper brain development as
69 homozygous (HOM) KO caused lethality by embryonic day 18.5 (Blackshear et al. 2003; Ashique et al.
70 2009; Xu et al. 2018). Rfx4 heterozygous (HET) knockdown animal models exhibited altered dorsal-
71 ventral brain patterning associated with dysregulation of WNT, BMP, and SHH signaling (Zhang et al.
72 2006; Zhang et al. 2007; Ashique et al. 2009; Sedykh et al. 2018). More recently, studies using hPSC
73 models have provided conflicting evidence for the role of RFX4 in cortical neurogenesis, suggesting that
74 RFX4 overexpression could either produce a more homogenous neuronal progenitor population (Joung et
75 al. 2023) or instead induce neuronal differentiation (Choi et al. 2024). Therefore, systematically defining
76 the role RFX4 plays in regulating human cortical neurogenesis across cEX and cIN development is a
77 necessary precursor to defining how pathogenic RFX4 mutations may alter cortical neurogenesis to disrupt
78 E/I balance and contribute to NDDs.

79 Here, we used hPSC models with RFX4 loss of function (LOF) mutation to demonstrate that RFX4
80 LOF triggers dose-dependent precocious neurogenesis during both cIN and cEX development, ultimately
81 causing deficits in cortical neuron layer stratification in organoids. We derived data for endogenous RFX4
82 binding, demonstrating that RFX4 commonly represses the expression of pro-neuronal and synaptic genes
83 during both cEX and cIN development, while also activating proliferative gene expression, specifically in
84 medial ganglionic eminence-like progenitors during cIN development. We further demonstrated that
85 regulation of cortical neurogenesis by RFX4 involves both functional interplay with NOTCH signaling
86 and with RFX3-mediated activation of neuronal gene expression. Finally, we modeled a patient specific
87 missense mutation in the RFX4 DNA binding domain, demonstrating that this causes a dramatic loss of

88 RFX4 genome binding, but results in unique transcriptomic dysregulation and cellular phenotypes.
89 Together, this defines the requirements for RFX4 and underlying mechanisms in regulating cortical
90 neurogenesis and demonstrates that the etiology of RFX4-associated NDDs is more complex than
91 haploinsufficiency-related LOF, as previously hypothesized. These findings provide a foundation for
92 elucidating the basis of *RFX4* variant pathogenicity and targeting these mechanisms for reversal to
93 ameliorate NDD-related consequences.

Results

Loss of RFX4 causes dosage-dependent induction of cortical neurogenesis

To characterize the effects of RFX4 loss of function (LOF) on human cortical development, we developed human pluripotent stem cell (hPSC) models with heterozygous (HET) or homozygous (HOM) RFX4 gene disruption (Fig. 1a, Supplementary Fig. S1a). Using our established protocols, we specified these hPSCs into neuronal progenitors (NPCs) with a dorsal (D-) or ventral (V-) telencephalic character (Fig. 1b). We then tested the validity of our LOF models in D-NPCs, confirming a ~60% and >90% loss of both RFX4 mRNA and protein in the HET and HOM models, respectively (Supplementary Fig. S1b-c). We next assessed how RFX4 deficiency changed neurosphere outgrowth during D-NPC and V-NPC specification, observing a significant reduction of outgrowth from both V-NPC (Fig. 1c-d) and D-NPC neurospheres (Fig. 1f-g). Interestingly, while neurosphere size was reduced during V-NPC specification (Fig. 1e), it was not significantly affected by RFX4 LOF during D-NPC specification (Fig. 1h), suggesting lineage specific roles for RFX4.

To further investigate lineage-specific RFX4 requirements, we performed RNA-sequencing in WT versus HOM D-NPCs and V-NPCs, highlighting both shared effects of RFX4 LOF and transcriptomic differences between the WT versus HOM D-NPC and V-NPC comparisons (Fig. 1i). We identified similar numbers of differentially expressed genes (DEGs) between WT and HOM D-NPCs (2,718) and HOM V-NPCs (2,456), respectively, with ~50% of DEGs upregulated upon RFX4 LOF (Fig. 1j, Supplementary Data 1). Notably, DEGs downregulated in both HOM D-NPCs and V-NPCs were enriched predominantly for genes involved in early developmental processes (Fig. 1k, Supplementary Data 2), while genes jointly upregulated upon RFX4 LOF were instead enriched for 'synaptic signaling' genes (Fig. 1l, Supplementary Data 2). Together, this work suggested that RFX4 may regulate the onset of neuronal differentiation similarly in both D-NPCs and V-NPCs.

117 We next validated altered expression of 10 upregulated (Fig. 1m-n) and 10 downregulated
118 (Supplementary Fig. S1d-e) DEGs, using RT-qPCR in WT, HET, and HOM D- and V-NPCs and
119 confirming that RFX4 LOF caused dosage-dependent changes in expression of these genes (Fig. 1m-n,
120 Supplementary Fig. S1d-e). We further validated these findings by assessing the fraction of cells
121 expressing ASCL1, a neurogenesis-promoting bHLH transcription factor, finding dose-dependent
122 increases in the ASCL1 immunopositive cell fraction upon RFX4 LOF in both V-NPCs and D-NPCs (Fig.
123 1o-p). The proportion of DCX positive cells, a protein essential for neuronal migration, was likewise
124 increased in both HET and HOM D-NPCs (Supplementary Fig. S1f). By contrast, we found that the
125 proportion of cells immunopositive for the early-born neuron marker TBR1 was unaltered in D-NPCs
126 (Supplementary Fig. S1g). Furthermore, while V-NPCs exhibited decreased proliferation, proliferation
127 remained unchanged in D-NPCs (Supplementary Fig. S1h-i), supporting lineage-specific requirements for
128 RFX4. Ultimately, these results indicated that RFX4 LOF triggers dosage-dependent neurogenesis, while
129 also highlighting lineage-specific RFX4 requirements.

130 Interestingly, these results appeared inconsistent with a previous publication, which concluded that
131 RFX4 overexpression (OE) promotes neuronal differentiation. Therefore, we tested whether RFX4 OE
132 alone was sufficient to induce neuronal differentiation (Supplementary Fig. S2a). After confirming RFX4
133 OE (Supplementary Fig. S2b), we assessed expression levels of multiple neuronal genes by RT-qPCR,
134 finding no significant increases (Supplementary Fig. S2c). Likewise, RFX4 OE was insufficient to induce
135 the expression of either the early neuronal marker TUJ1 or ASCL1 (Supplementary Fig. S2d-e) or to
136 change the proportion of cells immunopositive for pluripotency markers (Supplementary Fig. S2f-g).
137 Together, these results further supported our hypothesis that RFX4 is required to regulate cortical
138 neurogenesis by restraining premature neuronal differentiation, while demonstrating that RFX4 OE alone
139 is insufficient to induce neurogenesis.

140 **RFX4 directly restrains the onset of neuronal and synaptic gene expression**

141 To identify genes directly regulated by RFX4, we defined endogenous RFX4 genome-wide binding
142 using CUT&Tag, confirming an almost complete loss of RFX4 genome-wide binding in HOM D-NPCs
143 (Fig. 2a). We analyzed the quality of these data by footprinting, identifying a footprint under RFX4 bound
144 peaks in WT D-NPCs corresponding with a canonical RFX transcription factor binding site (TFBS) that
145 was lost in HOM D-NPCs (Supplementary Fig. S3a). We further compared our endogenous RFX4 binding
146 data to previously published chromatin immunoprecipitation (ChIP) data obtained by overexpressing
147 epitope-tagged RFX4, finding over 12,000 binding sites specific to endogenous RFX4 (Supplementary
148 Fig. S3b). Finally, we used a deep learning model to analyze the sequence syntax present at RFX4 bound
149 peaks, demonstrating that the presence of an RFX TFBS was highly correlated with sites of RFX4 binding
150 (Supplementary Fig. S3c). This work therefore identified the endogenous binding profile for RFX4 during
151 human cortical development, confirming both the data validity and quality.

152 We next generated similar genome-wide binding data for RFX4 in WT versus HOM V-NPCs (Fig. 2b),
153 identifying both D-NPC and V-NPC shared and lineage-specific RFX4 binding sites (Supplementary Fig.
154 S3d). We associated RFX4 peaks with their nearest transcription start site (TSS) to identify RFX4 bound
155 genes; we then defined which of these were also RFX4 DEGs (bound-DEGs) in D-NPCs and V-NPCs,
156 finding substantial RFX4 binding near promoters and introns of bound-DEGs (Supplementary Fig. S3e-
157 j). Bound-DEGs upregulated upon RFX4 LOF in both D-NPCs and V-NPCs were enriched for neuronal
158 and synaptic genes (Fig. 2c-d, Supplementary Data 3), supporting a role for RFX4-mediated repression in
159 regulating neurogenesis. By contrast, bound-DEGs downregulated upon RFX4 LOF uniquely in V-NPCs
160 were associated with cell proliferation (Fig. 2e, Supplementary Data 3), consistent with our previous
161 finding of reduced proliferation in HOM V-NPCs. These results suggest that while RFX4 has lineage-

162 specific functions, it broadly represses neuronal and synaptic gene expression in both D-NPCs and V-
163 NPCs.

164 As our work above highlighted shared cellular phenotypes across NPC lineages were more severe
165 during D-NPC specification (Fig. 1p), we next focused on understanding RFX4's regulatory function in
166 D-NPCs. We found that binding sites that were significantly lost upon RFX4 LOF (>2 fold, pValue<0.05)
167 and were associated with DEGs (Supplementary Data 4) were enriched for RFX motifs and TEAD,
168 homeodomain, and SOX family TFBSs (Fig. 2f, Supplementary Data 5), reminiscent of predictions made
169 by our deep learning model (Supplementary Data 4). We focused on assessing potential interplay between
170 RFX4 and TEAD1, given the latter's established role in neurogenesis and its upregulation upon RFX4
171 LOF (Mukhtar et al. 2020; Perry et al. 2025) (Supplementary Data 1), defining genome-wide TEAD1
172 binding in D-NPCs. This analysis showed minimal changes in TEAD1 binding upon RFX4 LOF
173 (Supplementary Data 4), but defined sites co-bound by both TEAD1 and RFX4 in D-NPCs. These
174 included sites associated with genes involved in WNT and NOTCH signaling, congruent with a potential
175 role for RFX4 and TEAD1 in cooperatively regulating genes critical for NPC maintenance.

