The following full text is a publisher's version.

For additional information about this publication click this link.
http://hdl.handle.net/2066/167380

Please be advised that this information was generated on 2020-09-19 and may be subject to change.
BCL11A Haploinsufficiency Causes an Intellectual Disability Syndrome and Dysregulates Transcription

Cristina Dias,1,17 Sara B. Estruch,2,17 Sarah A. Graham,2 Jeremy McRae,1 Stephen J. Sawiak,3,4 Jane A. Hurst,5 Shelagh K. Joss,6 Susan E. Holder,7 Jenny E.V. Morton,8 Claire Turner,9 Julien Thevenon,10,11 Kelly Mellul,12 Gabriela Sánchez-Andrade,1, Xiﬁne Ibarra-Soria,1 Pelagia Deriziotis,2 Rui E. Santos,13 Song-Choon Lee,1,14 Laurence Faivre,10,11 Tijitske Kleefstra,15 Pentao Liu,1 Mathew E. Hurles,1 DDD Study,1 Simon E. Fisher,2,16,18,* and Darren W. Logan1,19,*

Intellectual disability (ID) is a common condition with considerable genetic heterogeneity. Next-generation sequencing of large cohorts has identiﬁed an increasing number of genes implicated in ID, but their roles in neurodevelopment remain largely unexplored. Here we report an ID syndrome caused by de novo heterozygous missense, nonsense, and frameshift mutations in BCL11A, encoding a transcription factor that is a putative member of the BAF swi/snf chromatin-remodeling complex. Using a comprehensive integrated approach to ID disease modeling, involving human cellular analyses coupled to mouse behavioral, neuroanatomical, and molecular phenotyping, we provide multiple lines of functional evidence for phenotypic effects. The etiological missense variants cluster in the amino-terminal region of human BCL11A, and we demonstrate that they all disrupt its localization, dimerization, and transcriptional regulatory activity, consistent with a loss of function. We show that Bcl11a haploinsufﬁciency in mice causes impaired cognition, abnormal social behavior, and microcephaly in accordance with the human phenotype. Furthermore, we identify shared aberrant transcriptional proﬁles in the cortex and hippocampus of these mouse models. Thus, our work implicates BCL11A haploinsufficiency in neurodevelopmental disorders and deﬁnes additional targets regulated by this gene, with broad relevance for our understanding of ID and related syndromes.

Introduction

Currently, there are more than 820 genes known to contribute to intellectual disability (ID) and associated childhood neurodevelopmental disorders.1 Up to 2.5% of individuals are diagnosed with mild to severe ID,2 characterized by low cognitive ability and impaired adaptive behavior with onset during early development. Genes disrupted in ID and other neurodevelopmental disorders are enriched for those involved in chromatin remodeling and transcriptional regulation.3,4 Remarkably, more than 1% of cases are attributed to mutations disrupting genes of the BRG1/BRM-associated factor (BAF) swi/snf chromatin-remodeling complex, suggesting that BAFopathies represent an important recurrent cause of ID.2,5,6

The Deciphering Developmental Disorders (DDD) study has contributed signiﬁcantly to the understanding of ID through the identiﬁcation of novel associated genes on a large scale.6 The DDD identiﬁed de novo missense mutations in BCL11A (MIM: 606557, also known as CTIP1 and EVI9) in several individuals with ID. Its protein product has been implicated as a member of the mammalian BAF swi/snf chromatin remodeling complex in human T cells and post-natal mouse brain.7 BCL11A, a transcriptional factor with C2H2 zinc ﬁnger DNA-binding motifs, has been extensively studied for its role in hematopoiesis and malignancy8–12 and as a transcriptional repressor of fetal hemoglobin,13 but its contributions to neurodevelopment are more poorly understood.14–17 A role for BCL11A in normal human brain function is inferred by the presence of chromosomal microdeletions at 2p15–p16.1

1Wellcome Trust Sanger Institute, Wellcome Genome Campus, Hinxton CB10 1SA, UK; 2Language and Genetics Department, Max Planck Institute for Psycholinguistics, PO Box 310, 6500 AH Nijmegen, the Netherlands; 3Behavioural and Clinical Neuroscience Institute, University of Cambridge, Cambridge CB2 3EB, UK; 4Wolfson Brain Imaging Centre, University of Cambridge, Cambridge CB2 0QQ, UK; 5North East Thames Regional Genetics Service, Great Ormond Street Hospital for Children NHS Trust, London WC1N 3JH, UK; 6West of Scotland Regional Genetics Service, Level 2 Laboratory Medicine Building, Queen Elizabeth University Hospital, Glasgow G51 4TF, UK; 7North West Thames Regional Genetics Service, London North West Healthcare NHS Trust, Watford Rd, Harrow HA1 3JU, UK; 8West Midlands Regional Genetics Service, Birmingham Women’s NHS Foundation Trust, Birmingham Women’s Hospital, Edgbaston, Birmingham B15 2TG, UK; 9Peninsula Clinical Genetics Service, Department of Clinical Genetics, Royal Devon and Exeter NHS Foundation Trust, Clinical Genetics Department, Royal Devon & Exeter Hospital (Heavitree), Gladcstone Road, Exeter EX1 2ED, UK; 10Fédération Hospitalo-Universitaire Médecine Translationnelle et Anomalies du Développement (TRANSALD), Centre Hospitalier Universitaire Dijon, 21079 Dijon, France; 11Centre de Génétique et Centre de Référence Anomalies du Développement et Syndromes Malformatifs de l’Interrégion Est, Centre Hospitalier Universitaire Dijon, 21079 Dijon, France; 12Service de Génétique, Hôpital Necker-Enfants Malades, AP-HP, Institut Imagine, INSERM UMR1163, University Sorbonne-Paris-Cité, 75015 Paris, France; 13Children’s Radiology Department, Royal Manchester Children’s Hospital, Manchester M13 9WL, UK; 14Science Centre Singapore, 15Department of Human Genetics, Radboud University Medical Center, 6500 HB Nijmegen, the Netherlands; 16Donders Institute for Brain, Cognition and Behaviour, 6525 EN Nijmegen, the Netherlands

17These authors contributed equally to this work
18Twitter: @Genes_Speak
19Twitter: @darrenlogan
*Correspondence: simon.fisher@mpi.nl (S.E.F.), dls@sanger.ac.uk (D.W.L.)
http://dx.doi.org/10.1016/j.ajhg.2016.05.030.
© 2016 The Author(s). This is an open access article under the CC BY license (http://creativecommons.org/licenses/licenses/by/4.0/).
encompassing the gene in individuals with speech sound disorder\textsuperscript{18} and with more severe ID,\textsuperscript{19} as well as by a recent association with autism spectrum disorder (ASD).\textsuperscript{20} Moreover, biallelic ablation of Bcl11a in murine model brains at mid-gestation leads to deficient migration of cortical projection neurons.\textsuperscript{16,21} Notably, the microdeletions thus far associated with neurodevelopmental phenotypes have the potential to affect coding regions and non-coding regulatory elements of BCL11A\textsuperscript{18,19,22,23} and might also have effects on neighboring genes.\textsuperscript{24} Therefore, sufficiency of monoallelic BCL11A disruption alone has not been demonstrated to be a cause of ID to date, nor has its effects on brain development and regulation been explored.

In addition to missense mutations, we also identified several de novo nonsense and frameshift mutations in BCL11A in individuals with ID in the DDD and other cohorts. In this study, we aimed to address the following questions. What are the features of a putative clinical syndrome associated with disruption of BCL11A? What are the effects of BCL11A missense mutations on functions of the encoded proteins? Are the identified mutations sufficient to cause the clinical phenotype seen in affected individuals? And, as a transcriptional regulator, what are the molecular effects of heterozygous BCL11A mutations?

