An interaction network of mental disorder proteins in neural stem cells

MJ Moen¹, HH Adams¹, JH Brandsma¹, DHW Dekkers², U Akinci¹, S Karkampouna¹, M Quevedo¹, CEM Cockx³, Z Ozgür³, WJ van Uckén³, J Demmers² and RA Poot¹

Mental disorders (MDs) such as intellectual disability (ID), autism spectrum disorders (ASD) and schizophrenia have a strong genetic component. Recently, many gene mutations associated with ID, ASD or schizophrenia have been identified by high-throughput sequencing. A substantial fraction of these mutations are in genes encoding transcriptional regulators. Transcriptional regulators associated with different MDs but acting in the same gene regulatory network provide information on the molecular relation between MDs. Physical interaction between transcriptional regulators is a strong predictor for their cooperation in gene regulation. Here, we biochemically purified transcriptional regulators from neural stem cells, identified their interaction partners by mass spectrometry and assembled a protein interaction network containing 206 proteins, including 68 proteins mutated in MD patients and 52 proteins significantly lacking coding variation in humans. Our network shows molecular connections between established MD proteins and provides a discovery tool for novel MD genes. Network proteins preferentially co-localize on the genome and cooperate in disease-relevant gene regulation. Our results suggest that the observed transcriptional regulators associated with ID, ASD or schizophrenia are part of a transcriptional network in neural stem cells. We find that more severe mutations in network proteins are associated with MDs that include lower intelligence quotient (IQ), suggesting that the level of disruption of a shared transcriptional network correlates with cognitive dysfunction.

Translational Psychiatry (2017) 7, e1082; doi:10.1038/tp.2017.52; published online 4 April 2017

INTRODUCTION

Mental disorders (MDs) are categorized by Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition to include neurodevelopmental disorders such as intellectual disability (ID) and autism spectrum disorders (ASD), as well as psychiatric disorders such as schizophrenia.¹ ID, ASD and schizophrenia were shown to have a strong genetic component.²–⁴ Recently, many de novo gene mutations in patients with these MDs have been identified by high-throughput sequencing approaches.⁵ Recent evidence suggests that transcriptional regulators co-localize on the genome, depend on each other for their cooperation in gene regulation. We and others previously showed that physically interacting transcriptional regulators are essential for their cooperation in gene regulation. We and others previously showed that physically interacting transcriptional regulators co-localize on the genome. These interactions are important to appreciate that cooperating transcriptional regulators act together in the same gene regulatory network. Such information is important to appreciate that cooperating transcriptional regulators act together in the same gene regulatory network and regulators. Here we purify transcriptional regulators from neural stem cells, identified their interaction partners by mass spectrometry and assembled the first transcription factor interaction network in NSCs. Their high expression in NSCs suggested that these transcription factors are relevant for NSC biology and indeed Olig2, Npas3 and Sox2 were shown to be essential for NSC identity.¹⁷–¹⁹ On an MD-level, TCF4 haploinsufficiency causes Pitt Hopkins syndrome, which features severe ID, lack of speech, microcephaly and breathing abnormalities.²⁰,²¹ Several single-nucleotide polymorphisms in the TCF4 locus are genetic risk factors for developing schizophrenia.⁴,²² OLIG2 is triploid in Down syndrome patients. Restoring diploid gene dose for Olig2 and Olig1 in a mouse model for Down syndrome showed recovery of the normal balance of inhibitory and excitatory neuronal activity.²³ Npas3 mutations co-segregate with schizophrenia in two families,²⁴,²⁵ but its overall relevance for schizophrenia has remained unclear. SOX2 mutations cause an Anophthalmia syndrome with associated cognitive deficits in about half of the cases.²⁶,²⁷ The resulting interaction network of these four starting transcription factors and their interaction partners contains 206

¹Department of Cell Biology, Erasmus MC, Rotterdam, The Netherlands; ²Center for Proteomics, Erasmus MC, Rotterdam, The Netherlands and ³Center for Biomics, Erasmus MC, Rotterdam, The Netherlands. Correspondence: Dr RA Poot, Department of Cell Biology, Erasmus MC, Wytemaweg 80, 3015CN Rotterdam, The Netherlands.
E-mail: r.poot@erasmusmc.nl

²These two authors contributed equally to this work.

³Current address: Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands.

Received 15 August 2016; revised 6 February 2017; accepted 13 February 2017
proteins. We find that the network contains 68 proteins mutated in patients with ID, ASD or schizophrenia, as well as 52 proteins significantly lacking coding variation in the human population. We provide evidence that proteins associated with ID, ASD or schizophrenia can be part of the same transcription network and that within this network, mutation severity correlates with the level of cognitive dysfunction.

**MATERIALS AND METHODS**

Transcription factor purification and interaction partner identification

The NS-5 NSCs were derived from 46C embryonic stem cells and cultured, as described and regularly tested for mycoplasma contamination and for authenticity by expressed NSC markers Pax6, Sox2 and Nestin. The NSCs derived and cultured in this way have the capacity to...
differenicate into neurons and astrocytes, and still respond to signals to induce reversible quiescence. The NSC lines with stable expression of FLAG-V5-tagged Tcf4, Olig2 or Npas3 were created by electroporation with pcAG promoter-driven plasmids containing the appropriate cDNAs and puromycin selection for individual clones with moderate expression of the tagged proteins, as compared with endogenous levels. FLAG-tagged Tcf4, Olig2 or Npas3 was each purified from 1.5×10⁸ cells, equivalent to 2×10⁸ NSCs, by FLAG-affinity purification, as described. Two or three biologically independent purifications of each FLAG-tagged protein from separate NSC cultures and control purifications from separate parental NSC cultures were performed by the same experimenter(s). No samples of the above experiments were excluded. Identification of proteins by mass spectrometry was as described. Peptide spectra of purifications and control purifications of Npas3 and Tcf4 were searched against UniProt release 2012 update for protein identification. Interaction partner identification criteria are as described and applied in our previous publications. In short, a protein is included as interaction partner of a FLAG-tagged transcription factor if present in at least two of its purifications with a Mascot score of 50 or higher and at least threefold enriched by Mascot score over control purifications. The emPAI score, an estimate of the quantity of the identified protein in the purified protein sample, based on the number of peptide spectra identified by MS, normalized for the number of peptides that theoretically should be identifiable for that protein, is indicated for each identified protein in each experiment and average emPAI score is used to indicate the thickness of the edges in the interaction network in Figures 1a and 2a. Interaction network graphics were made with Cytoscape. Large-scale immunoprecipitations from 1 ml of nuclear extracts from NSCs or HEK293T cells were performed as described, using 10 μg Olig2 antibody (AB9610, Merck, Darmstadt, Germany, RRID:AB_10141047), 10 μg Sox2 antibody (Y-17, sc-17320) or 10 μg Npas3 antibody (HPA002892, Merck, RRID:AB_1079403). Each specific immunoprecipitation was performed once. The resulting western blots were probed with the same antibodies and Ep400 antibody (ab70301, Abcam, Cambridge, UK).

