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ARTICLE

Disruption of RFX family transcription factors causes autism, attention-deficit/hyperactivity disorder, intellectual disability, and dysregulated behavior

Holly K. Harris et al.[#]

PURPOSE: We describe a novel neurobehavioral phenotype of autism spectrum disorder (ASD), intellectual disability, and/or attention-deficit/hyperactivity disorder (ADHD) associated with de novo or inherited deleterious variants in members of the *RFX* family of genes. *RFX* genes are evolutionarily conserved transcription factors that act as master regulators of central nervous system development and ciliogenesis.

METHODS: We assembled a cohort of 38 individuals (from 33 unrelated families) with de novo variants in *RFX3*, *RFX4*, and *RFX7*. We describe their common clinical phenotypes and present bioinformatic analyses of expression patterns and downstream targets of these genes as they relate to other neurodevelopmental risk genes.

RESULTS: These individuals share neurobehavioral features including ASD, intellectual disability, and/or ADHD; other frequent features include hypersensitivity to sensory stimuli and sleep problems. *RFX3*, *RFX4*, and *RFX7* are strongly expressed in developing and adult human brain, and X-box binding motifs as well as *RFX* ChIP-seq peaks are enriched in the *cis*-regulatory regions of known ASD risk genes.

CONCLUSION: These results establish a likely role of deleterious variation in *RFX3*, *RFX4*, and *RFX7* in cases of monogenic intellectual disability, ADHD and ASD, and position these genes as potentially critical transcriptional regulators of neurobiological pathways associated with neurodevelopmental disease pathogenesis.

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INTRODUCTION

Autism spectrum disorder (ASD), marked by deficits in social communication and the presence of restricted interests and repetitive behavior, is highly heritable and genetically heterogeneous, with de novo loss-of-function variants as known contributors to ASD risk.¹ ASD is often comorbid with other neurodevelopmental diagnoses, including attention-deficit/hyperactivity disorder (ADHD). Emerging evidence also points to a role of de novo loss-of-function variants in ADHD.²

RFX3 is a member of the regulatory factor X (*RFX*) gene family that encodes transcription factors with a highly conserved DNA binding domain. *RFX3* is expressed in several tissues including developing and adult brain, and other *RFX* family members (*RFX1*, 4, 5, and 7) are also highly expressed in brain tissue, with expression patterns of *RFX1*, 3, 4, and 7 clustering tightly.³

We report a series of 38 individuals from 33 families with deleterious, mostly de novo variants in three brain-expressed members of the *RFX* family: *RFX3*, *RFX4*, or *RFX7*. *RFX3* was among 102 genes recently identified as statistically enriched for de novo variants in a large-scale analysis of trio exome data from individuals with ASD,⁴ but to date *RFX4* and *RFX7* have not been previously associated with human disease. Analysis of case clinical data reveals common features including intellectual disability (ID), ASD, and/or ADHD, delineating a novel neurobehavioral phenotype associated with *RFX* haploinsufficiency.

MATERIALS AND METHODS

Case ascertainment and data collection

We obtained phenotypic data from 15 unrelated individuals with loss-of-function variants in *RFX3*, 4 unrelated individuals with loss-of-function variants in *RFX4*, and 14 unrelated individuals with loss-of-function variants in *RFX7*. Individual case summaries for all individuals are provided (Supplemental Data). Variants arose de novo with the exception of four related individuals from the same nuclear family with the same heterozygous loss-of-function variant in *RFX3*, and three other related cases in *RFX4* (homozygous for an inherited missense variant). Pedigree information and contributed photographs are shown in Fig. 1. Diagnoses of ASD were reported in the medical record, but not uniformly evaluated by standardized measures such as the Diagnostic and Statistical Manual, Fourth or Fifth Edition (DSM-IV and 5), Autism Diagnostic Observation Schedule (ADOS), or Autism Diagnostic Interview, Revised (ADI-R). Similarly, ID and ADHD diagnoses were accepted per clinician report and not always accompanied by standardized cognitive or behavioral testing measures.

Exome sequencing

Individuals included underwent exome sequencing on a clinical or research basis. Seven of the individuals were sequenced through GeneDx using genomic DNA from the proband or proband plus parents, captured using either the Clinical Research Exome kit (Agilent Technologies, Santa Clara, CA) or the IDT xGen Exome Research Panel v1.0, and sequenced on an Illumina system with 100 bp or greater paired-end reads. Reads were aligned to human genome build GRCh37/UCSC hg19, and variants were analyzed and interpreted as previously described using variant classification criteria publicly available on the GeneDx ClinVar submission page (see Web Resources). Two cases of *RFX7* were sequenced through Ambry Genetics whose gene and variant classification process are available on the

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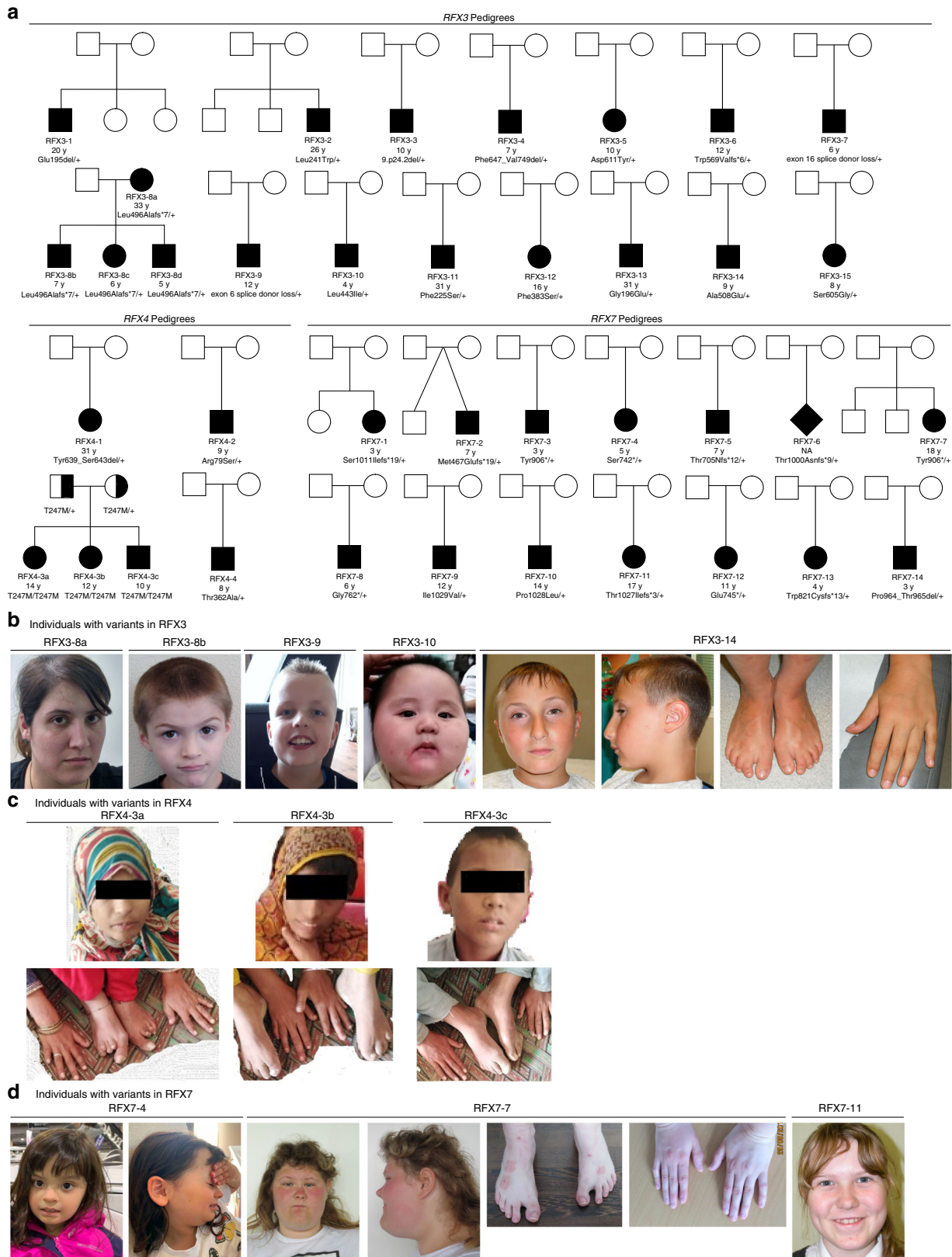