We identify shared clinical features in all individuals, including persistence of fetal hemoglobin (HbF). Our assays in cell-based models support the hypothesis that the missense mutations result in loss of function of the mutated proteins in vivo. Hence, we determine that ID is the result of haploinsufficiency of BCL11A through different mutational mechanisms. We show that haploinsufficiency of Bcl11a alone in a mouse model is sufficient to recapitulate key cognitive, behavioral, and neuroanatomical phenotypes present in affected individuals. In this haploinsufficient mouse model, we identify transcriptional dysregulation of the hippocampus and cortex, brain regions that correlate with neuroanatomical and behavioral phenotypes.

Together, we present an integrated approach to disease modeling in rare ID syndromes including cellular, behavioral, neuroanatomical, and molecular characterization, and further implicate the BAF complex in neurodevelopmental disorders.

Subjects and Methods

Human Subjects

The Deciphering Developmental Disorders (DDD) study has UK Research Ethics Committee approval (10/H0305/83, granted by the Cambridge South REC, and GEN/284/12 granted by the Republic of Ireland REC). Written informed consent was received from participants prior to inclusion in the study. The PARI (Regional Action Plan for Innovation) 2011 study was approved by the regional ethics committee and funded by the Regional Council of Burgundy and Dijon University Hospital. Informed consent was received from individuals prior to inclusion in the study.

Where exome sequencing was performed in the context of routine healthcare services, informed consent for the diagnostic procedure was obtained.

Exome Sequencing and Statistical Assessment

Affected individuals were identified through the DDD project (individuals 1–6), through clinical exome sequencing (individuals 7 and 8), and through the Dijon University Hospital PARI 2011 study (individual 9). Whole-exome sequencing and de novo variant annotation were performed as previously described.\textsuperscript{8,25} Functional annotation of variants was performed using the Ensembl Variant Effect Predictor (VEP)\textsuperscript{26} consequence predictions. The BCL11A de novo variants studied here, together with two loss-of-function de novo variants identified in individuals with ASD,\textsuperscript{20} were analyzed for significance in the context of 4,295 DDD study trios, 2,206 clinical exome trios, 50 PARI 2011 trios, and 6,138 trios from other reported exome-sequencing studies of developmental disorders.\textsuperscript{20,25,27–36} Statistical assessment of the BCL11A variants was performed using an analytical method combining evidence for enrichment for de novo mutations over that expected for gene mutation rate and cohort size,\textsuperscript{6} clustering of mutations,\textsuperscript{6} and Human Phenotype Ontology (HPO) term similarity between DDD individuals, as described.\textsuperscript{37}

Assessment of Enrichment of De Novo Mutations

Expected null mutation rates for BCL11A for different functional classes of variants were obtained from reported estimates.\textsuperscript{20} The loss-of-function mutation rate was estimated by summing the mutation rates of nonsense, canonical splice sites, and frameshift variants. The functional mutation rate was estimated by summing the loss-of-function mutation rate with the rate of missense variants and in-frame indels. The loss-of-function and functional mutation rates were multiplied by the number of gene transmissions (twice the number of probands) to give the total expected number of mutations given the number of probands sequenced. The expected number of mutations in each class was assumed to be the mean of a Poisson distribution, and the probability of drawing from that distribution a number of mutations equal or greater than the observed number of mutations was calculated. A combined functional de novo statistic was calculated by using Fisher’s method to combine p values from enrichment of functional mutations with clustering of missense mutations. The loss-of-function enrichment and the combined functional statistic were compared and the better performing model was selected. The resulting p value was adjusted for the use of two models by Bonferroni correction.

Assessment of Clustering of De Novo Mutations

Exon coordinates and sequences for BCL11A were retrieved from Ensembl. Nucleotide mutation rates in trinucleotide contexts were provided by Kaitlin Samocha and Mark Daly. De novo missense mutations were randomly sampled, weighted by the context-specific mutation rates, matching the number of sampled mutations to the number of known de novos. The de novos were assessed for their tendency to cluster within close proximity to each other. The proximity was calculated as the geometric mean coding distance between all the possible de novo pairs. The expected distribution of proximity was assumed as the distribution of proximity for sampled de novos from 1,000,000 simulations. The p value was estimated as the proportion of simulated proximities less than or equal to the observed proximity.
Assessment of Phenotypic Similarity
Probands in the DDD study had phenotypes systematically recorded by clinical geneticists using terms from the Human Phenotype Ontology (HPO). For pairs of HPO terms, we determined the information content (IC) for the most informative common ancestor of the two terms. The IC was calculated as the negative logarithm of the probability of the terms’ usage within the 4,295 DDD probands (including descendant terms’ usage). The similarity of terms between two individuals was estimated as the maximum IC from pairwise comparisons of the individuals’ HPO terms. The score for a set of n probands was estimated as the sum of all the pairwise scores. The null distribution was simulated by randomly sampling 100,000 sets of n probands and calculating scores as above. The p value was estimated as the proportion of simulated scores greater than or equal to the observed score.

DNA Constructs
The coding sequences of BCL11A-S (GenBank: NM_138559), BCL11A-L (GenBank: NM_018014), and NONO (GenBank: NM_001145408) were amplified from human fetal brain cdNA using the primers in Table S1 and cloned into pCRII-TOPO (Invitrogen). The missense mutations were introduced using the Quick-Change Lightning SDM kit (Agilent) and the primers in Table S2. For expression of fusion proteins with Renilla luciferase, YFP, and mCherry, cdNAs were subcloned into the pLuc, pYFP, and pmCherry expression vectors, respectively, which have been described previously, using the BamHI and XbaI sites.

Mammalian One-Hybrid Assay
Cells were transfected in white, clear-bottomed 96-well plates, in triplicate, with 8.5 fmol Renilla luciferase reporter plasmid, and 2 fmol Renilla luciferase normalization plasmid (total mass of DNA was adjusted to 60 ng with filler plasmid). Renilla luciferase or YFP with a nuclear localization signal were used as controls. Cells were cultured for 48 hr post-transfection. Enduren luciferase substrate (Promega) was added at a final concentration of 60 M, and cells were cultured for a further 4 hr. Luminescence was measured in a TECAN Infinite F200PRO microplate reader using the Blue1 and Green1 filters.

Animal Models
Housing and breeding of mice and experimental procedures were carried out under the authority of a UK Home Office license (80/2472), after review by the Animal Welfare and Ethical Review Body of the Wellcome Trust Sanger Institute.

Imaging
Adult mice (aged 16 ± 1 week) were anesthetized and then transcardially perfused with 20 ml of ice-cold PBS followed by 4% paraformaldehyde (PFA). The skull was detached and skin removed. The brain was kept in loco. Skulls were transferred into PBS after 24 to 48 hr and kept at 4°C before imaging.

MRI Image Acquisition
Brains were scanned using a Bruker PharmaScan 47/16 system at 4.7T with a manufacturer-provided birdcage transmit-receive coil. The imaging protocol was fast spin echo (scan parameters: repetition time 2,000 ms, effective echo time 16 ms, echo train...
length 4, bandwidth 32 kHz, matrix 256 × 192 × 128, field of view 1.79 × 1.34 × 0.90 cm³, resolution 70 μm isotropic with two averages).