Chromatin immunoprecipitations A total 1.5×10⁸ NSCs were used per chromatin immunoprecipitation (ChIP). For Olig2 ChIP, NSCs were washed three times with phosphate-buffered saline, crosslinked with 1/10 volume of fresh 11% buffered formaldehyde solution for 12 min, quenched with 1/20 volume of 2.5 M glycine for 5 min, washed with ice-cold phosphate-buffered saline and the cell pellets frozen with N₂ (l) and resuspended and washed two times in ice-cold cell lysis buffer (10 mM Tris-Cl pH 7.5, 100 mM NaCl, 3 mM MgCl₂, 0.5% NP40 and 0.5% NaPO₄) and sonicated, as described. ChIP was performed, as described using 15 μg of Olig2 antibody (AB9610, Merck, RRID:AB_10141047) or rabbit IgG for the control ChIP. For Tcf4 ChIP, FLAG-V5-Tcf4 expressing NSCs were crosslinked with 2 ml disuccinimidyl glutarate (Thermo Fisher Scientific, Waltham, MA, USA) and 1% formaldehyde, nuclei isolated, chromatin prepared and ChIP performed, as described, with 20 μl V5-antibody agarose beads (Merck). DNA was eluted from the V5-beads, as described. The NSCs not expressing FLAG-V5-Tcf4 were used as a control. For Npas3 ChIP, NSCs were crosslinked with disuccinimidyl glutarate and formaldehyde and ChIP performed as described using 15 μg of Npas3 antibody (HPA002892, Merck, RRID:AB_1079403) or rabbit IgG as control, and 60 μl prot-G beads (GE Healthcare, Eindhoven, The Netherlands), without crosslinking the anti-body to the beads. Smad4 ChIP was on NSCs crosslinked with 2 mM disuccinimidyl glutarate and formaldehyde, with 15 μg of Smad4 antibody (R&D Systems, Minneapolis, MN, USA, AF2097, RRID:AB_355150) or goat IgG and 150 μl prot-G Dynabead solution (Life Technologies, Waltham, MA, USA), without crosslinking the antibody to the beads. Each ChIP-seq for a transcription factor was performed. Each ChIP DNA library preparation and ChIP sequencing on Illumina GAII or HiSeq2500 (San Diego, CA, USA) platforms was performed at the Erasmus MC Center for Biomics on a HiSeq2500 sequencer (Illumina) according to manufacturer’s instructions. The RNA samples were sequenced for 36 bp. RNA-seq was mapped to mouse reference NCBIg37.67 (mm9) using TopHat2 v2.0.11 with default settings and a segment length of 20. The aligned exon reads were normalized and differential expression was calculated using Bioconductor DESeq2 package in R. The Tcf4 target genes were defined as having at least a 1.5-fold change in expression (adjusted P-value ≤0.01) upon Tcf4 knockdown, at least one significant Tcf4 binding site (P-value ≤1×10⁻¹⁰) within 100 kb of its transcription start site and at least 0.1 RPMK expression in untreated NSCs. The RNA sequencing data are available through the Gene Expression Omnibus (NCBI), accession code GSE70872.

Gene regulation experiments The pSuper-puro constructs encoding Tcf4 short hairpin RNA (shRNA sequence: 5′-GACCTGGCAAGCTACACAG-3′), Tcf4 shRNA2 (5′-GGTTAGCGAAGTACCTTTC-3′), Smad4 shRNA (5′-GCTCGACGTCTAGGATG-3′) or Sox2 shRNA15 were electroporated into NSCs, as described, with puromycin (2 μg ml⁻¹) was added after 18 h and NSCs were collected for analyses at 44 h after electroporation. Three biologically independent electroporations were performed per condition. RNA-seq of untreated NSCs and NSCs transfected with Tcf4 shRNA construct or control shRNA (Omarhain, Eindhoven, The Netherlands) construct was performed in biologically independent triplicates. poly(A) RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany), tested for quality with the Bioanalyzer and prepared using the TruSeq RNA sample prep kit v2, as described. RNA-seq was performed on the Erasmus MC Center for Biomics on a HiSeq2500 sequencer (Illumina) according to manufacturer’s instructions. The RNA samples were sequenced for 36 bp. RNA-seq was mapped against mouse reference NCBIg37.67 (mm9) using TopHat2 v2.0.11 with default settings and a segment length of 20. The aligned exon reads were normalized and differential expression was calculated using Bioconductor DESeq2 package in R. For gene expression analysis, total RNA was isolated from cells using the RNAeasy protocol (Qiagen). cDNA was made with Superscript II reverse transcriptase and RT-PCR was performed on a DNA engine Opticon2/CFX96 (Bio-Rad, Hercules, CA, USA) and normalized on CalR expression. Each gene expression analysis consisted of three biologically independent experiments. The s.e.m. of these three experiments is shown in Figures 3d, 4b, d and Supplementary Figures 3a-c. No samples were excluded from experiments in this paragraph.
Figure 2. Overlap protein interaction network with constrained human genes. (a) Protein interaction network in neural stem cells. Green color indicates overlap of network protein or protein complex subunit(s) with a set of 1003 constrained genes in humans. (b) Percentage overlap of the indicated categories with constrained genes. Between brackets is the number of human genes equivalent to network proteins in each category (network protein genes) or total number of genes in each category (all genes). ASD, autism spectrum disorder; ID, intellectual disability; IQ, intelligence quotient; MD, mental disorder.
Data analysis