Fig. 1 Pedigrees of reported individuals with *RFX3*, *RFX4*, and *RFX7* variants. Pedigrees and clinical photographs of individuals with variants in *RFX3*, *RFX4*, and *RFX7*. (a) *RFX3*, *RFX4*, and *RFX7* case pedigrees. All pedigrees show de novo origin of variants except for *RFX3*-8a–d: a 33 year-old affected mother carrying the variant p.(Leu496Alafs*7) with transmission to three children, and pedigree *RFX4*-3a–c: three affected children homozygous for p.(Thr247Met). (b) Individuals with *RFX3* variants. (c) Individuals with *RFX4* variants. (d) Individuals with *RFX7* variants.

Ambry Genetics web page. The remainder of the individual's exome sequencing was performed through the clinicians' institutions or an external laboratory or research program (see Acknowledgments).

Variant analyses

Variant genomic coordinates are reported in relation to the Human December 2013 (GRCh38/hg38) Assembly. The reference messenger RNA (mRNA) and protein sequences used are *RFX3* NM_134428.2, NP_602304.1; *RFX4* NM_213594.2, NP_998759.1; and *RFX7* NM_022841.5, NP_073752.5. The variant databases gnomAD v2.1.1 and v3 were examined for the presence of each variant.⁵ Predictions of the functional effects for all variants were assessed using MutationTaster, SIFT, PolyPhen-2, PROVEAN, LRT, and MutationAssessor, and the total number of algorithms out of six with a deleterious prediction is referred to as the Nonsynonymous Damaging score (NsynD) as previously described.⁶

Cell transfection and culture

Human *RFX3* (NM_134428.2; Human *RFX3* complementary DNA [cDNA]) was cloned into V5-tagged mammalian expression vectors using the Gateway cloning system (Thermo Fisher Scientific). Point mutations were introduced with the QuikChange Lighting Site-Directed Mutagenesis kit (Agilent Technologies) to incorporate variants from affected individuals. To quantify the expression level of exogenous *RFX3*, equal amounts of tagged-*RFX3* expression vectors were transfected into HeLa cells using Lipofectamine 3000 (Thermo Fisher Scientific). The transfected cells were cultured for 48 hours before harvesting. Cell extracts were analyzed by immunoblotting, using antibodies raised against *RFX3* (HPA035689, Sigma-Aldrich), V5 (R960-25, Thermo Fisher Scientific), or beta actin (ab6276, Abcam). Blots were scanned on a Li-Cor Odyssey imager (Li-Cor). Signal intensities were quantified using Image Studio Lite (Li-Cor). Each immunoblot analysis was replicated six times. One-way analysis of variance (ANOVA) by repeated measures was employed. Multiple comparison correction was performed by using Dunnett statistical testing.

KEGG pathway and ASD gene set overrepresentation analysis

ChIP-seq and eCLIP-seq narrowPeak bed files for RFX family members, CREBBP, EP300, FMR1, FXR1, and FXR2 were obtained from the ENCODE portal,⁷ and additional ChIP-seq data for *RFX3_K562* were obtained from RegulomeDB⁸ (Table S6). Functional binding genes (1 kb upstream/downstream of transcriptional start site [TSS]) were annotated using ChIPseeker.⁹ ASD risk gene lists included 102 TADA genes from Satterstrom et al. and 253 ASD/ID genes from Coe et al. (Table S7).^{4,10} Differentially expressed genes (DEGs) in ASD brains were extracted from Velmeshev et al. excluding endothelial DEGs that could originate from vascular cells in the brain (Table S7).¹¹ The SYSCILIA Gold Standard (SCGSv.1) was used as a gold standard of known ciliary genes in human.¹² Customized Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed using clusterProfiler¹³ to determine the enrichment for KEGG pathways, ciliary genes, ASD risk gene sets, and ASD DEGs. Multiple testing correction was performed using Benjamini–Hochberg correction (Table S8). Annotations, statistical analyses, and plots were implemented in R.

Motif analysis

For motif occurrence analysis, FIMO was used to scan promoter sequences for individual occurrences of RFX motifs.¹⁴ For all analyses, motif models were obtained from the JASPAR 2020 database.¹⁵ Motifs searched for included *RFX3* (MA0798.1), *RFX4* (MA0799.1), and *RFX7* (MA1554.1). Promoter sequences were defined as -1,000 base pairs and +500 base pairs relative to the transcription start site. Motif occurrences were classified as significant based on a reporting threshold of p value <0.00001 and q value (Benjamini) <0.10. For motif enrichment analysis, we used the HOMER findMotifs.pl and findMotifsGenome.pl scripts. Motifs were classified as enriched based on fold-enrichment >1.5 over randomly selected background sequences with matched GC% content, and q value (Benjamini) <0.01. Enhancer sequences associated with genes of interest were obtained from Enhancer Atlas 2.0.¹⁶ ASD risk gene lists were obtained as noted previously to include 102 TADA genes and 124 ASD/ID genes reaching exome-wide significance.^{4,10}

RESULTS

Case series of individuals with de novo or inherited *RFX3* variants We identified and obtained clinical information from 18 individuals bearing loss-of-function variants in *RFX3* via GeneMatcher.¹⁷ Genotypic information is provided in Table 1, clinical phenotypes are summarized in Table 2 and S1, and predicted variant impacts are summarized in Supplemental Table S2. A total of 15 distinct variants were identified: 2 frameshift variants, 2 canonical splice donor variants, 8 missense variants, 1 in-frame deletion, 1 42-kb deletion removing the last two exons of *RFX3*, and 1 227-kb deletion involving only *RFX3*. In one family, an affected parent transmitted a frameshift variant to three affected children; all other variants were de novo and novel (Fig. 1a).

There were 13 males and 5 females, with no sex-based differences in severity of phenotype. All individuals had neurodevelopmental delays, with formally recorded clinical diagnoses of ASD (72%) and ID of varying severity (borderline to moderate) or global developmental delay in young children (78%) and ADHD (56%) (Table 2 and Table S1). Many showed a distinct behavioral pattern marked by easy excitability/overstimulation, hypersensitivity to sensory (particularly auditory) stimuli, anxiety, emotional dysregulation and/or aggression (13/15 [87%] with specific behavioral information provided). Three individuals were reported to have seizures (17%). Some individuals had sleep difficulties (44%) including limited total duration of sleep, frequent awakenings, or early morning awakenings. Subtle nonspecific and nonrecurrent dysmorphisms were commonly reported (61%), including broad nasal bridge, high, arched palate, and hand and foot abnormalities (tapered fingers, widely spaced toes), but no consistent recognizable features were shared by all individuals (Fig. 1b). Both macrocephaly (six individuals) and microcephaly (two individuals) were reported (8/11 individuals [73%] with a head circumference measurement or percentile provided). Magnetic resonance imaging (MRI) of the brain was available for eight individuals, with reports of nonspecific findings in four, including white matter changes, uncus asymmetry, partially empty sella, or prominent ventricles. One individual had mild thinning of the corpus callosum (Table 2, individual RFX3-10). Five of seven individuals (71%) who were past the onset of puberty (ages 12–30 years) had reports of behavioral and/or cognitive worsening at the time of puberty/adolescence. Three had increased aggression specifically noted. Three were described as having psychotic s/or psychotic symptoms, two were described as having hallucinations (one requiring psychiatric hospitalization) and another was described as having conversations with imaginary friends. Three were reported to have had decline in cognition, one in adolescence and another around 28 years of age.