**MRI Tensor-Based Morphometry**

Brains were segmented into gray and white matter portions and registered using the SPMMouse toolbox with SPM8 (Wellcome Trust Centre for Neuroimaging, University College London) and the DARTEL registration toolbox. Jacobian determinants from the registration process were smoothed with a 400 μm Gaussian kernel and tested with an F-test between groups to produce voxel-wise maps for tensor-based morphometry. The scaling factor from the affine matrix was used as a covariate to find differences in volume that could not be explained by overall brain size. To control the type I error rate due to multiple comparisons, an adjusted p value was used for a false-discovery rate at p < 0.05.

**μCT**

Image acquisition and reconstruction were performed on the SkyScan 1172 high-resolution micro-CT (μCT) using the standard software provided by the manufacturer (Bruker micro-CT). 3D image and video generation was performed with DataViewer software (Bruker, v.1.5.1.2, May 27, 2014) using the same parameters for all skulls (opacity adjusted, luminance adjusted, 96% red, 90% green, 84% blue, 5% shadows, 100% emission, 50% diffuse, 25% specular, 40° camera viewing). Measurements were obtained blind to genotype as described by de Carlos et al. and corrected using a multplanar visualization of reference points in sagittal, axial, and coronal planes (described in Figure S2). Comparison of lengths and statistical analyses (Mann-Whitney test) were performed using GraphPad Prism v.6.00 (GraphPad Software).

**Histology and Immunohistochemistry**

All brain tissue samples were fixed through transcardial perfusion of 4% PFA as described above. Samples were dehydrated, paraffin embedded, and sectioned before hematoxylin and eosin, luxol fast blue, and cresyl violet staining using standard histological techniques.

For immunohistochemistry, perfused brain samples were cryoprotected in 30% sucrose, embedded in OCT, and stored at -80°C. Samples were sectioned at 16 μm using a LeicaCM3050S cryostat. Sections were permeabilized with 1% Triton X and blocked with BlockAid Blocking Solution (Thermo Fisher Scientific cat# B10710) or rabbit serum (Jackson Immunoresearch cat# R-37116, RRID: AB_2556544), and Alexa Fluor-488 rabbit anti-GFP (Torrey Pines Biolabs cat# TP-401, RRID: AB_10013661; anti-β-gal (Abcam cat# ab19489, RRID: AB_2063996; 1:250), anti-β-gal (Abcam cat# ab9361, RRID: AB_307210; 1:1,000), and anti-GFP (Torrey Pines Biolabs cat# TP-401, RRID: AB_10013661; 1:500). Secondary antibodies included Alexa Fluor-564 goat anti-mouse-IgG1 (Thermo Scientific cat# A-21123, RRID: AB_2553765), Alexa Fluor-488 goat anti-rabbit IgG (Thermo Scientific cat# R-37116, RRID: AB_2556544), and Alexa Fluor-488 rabbit anti-chicken-IgY (Jackson Immuno Research cat# 303-545-003, RRID: AB_2339327). Slides were mounted with ProLong Gold Antifade reagent with DAPI nuclear counterstain (Life Technologies cat# P36935). Sections were visualized and photographed on a Leica TCS SP5/DM6000 confocal microscope with Leica Application Suite Advanced Fluorescence software or a Zeiss Axiolander 200M microscope with Axiosovision software.

**Social Recognition Assay**

For all behavioral assays described, mice were habituated to the behavior test room for ≥1 hr under same light conditions as the test. For the social recognition assay, group-housed test mice (mutant and littermate wild-type controls) were habituated to the 39 × 21 cm test arena for 10 min. On day 1 (habitation-dis-habitation test), a conspecific anesthetized stimulus was placed on the center of the test arena for 1 min, repeated 4 times at inter-trial intervals of 10 min (Movie S1). On the 5th trial, a new stimulus mouse was presented. On day 2, after a 24 hr interval, the discrimination test was performed (Figure S4). The familiar stimulus animal from trials 1–4 and a new unfamiliar mouse were placed on opposite sides of the test arena for 2 min (Movie S2). The amount of time the test animal spent investigating by close-proximity sniffing, oronasal contact, or approaching within 1–2 cm was recorded. For day 2, social discrimination preference index was calculated according to the following equation: preference index (PI) = (investigation time [s] of novel unfamiliar stimulus – familiar stimulus)/(investigation time [s] of familiar + unfamiliar stimulus).

Trials were performed under red light and recorded with an overhead camera. The trials were scored blind to genotype by two observers, and the average of both observations was used as the time. Stimulus animals were subject to non-terminal anesthesia with ketamine/xylazine (i.p. 1 g/0.1 g per kg of body weight). Familiar stimuli were recovered with atipamezole for use in the 24 hr discrimination test. The stimulus animals were gender matched, equal or lower weight, and different strain. 129P2/OlaHsd,129S5/SvEvBrd mice were used for trials1–4 and 24 hr discrimination familiar stimulus; C57BL/6 or C57BL/6;129 mice from different breeding colonies were used for trial 5 and 24 hr discrimination unfamiliar stimulus. Two-way ANOVA and unpaired t test (after a D’Agostino & Pearson omnibus normality test) were employed where indicated. Statistical analysis for all mouse behavior experiments was performed with GraphPad Prism v.6.00 (GraphPad Software).

**Open Field Assay**

Mice were placed in a 37 × 37 cm open field for 5 min under red light. Their movements were tracked by detection of the mouse center point using overhead infrared video cameras and automated video tracking software (Ethovision XT 8.5, Noldus Information Technology). A 24 × 24 cm “center zone” was designated with equidistant borders to the open field walls. The frequency and time each mouse was within this center zone was recorded. A period of movement was defined when the mouse reached a velocity of 2 cm/s over two frames; a period of non-movement was defined when velocity was lower than 1.75 cm/s over two frames.

**Three-Chamber Social Behavior**

The three-chamber social approach task was performed under red light, modified from Yang et al. The chamber layout is presented in Figure S4F. In brief, a test arena was divided into four quadrants; one quadrant was sealed off, and the remaining three chambers formed the L-shaped arena used for the test. Innate chamber side preference was controlled for during the habituation phase and did not show difference between genotypes (t test, p = 0.2144). Two arenas were used for increased throughput of behavior experiments, and mice were randomly assigned to the test arena. Differences between arenas were tested. In one arena, mice showed an innate preference for the left chamber (0.15
increase in preference index in habituation) independent of genotype (2-way ANOVA, per genotype \( p = 0.2004 \); per arena \( p = 0.0262 \). Subsequent times were normalized to the innate preference index. Mouse movements were tracked via overhead infrared video cameras and automated video tracking software.

Test mice were habituated to the center chamber for 5 min. Doors were then opened to the two empty side chambers and mice were allowed to explore all three chambers for a further 5 min. Doors were re-closed and the mice were contained briefly in the center chambers as objects were placed in the side chambers. Identical objects—stainless steel cylindrical containers with holes sufficiently large to contain a mouse nose—were placed upside down in the side chambers. One object contained a live conspecific (novel sex-matched stimulus previously habituated to the object) that could freely move inside the object. Test mice then explored all three side chambers for 10 min with automated movement tracking (Movie S3). The preference index was calculated as follows: \( PI = (\text{time in chamber with object containing a conspecific stimulus} - \text{time in chamber with object only})/(\text{time in chamber with object containing a conspecific stimulus} + \text{time in chamber with object only}) \).