Known ID genes (528 genes), mutated in five or more ID patients were categorized in Gilissen et al., Supplementary Table 10. Genes with de novo non-synonymous mutations in patients with ASD with lower intelligence quotient (IQ; ≤90) and patients with ASD with normal IQ (>90) were published in Iossifov et al., Supplementary Table 7. Likely gene-disrupting mutations6 were classified as loss-of-function (LOF) mutations. Genes with LOF mutations in unaffected siblings and genes with only missense (not LOF) mutations in ASD patients and missense mutations in unaffected siblings were removed, as these are less likely to be relevant for ASD pathology, resulting in a list of 1584 genes. Males with ASD and lower IQ (≤90) and females with ASD (which nearly always have lower IQ)6,46 were classified as ASD with lower IQ (ASD-lowIQ). Males with ASD and normal IQ (>90) were classified as ASD-normIQ. Genes with de novo LOF and missense mutations in schizophrenia patients (662 genes, Supplementary Table 10) were categorized from literature.8,9,47,48 Frame-shift mutations, nonsense mutations and splice-site mutations (within two nucleotides) from the splice donor site or splice acceptor site9 were taken as LOF mutations. LOF mutations were calculated as a percentage of all de novo coding mutations (LOF+missense) in patients with ASD-lowIQ, ASD-normIQ or schizophrenia, either in genes with the equivalent mouse protein in the interaction network (network protein genes) or in the total mutation data sets (all genes). For known ID genes with the equivalent mouse protein in the interaction network, the type of mutation in the network transcription factor co-localization on the genome of neural stem cells. (a) Percentage overlap of genome-wide binding sites of pairs of transcription factors (TF pairs). Network transcription factors and percentage overlap of TF pair are indicated. (b) Transcription factors are both in the network (left) or one is not in the network (right). Each TF pair is indicated by a black dot, average overlap in each category is indicated by black bar. (c) Transcription factors are interacting (left) or not interacting (right). Each TF pair is indicated by a black dot, average overlap in each category is indicated by black bar. (d) Relative mRNA levels by RT-PCR of indicated genes in NSCs treated with the indicated shRNAs, s.e.m. of three independent experiments is indicated. (e) Binding site profile of indicated transcription factors and indicated histone modification profiles at Nrxn1. Ep300, H3K4me1 mark enhancers, H3K27ac marks active enhancers, arrows mark transcription factor co-localization. mRNA, messenger RNA; NSC, neural stem cell; shRNA, short hairpin RNA.
Figure 4. Gene regulation by Tcf4 and its interaction partners. (a) Gene Ontology (GO) analysis of putative Tcf4 target genes. Ranking is by Bonferroni-corrected p-value. P-value (as $-\log_{10}$) and number of Tcf4 target genes in each category is indicated. (b and d) Regulation of primary microcephaly genes by Tcf4 (b) or by Smad4 (d). Relative mRNA levels by RT-PCR of indicated genes in NSCs treated with the indicated shRNAs, s.e.m. of three independent experiments is indicated. (c) Interaction network of Tcf4 with network transcription factors that are associated with microcephaly. (e) Binding site profile of indicated transcription factors and indicated histone modification profiles at Mcph1 (upper panel) or Wdr62 (lower panel). Angpt2, internal to Mcph1 and not regulated by Tcf4, is not indicated. Ep300, H3K4me1 mark enhancers, H3K27ac marks active enhancers, arrows mark transcription factor co-localization. mRNA, messenger RNA; NSC, neural stem cell; shRNA, short hairpin RNA.
Table 1. Network proteins with mutations in the equivalent human gene in patients with a mental disorder

| Human homolog of network protein | ID | ASD-lowIQ | ASD-normIQ | Schizophrenia | Constrained gene | Severity mutation vs IQ in MD |
|----------------------------------|----|-----------|------------|--------------|------------------|-----------------------------|
| TCF4                             | LOF|           | Missense   | X            |                  | X                          |
| ZEB2                             | LOF|           | Missense   | X            |                  | >                          |
| SMC1A                            | Missense|         |            | X            |                  | X                          |
| SATB2                            | LOF|           |            | X            |                  | X                          |
| SOX2                             | LOF|           |            | X            |                  | X                          |
| CHD7                             | LOF|           | Missense   | X            |                  | X                          |
| RXF3                             | LOF|           | Missense   | X            |                  | >                          |
| HCFC1                            | LOF|           |            | Missense     |                  | X                          |
| CUL3                             | LOF|           | LOF        | X            |                  | >                          |
| SATB2                            | LOF|           |            | X            |                  | X                          |
| EMT1                             | LOF|           |            | X            |                  | X                          |
| SOX5                             | LOF|           |            | X            |                  | X                          |
| KANSL1                           | LOF|           | Missense   | Missense     |                  | >                          |
| TWIST1                           | LOF|           |            | Missense     |                  | X                          |
| KDM6A                            | LOF|           |            | Missense     |                  | X                          |
| NFIA                             | LOF|           | LOF        | Missense     |                  | X                          |
| SALL1                            | LOF|           |            | Missense     |                  | X                          |
| EHMT1                            | LOF|           |            | Missense     |                  | X                          |
| SMARCA4                          | Missense|         |            | Missense     |                  | X                          |
| SMARCA1                          | Missense|         |            | Missense     |                  | X                          |
| CUL4B                            | LOF|           |            | Missense     |                  | X                          |
| ADNP                             | LOF|           | Missense   | Missense     |                  | X                          |
| AHCDC1                           | LOF|           | Missense   | Missense     |                  | X                          |
| SETD2                            | LOF|           | Missense   | Missense     |                  | X                          |
| UBA2P2L                          | LOF|           | Missense   | Missense     |                  | X                          |
| UB5R                             | LOF|           | Missense   | Missense     |                  | X                          |
| BRC1                             | LOF|           |            | Missense     |                  | X                          |
| CNOT3                            | LOF|           |            | Missense     |                  | X                          |
| ILF2                             | LOF|           |            | Missense     |                  | X                          |
| CDC28                            | LOF|           |            | Missense     |                  | X                          |
| NACC1                            | LOF|           |            | Missense     |                  | X                          |
| SOX6                             | Missense|        | Missense   | Missense     |                  | X                          |
| ZNF219                           | Missense|        | Missense   | Missense     |                  | X                          |
| ALRJAP                           | Missense|        | Missense   | Missense     |                  | X                          |
| CHD4                             | Missense|        | Missense   | Missense     |                  | X                          |
| EP400                            | Missense|        | Missense   | Missense     |                  | X                          |
| NCO1                             | Missense|        | Missense   | Missense     |                  | X                          |
| NR2F1                            | Missense|        | Missense   | Missense     |                  | X                          |
| ZFHX3                            | Missense|        | Missense   | Missense     |                  | X                          |
| KDM1A                            | Missense|        | Missense   | Missense     |                  | X                          |
| RUVBL1                           | Missense|        | Missense   | Missense     |                  | X                          |
| HDAC1                            | Missense|        | Missense   | Missense     |                  | X                          |
| MBD2                             | Missense|        | Missense   | Missense     |                  | X                          |
| ROR2                             | Missense|        | Missense   | Missense     |                  | X                          |
| SMC3                             | Missense|        | Missense   | Missense     |                  | X                          |
| TCF3                             | Missense|        | Missense   | Missense     |                  | X                          |
| ETV6                             | LOF|           |            | Missense     |                  | X                          |