Variants in additional RFX family genes are associated with similar neurodevelopmental phenotypes

Additional individuals were ascertained who harbored loss-of-function variants in other closely related genes of the *RFX* family. Fourteen individuals bearing de novo loss-of-function variants in *RFX7* were identified (Tables 1 and 2), including four frameshift variants, five stop-gain variants, one in-frame deletion, and two missense variants (Table 1, Table S2). Slightly more males were identified than females (eight males, six females) without differences in phenotype based on sex. All individuals had language delay, and most had ID/global developmental delay (93%) (Table 2, Supplemental Table 1). While formal diagnoses of ASD (36%) and/or ADHD (29%) were less consistent, autistic features and/or significant behavioral challenges akin to those seen in *RFX3* individuals were reported in the majority of cases, including excitability/overstimulation, sensitivity to sensory (particularly auditory) stimuli, a high pain threshold, emotional dysregulation, aggression, and anxiety (8/8, 100% of those with specific behavioral information provided). Abnormal head size

Table 1. Molecular findings in individuals with ASD, ADHD, and/or ID and variants in *RFX3*, *RFX4*, or *RFX7*.

Gene	Individual	Inheritance	gDNA (GRCh38)	cDNA	Protein	Category	Domain
<i>RFX3</i>	RFX3-1	<i>De novo</i>	chr9:g.3293222_3293224del	c.584_586del	p.(Glu195del)	Inframe deletion	DBD
	RFX3-2	<i>De novo</i>	chr9:g.3293086A>C	c.722T>G	p.(Leu241Trp)	Missense	DBD
	RFX3-3	<i>De novo</i>	chr9:g.9p24.2del	NA	(gene deletion)	Deletion	(all)
	RFX3-4	<i>De novo</i>	chr9:g.3197716_3239762del	c.1968+8270_*27326del	(exon 17 and exon 18 deleted) p.Phe647_Val749del	Deletion	DD
	RFX3-5	<i>De novo</i>	chr9:g.3248169C>A	c.1831G>T	p.(Asp611Tyr)	Missense	DD
	RFX3-6	<i>De novo</i>	chr9:g.3257101dupG	c.1704dup	p.(Trp569Valfs*6)	Frameshift	DD
	RFX3-7	<i>De novo</i>	chr9:g.3248031C>T	c.1968+1G>A	exon 16 splice donor loss	Splicing	DD
	RFX3-8a-d	Inherited	chr9:g.3263053_3263054del	c.1486_1487del	p.(Leu496Alafs*7)	Frameshift	DD
	RFX3-9	<i>De novo</i>	chr9:g.3301541C>T	c.549+5G>A	exon 6 splice donor loss	Splicing	DBD
	RFX3-10	<i>De novo</i>	chr9:g.3270401G>T	c.1327C>A	p.(Leu443Ile)	Missense	EDD
	RFX3-11	<i>De novo</i>	chr9:g.3293134A>G	c.674T>C	p.(Phe225Ser)	Missense	DBD
	RFX3-12	<i>De novo</i>	chr9:g.3271057A>G	c.1148T>C	p.(Phe383Ser)	Missense	EDD
	RFX3-13	<i>De novo</i>	chr9:g.3293221C>T	c.587G>A	p.(Gly196Glu)	Missense	DBD
	RFX3-14	<i>De novo</i>	chr9:g.3263017G>T	c.1523C>A	p.(Ala508Glu)	Missense	DD
	RFX3-15	<i>De novo</i>	chr9:g.3256992T>C	c.1813A>G	p.(Ser605Gly)	Missense	DD
<i>RFX4</i>	RFX4-1	<i>De novo</i>	chr12:g.106750773_106750787del	c.1915_1929del	p.(Tyr639_Ser643del)	Inframe deletion	NA
	RFX4-2	<i>De novo</i>	chr12:g.106654271C>A	c.235C>A	p.(Arg79Ser)	Missense	DBD
	RFX4-3a-c	Recessive (homozygous)	chr12:g.106696353C>T	c.740C>T	p.(Thr247Met)	Missense	NA
	RFX4-4	<i>De novo</i>	chr12:g.106715490A>G	c.1084A>G	p.(Thr362Ala)	Missense	DD
<i>RFX7</i>	RFX7-1	<i>De novo</i>	chr15:g.56094696del	c.3032del	p.(Ser1011Ilefs*19)	Frameshift	NA
	RFX7-2	Unknown (adopted)	chr15:g.56096328_56096329del	c.1399_1400del	p.(Met467Glufs*19)	Frameshift	NA
	RFX7-3	<i>De novo</i>	chr15:g.56095010G>T	c.2718C>A	p.(Tyr906*)	Stop Gain	NA
	RFX7-4	<i>De novo</i>	chr15:g.56095503G>C	c.2225C>G	p.(Ser742*)	Stop Gain	NA
	RFX7-5	<i>De novo</i>	chr15:g.56095615dupT	c.2113dup	p.(Thr705Asnfs*12)	Frameshift	NA
	RFX7-6	<i>De novo</i>	chr15:g.56094730dupT	c.2998dup	p.(Thr1000Asnfs*9)	Frameshift	NA
	RFX7-7	<i>De novo</i>	chr15:g.56095010G>C	c.2718C>G	p.(Tyr906*)	Stop Gain	NA
	RFX7-8	<i>De novo</i>	chr15:g.56095444C>A	c.2284G>T	p.(Gly762*)	Stop Gain	NA
	RFX7-9	<i>De novo</i>	chr15:g.56094643T>C	c.3085A>G	p.(Ile1029Val)	Missense	NA
	RFX7-10	<i>De novo</i>	chr15:g.56094645G>A	c.3083C>T	p.(Pro1028Leu)	Missense	NA
	RFX7-11	<i>De novo</i>	chr15:g.56094648del	c.3080del	p.(Thr1027Ilefs*3)	Frameshift	NA
	RFX7-12	<i>De novo</i>	chr15:g.56095495C>A	c.2233G>T	p.(Glu745*)	Stop Gain	NA
	RFX7-13	<i>De novo</i>	chr15:g.56095266_56095269dup	c.2459_2462dup	p.(Trp821Cysfs*13)	Frameshift	NA
	RFX7-14	<i>De novo</i>	chr15:g.56094864_56094869del	c.2859_2864del	p.(Pro964_Thr965del)	Inframe deletion	NA

ADHD attention-deficit/hyperactivity disorder, ASD autism spectrum disorder, cDNA complementary DNA, DBD DNA binding domain, DD dimerization domain, gDNA genomic DNA, ID intellectual disability.

Molecular characterization of *RFX3*, *RFX4*, and *RFX7* variants reported in this study. Chromosome structure is described according to the Human December 2013 (GRCh38/hg38) Assembly. RefSeq identifiers: *RFX3* NM_134428.2, NP_602304.1; *RFX4* NM_213594.2, NP_998759.1; *RFX7* NM_022841.5, NP_073752.5. Protein domains were obtained from Sugiama-Trapman et al.³ Italics indicates individual has a variant of uncertain significance.