176 To identify a subset of key RFX4 direct targets for further experimental validation, we next focused on
177 RFX4 bound sites that were both associated with a DEG and contained an RFX4 TFBS (Fig. 2g), finding
178 that this DEG subset was enriched for synapse-associated genes upregulated upon RFX4 LOF (e.g. *SYT5*,
179 *CAMK2A*; Fig. 2h-i, Supplementary Data 3). We cloned a set of RFX4 putative cis-regulatory elements
180 (CREs) associated with these bound-DEGs into a luciferase reporter construct (Fig. 2j), finding that
181 luciferase expression driven by a majority of these putative CREs increased significantly upon RFX4 LOF
182 in D-NPCs (Fig. 2k). Together, this work confirmed RFX4's ability to repress gene expression at these
183 sites, identifying new requirements for RFX4-mediated transcriptional repression of synaptic gene
184 expression during cortical neurogenesis.

185 **RFX4 regulates the onset of cortical neurogenesis downstream of NOTCH signaling**

186 Our findings of TFBS co-enrichment at RFX4 bound sites above suggested functional interplay with
187 other factors and/or signaling pathways in regulating human cortical neurogenesis. Therefore, to identify
188 chemical modifiers of RFX4 LOF phenotypes, we performed an unbiased drug screen in WT and HOM
189 D-NPCs. This screen identified mechanisms of action (MoAs) that normalized the increased proportion
190 of LOF D-NPCs expressing the neurogenic bHLH TF OLIG2 (Supplementary Data 6). Interestingly,
191 significant hits included γ-secretase inhibitors; this was of particular interest as NOTCH signaling is
192 blocked by γ-secretase inhibitors and restrains neurogenesis. Therefore, we assessed if NOTCH inhibition
193 (using DAPT, Fig. 3a) phenocopied RFX4 LOF. Transcriptomic analysis highlighted distinct differences
194 between DAPT-treated WT (WT-DAPT) and HOM D-NPCs (Fig. 3b, Supplementary Data 7), despite the
195 observation that RFX4 expression was reduced upon DAPT-treatment of WT D-NPCs (Supplementary
196 Fig. S4a). Specifically, we found a more substantial upregulation of neuronal and synaptic gene expression
197 in WT-DAPT versus untreated HOM D-NPCs (Fig. 3c-d, Supplementary Data 1, 8), a phenomenon that
198 was further exacerbated when HOM D-NPCs were also DAPT treated (HOM-DAPT) (Fig. 3e,
199 Supplementary Fig. S4b). DAPT treatment caused a similar increase of the fraction of TBR1
200 immunopositive cells in both WT and HOM D-NPCs, while RFX4 LOF alone instead reduced the TBR1
201 immunopositive cell fraction (Fig. 3f); these data suggested that NOTCH inhibition is sufficient to
202 promote terminal neuronal differentiation, while RFX4 LOF is not. Based upon these findings, we
203 hypothesized that RFX4 acts downstream of NOTCH signaling to restrain initiation of cortical
204 neurogenesis, in part by directly repressing the expression of neurogenic TFs including ASCL1
205 (Supplementary Fig. S4c).

206 We next assessed if changes in chromatin state unique to NOTCH inhibition could be used to identify
207 TFs that could potentially co-function with RFX4 downstream of NOTCH signaling. Specifically, we

208 assessed changes in the repressive modification histone 3 lysine 27 tri-methylation (H3K27me3) and
209 primed enhancer marker histone 3 lysine 4 mono-methylation (H3K4me), observing substantive changes
210 in H3K4me (Supplementary Fig. S4d-e) but minimal changes in H3K27me3 (Supplementary Fig. S4f-g)
211 upon either RFX4 LOF or DAPT treatment. Further analysis highlighted increased H3K4me association
212 with synaptic and neuronal genes after either RFX4 LOF or DAPT treatment (Supplementary Data 8-9),
213 which increased upon DAPT treatment of HOM D-NPCs (Fig. 3g, Supplementary Data 9). We also
214 assessed H3K4me changes specific to DAPT treatment alone, finding that sites that lost H3K4me upon
215 NOTCH inhibition in D-NPCs were enriched for high mobility group (HMG) TFBSs (Fig. 3h,
216 Supplementary Data 10). Among HMG family TFs, SOX2 was most substantially and specifically
217 downregulated upon NOTCH inhibition (Supplementary Data 1,7), suggesting that continued SOX2
218 expression upon RFX4 LOF could facilitate maintenance of NPC gene expression. To identify other TFs
219 that could restrain terminal neuronal differentiation specifically in RFX4 LOF D-NPCs, we turned to our
220 in-silico footprinting analysis, finding a HES TFBS footprint was gained upon RFX4 LOF
221 (Supplementary Fig. S4h). Among this TF family, HES5 was most strongly downregulated specifically by
222 NOTCH inhibition (Supplementary Data 1,7), suggesting that both SOX2 and HES5 function with RFX4
223 to regulate cortical neurogenesis downstream of NOTCH signaling.

224 To test potential functional interplay between these TFs during cortical neurogenesis, we performed
225 siRNA knockdown (KD) of either SOX2 and/or HES5 in WT and HOM D-NPCs. SOX2 and HES5 levels
226 were significantly reduced specifically in the presence of siRNAs targeting these genes, with a single
227 siRNA targeting each gene selected for further study (siRNA 3; Fig. 3i-j, Supplementary Fig. S4i-k).
228 Following either SOX2 or HES5 KD, RFX4 mRNA levels decreased significantly (Supplementary Fig.
229 S4l), suggesting direct or indirect transcriptional regulatory control of RFX4 expression by SOX2 and
230 HES5. SOX2 KD reduced neuronal marker expression similarly in both WT and HOM D-NPCs (Fig. 3k-

231 l, Supplementary Fig. S4m-o). However, HES5 KD caused differential effects in WT versus HOM D-
232 NPCs (Fig. 3k-l, Supplementary Fig. S4m-o), promoting upregulation of the cortical deep layer neuron
233 markers TBR1 and CTIP2 in WT but not HOM D-NPCs (Fig. 3k-l). These results demonstrate that, while
234 SOX2 KD alters neuronal gene expression changes similarly regardless of RFX4 levels, TBR1
235 upregulation upon HES5 KD is hampered by RFX4 LOF, suggesting that RFX4 and HES5 function
236 cooperatively to restrain neurogenesis and differentiation. Together, these results highlight a role for RFX4
237 in regulating the onset of cortical neurogenesis downstream of key NOTCH effectors, specifically by
238 regulating the formation of early born, deep layer neurons in the developing cortex.
239

240 **RFX3 binding is contingent on RFX4 but plays a unique neurodevelopmental role**

241 Our work above highlighted a requirement for functional interplay between RFX4 and other TFs to
242 restrain cortical neurogenesis. Given that RFX family TFs can both homo- and hetero-dimerize
243 (Morotomi-Yano et al. 2002) and RFX3 deletion is also associated with NDDs (Banerjee et al. 2019;
244 Ajami et al. 2023), it is plausible that RFX4 acts with other RFX TFs to co-regulate human cortical
245 development. To assess this potential functional interplay, we first compared their expression, finding that
246 both RFX3 and RFX4 expression peaked in NPCs, while only RFX3 expression persisted following
247 neurogenesis (Fig. 4a). We next examined how RFX4 LOF affected RFX3's ability to bind DNA, finding
248 a substantial loss of RFX3 genome-wide binding in HOM D-NPCs (Fig. 4b, Supplementary Data 11),
249 particularly at cis-regulatory regions of neuronal and synaptic genes (Fig. 4c, Supplementary Fig. S5a,
250 Supplementary Data 12). Furthermore, we identified RFX3 and RFX4 co-bound sites (Fig. 4d); these were
251 associated with RFX4 DEGs, were enriched for RFX, TEAD, and SOX TFBSs (Fig. 4e, Supplementary
252 Data 13), and were predominately involved in synapse development (Fig. 4f, Supplementary Data 12).

253 Together, these results indicate that RFX3 binding is contingent upon RFX4 expression and suggest that
254 both RFX TFs play roles in direct regulation of synapse-related gene expression.

255 To assess if the RFX4-dependent RFX3 binding we observed above was reciprocal, we next generated
256 hPSC models with homozygous RFX3 LOF (RFX3-HOM, Fig. 4g, Supplementary Fig. S5b) and validated
257 loss of RFX3 expression in RFX3-HOM D-NPCs (Supplementary Fig. S5c-d). We assessed RFX3
258 genome-wide binding in WT and RFX3-HOM D-NPCs, demonstrating that RFX3 binding was enriched
259 at gene promoters and was lost upon RFX3 LOF (Supplementary Fig. S5e-f, Supplementary Data 11).
260 However, RFX3 LOF resulted in minimal alteration of RFX4 binding (Supplementary Fig. S5g),
261 indicating that RFX4 genome-binding does not reciprocally depend upon RFX3. Additionally, RFX3 LOF
262 resulted in unique cellular phenotypes; for example, unlike RFX4 LOF, RFX3 LOF caused no change in
263 neurosphere size, outgrowth, or proliferative and neurogenic marker immunopositivity (Supplementary
264 Fig. S5h-m), suggesting that RFX3, unlike RFX4, is not required to restrain cortical neurogenesis.
265 Interestingly, transcriptomic analysis confirmed both distinct and shared gene expression changes upon
266 either RFX3 LOF or RFX4 LOF (Fig. 4h, Supplementary Data 14). Specifically, LOF of each TF resulted
267 in shared upregulation of apoptosis associated genes (Fig. 4i, red box, Supplementary Data 12) and shared
268 downregulation of early neuronal genes (Fig. 4i, blue box, Supplementary Data 12). However, RFX3 LOF
269 uniquely caused the downregulation of synapse-related genes, many of which were bound by RFX3 at
270 promoters under WT conditions (Fig. 4j, Supplementary Fig. S5n-o, Supplementary Data 12). Many of
271 these genes were instead upregulated by RFX4 LOF (Supplementary Data 12). These results highlight
272 both shared and antagonistic roles for RFX3 and RFX4 during cortical neurogenesis, specifically in
273 restraining synaptic gene expression, with RFX4 having a more substantial role in regulating this process.