**RNA-Sequencing Processing and Analysis**

Mice were sacrificed at 16 weeks of age. The cortex and hippocampus were dissected from male animals, snap frozen in liquid nitrogen, and stored at \(-80^\circ C\). Tissue was homogenized in buffer RLT plus \( \beta \)-mercaptoethanol (\( 10 \mu L/mL \)) using the QIAGEN TissueLyser LT. RNA was pre-treated on gDNA eliminator columns and then extracted on RNeasy Plus columns as per manufacturer’s protocol (QIAGEN). Multiplexed libraries were prepared for sequencing using Illumina RNA Library Preparation Kits as per protocol (QIAGEN). TissueLyser LT. RNA was pre-treated on gDNA eliminator columns plus with m

**Gene Ontology**

Gene ontology enrichment analysis was performed with GeneTrail.\(^{50}\) Over-representation analysis of the differentially expressed genes was performed using parameters in Table S3 with significance threshold, 0.1 and \( p \) values adjusted for multiple testing using the Benjamini-Hochberg FDR adjustment. In all analyses a background comprised of only the expressed genes used for the relevant analysis was provided.

**Gene Enrichment Analysis**

Enrichment for voltage gated ion channels (VGIC) was performed using genes annotated as one of the 141 mouse VGIC in the IUPHAR/BPS database (accessed 30 September 2015).\(^{34}\) ASD-related genes were selected from the SFARI gene web portal.\(^{52}\) Of the 740 genes downloaded from The Human Gene Module of SFARI gene database (September 2015), 485 were selected based on exclusion of genes with the following levels of evidence annotated in the SFARI database: functional (only), functional negative association (>1), genetic association (only). Of these, 481 mouse orthologs were identified through Ensembl.\(^{53}\) We generated a second subset of 75 unique human genes and 1 pseudogene from the Gene Scoring Module of SFARI, which were annotated in the following categories defined in the database: S (syndromic), 1 (high confidence), and 2 (strong candidate). 75 mouse orthologs were identified through Ensembl. Analysis was performed in R v.3.2.2.; for statistical analysis, hypergeometric test was employed with a significant \( p \) value < 0.05. Genes used in gene enrichment analyses above are available in Table S4.

**Transcriptome Assembly**

For a qualitative analysis of \( Bcl11a \) isoform and transcription start site usage, the paired end reads aligned using STAR v.2.4.0 were assembled into transcripts via Cufflinks v.2.2.1.\(^{54}\) Assembled gtf files from different replicates for each tissue were merged via cuffmerge including the reference annotation. Cuffquant was employed for isoform quantification. Data visualization and graphical representation were performed with CummelBund v.2.12.0.\(^{54}\)

**Results**

**Identification of Mutations in BCL11A in Individuals with ID**

We identified nine individuals with intellectual disability with de novo mutations in \( BCL11A \): three missense and six loss of function (LoF). In the DDD study\(^6\) we identified six individuals with de novo heterozygous variants in \( BCL11A \), from a total of 4,295 affected individuals studied using whole-exome sequencing (Figure 1A; Table 1). Three missense variants were first identified; they cluster together in exon 2 of \( BCL11A \) (individuals 1, 2, and 3).\(^{14,15}\) Subsequently, we identified three novel variants (one nonsense [individual 4] and two frameshift [individuals 5 and 6]) classified\(^{25}\) as LoF (Figure 1C). Individual 6 also has a probably pathogenic 4.3 Mb duplication but
Additional individuals with de novo LoF variants were identified in other developmental disorder cohorts (individuals 7–9). To evaluate their pathogenicity, we performed a statistical enrichment analysis of all these variants, along with two further LoF variants recently identified in ASD20,36 (individuals 10 and 11; Table 1). We find compelling statistical support for an excess of mutations in BCL11A (p = 6.4 × 10^{-15}; see Subjects and Methods).

All individuals that we identified with BCL11A mutations presented with global delay in developmental milestones, including speech and language delay. Most individuals exhibited moderate ID, though cognitive dysfunction varies from mild (individual 1) to severe (individual 2) (Table 1). One of the two individuals recently ascertained through an ASD study20,36 also has severe ID (individual 10); intellectual capacity was not reported in the other. Individual 2 has likewise received a diagnosis of ASD, and four other individuals present a spectrum of behavioral abnormalities including repetitive behavior and sensory problems. Shared physical features among affected individuals (Figure 1D) include joint laxity (87%), strabismus...
HbF was significantly elevated in all affected individuals in whom it was assessed, including all those with missense mutations in \textit{BCL11A} (Figure 1D, Table 1). Notably, individual 1, carrying a missense mutation, had HbF levels similar to those seen for individual 5, carrying a frameshift mutation.

All LoF variants are predicted to deleteriously affect isoforms L (GenBank: NM_018014.3, Ensembl: ENST00000356842) and XL (NM_022893.3, EnST0000335712) of \textit{BCL11A} (Figure 1A) through premature truncation or frameshift and nonsense-mediated decay (annotated isoforms are summarized in Figure S5). All missense variants are located in the N-terminal region of \textit{BCL11A}, which is required for homo- and heterodimerization of \textit{BCL11A} isoforms, as well as for interaction with repressive nucleosome-remodeling complexes. Given the known role of the gene as a regulator of stage-specific hemoglobin expression, we hypothesized that the missense mutations impair function of \textit{BCL11A} and investigated functional consequences using cell-based assays.

### Missense Mutations Disrupt \textit{BCL11A} Function

Our cellular assays focused on \textit{BCL11A-L} and \textit{BCL11A-S} (GenBank: NM_138559, Ensembl: ENST00000359629), the two isoforms of \textit{BCL11A} reported in human brain. We generated mCherry and YFP-tagged versions of these isoforms containing each of the three missense mutations identified in affected individuals and expressed them in HEK293 cells (Figure 1A). \textit{BCL11A-L} binds to DNA in a sequence-specific manner via two C2H2 zinc finger domains (Figure 1A) and localizes to the nucleus. \textit{BCL11A-S} lacks the zinc finger domains and is unable to bind to DNA, but can form heterodimers with \textit{BCL11A-L} and -XL that mediate the translocation from its predominantly cytoplasmic localization into the nucleus. \textit{BCL11A-L} has predominant nuclear localization, whereas isoform S localizes to the cytoplasm in the absence of interaction with L and XL isoforms.

The mutant forms of \textit{BCL11A-L} and \textit{BCL11A-S} show similar protein levels to the wild-type (Figure S6). We found that both wild-type and mutant \textit{BCL11A-S} isoforms are predominantly localized to the cytoplasm (Figure 2A). In contrast, wild-type \textit{BCL11A-L} is found in nuclear paraspeckles, as demonstrated by its co-localization with the paraspeckle-specific protein NONO, also recently implicated in ID. Strikingly, all three missense mutations disrupt the paraspeckle distribution of \textit{BCL11A-L}, as well as its co-localization with NONO (Figures 2B and 2C). In addition, using a bioluminescence resonance energy transfer (BRET) assay, we found that the mutations reduce, but do not completely abolish, the interaction of \textit{BCL11A-L} with NONO (Figure 2D).

The three missense mutations identified in affected individuals all lie within a region encoding a putative dimerization site in \textit{BCL11A} (Figure 1A). We confirmed that wild-type \textit{BCL11A-L} and \textit{BCL11A-S} form homo- and heterodimers (Figures 3A, 3B, S7A, and S7B) and that co-expression of \textit{BCL11A-L} causes \textit{BCL11A-S} to translocate from the cytoplasm into nuclear paraspeckles (Figure 3C). We found that \textit{BCL11A-L} isoforms carrying mutations have a substantially reduced interaction with both L and S wild-type \textit{BCL11A} isoforms (Figures 3A and 3B) and a reduced capacity to translocate wild-type \textit{BCL11A-S} into the nucleus (Figure 3C). Similar effects were observed when the mutations were introduced into \textit{BCL11A-S} (Figure S7).