(five individuals with microcephaly and three with macrocephaly) was noted in 7/11 (64%) that provided head circumference measurements. In 5/11 patients (45%) who had neuroimaging, MRI abnormalities were observed (Dandy–Walker malformation, cerebellar tonsillar herniation, an abnormality of the basal ganglia, and a fourth case with limited information but an “abnormal brain MRI” noted). Subtle clinical dysmorphisms were reported in 86% including abnormalities of the hands and feet such as widely spaced toes, syndactyly, or long tapered fingers (50%) (Table 2).

Again, no consistent dysmorphisms were evident across individuals (Fig. 1d).

Six individuals with probable loss-of-function *RFX4* variants were also identified (Tables 1 and 2). Three were individuals who harbored de novo *RFX4* variants, including an in-frame deletion (*RFX4* p.[Tyr639_Ser643del]), and two predicted damaging missense variants (*RFX4* p.[Arg79Ser] and p.[Thr362Ala]) (Table 1, Supplemental Table S2). We also report a pedigree in which three additional related individuals (siblings) were homozygous for a

Table 2. Clinical features of individuals with pathogenic variants in RFX3, RFX4, or RFX7.

Individual	Age	Sex	Presentation	Variant	Language delay	Motor delay	ASD*	ID	ADHD	Behavioral Profile	Sleep issues	Seizures	Hypotonia; Other Neurologic Findings	Dysmorphism	Micro or macrocephaly	Neuroimaging Findings	Other Medical or Neuropsychiatric Features	
RFX3																		
RFX3-1	20 years	M	ASD, ID, ADHD	Inframe deletion	Yes	Yes	Yes	Yes	Yes	Mood swings, anxiety, aggression, sensory hypersensitivity, rocking	Yes	No	Yes	Yes	No	NA	Behavioral decline in adolescence	
RFX3-2	26 years	M	ASD, ID, ADHD	Missense	Yes	Yes	Yes	Yes	Yes	Sensory seeking behavior, aggression, biting, pica, self-injury, sensory hypersensitivity	Yes	No	Yes	Yes	No	Normal/nonspecific findings	Hypogonadism, strabismus, bipolar, behavioral and mild cognitive decline in adolescence	
RFX3-3	10 years	M	ASD, ADHD	Deletion	No	Yes	Yes	No	Yes	Sensory hypersensitivity	Yes	Yes	NA	Yes	Macrocephaly	NA	Strabismus	
RFX3-4	7 years	M	ADHD, anxiety	Deletion	Yes	Yes	Yes	Yes	Yes	NA	NA	No	NA	Yes	mild Macrocephaly	Normal/nonspecific findings	Myopia	
RFX3-5	10 years	F	GDD	Missense	No	Yes	Yes	No	yes	Oppositional behavior, aggression	NA	No	NA	Yes	Mild macrocephaly	NA	NA	
RFX3-6	12 years	M	ASD, GDD, ADHD	Frameshift	No	No	Yes	No	Yes	Anxiety, aggression, emotional dysregulation	NA	No	Yes	No	Macrocephaly	PVL	Anxiety, mild cognitive and behavioral decline at puberty	
RFX3-7	6 years	M	ASD, ADHD	Splicing	Yes	No	No	Yes	NA	Aggression, sensory-seeking behavior, elopement, and impulsivity	Yes	No	Yes	No	No	Partially empty sella	Myopia	
RFX3-8a	33 years	F	ASD, ID	Frameshift	Yes	NA	Yes	Yes	NA	NA	NA	NA	NA	Yes	NA	NA	NA	
RFX3-8b	7 years	M	ASD, ID, ADHD	Frameshift	Yes	NA	Yes	Yes	Yes	Aggression, biting	NA	No	NA	No	NA	NA	NA	
RFX3-8c	6 years	F	GDD	Frameshift	Yes	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
RFX3-8d	5 years	M	GDD	Frameshift	Yes	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	
RFX3-9	12 years	M	ASD, ID	Splicing	Yes	NA	Yes	Yes	No	Sensory hypersensitivity	Yes	No	Yes	Yes	Mild macrocephaly	Normal/nonspecific findings	NA	
RFX3-10	4 years	M	GDD	Missense	NA	Yes	NA	NA	NA	NA	NA	Yes	Yes	Yes	microcephaly	Thin corpus callosum	NA	
RFX3-11	31 years	M	ASD	Missense	NA	NA	Yes	NA	Yes	NA	Yes	NA	NA	NA	NA	NA	Crohn's disease, anxiety, depression	
RFX3-12	16 years	F	ID, ASD	Missense	Yes	No	Yes	Yes	Yes	Impulsivity, mood swings	No	No	NA	Yes	Macrocephaly	Normal	Hallucinations, mania, behavioral decline	
RFX3-13	31 years	M	ASD	Missense	No	No	Yes	No	No	Aggression, elopement	Yes	Yes	No	No	NA	Uncal asymmetry	Cognitive and behavioral decline, hallucinations	
RFX3-14	9 years	M	GDD	Missense	Yes	Yes	No	Yes	No	no	No	No	No	Yes	NA	NA	Bilateral conductive hearing loss	
RFX3-15	8 years	F	ASD, ID, ADHD	Missense	Yes	Yes	Yes	Yes	Yes	Aggression, anxiety, impulsivity	Yes	No	No	Yes	Mild macrocephaly	NA	Asthma	

Table 2 continued

Individual	Age	Sex	Presentation	Variant	Language delay	Motor delay	ASD*	ID	ADHD	Behavioral Profile	Sleep issues	Seizures	Hypotonia; Other Neurologic Findings	Dysmorphism	Micro or macrocephaly	Neuroimaging Findings	Other Medical or Neuropsychiatric Features	
RFX4																		
RFX4-1	31 years	F	ASD, ID, epilepsy	Inframe deletion	NA	NA	Yes	Yes	NA	Hand flapping, hand wringing, inappropriate laughter	NA	Yes, generalized intractable	NA	NA	NA	Asymmetric volume loss	NA	
RFX4-2	9 years	M	ID, ASD, epilepsy	Missense	NA	NA	NA	NA	NA	NA	NA	Yes	NA	NA	NA	NA	NA	
RFX4-3a	14 years	F	ID, ASD, behavior problems	Missense	Yes	Yes	Yes	NA	NA	Yes, behavior challenges	NA	No	NA	No	No	NA	Skeletal abnormalities	
RFX4-3b	12 years	F	ID, ASD, behavior problems	Missense	Yes	Yes	Yes	NA	NA	Yes, behavior challenges	NA	No	NA	No	No	NA	Skeletal abnormalities	
RFX4-3c	10 years	M	ID, ASD, behavior problems	Missense	Yes	Yes	Yes	NA	NA	Yes, behavior challenges	NA	No	NA	No	No	NA	None	
RFX4-4	8 years	M	GDD, ASD, behavior problems	Missense	Yes	Yes	Yes	NA	NA	Sensory hypersensitivity, impulsivity, anxiety, mood swings	NA	No	NA	Cleft lip and palate	Microcephaly	Absent pituitary	Hypopituitarism	
RFX7																		
RFX7-1	3 years	F	ID	Frameshift	Yes	Yes	No	Yes	No	NA	No	No	No	Yes	Microcephaly	Normal	NA	
RFX7-2	7 years	M	ID, ASD	Frameshift	Yes	Yes	Yes	No	No	Sensory seeking behavior, sensory hypersensitivity, attention seeking behavior	No	NA	No	Yes	Macrocephaly	NA	Underdeveloped scrotum	
RFX7-3	3 years	M	GDD, ASD	Stop-gain	Yes	No	Yes	Yes	Yes	Low frustration tolerance, hair pulling, high pain threshold	NA	No	NA	Yes	NA	NA	Eczema	
RFX7-4	5 years	F	GDD, ASD	Stop-gain	Yes	Yes	Yes	No	No	Sensory hypersensitivity and sensory seeking, excitable, high pain threshold	No	No	Yes	Yes	No	Normal	Hydronephrosis, constipation	
RFX7-5	7 years	M	ASD, GDD	Frameshift	Yes	Yes	Yes	No	No	Excitable, Laughs Easily, Sensory Hypersensitivity, aggressive when younger	Yes	No	Yes	No	Macrocephaly	Normal	None	
RFX7-6	NA	M	ID, ASD	Frameshift	Yes	Yes	Yes	Yes	Yes	mood swings, anxiety, aggression, self-injury	Yes	No	No	Yes	NA	Cerebellar tonsillar herniation, abnormal 4th ventricle	Polyphagia	
RFX7-7	18 years	F	ID, ADHD	Stop-gain	Yes	Yes	No	Yes	Yes	Sensory hypersensitivity, aggression, anxiety, hair pulling, skin picking, nail biting	No	No	No	Yes	Microcephaly	Normal (CT)	Obesity, mild scoliosis	
RFX7-8	6 years	M	ID	Stop-gain	Yes	Yes	No	NA	NA	Sensory hypersensitivity	No	No	Yes	Yes	Macrocephaly	NA	Neuroblastoma	
RFX7-9	12 years	M	GDD	Missense	Yes	Yes	No	Yes	Yes	NA	No	Febrile seizure x 1	Hyperreflexia	no	Microcephaly	Normal	Hypocalcemia	