274 **Pathogenic *RFX4* missense mutation disrupts genome-wide binding, but has distinct effects from**
275 **LOF on transcriptional regulation**

276 As RFX4 was recently classified as an NDD risk gene with pathogenic mutations hypothesized to act
277 by LOF (Harris et al. 2021), we next generated hPSC models heterozygous or homozygous for a
278 pathogenic p.R79C missense mutation in the RFX4 DNA binding domain (R79C-HET and R79C-HOM,
279 respectively; Fig. 5a, Supplementary Fig. S6a). Levels of RFX4 mRNA and protein were assessed in
280 R79C-HET and R79C-HOM D-NPCs, highlighting substantially reduced RFX4 protein levels specifically
281 in the R79C-HOM but not R79C-HET D-NPCs (Supplementary Fig. S6b-c); these data suggest that this
282 mutation could disrupt the expression and/or protein stability of RFX4 and may alter its function.
283 Consistent with pathogenic disruption of the DNA binding domain of RFX4, we found an almost complete
284 loss of RFX4 genome-wide binding in R79C-HOM D-NPCs (Fig. 5b, Supplementary Fig. S6d,
285 Supplementary Data 15). This occurred at similar locations and was associated with a loss of binding at
286 many of the same genes defined in the LOF HOM D-NPCs (e.g. *DLG4* and *SCRN1*; Fig. 5c-e,
287 Supplementary Data 16), suggestive of LOF as a potential mechanism of pathogenicity.

288 We next assessed transcriptomic changes in R79C-HOM versus WT D-NPCs, surprisingly finding
289 substantially different DEGs in R79C-HOM versus LOF HOM versus matched control D-NPC data
290 comparisons (Fig. 5f). Likewise, unlike the LOF model, R79C-HOM D-NPCs did not alter neurosphere
291 outgrowth (Supplementary Fig. S6e-g). Further analysis identified many R79C-HOM specific DEGs
292 (Supplementary Data 16), with predominant downregulation of neuronal and synaptic gene expression by
293 contrast with HOM D-NPCs (Supplementary Data 1,16). We also identified a small set of shared DEGs
294 with similarly dysregulated expression in both R79C-HOM and HOM D-NPCs (Fig. 5g); these shared
295 downregulated DEGs were enriched for cell migration genes, while shared upregulated DEGs were
296 instead enriched for early forebrain development genes (Fig. 5h, Supplementary Data 16). Together, these

297 results highlight the unexpected finding that, while p.R79C pathogenic mutation disrupts genome-wide
298 RFX4 binding like LOF, it causes distinct transcriptomic changes.

299 We next tested whether these transcriptional consequences were dependent upon homozygosity for the
300 p.R79C mutation, finding that, unlike R79C-HOM, R79C-HET had minimal consequences on RFX4
301 genome-wide binding in D-NPCs (Fig. 5i, Supplementary Fig. S6h, Supplementary Data 15). We also
302 examined a selection of DEGs that were differentially regulated in the R79C versus the LOF models,
303 finding that both R79C-HET and R79C-HOM D-NPCs exhibited similar gene expression changes, while
304 these differed from those seen in the HET and HOM LOF models (Fig. 5j). Furthermore, while LOF D-
305 NPC models increased ASCL1 immunopositivity (Fig. 1p), neither R79C-HET or R79C-HOM D-NPCs
306 did (Fig. 5k). Likewise, the KI67, FOXG1, and PAX6 immunopositive cell fractions were unchanged upon
307 p.R79C mutation (Supplementary Fig. S6i-l). These findings demonstrate that, despite similarly reducing
308 genome-wide binding, the p.R79C mutation disrupts transcriptional regulation distinctly from
309 haploinsufficiency-related LOF.

310

311 **RFX4 expression during neurogenesis is necessary for stratification of neuronal layers in cortical**
312 **organoids**

313 To examine the neuronal consequences of altered cortical neurogenesis resulting from RFX4 LOF, we
314 next generated both cortical excitatory neurons (cEXs) by differentiating WT and HOM D-NPCs (Fig. 6a)
315 and glutamatergic-like neurons (iGluts, Fig. 6b) by overexpressing Neurogenin-2 (NGN2), in WT and
316 HOM hPSCs. As the generation of iGluts bypasses the formation of neuronal progenitors and RFX4
317 expression is temporally restricted to D-NPCs (Fig. 6c, Supplementary Fig. S7a-b), we tested whether
318 transcriptomic changes caused by RFX4 LOF specifically in D-NPCs were detected in cEXs but not
319 iGluts. We examined neuronal genes directly regulated by RFX4 in D-NPCs, observing continued

320 upregulation of these genes in HOM cEXs, while HOM iGluts exhibited no consistent changes in
321 expression of these genes (Fig. 6d). This assessment confirmed that neuronal consequences from RFX4
322 LOF require transit through a prior progenitor stage. To further examine the neuronal consequences of
323 LOF, we performed RNA-sequencing in WT and HOM cEXs (Supplementary Fig. S7c) identifying
324 consistently dysregulated DEGs common to both HOM D-NPCs and HOM cEXs (Supplementary Tabel
325 1,18). This analysis identified a persistent upregulation of TFs involved in neuronal specification (e.g.
326 ASCL1, OLIG2, Fig. 6e-f, Supplementary Data 19) in both HOM D-NPCs and cEXs. These data confirm
327 that transcriptomic disruptions caused by RFX4 LOF originate during D-NPC specification and persist
328 through cEX differentiation, resulting in persistently elevated pro-neurogenesis gene expression in
329 neurons that could potentially impact their later differentiation or maturation.

330 To examine if these disruptions altered the differentiation capacity, identity, and/or maturation of HOM
331 cEXs, we next profiled expression of cortical layer markers and synaptic genes critical for glutamatergic
332 neuron function, finding that many of these genes were downregulated in HOM cEXs (Fig. 6g). This was
333 further supported by our RNA-sequencing data, which revealed the predominant downregulation of genes
334 involved in the generation of neurons in HOM cEXs (Supplementary Data 19). As TBR1+ (deep cortical
335 layer 6) neurons are the first subtype to be generated from D-NPCs during neurogenesis, we also examined
336 the production of these neurons by immunofluorescence, finding that HOM cEX cultures failed to generate
337 TBR1 immunopositive cells (Fig. 6h), although there was no significant difference in production of
338 neurons expressing another deep layer 5/6 cortical neuron marker, CTIP2 (Fig. 6h). Finally, we examined
339 synapse production in WT and HOM cEX cultures by examining the density of SYN1+ and PSD95+
340 puncta colocalized to TUJ1+ neurites, finding no alteration in the density of these synaptic markers in
341 HOM cEXs (Fig. 6i). Together, these data suggest that, while these neurons initiate neurogenesis
342 prematurely, some aspects of their later differentiation capacity are impaired.

343 The reduction of some neuronal marker expression in cEXs suggested that LOF effects on neurogenesis
344 could persistently alter later neuronal differentiation and layering of the developing cortex. To examine
345 this, we generated dorsal telencephalic patterned 3-D cortical organoids (D-ORGs, Fig. 6j). We first tested
346 key neurogenesis-related phenotypes observed above in D-NPCs, focusing on approximately analogous
347 D-ORG timepoint, day 30 (D30). Like our prior findings for D-NPCs, HOM D-ORGs exhibited
348 precocious neurogenesis, as marked by an increased area expressing the newborn neuron marker TUJ1,
349 with no change to the fraction of SOX2-expressing progenitors (Fig. 6k). This was congruent with a
350 decreased KI67- and increased TBR1-immunopositive cell fraction (Supplementary Fig. S7d-e). We
351 further detected an increased proportion of cells immunopositive for FOXG1, a cortical TF expressed
352 throughout differentiation (Supplementary Fig. S7f), while neither the intermediate progenitor marker
353 TBR2 (Supplementary Fig. S7g) nor ASCL1 were affected in organoid models (Supplementary Fig. S7h).
354 Together these results demonstrate that RFX4 restrains cortical neurogenesis, while LOF causes
355 precocious neurogenesis in both 2-D and 3-D models of cortical development.

356 As TBR1+ neuron production in cEX cultures was significantly reduced by RFX4 LOF, we further
357 differentiated D-ORGs to day 60 (D60) to examine if cortical stratification into different neuronal
358 subtypes/layers is similarly altered by RFX4 LOF. We found significant increases in the production of
359 TBR1+ (Fig. 6l) and CTIP2+ (Fig. 6m) neurons upon RFX4 LOF and increases to the area expressing
360 TUJ1 in D60 D-ORGs, with no change in the apoptosis marker Cleaved Caspase 3 (CC3) (Supplementary
361 Fig. S7i). These results demonstrate that the increased production of early-born neurons persists to affect
362 3-D organoid cortical neuron layering. Together, these results support a progenitor-specific requirement
363 for RFX4 to restrain cortical neurogenesis and differentiation, with its disruption causing persistent effects
364 in neuron differentiation and stratification into layers in these hPSC-based models of human cortical
365 development.