Abbreviations: ASD, autism spectrum disorder; ID, intellectual disability; IQ, intelligence quotient; LOF, loss of function; MD, mental disorder. Human equivalent genes of network proteins with mutations in patients with the indicated mental disorders are listed. Predominant type of gene mutations, LOF or missense, in ID patients is listed. Type and number (if more than one) of gene mutations in patients with ASD-lowIQ, patients with ASD-normIQ or patients with schizophrenia are listed. Overlap with a list of 1003 constrained genes in the human population is indicated. Network proteins with a missense mutation in the human equivalent gene in patients with ASD-normIQ or schizophrenia and a LOF mutation in patients with ID or ASD-lowIQ are marked by $. The opposite pattern is not observed.
majority of patients was assessed per gene and assigned in Table 1 as LOF or missense. Enrichment in the network of ID genes equivalent to network proteins was calculated over the expected value in case of a random overlap, which was corrected for protein length and expression in our NSCs by using average protein length from Ensembl genes of network proteins over NSC-expressed genes. Protein length was calculated from ensembl GRCm38 by counting the number of amino acids for each protein. A gene was regarded as expressed in our NSCs, if its expression was equal or above that of Zeb1 (0.127 RPKM in our RNA-seq data set), which is the network protein with the lowest messenger RNA (mRNA) expression in our NSCs. Genes significantly devoid of coding variants in the human population (1003 genes), also called constrained genes, were reported.49 Enrichment in the network of network proteins encoding constrained genes was calculated over the expected value in case of a random overlap, which was corrected for expression in our NSCs. Network-enrichment P-values for network proteins encoding known ID genes or constrained genes are obtained from two-sided binomial tests on the observed and expected values. Enrichments and enrichment P-values of de novo non-synonymous mutations in ASD patients, their healthy siblings or schizophrenia patients in human genes equivalent to network proteins were calculated by dnenrich,9 corrected for gene length, sequence context and expression of the mouse homolog in our neural stem cells. Equivalent human genes of network proteins were provided to the dnenrich program as ‘Gene set’ and human equivalents of genes expressed in mouse NSCs were provided as ‘Background list’.

Significance of differences in the percentages of proteins associated with ID, ASD-lowIQ, ASD-normIQ or SZ in the interactomes of Tcf4, Olig2, Npas3 and Sox2 and significance of differences in percentages LOF between network protein mutations in patients with ASD-lowIQ, ASD-normIQ or schizophrenia were calculated using Fisher’s exact test, as some of the expected counts in the contingency tables were below 5. To determine whether the percentages LOF in network protein mutations in patients with ASD-lowIQ, ASD-normIQ or schizophrenia were differently distributed than equivalent percentages LOF in the total mutation sets, we performed a permutation test by sampling 10 000 random subsets of 60 mutations; the number of mutations in patients with ASD or schizophrenia, identified in our interaction network. The resulting permutation P-value was calculated by dividing the number of observed P-values that were more significant than the P-value (0.002) for our interaction network (34 observations) by the total number of observations (10 000). Significance of differences in LOF percentages in mutations in ASD-lowIQ, ASD-normIQ and schizophrenia categories between network proteins and the total mutation data sets were calculated by Fisher’s exact test. Thirteen human primary microphygal genes (MCPH1, WDWR62, ASPM, CASC5, CENPJ, CDKRAP1, CEP135, CEP152, STIL, CDK6, ZNF533, PHC1) are known,63 which were overlapped with Tcf4 target genes (see below). Microphygal genes were retrieved from the Online Mendelian Inheritance in Man (http://www.omim.org) database by scoring for genes in which mutations cause human monogenic conditions or syndromes that include microphygal.

RESULTS
Identification of a protein interaction network in NSCs
We recently improved the FLAG-tag affinity protocol to purify transcription factors and their interacting proteins with high efficiency and low background.12,15 The accuracy of interaction partner identification by this protocol was extensively validated by independent immunoprecipitations.12,15 Importantly, many identified interaction partners were shown by us (Supplementary Table 1) and others (Supplementary Table 2) to be biologically relevant and uncover novel functions of the target protein or provide insight into the molecular cause of malformations associated with human syndromes.15 Here, we applied this protocol to purify transcription factors Tcf4, Olig2 and Npas3 from mouse NSCs. Tcf4, Olig2 and Npas3 have relatively high endogenous expression levels, as compared with the median expression level in our NSCs, determined by RNA-seq (Supplementary Table 3). NSC lines with stable expression of FLAG-tagged Tcf4, Olig2 or Npas3 (Supplementary Figure 1) were grown to large scale and two or three independent purifications of the FLAG-tagged proteins were performed. Interacting proteins, identified by mass spectrometry, present in at least two purifications of the target protein were included (Supplementary Tables 4, see the ‘Materials and methods’ section for inclusion criteria) and combined with the interaction partners of previously purified Sox2 (ref. 15; Supplementary Table 7). This resulted in a protein interaction network of 206 proteins and 401 protein–protein interactions (Figure 1a, Supplementary Tables 8 and 9), including 13 protein–protein interactions that were previously shown to be biologically relevant (Supplementary Table 9). The interaction network contains multiple chromatin modifying complexes, such as NuRD, SWI-SNF and Ncor, and transcription factors such as Rfx3 and Salt3 that interact with all four purified transcription factors (Figure 1a). However, other identified interaction partners were found to be specific for one purified transcription factor, such as Ascl1, Neurod1, Kdm6a (Tcf4), Satb2, Yy1, Adreh (Olig2), Amt2, Lmo7 (Npas3) and Xpo4 (Sox2) (Figure 1a). We confirmed by immunoprecipitations the interaction of endogenous Olig2 with Sox2 (Supplementary Figure 2a) and the interaction of endogenous Npas3 with Sox2 and Ep400 (Supplementary Figure 2b).