Table 2 continued

Individual	Age	Sex	Presentation	Variant	Language delay	Motor delay	ASD*	ID	ADHD	Behavioral Profile	Sleep Issues	Seizures	Hypotonia; Other Neurologic Findings	Dysmorphism	Micro or macrocephaly	Neuroimaging Findings	Other Medical or Neuropsychiatric Features
RFX7-10	14 years	M	ID	Missense	Yes	Yes	No	Yes	No	No	Yes	Epilepsy with myoclonic seizures	Dystonic movements, mixed hypo/hypertonia	YES	Microcephaly	Abnormality of the basal ganglia, delayed CNS myelination	Optic nerve hypoplasia, cataract, mild hearing loss, laryngomalacia, recurrent bronchitis/pneumonia, cryptorchidism
RFX7-11	17 years	F	ID	Frameshift	Yes	No	No	Yes	No	Short attention span, excessive fears/phobias, fixated interests, sensory hypersensitivity, poor social interactions	No	Yes; absence during day and grand mal seizures in sleep but none since 10 years	No	Long fingers	No	Normal	None
RFX7-12	11 years	F	GDD	Stop-gain	Yes	Yes	NA	NA	NA	Potential anxiety	NA	No	Yes	Yes	NA	Abnormal myelination but no structural abnormality	Loose anagen hair syndrome
RFX7-13	4 years	F	ID	Frameshift	Yes	Yes	No	Yes	No	Stereotypies	Yes	No	No	Yes	No	Subcortical hypersignal in the left temporal pole (cortical dysplasia or developmental venous anomaly)	Ventricular septal defect, mild unilateral hearing loss
RFX7-14	3 years	M	Abnormal brain MRI	Inframe deletion	Yes	NA	NA	NA	NA	NA	NA	NA	NA	Yes	No	Cerebellar vermis hypoplasia with marked apraxia, cystic enlargement of 4th ventricle	Asymptomatic atrial septal and ventricular septal defects

ADHD attention-deficit hyperactivity disorder, *ASD* autism spectrum disorder, *CNS* central nervous system, *CT* computerized tomography, *GDD* global developmental delay, *ID* intellectual disability, *MRI* magnetic resonance imaging, *NA* not available, *PVL* periventricular leukomalacia.
 Regarding *ASD*: we recognize the heterogeneity in *ASD* diagnoses. For our table, individuals were considered to have *ASD* if documented in the clinical note as having *ASD* diagnosed via formal measure, according to clinical expertise, or documented as having clear “autistic features,” a designation we considered equivalent to a diagnosis of *ASD* for purposes of this report. Granular description of social communication or restrictive and repetitive behavior data to determine *DSM-5* diagnosis was not uniformly available. Italics indicates individual has a variant of uncertain significance.

missense variant in *RFX4* (p.Thr247Met) altering a well-conserved threonine residue. Parents of these siblings were first cousins, each heterozygous for the variant and without any known neurobehavioral phenotype, and a heterozygous sibling was similarly reported as neurotypical; this pedigree therefore raises the possibility that the *RFX4* phenotype may be associated with both monoallelic and biallelic inheritance as has been described for several other genetic conditions.¹⁸ Of these six individuals, three were female and three were male. All were noted to have ID or global developmental delay (100%) and most had documented ASD (83%). Four individuals were normocephalic, and one was microcephalic. Neuroimaging was performed in two and demonstrated asymmetric volume loss in one individual and absent pituitary gland in another individual with hypopituitarism. The latter individual also presented with cleft lip and palate. Seizures were described in two individuals (33%). No consistent dysmorphisms were evident (Fig. 1c).

RFX3, *RFX4*, and *RFX7* variant analyses

In total, 33 distinct variants in *RFX* family members (15 *RFX3*, 4 *RFX4*, and 14 *RFX7*) were identified (Table 1). Excluding related individuals, each case involved a novel variant (e.g., there were no recurrent variants). *RFX3*, *RFX4*, and *RFX7* each exhibit intolerance to loss-of-function variation in human population databases (gnomAD, pLI scores = 1.00). All variants were absent from gnomAD except for *RFX7* p.Pro964_Thr965del, which is detected at a very low frequency in gnomAD v2.1.1 (AF 0.00007677) leading us to formally classify it as a variant of uncertain significance (VUS) (see Supplemental *RFX7* Case Descriptions Individual 14 for further details). The fact that the majority of variants identified are predicted to cause outright protein truncation or gene deletion (20/33) strongly supports a loss-of-function/haploinsufficiency model. Of the 13 missense variants, 11 were predicted to be damaging by at least four of six algorithms (NsynD score ≥ 4) and two missense variants were predicted to be damaging by at least two algorithms (*RFX4* p.[Thr247Met] and p.[Thr362 Ala]) (Table S2). All missense variants affect highly conserved amino acids (PhastCons vertebrate, mammalian, and primate scores ranging from 0.99 to 1.00) (Table S2).

RFX transcription factors are defined by a conserved, specialized winged-helix type DNA binding domain (DBD) that recognizes the X-box motif. In addition to the DBD, *RFX3* and *RFX4* have three known domains that are associated with dimerization (DD).³ *RFX4* and *RFX7* variants did not exhibit clustering to specific functional domains, but all of the nontruncating (missense or in-frame deletion) variants identified in *RFX3* were found to be located in the DBD or one of the dimerization domains (Fig. 2a, Table 1). We engineered five of the nontruncating variants—p.(Glu195del), p.(Leu241Trp), p.(Phe383Ser), p.(Leu443Ile), and p.(Asp611Tyr)—into a V5-*RFX3* heterologous expression vector for protein stability analyses in HeLa cells (Figure S1). The majority of these variants resulted in significant decreases in detectable *RFX3* levels, consistent with a destabilizing impact on protein expression. Two missense variants, p.(Leu241Trp) and p.(Leu443Ile) (residing in the DNA binding domain or the second extended protein dimerization domain, respectively), did not appear to impact protein stability, raising the possibility that they might disrupt more specific functional interactions of *RFX3* to be investigated more thoroughly in the future.