366 **Discussion**

367 In this work, we derived new hPSC models and used these to identify requirements for RFX4 and
368 consequences of pathogenic mutation during human cortical development. We demonstrated that RFX4
369 restrains cortical neurogenesis in both 2-D and 3-D models and during both cortical excitatory (cEX) and
370 inhibitory neuron (cIN) differentiation. While RFX transcription factors were previously assumed to
371 behave as transcriptional activators (Harris et al. 2021; Choi et al. 2024), this work demonstrates a
372 requirement for RFX4 to directly repress neuronal and synaptic gene expression during this process. We
373 further found that both molecular and cellular phenotypes associated with RFX4 LOF in progenitors
374 persist through neuronal differentiation, despite the cessation of RFX4 expression. RFX4 overexpression
375 alone was insufficient to induce neuronal differentiation without concomitant or prior neuronal patterning;
376 these results clarify prior conflicting studies suggesting that RFX4 could both stabilize neuronal progenitor
377 production (Joung et al. 2023) and induce neuronal differentiation (Choi et al. 2024). Our work
378 demonstrates that RFX4 is required repress neuronal gene expression to maintain the neuronal progenitor
379 state, while LOF induces neurogenesis.

380 To investigate the mechanisms by which RFX4 regulates cortical neurogenesis, we identified
381 endogenous genome-wide RFX4 binding during both cEX and cIN development, confirming the validity
382 and quality of these data across multiple paradigms. We also identified other TFs that colocalized on the
383 genome with RFX4 (e.g. TEAD1, RFX3) and tested whether these were affected by RFX4 LOF, to
384 determine whether they could also act at RFX4-bound cis-regulatory elements (CREs) to regulate cortical
385 neurogenesis. Specifically, we defined putative CREs that co-bound RFX4 and TEAD1; this was notable
386 because TEAD TFs play known roles in regulating cortical neurogenesis (Mukhtar et al. 2020). Some of
387 these co-bound CREs were associated with NOTCH pathway genes such as JAG1, congruent with the

388 recent observation that TEAD transcription factors can modulate NOTCH signaling during mouse cortical
389 neurogenesis (Perry et al. 2025).

390 Our data also demonstrated that RFX3 and RFX4 co-occupy CREs associated with synaptic genes and
391 functionally interact to regulate gene expression, with RFX3 relying on RFX4 to bind most of these shared
392 enhancers. Despite co-occupying putative CREs at synaptic genes, RFX3 and RFX4 requirements for
393 cortical neuron development differ; RFX4 expression specifically during neurogenesis is required to
394 represses neuronal gene expression, while RFX3 can activate later neuronal and synaptic gene expression
395 during differentiation, a finding substantiated by Lai and colleagues using RFX3 LOF iGlut models (Lai
396 et al. 2025). RFX3 was also previously shown to promote activity-dependent gene expression in cortical
397 neurons through interactions with CREB1 (Lai et al. 2025; Neumann et al. 2025), versus the distinct role
398 we describe here for RFX4 specifically in neural progenitors, suggesting distinct temporal and
399 transcriptional activities for these TFs in cortical development. These divergent roles are congruent with
400 the temporal restriction of RFX4 expression to progenitors, while RFX3 continues to be expressed in
401 neurons (Wang et al. 2025). While our work here supports co-occupancy of RFX4 with TEAD1 or RFX3
402 at enhancers during cortical neurogenesis, future work is needed to further define the mechanistic
403 relationships between the activities of these TFs and other TFs with enriched binding motifs under the
404 RFX4 bound peaks we defined here.

405 We also used an unbiased drug screen to assess mechanisms of action that could reverse RFX4 LOF
406 phenotypes, identifying functional interplay between RFX4 LOF and NOTCH signaling blockade.
407 Specifically, we found that RFX4 functions downstream of NOTCH signaling and functionally cooperates
408 with SOX2 and HES5 to regulate cortical neurogenesis. Our findings are congruent with a cooperative
409 role for SOX2 in neuroectoderm and neural progenitor formation and maintenance (Graham et al. 2003;
410 Bani-Yaghoub et al. 2006; Amador-Arjona et al. 2015; Batchuluun et al. 2017), while we also identified a

411 previously undefined relationship between RFX4 and HES5, a NOTCH effector that, like RFX4, restrains
412 neurogenesis (Ohtsuka et al. 2001; Bansod et al. 2017; Luiken et al. 2020). Our data indicate that RFX4
413 and HES5 activity are both required to cooperatively restrain neurogenesis and differentiation into early-
414 born neurons, highlighting the importance of strict regulation of this process.

415 Examining the persisting consequences in neurons of altered neurogenesis resulting from RFX4 LOF,
416 we found that these were specific to neurons which underwent a progenitor stage rather than being directly
417 differentiated into iGluts. Similar findings have been reported for other NDD hPSC models (Schafer et al.
418 2019), supporting a developmental etiology involving disrupted neurogenesis for NDDs resulting from
419 RFX4 mutation. Studies exploring other NDD-associated mutations have also reported altered cortical
420 neurogenesis (Kaushik and Zarbalis 2016; Adam and Harwell 2020; Niu et al. 2024; Chapman et al. 2025;
421 Kaushik et al. 2025; Prakasam et al. 2025) that can cause E/I functional neuron imbalance, a central
422 hypothesis for the cellular etiology of NDDs (Siegel-Ramsay et al. 2021; Hollestein et al. 2023). For
423 example, EZH1 LOF disrupts neurogenesis (Gracia-Diaz et al. 2023) while conditional Ezh2 knockout in
424 mouse models enhances formation of TBR1+ and CTIP2+ neurons during peak cortical neurogenesis
425 (Pereira et al. 2010). EZH2 has also been shown to act as an epigenetic barrier during neuronal
426 differentiation of hPSC models (Ciceri et al. 2024). As mutations in each of these genes can cause NDDs,
427 disruption of neurogenesis represents a critical developmental window sensitive to perturbation to cause
428 NDDs.

429 Our cEX and cortical organoid models both exhibited precocious neurogenesis with persistent neuronal
430 consequences, with LOF altering expression of lineage-specific neuronal markers. Interestingly, LOF in
431 D-NPCs caused a persistent upregulation some TFs specific to cINs in cEXs, while the increase in FOXG1
432 immunopositivity in LOF organoid models may also indicate altered progenitor identity, as FOXG1
433 overexpression in organoids was previously reported to increase GABAergic neuron production (Mariani

434 et al. 2015). Our observations of perturbed progenitor identity here are reminiscent of the altered dorsal-
435 ventral brain patterning seen in Rfx4 knockout mouse and zebrafish models (Blackshear et al. 2003; Zhang
436 et al. 2006; Ashique et al. 2009; Sedykh et al. 2018), suggesting that RFX4 mutations may affect both
437 neurogenesis and neuronal lineage allocation.

438 Finally, having established requirements for RFX4 during cortical development, we examined how the
439 pathogenic mutation p.R79C, a missense mutation in the DNA-binding domain predicted to act via LOF,
440 affected regulation of cortical neurogenesis. Surprisingly, while this mutation largely abrogated RFX4
441 genome-wide binding like LOF models, it caused unique and substantial transcriptional changes,
442 suggesting a distinct mechanism of pathogenicity. As RFX TFs can both homo- and heterodimerize
443 (Morotomi-Yano et al. 2002) and can co-bind shared genomic targets, pathogenic mutation may alter these
444 dynamics. Furthermore, it has been shown that two RFX family TFs can co-bind a single canonical X-box
445 motif (Gajiwala et al. 2000), suggesting that RFX family TF heterodimerization may alter their
446 transcriptional regulatory activity. Studying the basis of pathogenicity of additional mutations will be
447 required to understand the etiology of RFX4-associated NDDs and underlying mechanisms. Together, this
448 study elucidated previously unknown roles for and mechanisms of transcriptional repression by RFX4
449 during cortical neurogenesis, highlighting their disruption upon both LOF and pathogenic missense
450 mutation and providing a necessary foundation to develop approaches for reversal of pathogenesis-
451 associated phenotypes.

452 **Methods**

453 **hPSC Model Generation and Culture**

454 Work with human pluripotent stem cells (hPSCs) was performed under our approved Washington
455 University Embryonic Stem Cell Research Office (ESCRO) under protocol #12-002. RFX4 heterozygous
456 (HET) and homozygous (HOM) knockout hPSCs were generated in both the human embryonic stem cell
457 (hESC) line WA09 and human induced pluripotent stem cell (hiPSC) line AN1.1 by targeting the first
458 canonical exon (6) with CRISPR-Cas9-based genome engineering (Supplementary Fig. 1a). The same
459 process was employed to generate RFX3 homozygous (RFX3-HOM) knockout AN1.1 hiPSC models by
460 targeting exon 8 (Supplementary Fig. 5b). Finally, a patient-specific *RFX4* mutation (c.235C>T) was
461 genetically engineered into the WA01 hESC line targeting one or both alleles of the *RFX4* gene with
462 CRISPR-Cas9-based genome editing (Supplementary Fig. 6a). The validity of all models was confirmed
463 by NGS-based amplicon sequencing (Sentmanat et al. 2018), with all model generation and validation
464 conducted by the Washington University Genome Engineering & Stem Cell Center (GESC). All hPSCs
465 were maintained under feeder-free conditions on vitronectin (Gibco™) coated plasticware in StemFlex
466 Medium (Gibco™) at 37°C with 5% CO_2. Experiments were carried out between passages 15-60 for all
467 hPSC lines. Details of cell backgrounds used for each experiment are detailed in Supplementary Data 20.
468

469 **Modeling cortical inhibitory and excitatory neuron development**

470 To model cortical inhibitory neuron development, we specified hPSCs as medial ganglionic eminence
471 (MGE)-like progenitors with a ventral telencephalic progenitor (V-NPC, day 15) identity, as previously
472 described (Meganathan et al. 2017) with minor modifications. Modeling of cortical excitatory neuron
473 development was carried out as described in our prior work (Chapman et al. 2022; Chapman et al. 2024)
474 by specifying hPSCs as progenitors with a dorsal telencephalic progenitor (D-NPC, day 20) identity and