The interaction network is enriched for proteins mutated in patients with ID, ASD or schizophrenia and proteins significantly lacking coding variation in humans
We investigated whether the proteins in our interaction network are associated with ID, ASD or schizophrenia. To our knowledge, the most extensive and best validated list of genes associated with (often syndromic) ID is a list of 528 ‘known ID genes’,5 which are mutated in at least five ID patients. We find 26 network proteins encoded by ID genes (Figure 1a, Table 1). Taking into account only genes expressed in our NSCs and correcting for protein length, a random overlap would give 9.6 proteins encoded by ID genes in the network. We therefore find a 2.7-fold enrichment of proteins encoded by ID genes in the network, over the expected value (enrichment P-value 7.2 × 10−4).

To probe for genes in which mutations are unambiguously associated with (non-syndromic) ASD or schizophrenia is a difficult task as few such genes are currently identified. Therefore, as a source for candidate ASD genes, we used a recent large exome sequencing study in 2500 ASD families,6 which has the additional benefit to separate ASD patients by low IQ (≤90, ASD-lowIQ) or normal IQ (>90, ASD-normIQ). The study identified de novo LOF (frameshift, nonsense, splice-site) mutations or missense mutations in 1584 genes specifically in patients with ASD-lowIQ or ASD-normIQ.6 We find that the interaction network contains 38 proteins encoded by such putative ASD-associated genes with, in total, 42 mutations (Figure 1a, Table 1). A random overlap with the network, corrected by dnenrich9 for gene length, sequence context and expression in neural stem cells, would expect 31.5 of such mutations in the network (enrichment P-value 0.04). Mutations in unaffected siblings (24 mutations observed in the network; 22.5 expected, P-value 0.40) are not enriched in the network. To overlap with schizophrenia candidate genes, we merged mutation data from four exome sequencing studies8,47,48 and curated a list of 662 genes with LOF or missense de novo mutations in schizophrenia patients (Supplementary Table 10). We find 18 network proteins encoded by putative schizophrenia-associated genes with 18 mutations (Figure 1a, Table 1), where the corrected expectation would be an overlap of 12.2 mutations (enrichment P-value 0.07). In total, the network contains 68 proteins mutated in patients with ID, ASD and/or schizophrenia and these 68 MD-associated proteins have 260 interactions with other proteins in the network (Figure 1a, Table 1, Supplementary Tables 4). We identified 47 interactions between ID-associated network proteins and network proteins mutated in patients with ASD or schizophrenia (Figure 1a, Supplementary Table 11). We do not find significant differences in the percentages of proteins
associated with ID, ASD or schizophrenia between the interactomes of starting transcription factors Tcf4, Olig2, Npas3, Sox2 (P-value > 0.2 for all MD categories, Figure 1b). Together, our results show that the network is enriched for MD-associated proteins and that the different categories of MD-associated proteins appear homogenously distributed in the network.

To have an indication of the potential of our interaction network to contain yet undiscovered MD proteins, we overlapped our network with a recently reported list of 1003 genes that are significantly devoid of missense variants in the human population49 (Figure 2a). These genes are likely to be evolutionarily constrained and intolerant to mutation. Indeed, highly constrained genes were found to be far more often associated with dominant Mendelian disease than genes with an average constraint.49 Mutations in the above set of 1003 constrained genes were found to be overrepresented in ASD patients.49 Accordingly, we find that genes mutated in patients with ID, ASD-lowIQ, ASD-normIQ or schizophrenia are between two- and fourfold enriched for this set of constrained genes (Figure 2b). We find that a quarter (52 proteins) of the proteins in our interaction network overlap with the set of constrained genes (Figures 2a and b, Supplementary Table 8), a 4.3-fold enrichment over an NSC expression-corrected random expectation (12.2 proteins, P-value 1.9 × 10−12). Proteins encoded by constrained genes are still enriched in the network after removal of MD-associated proteins (21 observed, 7.9 expected, P-value 6.7 × 10−5, Figure 2b). Remarkably, in each of the four MD categories, around 50% of the network proteins with mutations in MD patients are encoded by constrained genes (Figure 2b, Supplementary Table 8). The observed enrichments suggest that network proteins, in particular, those encoded by constrained genes, would be good candidates for mutation screening in patients to identify novel MD genes.

Mutation severity in network proteins correlates with cognition levels in the associated MD.

Cognitive ability is more affected in patients with ASD-lowIQ than in patients with ASD-normIQ or schizophrenia. We were interested whether mutation severity in network proteins would correlate with cognitive ability, with LOF mutations, on average, affecting gene function more severely than missense mutations. We used mutations from our data sets on patients with ASD-lowIQ, ASD-normIQ and schizophrenia, which are three comparable sets of patients and their parents. We find that 43% of the network protein mutations in patients with ASD-lowIQ are LOF mutations, which is 2.5-fold higher than in network protein mutations in ASD-normIQ patients (17% LOF) and nearly fourfold higher than in network protein mutations in schizophrenia patients (11% LOF; Figure 1c, Table 1). The differences in %LOF in network protein mutations between the three MD categories are significant (P-value = 0.002). %LOF in the total mutation data sets were also different between ASD-lowIQ (24% LOF), ASD-normIQ (18% LOF) and schizophrenia (15% LOF; Figure 1c) but the fold differences were less distinct. Indeed, we find that percentages LOF in our network are differently distributed than percentages LOF in equally sized subsets from the total mutation sets (P = 0.0034, 10 000 permutations). The difference in mutation distribution between the network and the total data sets is mostly due to the different LOF percentages in ASD-lowIQ (Figure 1c). We find that LOF mutations are significantly overrepresented in the ASD-lowIQ category in our network, as compared with the total mutation set (odds ratio 4.2, P-value 1.5 × 10−4), whereas this is not the case for ASD-normIQ (odds ratio 0.94, P-value 1) and schizophrenia (odds ratio 0.72, P-value 1).