We used a mammalian one-hybrid assay to examine the effects of the missense mutations on the capacity of \textit{BCL11A} to regulate transcription. \textit{BCL11A-L} was fused to the DNA-binding domain of yeast GAL4 and co-transfected with a reporter plasmid containing five sequential GAL4 binding sites upstream of a luciferase gene. Wild-type \textit{BCL11A-L} produced a ~2.5-fold activation of reporter transcription, an effect that was significantly reduced for all three mutant isoforms (p < 0.05, one-way ANOVA followed by Bonferroni post hoc test; Figure 3D).

In summary, all three missense mutations identified in affected individuals are associated with persistence of HbF and show consistent deleterious effects on multiple aspects of \textit{BCL11A} molecular function, including localization, dimerization, and transcriptional regulatory activity. These findings indicate that the mutations yield a loss of function of the mutant proteins in vivo, suggesting that the associated neurodevelopmental syndrome may result from a haploinsufficiency mechanism.

### Mice with \textit{Bcl11a} Haploinsufficiency Phenocopy Features of the Human ID Syndrome

To test the hypothesis that haploinsufficiency of \textit{BCL11A} is sufficient to cause a specific neurodevelopmental syndrome, and to gain further insights into pathogenic mechanisms, we generated a \textit{Bcl11a} heterozygous mouse line containing a LacZ reporter (Figure S1, herein termed \textit{Bcl11a}+/−; see Methods). Mouse \textit{Bcl11a} (also known as \textit{Evi9} and \textit{Ctip1}) is highly homologous to human \textit{BCL11A} (the XL isoform shows 97% cDNA sequence identity and 100% protein sequence identity between species). Germine biallelic loss of \textit{Bcl11a} leads to perinatal lethality in mouse models, though its neurodevelopmental phenotype has not been explored. In utero knockdown experiments and conditional knockout models show that \textit{Bcl11a} is necessary for normal mouse cortical development. However, global haploinsufficiency of \textit{Bcl11a} in...
<table>
<thead>
<tr>
<th>Individual</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6*</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10b</th>
<th>11c</th>
<th>Summary of Features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCL11A</strong></td>
<td>mutation</td>
<td>c.139A&gt;C</td>
<td>c.143G&gt;T</td>
<td>c.198C&gt;A</td>
<td>c.529C&gt;T</td>
<td>c.2035_2037delinsC</td>
<td>c.154G&gt;T</td>
<td>c.1775_1776insTGG</td>
<td>c.514C&gt;T</td>
<td>c.1325_1325del</td>
<td>c.792_793insC</td>
<td>3 missense; 8 nonsense/frameshift</td>
</tr>
<tr>
<td>Decipher ID</td>
<td>262471</td>
<td>262952</td>
<td>261658</td>
<td>268026</td>
<td>275695</td>
<td>280953</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Mutation class</td>
<td>missense</td>
<td>missense</td>
<td>missense</td>
<td>loss of function</td>
<td>loss of function</td>
<td>loss of function</td>
<td>loss of function</td>
<td>loss of function</td>
<td>loss of function</td>
<td>loss of function</td>
<td>loss of function</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>3 M, 8 F</td>
<td></td>
</tr>
<tr>
<td>Microcephaly</td>
<td>−*</td>
<td>−*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−*</td>
<td>−*</td>
<td>NA</td>
<td>NA</td>
<td>5/9</td>
</tr>
<tr>
<td>Intellectual disability</td>
<td>mild-moderate</td>
<td>moderate-severe</td>
<td>moderate</td>
<td>moderate</td>
<td>+*</td>
<td>moderate</td>
<td>moderate</td>
<td>moderate</td>
<td>severe</td>
<td>NA</td>
<td>10/10 (average moderate)</td>
<td></td>
</tr>
<tr>
<td><strong>Developmental Milestones (Age of Achievement in Months)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sat independently</td>
<td>7</td>
<td>10</td>
<td>12</td>
<td>NA</td>
<td>11</td>
<td>NA</td>
<td>NA</td>
<td>14</td>
<td>12</td>
<td>NA</td>
<td>NA</td>
<td>~11</td>
</tr>
<tr>
<td>Walked independently</td>
<td>20</td>
<td>36</td>
<td>24</td>
<td>22</td>
<td>45</td>
<td>NA</td>
<td>NA</td>
<td>23</td>
<td>36</td>
<td>30</td>
<td>NA</td>
<td>~29.5</td>
</tr>
<tr>
<td>First words</td>
<td>22</td>
<td>27</td>
<td>36</td>
<td>16</td>
<td>NA* (80 words at 100 months)</td>
<td>24–30</td>
<td>NA (few words at 33 months)</td>
<td>60</td>
<td>36</td>
<td>NA (2 words at 6 years)</td>
<td>NA</td>
<td>~32</td>
</tr>
<tr>
<td><strong>Craniofacial Features</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Downsloping palpebral fissures</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>NA</td>
<td>NA</td>
<td>4/8</td>
</tr>
<tr>
<td>Epicantus</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Strabismus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
<td>8/8</td>
</tr>
<tr>
<td>Blue sclera in infancy</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>NA</td>
<td>NA</td>
<td>3/9</td>
</tr>
<tr>
<td>Flat midface</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>NA</td>
<td>6/8</td>
<td></td>
</tr>
<tr>
<td>Thin upper lip</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>NA</td>
<td>7/8</td>
<td></td>
</tr>
</tbody>
</table>

(Continued on next page)
Table 1. Continued

<table>
<thead>
<tr>
<th>Individual</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6(^a)</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10(^b)</th>
<th>11(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Everted lower lip</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Nose</td>
<td>antverted small nares</td>
<td>antverted; full tip</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>NA</td>
<td>full tip</td>
<td>small nares; full tip</td>
<td>small nares; full tip</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Micro/retrognathia</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>retro</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Additional craniofacial features</td>
<td>frontal upsweep, cleft uvula</td>
<td>coarse hair</td>
<td>−</td>
<td>small mouth, high palate, pointed chin</td>
<td>small mouth, plagiocephaly, synophrys</td>
<td>−</td>
<td>large tip of the nose, broad bridge, flared eyebrows, telecanthus</td>
<td>large tip of the nose, high palate</td>
<td>high nasal bridge</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>External ear anomalies</td>
<td>−</td>
<td>−</td>
<td>+(^i)</td>
<td>+(^j)</td>
<td>+(^k)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

Additional Physical and Neurologic Features

| Joint hypermobility | + | − | − | − | + | + | NA | + | + | + | NA | NA | 7/8 |
| Short stature | − | + | − | − | − | − | − | − | − | − | − | − | 2/9 |
| Gait abnormalities | broad based | broad based, truncal ataxia | − | − | − | NA | − | − | − | ataxia | NA | NA | 3/8 |
| Other | anteriorty placed anus, dyspraxia | fetal pads, bilateral coxa valga, valgus foot deformity | hernia repair | congenital hip dislocation, delayed bone age | GE reflux | large 2\(^{nd}\) metacarpals, scoliosis | pectus excavatum | NA | NA | NA | |

Behavioral Features

| ASD | − | + | − | − | − | − | − | − | − | − | + | + | 3/10 |
| Repetitive behavior | + | + | + | − | − | − | − | − | − | − | − | − | 4/9 |
| Other behavior problems | emotional lability | recurrent hand flapping | sensory abn., self-injurious behavior | recurrent hand flapping/biting | none reported | none reported | anxiety, eating disorder | none reported | attention deficit | NA | NA | 6/9 |
| Sleep disturbance | − | + | + | − | − | − | − | − | − | − | − | − | 4/9 |