We examined 35 additional reported variants in *RFX3*, *RFX4*, and *RFX7* from prior studies of de novo or inherited variants in ASD and neuropsychiatric conditions (Table S3, Figure S2).^{4,19–22} Missense variants from the literature tended to be of milder predicted deleteriousness than those reported here (Fig. 2b). Sixteen were in *RFX3*, including five de novo variants (four protein truncating and one missense variant predicted damaging by all six algorithms, NsynD6, supportive of likely deleteriousness), seven

inherited variants (four copy-number variants [CNVs], one frameshift variant (p.[Pro408fs]), and three missense variants (p.[Thr151Ala], p.[Ala101Thr], p.[Arg615His]; NsynD scores 3–6), and four CNVs (all microdeletions) were reported for which parental inheritance was not established (Table S3). Among previously reported *RFX7* variants, one was a de novo frameshift and one was an inherited frameshift variant. There were also six reported inherited missense variants (6/6 with NsynD > 4), and two de novo missense variants that are likely benign. Finally, there were nine previously reported *RFX4* variants, only one of which was de novo (a missense variant lacking strong evidence of pathogenicity), and eight inherited missense variants of varying predicted deleteriousness (NsynD scores 3–6).

RFX expression is enriched in human brain

RFX3, *RFX4*, and *RFX7* have been reported to have relatively high expression in human fetal cortex.²³ To determine whether specific cell types are affected by *RFX* haploinsufficiency, we examined single-cell transcriptomes from developing and adult human cortex (Fig. 3a–f, Figure S3A, B).^{11,24} In developing human cortex, *RFX3* and *RFX7* exhibited the strongest brain expression, with *RFX3* most highly expressed in maturing excitatory upper enriched neurons, *RFX4* most highly expressed in outer radial glia, and *RFX7* most highly expressed in interneurons from the medial ganglionic eminence (Fig. 3a–c). We also examined *RFX* expression patterns in the adult human cortex (Fig. 3d–f, Figure S3A, B).^{11,25} Again, *RFX3* and *RFX7* exhibited the highest expression. *RFX3* was most highly expressed in glutamatergic layer 2/3 neurons, followed by astrocytes. *RFX7* was expressed in both inhibitory and excitatory neurons. *RFX4* expression was much lower overall, but highest in astrocytes (Fig. 3f). These expression profiles suggest that *RFX* deleterious variants may lead to our observed neurodevelopmental phenotypes by altering early developmental cell fates or by impacting the function of upper-layer cortical neurons, astrocytes, and interneurons.

RFX binding motifs are present in ASD risk gene *cis*-regulatory regions

Dysregulated gene expression, especially in upper-layer cortical neurons, has been implicated in ASD pathogenesis.^{11,26} Given the expression of *RFX3* in layer 2/3 neurons and the autistic features of individuals reported here, we considered whether *RFX* family genes might be important transcriptional regulators of ASD risk genes. *RFX* family transcription factors bind to a characteristic consensus motif called an X-box (GTHNYY AT RRNAAC)²⁷ with individual family members having additional specificity for particular subsequences within this consensus. We therefore performed *RFX3*, 4, and 7 motif enrichment analysis in upstream regulatory sequences of 187 ASD risk genes (the union of 102 TADA genes from Satterstrom et al. and 124 genes meeting exome-wide significance from Coe et al.)^{4,10} and an additional set of 447 genes identified to be upregulated in ASD brains.¹¹ We found enrichment of X-box motifs (q value < 0.05) in human ESC-neuron specific enhancers for ASD risk genes (Table S5A). As a group, *RFX3* and *RFX4* motifs were particularly enriched (q value < 0.005), while the *RFX7* motif was not (q value 0.48). X-box, *RFX3* and *RFX4* motifs were similarly enriched in the enhancer regions of genes upregulated in ASD brains (Table S5B).¹¹ Enrichment of *RFX* motifs in promoter regions of ASD risk genes and DEGs did not emerge (data not shown). Last, we analyzed available *RFX* ChIP-seq data from the ENCODE project (Table S6) to determine enrichment for KEGG pathways, ciliary genes, ASD risk gene sets, and ASD DEGs (Table S7). *RFX* functional binding genes from most ENCODE cell lines were significantly enriched in ASD risk genes and DEGs after multiple testing correction (p.adjust < 0.05 ; Benjamini–Hochberg’s correction; Fig. 3g, Figure S5, Table S8). Across cell lines, there was a positive correlation between

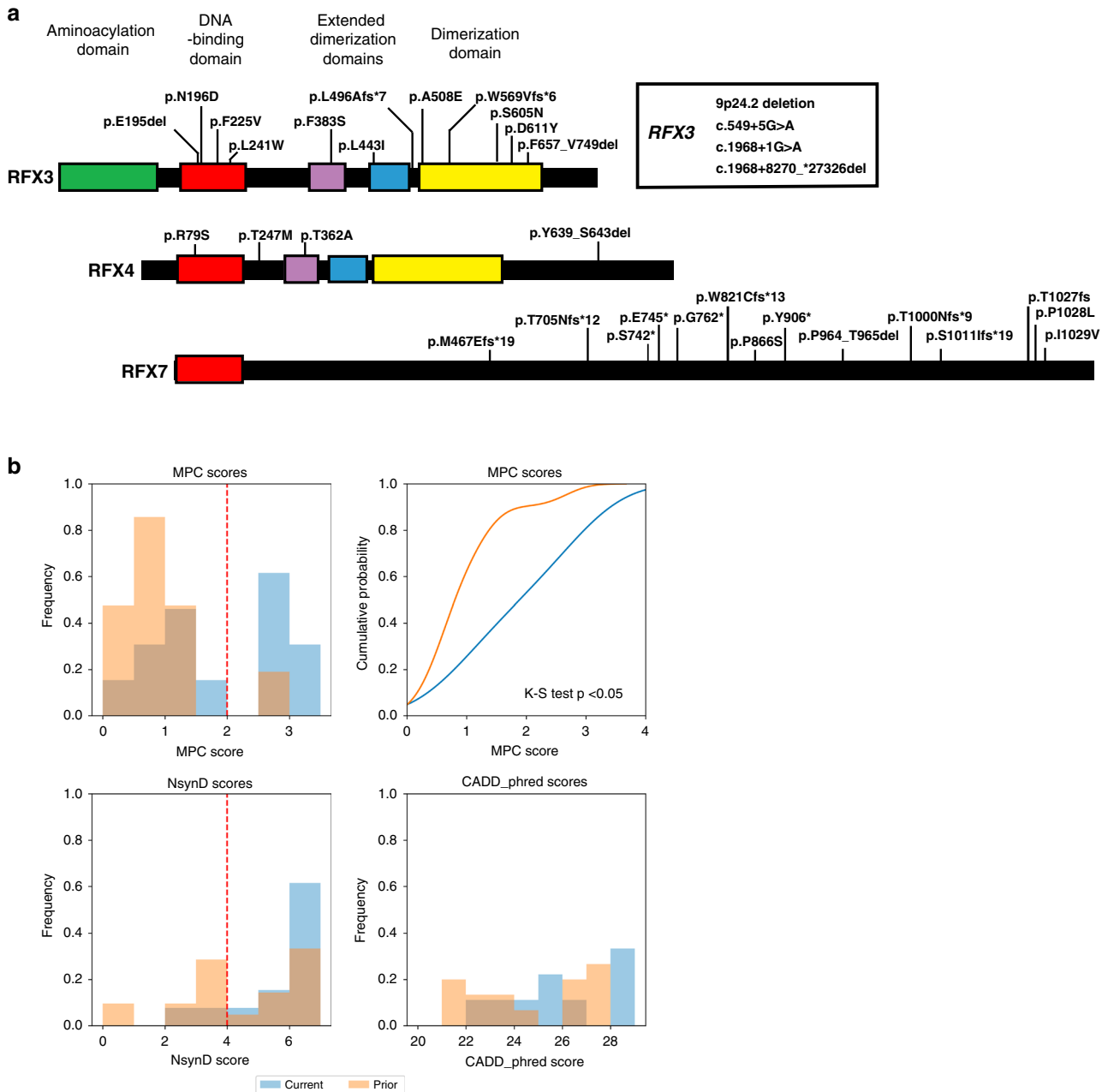


Fig. 2 Distribution and predicted deleteriousness of *RFX* variants. (a) Mapping of selected *RFX* variants to domains. Whole-gene deletion and intronic variants are not illustrated. *RFX3* (NP_602304.1), *RFX4* (NP_998759.1), *RFX7* (NP_073752.5). (b) Missense variant deleteriousness scores for the currently reported variants (current) and prior reported variants (prior) in *RFX3*, 4, and 7. The distribution of MPC scores for missense variants reported in this study is significantly different from that of prior reported missense variants, Kolmogorov–Smirnov (K-S) test p value <0.05 (p value = 0.015). CADD Combined Annotation Dependent Depletion, MPC Missense badness, PolyPhen-2, and Constraint, NsynD Nonsynonymous Damaging score.