One explanation for the exaggerated fold differences in percentages LOF between MD categories within the network could be that mutations in network protein genes more likely contribute to the MD, as supported by the high overlap of mutated network proteins with constrained genes (Figure 2b), a category of genes in which mutations more often cause disease.49 In this scenario, the total sets of mutations in the different MD categories (Figure 1c) would contain higher frequencies of non-contributing mutations, which by definition will not have a severity bias between the different MD categories. We also find a mutation bias in network proteins with multiple de novo mutations, associated with different MDs; six network proteins have missense mutations in patients with ASD-normIQ or schizophrenia and LOF mutations in patients with ID or ASD-lowIQ, whereas the opposite pattern is not observed (Table 1). Together, this suggests that particularly in network proteins, the severity of mutations increases in MDs with a low IQ.

Network transcription factors preferentially co-localize on the genome and cooperate in disease-relevant gene regulation

Having identified an interaction network containing MD-related proteins, we investigated whether network proteins preferentially overlap in their genome-wide binding sites, as a proxy for their cooperation in gene regulation.13,52 We determined the genome-wide binding sites in NSCs for network transcription factors Tcf4, Olig2, Smad4 and Npas3 by ChIP-seq and added published data for Chd7, Sox2, Ascl1 and Ep300. We also included published data on Brn2 and Max, two transcription factors with NSC expression levels similar to the tested network transcription factors (Supplementary Table 3) but not part of our interaction network. We find that overlaps in the top 5000 binding sites between transcription factors within the network are on average higher than with Max or Brn2 (Figures 3a and b). Overlaps above 30% are only observed within the network (Figures 3a and b) and overlaps above 35% are only observed between network transcription factors that interact with each other (Figures 3a, c and 1a).

We subsequently explored whether the interaction network can provide gene regulatory explanations for disease overlap. We performed RNAi-mediated knockdown of Tcf4 in NSCs, shortly followed by RNA sequencing. Identified Tcf4 target genes, which are misregulated on Tcf4 knockdown and binding by Tcf4 (Supplementary Table 12), include 71 ID genes, 210 genes de novo mutated in ASD patients and 85 genes de novo mutated in schizophrenia patients and include well-known MD genes Foxp2, Shank3 and Syngap1 (Supplementary Table 13). We find that Tcf4 maintains the expression of Nrxn1 and binds to several active enhancers in the Nrxn1 gene (Figures 3d and e, Supplementary Table 12). Patients with compound heterozygous mutations in Nrxn1 suffer from Pitt Hopkins-like syndrome.53 Regulation of Nrxn1 by Tcf4 provides a mechanistic explanation for the strong phenotypic overlap in patients with mutations in any of these two genes. Tcf4 and its interactor Sox2 regulate and co-localize on ID genes Gpr56, Tgfbr2 and Gli2 (Supplementary Figures 3a–d). Disruption of the regulation of GPR56 by RFX proteins causes cerebral cortex patterning defects and ID.54 Rfx proteins interact with Tcf4 and Sox2 (Figure 1a), suggesting their cooperative regulation of Gpr56.

Congenital or acquired microcephaly occurs in the majority of patients with Pitt Hopkins syndrome, caused by Tcf4 mutations.55 We found that target genes activated by Tcf4 have the highest Gene Ontology term enrichments for gene categories such as Cell cycle, M-phase, Chromosome centromeric region and Cell division (Figure 4a), related categories that are often affected in primary microcephaly.50 Indeed, Tcf4-activated target genes include 6 of the 13 known primary microcephaly genes (Supplementary Table 13, Figure 4b) and we find that Tcf4 also maintains the expression of primary microcephaly genes Cenpj and Cdk5rap2 (Figure 4b). Moreover, Tcf4 protein interacts with 10 transcriptional regulators
associated with microcephaly, including Smad4 (Figures 1a and 4c). Smad4, like Tcf4, regulates primary microcephaly genes (Figure 4d). Tcf4 binds together with microcephaly-associated transcription factors Smad4, Sox2, Chd7 and Ep300 to active enhancers at primary microcephaly genes Mcph1 and Wdr62 (Figure 4e). In conclusion, we identify a regulatory network in NSCs related to microcephaly, which may explain the association of this condition with the participating proteins.

**DISCUSSION**

A transcription factor interaction network in NSCs enriched for MD-associated proteins

Here we believe we describe the first transcription factor interaction network in a neural system. We find that the network is enriched for proteins associated with ID, ASD or schizophrenia. Accordingly, we provide a description of the molecular environment of such proteins, often for the first time, in a cell type highly relevant for neurodevelopment and its diseases. We carried out our studies in mouse NSCs, as the necessary scale of our proteomics and ChiP-seq experiments would be difficult to perform using human NSCs. Nevertheless, a recent comprehensive comparison of transcriptional networks and transcription factor target genes in mouse and human shows high inter-species conservation, making our work relevant for the human situation.

The enrichment in the network was highest for a set of established ID proteins. Enrichments were lower for ASD-associated and schizophrenia-associated mutations, which is possibly due to their origin from sets of de novo mutations in ASD or schizophrenia patients, where causality is less certain. Protein mutations in these sets that do not contribute to disease would not be enriched in the network but would increase the expected mutation score, reducing the observed enrichments. The network is highly enriched for proteins encoded by genes significantly lacking coding variation in the human population, a set of ~1000 constrained genes that is more frequently associated with disease, including ASD. The enrichment for constrained genes would suggest that the network has an above-average content of yet-to-be-discovered MD proteins. Indeed, recently three additional bona fide ID genes were discovered, Rlim, Zbtb20 and Jmjd1c, that are encoding network proteins and can be added to 26 ID proteins in the network from the overlap with the 2014 ID gene list.

Genome co-localization and cooperation in gene regulation by proteins in the network

Transcription factors that interact are more likely to cooperate in gene regulation. Another proxy for cooperation in gene regulation is co-localization on the genome. For example, we previously showed that Sox2 and its interaction partner Chd7 have a high binding-site overlap on the genome and indeed Sox2 and Chd7 have a large overlap (50%) in regulated genes. Accordingly, to have an additional indication that network proteins preferentially cooperate with each other in gene regulation, we showed that eight network transcription factors have, on average, more overlap in binding sites with each other than with two transcription factors, Bmi2 and Max, that are expressed in NSCs but are not part of the network. Tcf4 binds with a number of its interaction partners to primary microcephaly genes and (at least) Tcf4 and Smad4 also regulate such genes, providing an explanation for their shared microcephaly phenotype. In conclusion, our interaction network shows features of a transcriptional network, where proteins can cooperate to regulate disease-relevant genes.