(Continued on next page)
<table>
<thead>
<tr>
<th>Individual</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6&lt;sup&gt;a&lt;/sup&gt;</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10&lt;sup&gt;b&lt;/sup&gt;</th>
<th>11&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Summary of Features</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MRI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WM volume</td>
<td>slightly reduced</td>
<td>NA</td>
<td>normal</td>
<td>VM</td>
<td>NA</td>
<td>NA</td>
<td>atrophy of the superior CV</td>
<td>mild hypoplasia of the CC</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin F%</td>
<td>20.8%</td>
<td>8%</td>
<td>8.7%</td>
<td>NA</td>
<td>26.3%</td>
<td>NA</td>
<td>3.1%</td>
<td>8.6%</td>
<td>NA</td>
<td>NA</td>
<td>6/6</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations are as follows: GE, gastroesophageal; ASD, autism spectrum disorder; Abn, abnormalities; WM, white matter; VM, ventriculomegaly; CV, cerebellar vermis; CC, corpus callosum; NA, not available. Ascertainment: individuals 1 to 6, the DDD study (1 to 3 reported in previous DDD study, 4 to 6 identified subsequently); individuals 7 and 8, clinical exome sequencing; individual 9, PARI 2011 study.

<sup>a</sup>Individual 6 has an additional probable pathogenic copy number variation (4.3 Mb duplication: dup15q15.3q21.1).

<sup>b</sup>Mutation reported by de Rubeis et al.20

<sup>c</sup>Mutation reported by Iossifov et al.36

<sup>d</sup>BCL11A mutations are annotated to transcript NM_022893.3, ENST00000335712 (GRCh37).

<sup>e</sup>OFC measured between percentiles 9 and 25.

<sup>f</sup>Severity unknown.

<sup>g</sup>No words at 28 months.

<sup>h</sup>No microstomia, with intercomissural distance ≤ −2 SD.

<sup>i</sup>Unilateral (left) flat helix, prominent anti-crus.

<sup>j</sup>Bilateral microtia (< −2 SD), asymmetric low-set ears, with overfolded and cupped helix, attached earlobes.

<sup>k</sup>Low-set ears with overfolded helixes and attached earlobes.

<sup>l</sup>Thick, overfolded helixes.

<sup>m</sup>Dysplastic, posteriorly rotated ears.
brain development and function, most relevant to the human disorder described here, has not been previously investigated in animal models. We first used our reporter mouse model to confirm Bcl11a expression in developing brain from embryonic day E10.5, with highest expression in the forebrain between E12.5 and E14.5 (Figures S1A and S1B). Central nervous system expression persists throughout the post-natal period in the cortex, hippocampus, olfactory bulb, and, to a lesser extent, in the cerebellum (Figure S1C). In the hippocampus, BCL11A localizes intensely to granule cell layers of the dentate gyrus and principal cell layers CA1, CA2, and CA3 (Figures S1D and S1E). Within the adult neocortex, localization spans layers II to VI. Although they do not show overt morphological differences, Bcl11a/+/C0 mice appear to have a shift in Bcl11a localization toward deeper cortical layers relative to more superficial layers (Figure S8).

We next investigated whether murine haploinsufficiency of Bcl11a phenocopies the human individuals carrying BCL11A mutations. Microcephaly is a feature of BCL11A-associated ID (Figure 1, Table 1), particularly in individuals heterozygous for complete LoF mutations. We performed ex vivo imaging of whole mouse heads using magnetic resonance imaging (MRI) tensor-based morphometry followed by voxel-based quantification of brain volume (Figures 4A and 4B). Overall brain volume is significantly decreased in Bcl11a/+/C0 mice, affecting both gray and white matter (p = 1.3 × 10⁻⁵, two-tailed F-test corrected for multiple comparisons with FDR-adjusted p < 0.05) (Figures 4A and 4B). Next, we normalized by overall brain volume per genotype to investigate whether specific substructures had a greater effect. We found a greater decrease in the volume of regions that anatomically correspond to the limbic system—the hippocampus (corresponding to CA1, CA2, CA3, and the fimbria), corpus callosum, the posterior cortical amygdaloid nuclei, regions of the ventral and midline thalamic nuclei—and parts of the cerebellum, including regions of the superior vermis (see Allen Brain Atlas in Web Resources) (Figures 4C and S2 and Movie S4). Micro-computed tomography (μCT) revealed that skull morphology is similar in Bcl11a/+/C0 mice and their...
wild-type littermates, consistent with the absence of major congenital malformations (Figure 4D, Movies S5 and S6). However, the Bcl11a<sup>+/−</sup> skulls are significantly smaller in width (Mann Whitney $p = 0.008$ and $p = 0.032$, for bi-temporal and interzygomatic distances, respectively) but not in length ($p = 0.31$) (Figure 4E).

To investigate potential impairments in behavior, learning, and memory, we tested the Bcl11a<sup>+/−</sup> mice in...
social and activity paradigms. In mouse models a conspecific recognition assay can be used to test hippocampal-mediated cognitive processes, using their natural propensity to investigate unfamiliar over familiar animals (Figures 5A–5C and S4A; see Subjects and Methods). On day 1 of the assay, both Bcl11a+/−/C0 mice and wild-type littermates investigated, then habituated and dishabituated to anesthetized stimulus mice (Figure 5B). In a discrimination test 24 hr later, unlike wild-type mice, the Bcl11a+/−/C0 mice were unable to differentiate between the familiar and unfamiliar stimulus mice (t test p = 0.710; mean preference index [PI] = −0.0204 ± 0.099), indicating impairment of long-term social memory (Figures 5C and S4B).

Some affected individuals with heterozygous BCL11A mutations have exhibited increased physical activity (repetitive behavior and hand flapping), so we tested Bcl11a+/−/C0 mice in an open field environment. The Bcl11a+/−/C0 mice displayed hyper-locomotion across the open field, with significant increase in the number of transversals and time in center (Figures S4C and S4D).

Because 30% of individuals show ASD (and overall 67% present some type of behavioral phenotype; Table 1), we sought to investigate the effects of haploinsufficiency on social behavior in our mouse model. The conspecific recognition assay showed that the mutant mice did not display an overall decreased investigation time for novel stimuli (Figures 5B and 5C), and dishabituation is consistent with intact olfaction (Figure 5B). Therefore, we performed a three-chamber social approach assay, widely used as a test for ASD-like phenotypes in rodent models. Consistent with their behavior in the open field assay (Figures S4C and S4D), Bcl11a+/−/C0 mice show...
increased activity throughout the length of the test, with increased number of transversions between chambers (Figure S4G). Both genotypes have similar number of transversion in the first 2 min of the test, however, showing equal motivation to investigate novelty. Although overall time spent in the side chambers is not different between genotypes (Figure S4H), wild-type mice significantly prefer a chamber with a conspecific compared to an empty object (mean PI = 0.285 ± 0.04; paired t test per chamber for wild-types p < 0.0001; Figure 5). In contrast, Bcl11a+/+/
mice continue equal exploration throughout the test; they have a significantly decreased preference (t test for PI, mutants versus wild-types; p = 0.0005), spending a similar amount of time in both chambers (mean PI = 0.017; paired t test per chamber for mutants p = 0.063; Figures S5F, S4H, and S4I).