475 further differentiated into immature cortical excitatory neurons (cEXs, day 40). Induced glutamatergic-
476 like neurons (iGluts) were generated by overexpressing the bHLH transcription factor Neurogenin-2
477 (NGN2) and as described in our prior work (Chapman et al. 2025) with cells constituting iGluts at day 21.
478 For RFX4 overexpression experiments, wildtype hPSCs were transduced with an RFX4 open reading
479 frame plasmid (Genecopoeia, H2578-Lv203) and cultured in neuronal media for 6 days before collection.
480 Finally, to model 3-D human cortical development, dorsally patterned cortical organoids were generated
481 as previously described (Lunn 2022) with minor modifications and collected on days 30 and 60. All
482 protocols are detailed in Supplementary Materials.
483
484 **Cellular Phenotyping**
485 Changes in RFX4 in pathogenic and loss of function models was assessed by western blot and RT-
486 qPCR using protein and RNA isolated from D-NPCs; similarly, changes in RFX3 was assessed by western
487 blot and RT-qPCR using protein and RNA isolated from D-NPCs. Quantifications of neurosphere
488 outgrowth were performed in both D-NPCs and V-NPCs after 12 days (D12) of differentiation and changes
489 in immunopositive markers were assessed by immunocytochemistry 2 days after replating specified
490 progenitors. Upon specification into progenitors, RT-qPCR was used to examine changes in gene
491 expression in RFX4 HET LOF models. To examine interactions between SOX2, HES5, and RFX4, D-
492 NPCs were treated with siRNAs on day 21 and collected on day 24; confirmation of siRNA knockdown
493 and marker changes was assessed by RT-qPCR. To determine RFX4 protein function, luciferase assays
494 were performed in WT and RFX4 HOM D-NPCs on day 24 after transfection of luciferase vectors. For
495 our unbiased drug screen, we examined changes in OLIG2 immunopositivity in WT and HOM D-NPCs
496 on day 24 after 4 days of treatment.

497 To determine changes in differentiation and proliferation in D-ORGs, immunocytochemistry was
498 performed at days 30 (D30) and days 60 (D60). Examination of D-NPC RFX4-Bound genes was
499 performed by RT-qPCR at day 40 of cEX and day 21 of iGlut differentiation; assessment of cortical layer
500 and synaptic markers was performed similarly by RT-qPCR in day 40 cEXs. Finally,
501 immunocytochemistry assessment of neuronal differentiation and synaptic production in monolayer cEXs
502 was performed at day 40 and day 50, respectively. siRNA oligonucleotides are detailed in Supplementary
503 Data 21. All RT-qPCR primers and antibodies used in this study are listed in Supplementary Data 22 and
504 Supplementary Data 23, respectively. Details for luciferase oligonucleotides are listed in Supplementary
505 Data 24. For detailed cellular phenotyping methods, see Supplementary Material.
506
507 **RNA-sequencing and CUT&Tag**
508 Both RNA-sequencing and CUT&Tag was performed as previously described (Chapman et al. 2024)
509 on RFX4 LOF V-NPCs at day 15 and day 20 for RFX4 and RFX3 LOF and R79C D-NPCs. For NOTCH
510 experiments, CUT&Tag for H3K4me and H3K27me3 in WT and RFX4 HOM D-NPCs was performed at
511 day 24 following either DMSO or DAPT treatment; likewise, RNA-sequencing was performed in WT D-
512 NPCs treated with either DMSO or DAPT on day 24. Finally, RNA-sequencing was performed on day 40
513 WT and RFX4 HOM cEXs. All raw data (RNA and CUT&Tag) is available from the Gene Expression
514 Omnibus database under accession GSE316377. For full details on RNA-sequencing and CUT&Tag
515 methodologies and data processing, see Supplementary Material.
516
517 **Quantification and Statistical Analysis**
518 Where appropriate, statistical analysis was carried out using a combination of tools available in
519 GraphPad Prism version 9 (GraphPad Software; La Jolla, CA, USA, available from www.graphpad.com)

or RStudio version 3.5.1 (RStudio: Integrated development environment for R; Boston, MA, USA. Available from www.rstudio.org). All technical replicates were averaged before statistical analysis and statistical tests used for the analysis of each data set are detailed in the figure legends or in the methods section for specific analysis paradigms including differential gene expression and differential binding analysis. A minimum of 3 independent differentiations (3 biological replicates) were used for each time point and biological condition with the number of differentiations used for each sample listed in the figure legends as N. The results in figures are presented as group mean +/- standard error (SE) indicating each biological replicate used for the analysis, unless otherwise specified in figure legends. Statistical significance is indicated with asterisks as follows: ns, not significant; $*$, $p<0.05$; $**$, $p<0.01$; $***$, $p<0.001$; $****$, $p<0.0001$ unless otherwise specified in figure legends.

Competing Interests

The authors declare no competing interests.

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Author Contributions

J.J.D. and K.L.K. designed the study. J.J.D. performed developmentally patterned differentiations and downstream cellular phenotyping for RFX4 LOF, RFX3 LOF, R79C, and NOTCH experiments. G.C. performed iGlut experiments. J.J.D. generated dorsally patterned organoids, G.C. cryo-sectioned organoids, and J.J.D. and G.C. performed immunocytochemistry on organoids. S.C. performed RFX4 overexpression experiments and RT-qPCR. F.B. performed R79C western blots. S.M. performed RT-qPCR and organoid immunocytochemistry. F.B. and H.J. generated immunofluorescence images. J.J.D. and G.C. performed bioinformatic analyses. W.B., C.V., S.E., and T.S.G. performed the drug screen to identify mechanisms of action for RFX4 LOF. M.S. and X.C. generated and confirmed validity of RFX3 KO hPSC lines. J.J.D. and G.C. analyzed data and generated figures. J.J.D. and K.L.K. wrote the manuscript and all authors approved the manuscript.

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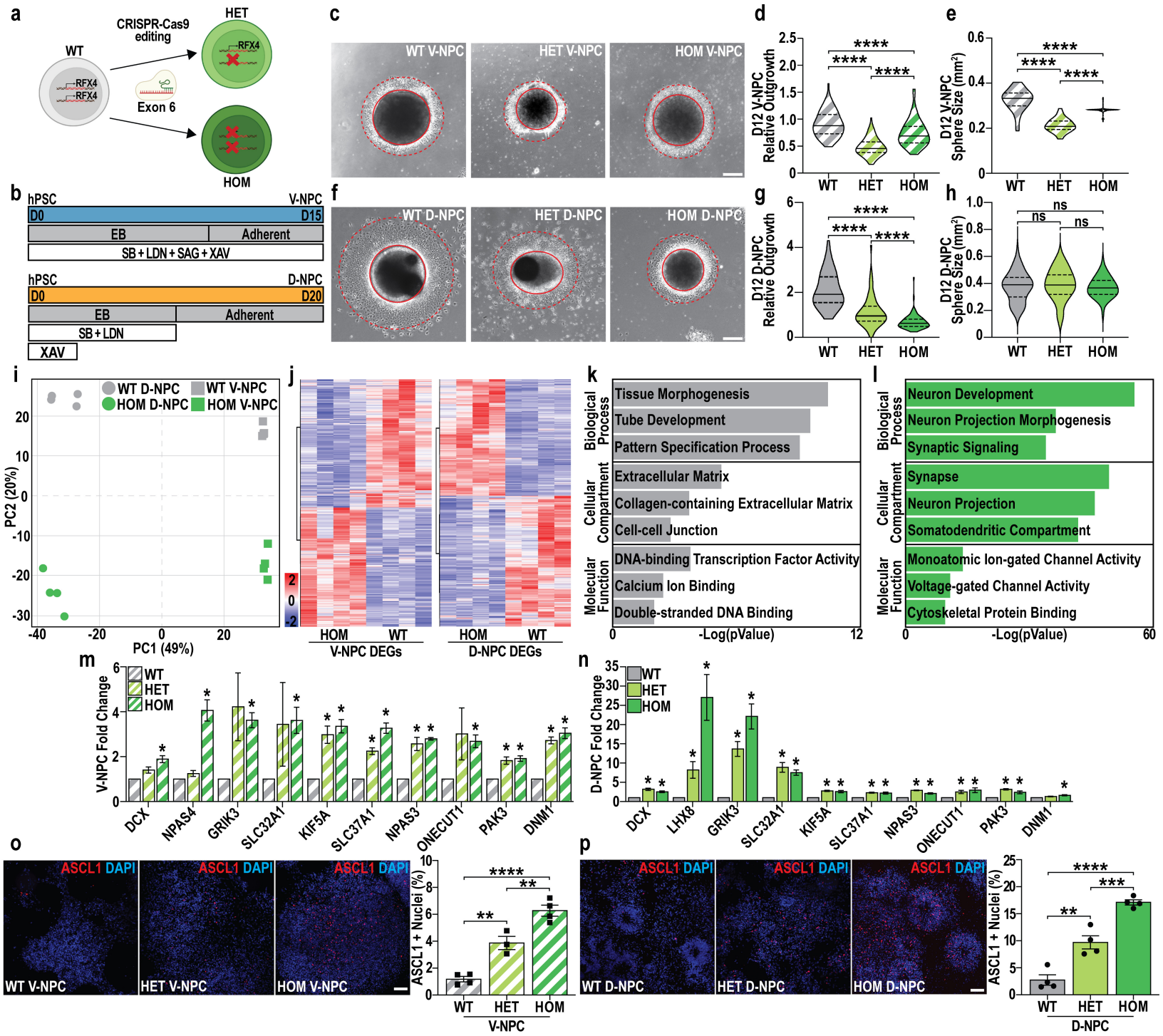

Fig. 1: Loss of RFX4 causes dosage-dependent induction of cortical neurogenesis.
(a-b) Schematic of **(a)** heterozygous (HET) and homozygous (HOM) RFX4 loss of function (LOF) hPSC model generation and **(b)** protocols used to specify hPSCs to ventral (V-) and dorsal (D-) neural progenitor cells (NPCs). **(c-h)** Quantification of **(c-e)** V-NPC and **(f-h)** D-NPC neurosphere outgrowth measuring **(d,g)** neuosphere body outgrowth (marked by dotted red circle) and **(e,h)** neurosphere size (marked by solid red circle) after 12 days of differentiation. **(i)** Principal component analysis (PCA), highlighting variation between WT D-NPCs (grey circles), RFX4 HOM D-NPCs (green circles), WT V-NPCs (grey squares), and RFX4 HOM V-NPCs (green squares). **(j)** Heatmaps of differentially expressed genes (DEGs) between WT and HOM V-NPCs (left) or D-NPCs (right). **(k-l)** Summary of gene ontology (GO) enrichment analysis for DEGs **(k)** downregulated or **(l)** upregulated by RFX4 LOF in both D- and V-NPCs. **(m-n)** RT-qPCR of genes significantly upregulated by RFX4 LOF; in WT, HET and HOM **(m)** V-NPCs and **(n)** D-NPCs. **(o-p)** Representative images and quantification of ASCL1 immunopositivity in **(o)** V-NPCs or **(p)** D-NPCs. Data are represented as **(d,e,g,h)** distributions with quartiles (dashed lines) and means (solid line) marked or **(d,e,g,h,m-p)** as mean +/- SEM, with **(m-p)** fold change calculated relative to GAPDH. Data in **(d,e,g,h)** was analyzed using one-way ANOVA (n=4) or **(m-n)** Student's t-test (n=4); ns = not significant, *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001, scale bars = **(c,f)** 100 µm and **(o,p)** 50 µm.