enrichment in ASD genes and *RFX* expression levels in that cell type (Figure S6, Table S9), indicating that higher *RFX* expression levels may be required to engage ASD relevant targets.

Finally, single-gene analyses showed enrichment of *RFX3* and *RFX4* motifs in the promoters of five ASD-associated genes (FIMO p value <0.0001 , q value <0.1): *AP2S1*, *KDM6B*, *ANK2*, *NONO*, and *MYT1L* (Figure S4B, D),¹⁴ and *RFX3* ENCODE ChIP-seq data from HepG2 cells confirmed *RFX3* binding peaks in the promoters of *AP2S1*, *KDM6B*, and *NONO* (Figure S4E–G). Notably, de novo loss-of-function variants in *KDM6B* (MIM 611577) cause a neurodevelopmental syndrome that has phenotypic overlap with *RFX3* haploinsufficiency as described in this report, namely mild global

delays, delayed speech, hypotonia, and features of ASD and ADHD, while loss-of-function variants in *NONO* (MIM 300084) and *MYT1L* (MIM 613084) are a cause of X-linked and autosomal dominant ID, respectively (Table S4). These cases support the model that *RFX* members may be transcriptional activators of a subset of ASD risk genes via actions at both enhancer and promoter sites.

DISCUSSION

Our results delineate a novel human neurobehavioral phenotype including ASD, ID, and/or ADHD due to deleterious variants in *RFX*

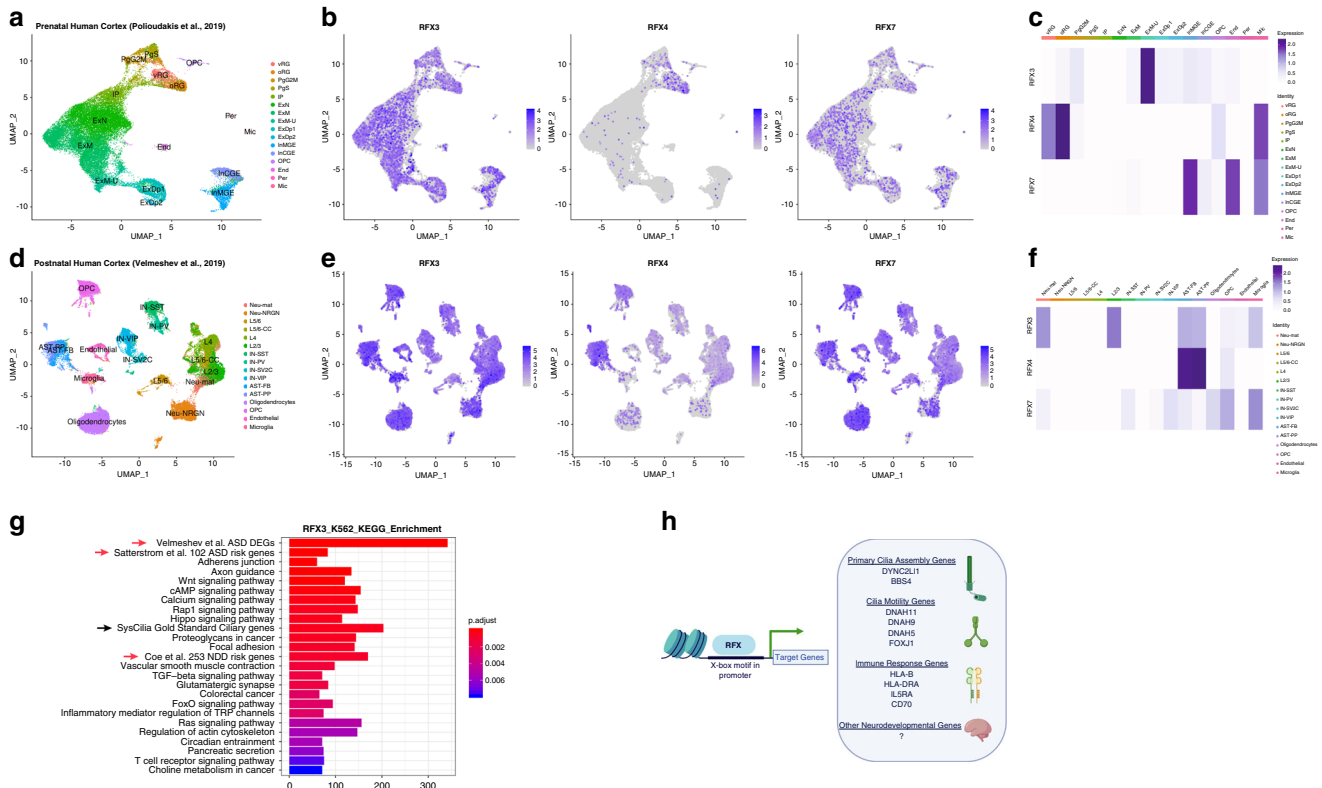


Fig. 3 *RFX3*, *RFX4*, and *RFX7* expression patterns in human cortex and haploinsufficiency gene dosage model. (a) Transcriptomic cell types in the prenatal human cortex identified by single-cell RNA-sequencing. (b) *RFX3*, 4, and 7 expression patterns in single cells of the prenatal human cortex. (c) Heatmap of *RFX3*, 4, and 7 expression levels among cell types in the prenatal human cortex. (d) Transcriptomic cell types in the postnatal human cortex identified by single-cell RNA-sequencing. (e) *RFX3*, 4, and 7 expression patterns in single cells of the postnatal human cortex. (f) Heatmap of *RFX3*, 4, and 7 expression levels among cell types in the postnatal human cortex. (g) The enrichment of KEGG pathways, ciliary genes, ASD risk gene sets, and ASD differentially expressed genes (DEGs) among *RFX3* ChIP-seq binding targets. Pathways and ASD gene sets are ranked by their statistical significance (p.adjust values, Benjamini–Hochberg’s correction). Red arrows indicate ASD risk gene sets and ASD DEGs. X-axis shows the number of genes bound by *RFX* in their promoter regions. (h) Binding of *RFX* family transcription factors bind to X-box motif in promoter regions of ciliary and immunologic genes. Target gene lists obtained from Piasecki, Durand, Reith, Sugiaman-Trapman.^{3,38–40} Model of *RFX* gene dose-dependent regulation of genes. In tissues with higher expression of *RFX* genes, ASD genes are activated. Lower levels of *RFX* genes are sufficient to activate ciliary genes. ASD autism spectrum disorder, AST-FB fibrous astrocytes, AST-PP protoplasmic astrocytes, End endothelial, ExDp1 excitatory deep layer 1, ExDp2 excitatory deep layer 2, ExM-U maturing excitatory upper enriched, ExM maturing excitatory, ExN migrating excitatory, IN-PV parvalbumin interneurons, IN-SST somatostatin interneurons, IN-SV2C SV2C expressing interneurons, IN-VIP VIP interneurons, InCGE interneuron CGE, InMGE interneuron MGE, IP intermediate progenitors, L2/3 layer 2/3 excitatory neurons, L4 layer 4 excitatory neurons, L5/6-CC layer 5/6 excitatory cortico-cortical projection neurons, L5/6 layer 5/6 excitatory neurons, Mic microglia, Neu-mat immature neurons, Neu-NRGN NRGN expressing neurons, OPC oligodendrocyte precursor cells, OPC oligodendrocyte precursor cells, ORG outer radial glia, Per pericyte, PgG2M cycling progenitors G2/M phase, PgS cycling progenitors S phase, vRG ventricular radial glia¹¹.