Mutation severity in network proteins correlates with IQ levels in the associated MD

We wondered why in our interaction network some mutations occur in patients with ASD-lowIQ, whereas others occur in patients with ASD-normIQ or schizophrenia, MDs without obligatory loss of IQ. One hypothesis would be that severe mutations disrupt the network more and have a worse outcome for IQ levels. Mutation severity within a network of interacting proteins has not been analyzed yet in relation to cognition levels. We find that %LOF rates in our interaction network of transcriptional regulators increase several fold from schizophrenia or ASD-normIQ, two MDs without IQ loss, to ASD-lowIQ, a significantly different distribution than in the total mutation data sets from which they originate (Figure 1c). In addition, in network proteins with multiple mutations across different MDs, mutation severity follows MD severity. A recent data analysis using de novo mutations across all genes in severe ID patients (IQ < 50), ASD patients and schizophrenia patients showed an increase in %LOF from 15% in schizophrenia patients, to 17% in ASD patients, to 24% in severe ID patients. These LOF percentages for ASD and schizophrenia do not deviate much from those in the total mutation data sets that we used, suggesting that also here %LOF increase with lower cognition is more modest than in the network. Interestingly, %LOF for network proteins associated with ASD-lowIQ is nearly twice the %LOF value in the set of de novo mutations in severe ID patients, a group with a more severe cognitive deficit. One has to take here into account that in these studies severe ID patients were counter selected for the occurrence of congenital anomalies, which likely impacts on the set of detected de novo gene mutations and %LOF. We argued above that the exaggerated increase of average mutation severity with lower IQ in the network may be caused by mutations often being in network proteins encoded by constrained genes and therefore more likely to be causal. This would imply that network mutations and their LOF percentages provide a better reflection of the real mutation spectra associated with the different MD categories than can currently be obtained from the total mutation data sets.

There are a number of limitations to our study. We investigated only the 68 proteins mutated in MD patients present in our network, whereas it is estimated that mutations in several hundreds of proteins may contribute to mental disorders. Therefore, our protein set represents only a fraction of the estimated number of MD genes. For ASD and schizophrenia, most of the included proteins are not necessarily causally linked to disease, as they were observed in a single patient. Although most MD-mutated transcriptional regulators are highest expressed in NSCs, we cannot exclude that they are relevant for disease in other stages of brain development, such as neuronal maturation, where our network may be less relevant.

Our data are consistent with a scenario where the level of in vivo disruption of a shared transcriptional network correlates with the level of cognitive dysfunction in the associated MDs. Our interaction network contains only a fraction of the total number of transcriptional regulators believed to be associated with ID, ASD or schizophrenia, but there is no a priori reason to assume that its principles would not apply to a larger network of MD-related transcriptional regulators. A shared underlying transcriptional network is in line with the significant comorbidity observed between ID, ASD and schizophrenia.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGMENTS**

We thank Richard Festenstein, Frank Grosveld and Danny Huylenbroeck for comments on the manuscript and Erik Engelen and Mike Dekker for technical assistance. MJM was supported by a grant from the Erasmus MC Stem Cell Institute, JHB was supported by an ALW-open program grant (no 821.02.004) from the Netherlands.
Organisation for Scientific Research (NWO) and RAP by a grant from the Dutch government to the Netherlands Institute for Regenerative Medicine (NIRM, grant no. FES0908) and the DevRepair (P7/07) IAP-VII network. DHWD and JD were funded by The Netherlands Proteomics Centre (Project Number 184.032.201), financed by NWO.

**AUTHOR CONTRIBUTIONS**

MJM performed plasmid construction, protein purifications, immunoprecipitations, ChIP experiments and gene regulation experiments. HHHA performed plasmid construction, protein purifications, proteomics data categorization and statistics. JHB processed ChiP-seq and RNA-seq data and performed bioinformatics analyses and statistics. DHWD and JD performed the mass spectrometry analyses. UA and SK performed plasmid construction and protein purifications. MQ performed immunoprecipitations. CEMK, ZO and WFW performed labeling and Illumina sequencing of ChIP and RNA material. RAP conceived the study and designed the experiments. RAP wrote the manuscript with help from co-authors.

**REFERENCES**

1. Association AP. DSM Diagnostic and Statistical Manual of Mental Disorders. APA: Arlington, TX, USA, 2013.
2. Ropers HH. Genetics of early onset cognitive impairment. Annu Rev Genomics Hum Genet. 2010; 11: 161–187.
3. Mefford HC, Batshaw ML, Hoffman EP, Genomics, intellectual disability, and autism. N Engl J Med 2014; 366: 733–743.
4. Schizophrenia Working Group of the Psychiatric Genomics C. Biological insights from 108 schizophrenia-associated genetic loci. Nature 2014; 511: 421–427.
5. Gilissen C, Heirh-Kwa JY, Thung DT, van de Vorst M, van Bon BW, Willemsen MH et al. Genome sequencing identifies major causes of severe intellectual disability. Nature 2014; 511: 344–347.
6. Liss fussof I, OR’ ORoak B, Sanders SJ, Ronemus M, Krumm N, Levy D et al. The contribution of de novo coding mutations to autism spectrum disorder. Nature 2014; 515: 216–221.
7. De Rubies S, He X, Goldberg AP, Poultney CS, Samocha K, Cicke AE et al. Synthetic, transcriptional and chromatin genes disrupted in autism. Nature 2014; 515: 209–215.
8. Gulisiner S, Walsh T, Watts AC, Lee MK, Thornton AM, Casadei S et al. Spatial and temporal mapping of de novo mutations in schizophrenia to a fetal prefrontal cortical network. Cell 2013; 154: 518–529.
9. Fromer M, Pocklington AJ, Kavanagh DH, Williams HJ, Dwyer S, Gormley P et al. De novo mutations in schizophrenia implicate synaptic networks. Nature 2014; 506: 179–184.
10. Sahin M, Sur M. Genes, circuits, and precision therapies for autism and related neurodevelopmental disorders. Science 2015; 350: 6263.
11. Ronan JL, Wu W, Crabtree GR. From neural development to cognition: unexpected roles for chromatin. Nat Rev Genet 2013; 14: 347–359.
12. van den Berg DL, Snoek T, Mullin NP, Yates A, Beztazaroi K, Demmers J et al. An Oct4-centered protein interaction network in embryonic stem cells. Cell Stem Cell 2010; 6: 369–381.
13. Chen X, Xu H, Yuan P, Fang F, Huss M, Vega VB et al. Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell 2008; 133: 1106–1117.
14. Kim J, Chu J, Shen X, Wang J, Orkin H. An extended transcriptional network for pluripotency of embryonic stem cells. Cell 2008; 132: 1049–1061.
15. Engelen E, Akinci U, Bryne JC, Hooi J, Gontan C, Moen MJ et al. Sox2 cooperates with ChiP to regulate genes that are mutated in human syndromes. Nat Genet 2011; 43: 607–611.
16. Parikhshak NN, Luo R, Zhang A, Won H, Lowe JK, Chandran V et al. Characterization of the neural stem cell gene regulatory network identifies OLIG2 as a multifunctional regulator of self-renewal. Genome Res 2015; 25: 41–56.
17. Pieper AA, Wu X, Han TW, Estill SJ, Dang Q, Wu LC et al. The neuronal PAS domain protein 3 transcription factor controls FGF-mediated adult hippocampal neurogenesis in mice. Proc Natl Acad Sci USA 2005; 102: 14052–14057.
18. Graham V, Khudyakov J, Ellis P, Pevny L. SOX2 functions to maintain neural progenitor identity. Neuron 2003; 39: 749–765.
19. Zweier C, Peippo MM, Hoyer J, Sousa S, Bottani A, Clayton-Smith J et al. Haplinsufficiency of TCF4 causes syndromal mental retardation with intermingled hyperventilation (Pitt-Hopkins syndrome). Am J Hum Genet 2007; 80: 994–1001.
Translational Psychiatry (2017), 1 – 12