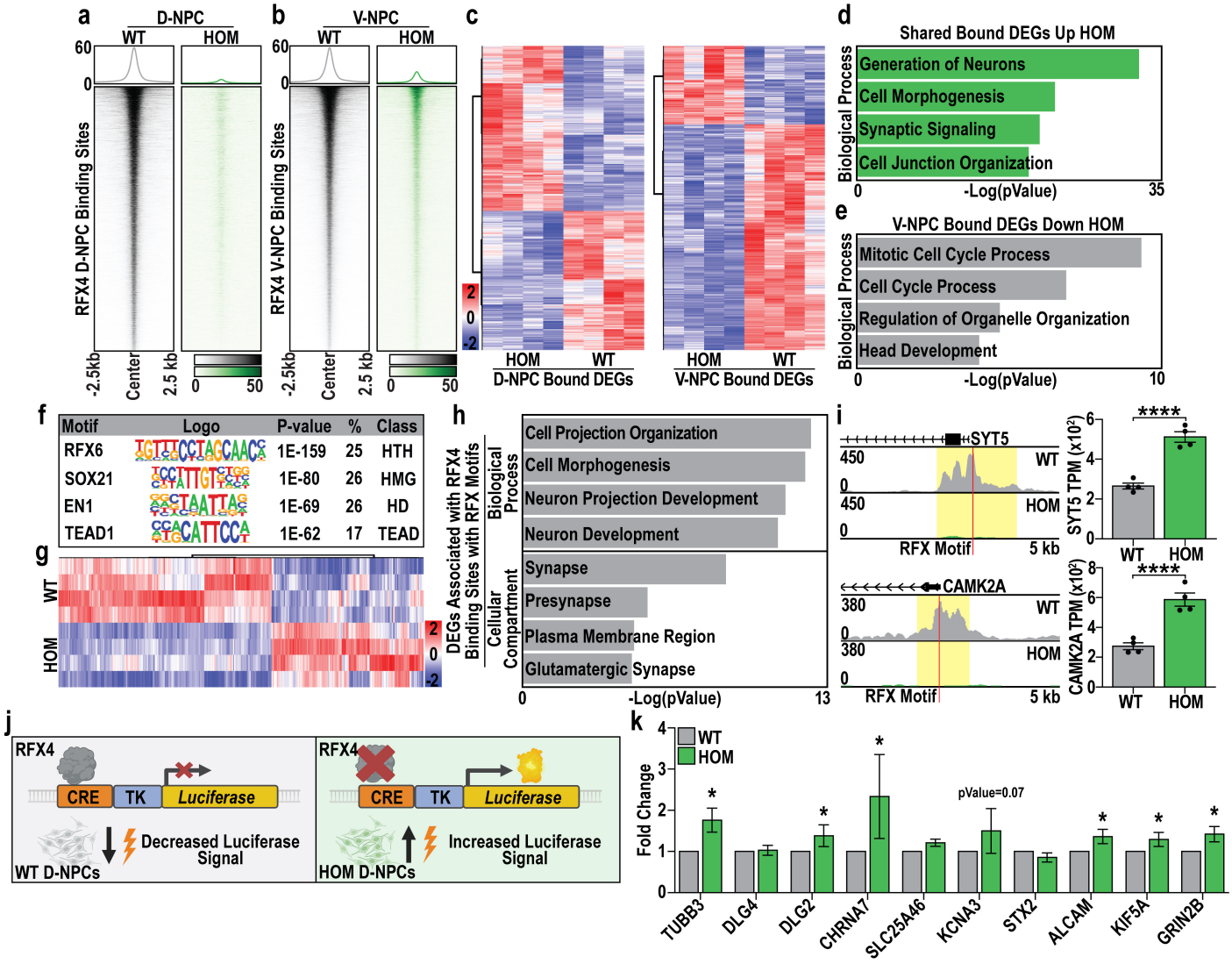

Fig. 2: RFX4 directly restrains the onset of neuronal and synaptic gene expression.
(a-b) Heatmaps of all RFX4 bound sites in WT and HOM **(a)** D-NPCs and **(b)** V-NPCs. **(c)** Heatmaps of DEGs associated with RFX4 binding (bound DEGs) in D-NPCs (left) and V-NPCs (right). **(d-e)** Summary of GO enrichment analysis for bound DEGs **(d)** upregulated in HOM V- and D-NPCs or **(e)** downregulated in HOM V-NPCs. **(f)** Summary of transcription factor binding site (TFBS) enrichment analysis of RFX4 bound DEGs. **(g)** Heatmap of RFX4 D-NPC bound DEGs associated with an RFX TFBS. **(h)** Summary of GO enrichment analysis for RFX4 bound DEGs associated with an RFX TFBS. **(i)** Examples of RFX4 D-NPC bound DEGs containing an RFX TFBS. **(j)** Experimental scheme and model for testing the hypothesized regulatory relationship between RFX4 and neuronal gene expression using luciferase assays. **(k)** Relative fold change in luciferase signal in HOM D-NPCs relative to the WT control. Data is represented as **(i,k)** mean +/- SEM and was analyzed by **(i)** differential gene expression analysis (n=4), or **(k)** Student's t-test (n≥3); **(i,k)** ns = not significant, *p<0.05 and ****p<0.0001.