family transcription factors. While presence of neuroimaging findings, seizures, and dysmorphisms varied between different *RFX* family members, the behavioral phenotypes of individuals with *RFX3*, *RFX4*, and *RFX7* were strikingly similar, and often included sensory hypersensitivity and impulsivity. Like ID/DD and ASD more generally,²⁸ individuals with *RFX* variants also exhibited a male bias.

This report complements accumulating statistical genetic evidence for *RFX3* as an ASD risk gene,^{4,21} and extends these findings to the closely related *RFX* family members *RFX4* and *RFX7*. Two-thirds of individuals with *RFX3* variants in our series carried an ASD diagnosis, half had ADHD, and just over half of individuals had ID. Several individuals with *RFX3* variants also exhibited postpubertal cognitive or behavioral regression sometimes accompanied by psychosis. *RFX3* CNVs have been previously reported in schizophrenia.^{20,29} *RFX3* also lies within the region of the chromosome 9p deletion syndrome (OMIM 158170), associated with developmental delay, ID, and ASD, although the size of

the deletions in this syndrome make *RFX3* unlikely to be the sole contributor.

This report also implicates both *RFX4* and *RFX7* as causes of human neurodevelopmental disorders. Individuals with *RFX4* or *RFX7* variants were somewhat more severely affected than those with *RFX3* variants, with *RFX7* less likely to be associated with ASD or ADHD, but showing almost uniform diagnoses of language delay and ID (92%). There were fewer individuals identified with *RFX4* variants, but those identified had high rates of ASD and ID.

RFX family members have been previously known for their biological roles in cilia development. The *RFX3* transcription factor activates core components necessary for development and maintenance of both motile and primary cilia,^{30–32} and biallelic *Rfx3* knockout in mice results in situs inversus, hydrocephalus, and deficits in corpus callosum formation.^{32–34} This raises the question of whether the neurodevelopmental phenotypes reported here may be mechanistically related to cilia development—e.g., a hypomorphic human ciliopathy. The majority of described genetic

ciliopathies are recessive, and therefore not due to haploinsufficiency, but some (e.g., Meckel syndrome, Joubert syndrome, Bardet-Biedl syndrome, oral-facial-digital syndrome type I) may be associated with neurodevelopmental abnormalities and/or brain malformations. On the other hand, the individuals described in this report lack systemic features of ciliopathies, suggesting the alternative hypothesis that *RFX* haploinsufficiency may directly dysregulate the expression of ASD risk genes while leaving ciliary genes intact (Fig. 3h). Future work, which may include analyzing cilia morphology and function in cellular or animal models of *RFX* haploinsufficiency, or characterizing transcriptome-wide effects of *RFX* gene disruption, may prove helpful in distinguishing these hypotheses.

Enriched expression of *RFX3* in upper cortical layer neurons places this gene in cells that are involved in communication between regions of the cortex important for higher cognition and social behavior,³⁵ raising the possibility that haploinsufficiency may disrupt either the developmental specification, synaptic connectivity, or electrophysiological function of this set of neurons. Projection neurons in this layer have been implicated in ASD by analyses of coexpression networks of autism genes,^{36,37} and superficial cortical neurons exhibit the strongest amount of differential gene expression in ASD brains compared with controls.^{11,26} Sun and colleagues in fact showed strong enrichment of *RFX* motifs in differentially acetylated peaks upregulated in ASD brains compared with controls.²⁶ Future studies aimed at understanding the downstream targets of *RFX* family members in human brain may shed new light on pathways important to the molecular pathogenesis of ASD, ADHD, and ID.

WEB RESOURCES

Ensembl Variant Effect Predictor, <https://uswest.ensembl.org/info/docs/tools/vep/index.html>. Online Mendelian Inheritance in Man (OMIM), <https://www.omim.org/>. GeneDx ClinVar submission page, <https://www.ncbi.nlm.nih.gov/clinvar/submitters/26957/>. Ambry Genetics, <https://ambrygen.com/clinician/our-scientific-excellence>.

DATA AND CODE AVAILABILITY

All data referred to in this paper are either provided in the main text/supplementary material, or appropriately referenced (where derived from pre-existing, publicly accessible data sets).

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AUTHOR CONTRIBUTIONS

T.W.Y., H.K.H., and T.N. conceptualized the paper, collected case information from collaborators, conducted and managed all functional studies, drafted the initial manuscript, and edited and revised the manuscript. T.W.Y. provided research study oversight. J.L. designed and performed *RFX* motif analysis, analyzed published brain transcriptome and single-cell RNA-sequencing data, conducted variant analyses, contributed to the manuscript, and edited and revised the manuscript. B.Z. collected ChIP-seq data and performed overrepresentation analysis, contributed to the manuscript, and edited and revised the manuscript. N.A. and C.S.G. conducted functional studies of the impact of *RFX3* variants on protein stability. A.S. performed *RFX* variant analyses. C.A.G. and V.S. coordinated research enrollment. L.H.R., R.P., T.G., B.B.A.d.V.,

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ETHICS DECLARATION

This series was compiled via an international collaborative effort involving Boston Children's Hospital, Kaiser Permanente, Lyon University Hospital, Nemours/A.I. DuPont Hospital for Children, University of Zurich, Johns Hopkins University, Peninsula Clinical Genetics at Royal Devon and Exeter NHS Foundation Trust, University Medical Centre Utrecht, Radboud University Medical Centre, Dell Children's Medical Group, Washington University School of Medicine in St. Louis, University Medical Center Groningen, Ciphergene, SUNY at Buffalo School of Medicine, Oslo University Hospital, Baylor College of Medicine, Bambino Gesù Children's Hospital, Odense University Hospital, Indiana University Health Neuroscience Center, Seattle Children's Research Institute, Children's Hospital of Philadelphia, Spectrum Health Helen DeVos Children's Hospital, Sapienza University and San Camillo-Forlanini Hospital, CHU Nantes et Service de Génétique Médicale, University of Erlangen-Nuremberg, The Islamia University of Bahawalpur, Brest University Hospital, Children's Minnesota, Università Cattolica del Sacro Cuore, University Hospital Heidelberg, University of Leipzig Medical Center, University of Washington, Oslo University Hospital, APHP.Sorbonne Université, Département de Génétique, Groupe Hospitalier Pitié-Salpêtrière, Centre de Référence Déficiences Intellectuelles de Causes Rares, and Weill Cornell Medical College. Collaboration was facilitated by the online genetics/genomics resource GeneMatcher. Affected individuals were clinically assessed by at least one clinical geneticist from one of the participating centers. De-identified clinical data from collaborating institutions (collected with local institutional review board [IRB] approval or deemed exempt from IRB review as per local institutional policy) was shared for analysis and publication under a study protocol approved by the Boston Children's Hospital IRB. Consent for the publication of full-face photographs was obtained from all appropriate individuals in Fig. 1.

PERMISSIONS

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION


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