Mice with Bcl11a Haploinsufficiency Have Significant Transcriptional Deregulation in the Cortex and Hippocampus

Next, we used a genome-wide transcriptomic approach to obtain evidence that BCL11A is involved in transcriptional regulation in the mouse brain and to identify candidate pathways involved in disease pathophysiology. We performed RNA sequencing of cortex and hippocampus, two brain structures that are enriched for Bcl11a expression and are implicated in the neuroanatomical and behavioral phenotypes that we describe above. Results of the transcriptomic analysis are detailed in Table S3. For the cortex, we identified 608 differentially expressed (DE) genes in Bcl11a+/− mice compared to wild-type littermates (Benjamini-Hochberg [BH] adjusted p value with 10% FDR), of which 157 (25.9%) were upregulated and 451 (74.2%) downregulated (Figure 6A) as a consequence of haploinsufficiency. Among the DE genes, we identified significant enrichment of genes involved with syndromic ASD (p = 0.047) from the Gene Scoring Module of SFARI, and genes with functional evidence for ASD from the SFARI Human Gene Module (p = 0.029; see Methods).

Figure 6. Bcl11a+/− Mice Have Altered Gene Expression Profiles in Cortex and Hippocampus

(A and B) MA plots of differential gene expression between Bcl11a+/− and Bcl11a+/+ cortex (A) and hippocampus (B). The x-axis is the log10 average expression of all samples (normalized counts); the y-axis is DESeq2’s shrinkage estimation of log2 fold changes between genotypes. Each gene is represented as a dot; significantly differentially expressed genes (BH-adjusted p value < 0.1) are highlighted in red. n = 9 per genotype for cortex; n = 6 per genotype for hippocampus.

(C) Comparison of DESeq2’s shrinkage estimation of log2 fold changes of genes in both tissues. Red dots represent genes with significant differential expression in both tissues; yellow dots represent genes differentially expressed in the cortex only, and blue in the hippocampus only; gray dots represent genes not differentially expressed in either tissue (BH-adjusted p value < 0.1).
For the hippocampus we identified 442 DE genes, including 183 (41.4%) upregulated and 259 (58.6%) downregulated (Figure 6B, Table S3) due to haploinsufficiency. Gene ontology analysis revealed overlapping categories in both tissues (Figure S9), including ion channel activity and transport. We analyzed specific enrichment for genes encoding voltage gated ion channels, as they are associated with both ASD and ID, and found significant enrichment in the hippocampus (p = 0.004) and an increase just below the significance threshold in the cortex (p = 0.051). These changes were predominantly downregulation (Table S3). DE genes from the BAF complex were also identified in the hippocampus: downregulation of Smarcd1 (padj = 0.0020) of the neural progenitors-specific BAF, and of Actl6b (padj = 0.0095), which encodes a neuronal specific BAF complex protein.

Within the gene ontology-enriched category of “cell recognition” in the cortex, we identified reciprocal upregulation of the Roundabout (Robo) receptor gene Robo1 (padj = 0.0744) and downregulation of Robo3 (padj = 0.002). In both tissues we also found evidence for involvement of other genes of the semaphorin-plexin pathway, putatively regulated by Bcl11a and involved in neuronal migration and polarity and development of neuronal circuitry. Upregulation of semaphorins Sema3d (cortex, padj = 0.0037) and Sema3e (hippocampus, padj = 0.0047) was accompanied by reciprocal downregulation of the class3 semaphorin cell surface receptor PlxnA1 in both tissues (padj = 0.0246, padj = 0.0038, respectively).

We performed de novo transcript assembly to test whether haploinsufficiency of Bcl11a alters the relative expression of its different gene isoforms. We identify transcript start sites (TSS) and sequence corresponding to the annotated isoforms: L (GenBank: NM_016707, Ensembl: ENSMUST00000008881, XL (NM_001242934, ENSMUST00000109514), XS (NM_001159290, ENSMUST0000118955), and S (NM_001159289, ENSMUST00000 109516), all of which are predicted to contain all or part of the putative N-terminal dimerization region. At the transcript level, isoforms L and XS are the most abundant transcripts in both mouse cortex and hippocampus (Figure S10 and Table S4), with a small relative increase of L in proportion to XS in Bcl11a/−/− mice. The S isoform expression level is low but fully preserved in both genotypes. We provide evidence for a previously underappreciated expression of the Bcl11a-XS isoform usage. Importantly, the apparent preservation of expression of the S isoform in haploinsufficient cortex and hippocampus suggests a fundamental role of this isoform in the adult brain.

Finally, given that both tissues share Bcl11a isoform usage and have well-correlated transcriptome-wide expression patterns, we compared the patterns of differential expression between the two brain loci (Figure S11). All 69 genes that showed significant differential expression in both structures are dysregulated in the same direction. Indeed, of the 981 DE genes in either the cortex or the hippocampus, 89.3% (876) show the same direction of change in both tissues (Figures 6C and S11). This shared gene expression repertoire in the brain regions implicated in morphological, cognitive, and behavioral phenotypes supports the biological significance of these DE genes underpinning the Bcl11a+/− phenotype.

Discussion
Here we identify and phenotypically characterize an ID syndrome in individuals with missense and truncating BCL11A mutations. We demonstrate that the missense variants have consistent deleterious effects on BCL11A localization, dimerization, and transcriptional regulatory activity. Together with the clinical observation that affected individuals with such mutations have elevated HbF similar to loss-of-function mutations and previously identified in individuals with chromosomal microdeletions encompassing BCL11A, our data support the hypothesis that these N terminus missense mutations result in a loss of BCL11A function in vivo.

The functional studies of the BCL11A missense mutations suggest that they are hypomorphic alleles, where the loss of function may not be complete. This could explain the similar but milder phenotypes seen in the individuals with missense mutations, as compared to what is observed in individuals with truncations, where loss of function of the affected allele is expected to be complete. These experiments also support the hypothesis that the N terminus of BCL11A is involved in protein dimerization. Notably, the DNA-binding domains of BCL11A isoforms remain intact with these mutations but functional transactivation of transcription is impaired. Thus, cell-based assays and clinical and hematological features indicate the importance of protein-protein interactions for BCL11A’s role in regulating gene expression. This implies that the N terminus region of BCL11A has an underappreciated significance for transcriptional repression of fetal hemoglobin.

The clinical and cellular findings are consistent with a dosage-mediated phenotypic spectrum, with increased severity and syndromic features in individuals with truncating mutations, including blue sclerae, microcephaly, and external ear dysplasia. Although the affected individuals do not present recognizable dysmorphic features, the presence of mild dysmorphism with ID and persistence of fetal hemoglobin define a clinical syndrome, with the latter providing a valuable diagnostic tool. BCL11A has previously been proposed as a susceptibility gene for ASD. However, we note that despite a high frequency of behavior abnormalities, only 30% of affected individuals fulfill diagnostic criteria for ASD, although all have cognitive impairment. Although it is possible that this reflects an over-representation due to ascertainment bias, it is important to note that individuals ascertained through ASD also have ID, whereby ASD is a variable feature of the BCL11A-intellectual disability syndrome.
Microdeletions at 2p15–p16.1 encompassing \textit{BCL11A} and adjacent genes/genomic regions have been associated with ID accompanied by variable additional features absent in, or of greater severity than, those in individuals with missense and LoF mutations. Genitourinary anomalies are identified in half of patients. and camptodactyly is also a common feature. Telecanthus, a feature of almost all individuals with microdeletions, is observed in only one individual with a LoF mutation. CNS anomalies are detected in half of individuals, and of greater severity than those seen in the individuals presented here, including cortical dysplasia, abnormalities of the corpus callosum, white matter involvement, cerebellar hypoplasia, hypoplasia of the pons, and optic nerve hypoplasia. The smallest microdeletions include, in one case, an adjacent miRNA gene, and in the other, a region centromeric to \textit{BCL11A} with a putative transcription enhancer site.