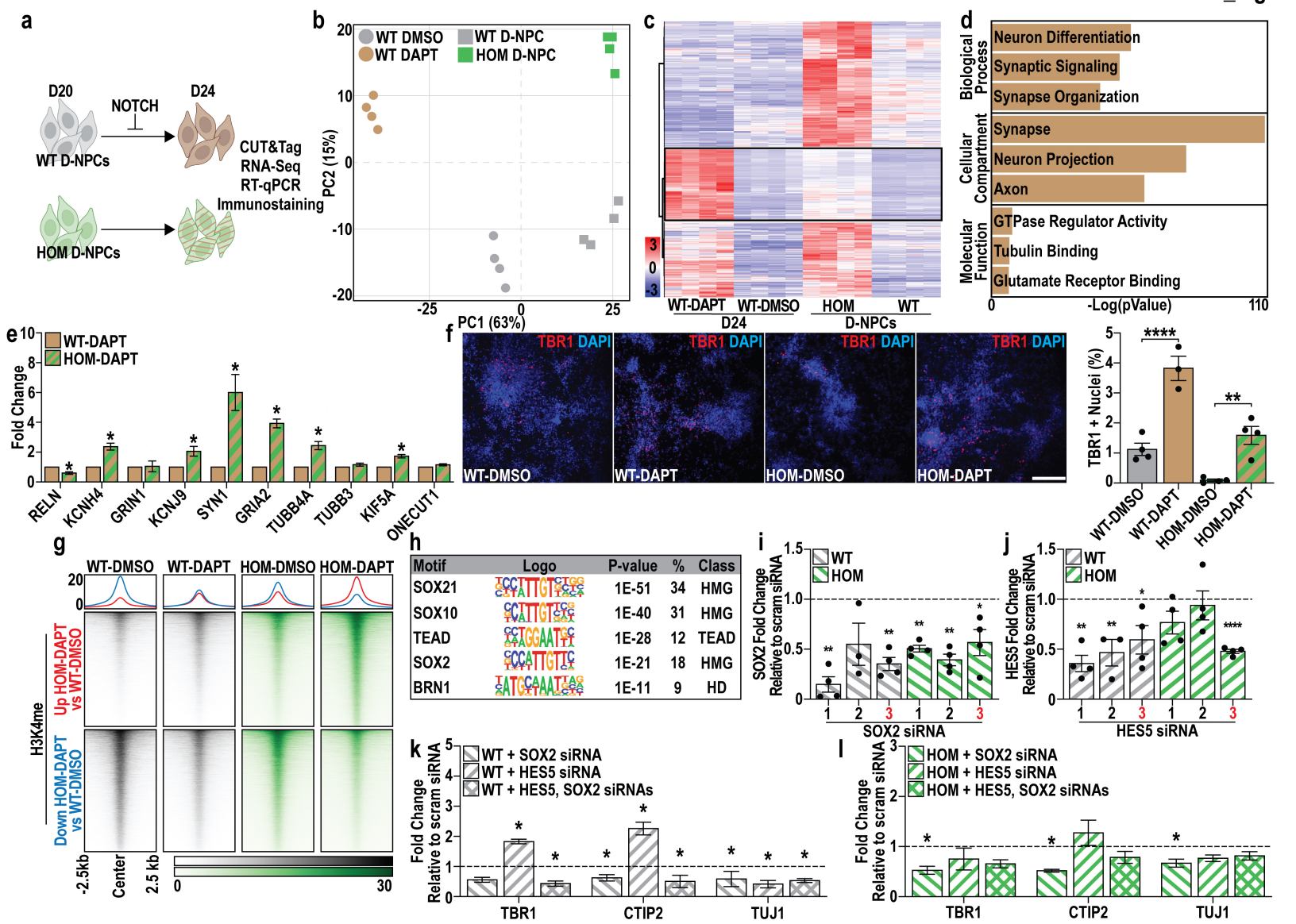

Fig. 3: RFX4 regulates the onset of cortical neurogenesis downstream of NOTCH signaling.
(a) Schematic of experimental approach for assessing the effect of NOTCH inhibition (using DAPT) on WT and RFX4 HOM D-NPCs. **(b)** PCA highlighting variation between, WT-DMSO vehicle treated D-NPCs (grey circles), WT-DAPT treated D-NPCs (brown circles), and untreated WT D-NPC (grey squares), and RFX4 HOM D-NPCs (green squares). **(c)** Heatmap of RFX4 HOM DEGs, highlighting similar gene expression increases upon DAPT treatment, with genes upregulated most strongly by NOTCH inhibition indicated (black rectangle). **(d)** Summary of GO enrichment analysis of genes upregulated by RFX4 LOF and further upregulated by NOTCH inhibition (black rectangle from c). **(e)** RT-qPCR of genes significantly upregulated by RFX4 LOF and further upregulated by NOTCH inhibition in WT-DAPT and HOM-DAPT cells. **(f)** Representative images and quantification of TBR1 immunopostitivity in WT-DMSO, WT-DAPT, RFX4 HOM-DMSO, and RFX4 HOM-DAPT treated D-NPCs, highlighting a significant effect of DAPT treatment in both WT and HOM D-NPCs ($F_{1,11}$=69.41). **(g)** Heatmap of H3K4me sites upregulated (top, red) or downregulated (bottom, blue) in RFX4 HOM-DAPT D-NPCs versus WT-DMSO D-NPCs. **(h)** Summary of TFBS enrichment analysis for H3K4me sites lost upon NOTCH inhibition in D-NPCs. **(i-j)** RT-qPCR quantification of **(i)** SOX2 mRNA or **(j)** HES5 mRNA expression levels in WT and HOM D-NPCs treated with SOX2 or HES5 siRNAs respectively, relative to scram siRNA control treated D-NPCs (red text indicates siRNA used for subsequent experiments). **(k-l)** RT-qPCR for neuronal markers in **(k)** WT or **(l)** HOM D-NPCs treated with SOX2 and/or HES5 siRNAs. Data are represented as **(e,f,i-l)** mean +/- SEM, with fold change calculated relative to **(e)** GAPDH or **(i-l)** RPL30. Data was analyzed by **(e,i-l)** Student's t-test (n=4) or **(f)** two-way ANOVA (n=4); ns = not significant, *p<0.05, **p<0.01, and ****p<0.0001, scale bar = 100 µm.

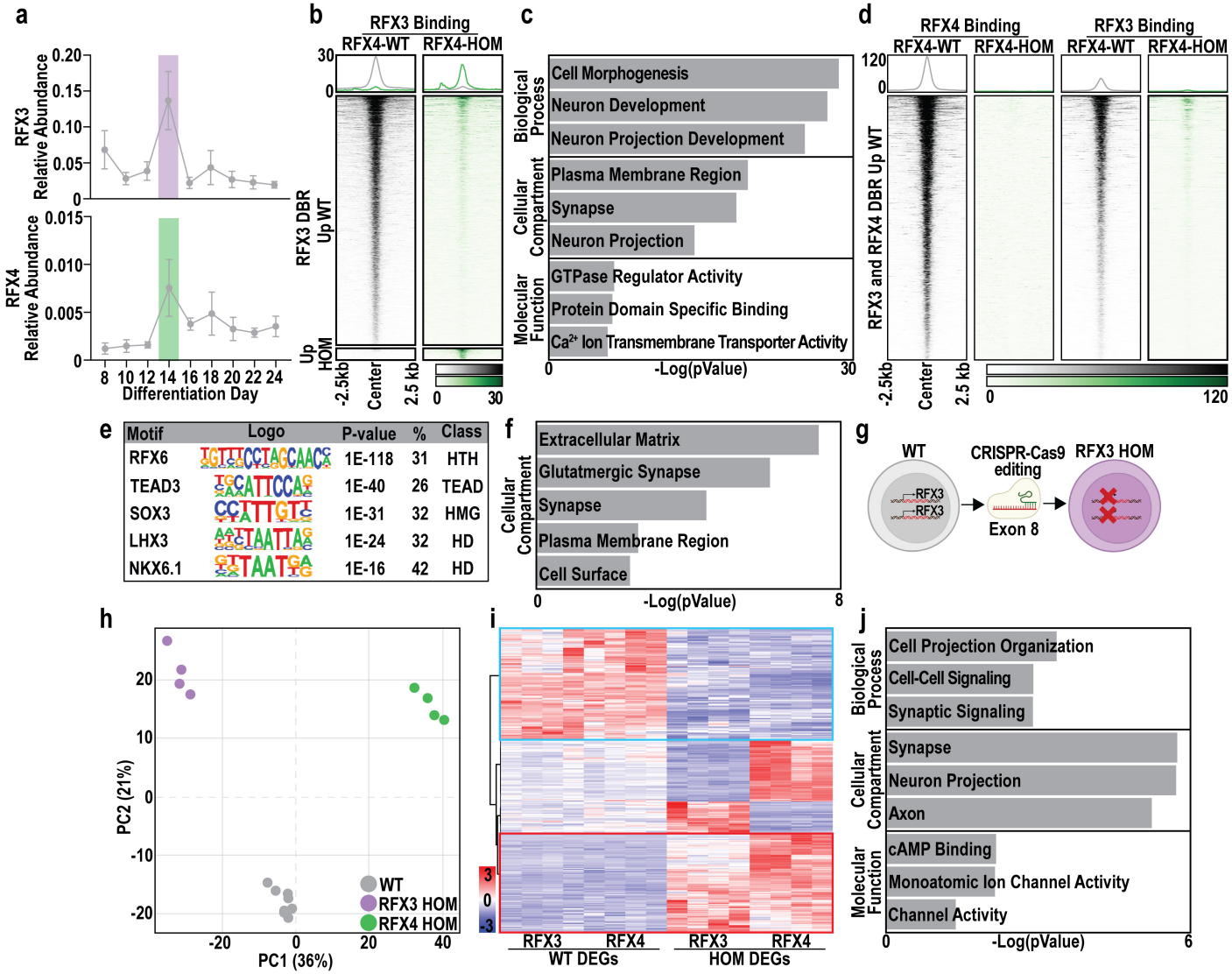

Fig. 4: RFX3 binding is contingent on RFX4 but plays a unique neurodevelopmental role.
(a) RT-qPCR quantifying RFX3 (top, purple) and RFX4 (bottom, green) mRNA expression levels from day 8-24 of WT D-NPC differentiation, with peak expression highlighted. **(b)** Heatmap of RFX3 binding in RFX4 WT and HOM D-NPCs, separating sites which lost (upper panel) or gained (lower) RFX3 binding in response to RFX4 LOF. **(c)** Summary of GO enrichment analysis for genes associated with an RFX3 bound site lost in RFX4 HOM D-NPCs. **(d)** Heatmaps of RFX3 and RFX4 binding for co-bound sites that lose RFX4 binding in RFX4 HOM D-NPCs. **(e)** Summary of TFBS enrichment analysis for RFX3/4 co-bound sites at which binding is lost in RFX4 HOM D-NPCs. **(f)** Summary of GO enrichment analysis of RFX4 DEGs that are associated with RFX3/4 co-bound sites lost in RFX4 HOM D-NPCs. **(g)** Schematic of homozygous (HOM) RFX3 loss of function (LOF) hPSC model generation. **(h)** PCA plot highlighting variation in gene expression between WT (grey circles), RFX3 HOM (purple circles), and RFX4 HOM (green circles) D-NPCs. **(i)** Heatmap of DEGs dysregulated upon both RFX3 LOF or RFX4 LOF, with genes similarly downregulated or upregulated in both RFX3 and RFX4 HOM models highlighted (blue and red boxes, respectively). **(j)** Summary of GO enrichment analysis for DEGs downregulated upon RFX3 LOF. Data represents **(a)** mean +/- SEM, with fold change calculated relative to GAPDH.