In the latter report, the individual had two additional CNVs (duplications at 2q13 and 6p25.3) of unknown significance. In individuals with microdeletions, the contribution of haploinsufficiency or disruption of contiguous genes, disruption of coding and non-coding regulatory elements, or positional effects is not clear. The lack of additional malformations in individuals with missense and LoF mutations in \textit{BCL11A} (Table 1) indicates that adjacent genes or regulatory regions contribute to those features and that \textit{BCL11A} may be a defining gene for the developmental delay/ID phenotypes of individuals with microdeletions. Mutations in \textit{BCL11A} therefore cause a distinct ID syndrome.

The recapitulation of affected individual phenotypes in a mouse model with heterozygous loss of function of \textit{Bcl11a} confirms our hypothesis that haploinsufficiency of this gene alone is sufficient to cause neurodevelopmental deficits, supporting its role in brain development.

Using social- and activity-based paradigms, we investigated potential effects on behavior, learning, and memory. We demonstrated that the \textit{Bcl11a}+/− mouse model displays normal novelty-seeking behavior but shows long-term social memory defects, impaired sociability, and increased physical activity. In addition to decreased overall brain size, we identified an overrepresentation of the limbic system (namely hippocampus and amygdala) among specific mouse brain regions that are more severely affected. These data together indicate that the individuals’ de novo \textit{BCL11A} mutations underlie their cognitive and behavioral phenotypes.

Given \textit{Bcl11a}’s established role as a known transcription factor and its association with the BAF swi/snf chromatin remodeling complex, we hypothesized that the phenotype could be mediated by transcriptional dysregulation. We combined cognitive assessment and morphometry to select brain structures most severely affected by haploinsufficiency for large-scale transcriptomic analysis to investigate the genes regulated by \textit{Bcl11a} in these structures. We identified large-scale transcriptional dysregulation in both cortex and hippocampus of \textit{Bcl11a}+/− mice, with broad over-representation of genes involved in ion transport, membrane trafficking, and neuronal signaling. Interestingly, even though \textit{BCL11A} is better known for its transcriptional repressor properties, we find more genes downregulated in haploinsufficient brains, particularly in the cortex. This may be the result of downstream effects on targets that are not directly regulated by \textit{BCL11A} DNA binding, or transcriptional regulation through a compromise of BAF complex function.

Several members of the BAF swi/snf complex have been implicated in mammalian neurodevelopment. The mutant hippocampus, we find downregulation of \textit{Smurdc1} of the embryonic stem cell and neuronal progenitors-specific BAF complexes (esBAF and npBAF, respectively), essential for embryonic stem cell differentiation to neuroectoderm, and of \textit{Actl6b}. Recruitment of \textit{Actl6b} accompanies the differentiation of progenitors into neurons. \textit{Actl6b} is highly specific to (but not essential for) the neuronal BAF complex (nBAF), associated with post-mitotic neuron morphogenesis and maturation. Deficiency of \textit{Actl6b} has also been associated with long-term, but not short-term, memory defects in mouse models. Given the microcephaly present in some of the affected individuals and the reduced brain size and skull width in the mouse model, this could be suggestive of decreased neuronal proliferation or reduced survival of the post-natal neurogenic population or of post-mitotic neurons as seen postnatally in biallelic loss of \textit{Bcl11a}. Further studies are required to distinguish between these possible mechanisms in the heterozygous brain.

The differentially expressed genes are significantly enriched for those annotated as involved in ASD, transmembrane transport, and ion channel genes (Table S3). There is a considerable overlap of genes involved in ID and ASD. These transcriptional changes in a \textit{Bcl11a}+/− mouse model support the hypothesis that converging molecular mechanisms underlie both disorders.

Several molecular pathways function to control axonal growth and guidance, providing cues through cell surface and secreted molecules. These include ephrins, netrins, repulsive guidance molecules, and proteins of the semaphorin-plexin and slit-robo signaling pathways (reviewed by Van Battum et al.). Importantly, they are also regulators of synaptic assembly and refinement, which highlights their role in postnatal brain regulation.

Our results further implicate regulation of class 3 semaphorins by \textit{BCL11A}. Class 3 semaphorins are secreted molecules that interact with plexin transmembrane receptors to regulate axon guidance, with additional roles in cell migration, vascular and lymphatic development, tumor growth and suppression, and immunologic response. \textit{BCL11A} transcriptionally represses the semaphorin Sema3c in radially migrating neurons during embryonic cortical development. However, \textit{Bcl11a}-dependent postnatal differentiation and survival does not require...
SEMA3C, suggesting that time-sensitive interactions of BCL11A with other targets or proteins may contribute to developmental regulation of cortical migration. We find evidence for dysregulation of semaphorin-plexin signaling in the post-natal cortex and hippocampus of Bcl11a−/− mice. Upregulation of Sema3d (in cortex) and Sema3e (in hippocampus) of our haploinsufficient mice is accompanied by reciprocal downregulation of the plexin receptor Ptlnd1. SEMA3D regulates axon-axon interaction, and SEMA3E/Plexin-D1 signaling has been implicated in forebrain axonal guidance, synapse formation of cortical neurons, and regulation of Cajal-Retzius cell migration in the developing cortex. Class 3 semaphorins act as Plexin-D1-mediated axonal repellents or attractants, requiring and dependent on the presence of different neuropilin co-receptors. Unlike SEMA3C and SEMA3D, SEMA3E appears to be able to also bind Plexin-D1 in the absence of neuropilin-1.

The slit-robo signaling pathway has equally been implicated in axon guidance and angiogenesis. We find opposing differential expression of Robo3 (up) and Robo1 (down), consistent with previous observations of suppression of ROBO1 by ROBO3 in commissural axons. ROBO3 regulates the number, migration, and differentiation of cortical interneurons, whereas ROBO1 has been shown to regulate migration of cortical neurons, notably through regulation of semaphorin signaling, and has been implicated in language-related disorders. Thus, we identify significant effects of Bcl11a haploinsufficiency on expression of guidance molecules. Although further studies are required to understand the role of the class3/semaphorin and slit/robo pathways in the post-natal brain, these findings suggest that BCL11A has a broader role in post-natal synaptic regulation and neuronal and/or vascular morphogenesis of the brain than previously recognized.

Overall, our data suggest that there is an ongoing molecular phenotype in Bcl11a-associated ID that is not restricted to early development and involves multiple post-natal molecular pathways underlying cognitive impairment and behavioral defects. These findings raise both enthusiasm and caution with regards to therapeutic opportunities. On the one hand, the identification of dynamic post-natal transcriptional dysregulation suggests that there may be therapeutic potential to modulate the ongoing phenotype. On the other, it raises concern with regards to the inactivation of BCL11A as a target for hemoglobinopathies. Given the broad transcriptional effects of reducing BCL11A dose in the brain, any therapeutic, even if restricted to erythroid lineages, would require caution.

In summary, we identify genes and pathways altered in the BCL11A haploinsufficient post-natal brain, suggesting non-linear dosage sensitivity of transcriptional targets or of interacting protein complexes such as the BAF complex. Together, our observations underscore the importance of BCL11A dosage in mammalian brain development and as a cause of a previously undescribed BAFopathy syndrome.
References