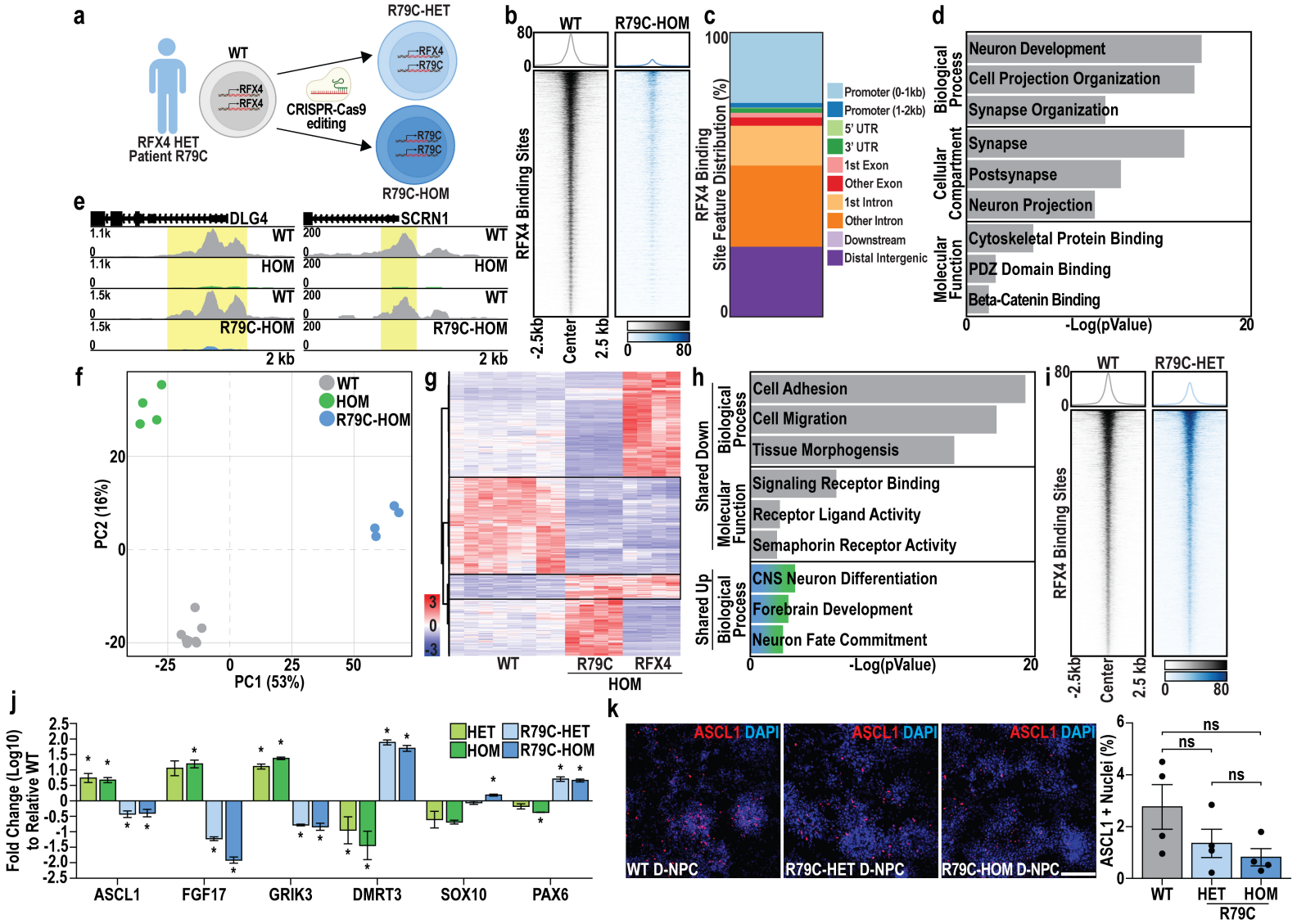

Fig. 5: Pathogenic RFX4 missense mutation disrupts genome-wide binding, but has distinct effects from LOF on transcriptional regulation.

(a) Variant knock-in of a heterozygous (R79C-HET) or homozygous (R79C-HOM) RFX4 missense mutation into WT hPSCs to model RFX4 pathogenic mutation. **(b)** Heatmap of RFX4 binding in WT and R79C-HOM D-NPCs. **(c)** Distribution of RFX4 binding sites relative to key genomic features. **(d)** Summary of GO enrichment analysis for genes associated with RFX4 binding sites lost in R79C-HOM D-NPCs. **(e)** Examples of RFX4 binding in browser track views, highlighting similar losses in RFX4 HOM and R79C-HOM D-NPCs at two genes. **(f)** PCA plots highlighting distinct alterations in gene expression between the WT control (grey circles), RFX4 HOM (green circles), and R79C-HOM (blue circles) D-NPCs. **(g)** Heatmap of DEGs dysregulated upon RFX4 LOF and pathogenic mutation, highlighting genes similarly dysregulated by both perturbations (black boxes). **(h)** Summary of GO enrichment analysis for DEGs downregulated (top, grey) or upregulated (bottom, blue/green) in both R79C-HOM and RFX4 HOM D-NPCs. **(i)** Heatmap of RFX4 bound sites associated with R79C-HET D-NPCs DEGs. **(j)** RT-qPCR for a selection of R79C-HOM and RFX4 HOM DEGs that showed opposing gene expression changes across the LOF and pathogenic mutant HET and HOM models. **(k)** Representative images and quantification of the ASCL1 immunopositivity in WT, R79C-HET and -HOM D-NPCs. Data are represented as **(j,k)** mean +/- SEM with fold changes calculated relative to RPL30. Data was analyzed by **(j)** Student's t-test (n=4) or **(k)** one-way ANOVA (n=4); ns = not significant and *p<0.05. **(k)** Scale bar = 50 µm.

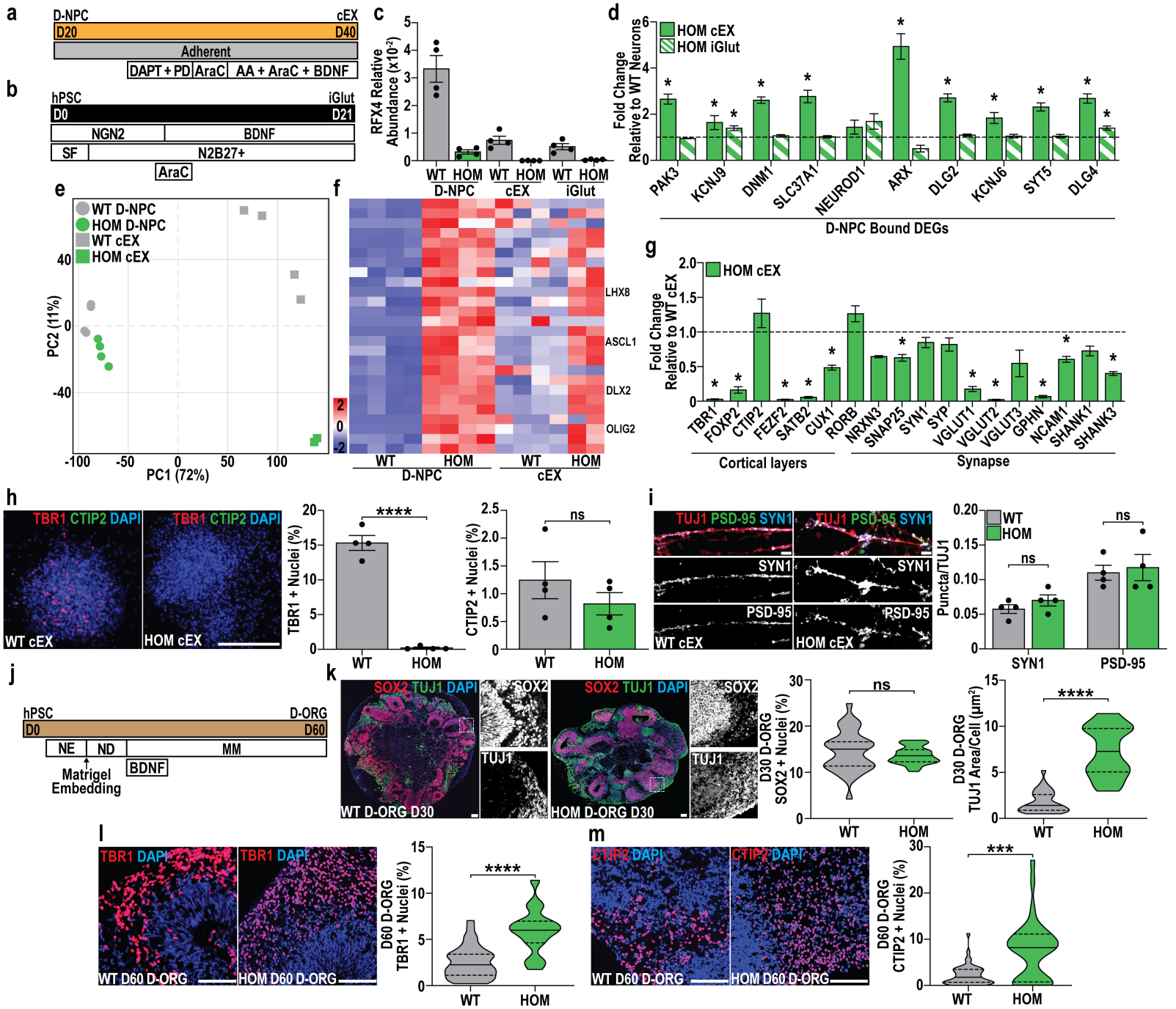

Fig. 6: RFX4 expression during neurogenesis is necessary for stratification of neuronal layers in cortical organoids. **(a-b)** Differentiation schematic for generating **(a)** cortical excitatory neurons (cEXs) and **(b)** glutamatergic-like neurons (iGluts). **(c)** RT-qPCR of RFX4 mRNA expression in WT and HOM D-NPCs, cEXs, and iGluts relative to RPL30 endogenous control, highlighting restriction of RFX4 expression to D-NPCs. **(d)** RT-qPCR for a selection of RFX4 D-NPC bound DEGs upregulated in RFX4 HOM D-NPCs, showing fold change in expression across RFX4 HOM cEXs and iGluts relative to expression levels in the corresponding WT cells. **(e)** PCA plot highlighting an increased divergence between the transcriptomic profiles of WT (grey) and HOM (green) cEXs (squares) versus D-NPCs (circles). **(f)** Heatmap of transcription factors upregulated upon RFX4 LOF in both D-NPCs and cEXs. **(g)** RT-qPCR for cortical layer and synaptic markers in HOM cEXs relative to WT cEXs. **(h-i)** Representative images and quantification of **(h)** TBR1 and CTIP2 immunopositivity and **(i)** PSD-95+ and SYN1+ puncta localized to TUJ1 immunopositive neurites in WT and HOM cEXs. **(j)** Schematic of the protocol used to generate dorsal cortical organoids (D-ORG). **(k-m)** Representative images and quantification of **(k)** SOX2 immunopositivity and the area of TUJ1 immunopositivity in D30 D-ORGs and **(l)** TBR1 and **(m)** CTIP2 immunopositivity in D60 D-ORGs. Data is represented as **(c,d,g-i)** mean +/- SEM, with fold changes calculated relative to RPL30, or **(k-m)** as data distributions with quartiles (dashed lines) and median (solid line) marked. Data was analyzed by **(d,g-i)** Student's t-test (n=4) or **(k-m)** Mann-Whitney test (n≥12); ns = not significant, *p<0.05, ***p<0.001, and ****p<0.0001. Scale bars = **(h,i)** 200 µm or **(k-m)** 100 µm.