Mental disorder protein network in neural stem cells
MJ Moen et al

48 Xu B, Ionita-Laza I, Roos J, Boone B, Woodrick S, Sun Y et al. De novo gene mutations highlight patterns of genetic and neural complexity in schizophrenia. Nat Genet 2012; 44: 1365–1369.

49 Samocha KE, Robinson EB, Sanders SJ, Stevens C, Sabo A, McGrath LM et al. A framework for the interpretation of de novo mutation in human disease. Nat Genet 2014; 46: 944–950.

50 Faheem M, Naseer MI, Rasool M, Chaudhary AG, Kumosani TA, Ilyas AM et al. Molecular genetics of human primary microcephaly: an overview. BMC Med Genomics 2015; 8: 54.

51 Mizrak GM, Vitre B, Carpenter G, Abramowicz I, Gleeson JG, Paciorkowski AR et al. Mutations in CENPE define a novel kinetochore-centromeric mechanism for microcephalic primordial dwarfish. Hum Genet 2014; 133: 1023–1039.

52 Ouyang Z, Zhou Q, Wong WH. ChiP-Seq of transcription factors predicts absolute and differential gene expression in embryonic stem cells. Proc Natl Acad Sci USA 2009; 106: 21521–21526.

53 Zweier C, de Jong EK, Zweier M, Ousager LB, Collins AL et al. CNTNAP2 and NRXN1 are mutated in autosomal-recessive Pitt-Hopkins-like mental retardation and determine the level of a common synaptic protein in Drosophila. Am J Hum Genet 2009; 85: 655–666.

54 Bae BL, Tietjen I, Atabay KD, Evrony GD, Johnson MB, Asare E et al. Evolutionarily dynamic alternative splicing of GPR56 regulates regional cerebral cortical patterning. Science 2014; 343: 764–768.

55 Zweier C, Sticht H, Bijlsma EK, Clayton-Smith J, Boonen SE, Fryer A et al. Further delineation of Pitt-Hopkins syndrome: phenotypic and genotypic description of 16 novel patients. J Med Genet 2008; 45: 738–744.

56 Yue F, Cheng Y, Breschi A, Vienstra J, Wu W, Ryba T et al. A comparative encyclopedia of DNA elements in the mouse genome. Nature 2014; 515: 355–364.

57 Tonne E, Holdhuis R, Stansberg C, Stray-Pedersen A, Petersen K, Brunner HG et al. Syndromic X-linked intellectual disability segregating with a missense variant in RLIM. Eur J Hum Genet 2015; 23: 1652–1656.

58 Coreddu V, Redeker B, Stellacci E, Jongejans A, Fragale A, Bradley TE et al. Mutations in ZBTB20 cause Primrose syndrome. Nat Genet 2014; 46: 815–817.

59 Hu H, Haas SA, Chelly J, Van Esch H, Raynaud M, de Brouwer AP et al. X-exome sequencing of 405 unresolved families identifies seven novel intellectual disability genes. Mol Psychiatry 2016; 21: 133–148.

60 Saez MA, Fernandez-Rodriguez J, Moutinho C, Sanchez-Mut JV, Gomez A, Vidal E et al. Mutations in JMJ1D1C are involved in Rett syndrome and intellectual disability. Genet Med 2016; 18: 378–385.

61 de Ligt J, Willemsen MH, van Bon BW, Kleefstra T, Yntema HG, Kroes T et al. Diagnostic exome sequencing in persons with severe intellectual disability. N Engl J Med 2012; 367: 1921–1929.

62 Rauch A, Wexzerek D, Graf E, Wieland T, Endele S, Schwarzmayr T et al. Range of genetic mutations associated with severe non-syndromic sporadic intellectual disability: an exome sequencing study. Lancet 2012; 380: 1674–1682.

63 Iossifov I, Ronemus M, Levy D, Wang Z, Hakker I, Rosenbaum J et al. De novo gene disruptions in children on the autistic spectrum. Neuron 2012; 74: 285–299.

64 Sanders SJ, Murtha MT, Gupta AR, Murdoch JD, Raubeson MJ, Willsey AJ et al. De novo mutations revealed by whole-exome sequencing are strongly associated with autism. Nature 2012; 485: 237–241.

65 Neale BM, Kou Y, Liu L, Ma’ayan A, Samocha KE, Sabo A et al. Patterns and rates of exonic de novo mutations in autism spectrum disorders. Nature 2012; 485: 242–245.

66 Kohane IS, McMurry A, Weber G, MacFadden D, Rappaport L, Kunkel L et al. The co-morbidity burden of children and young adults with autism spectrum disorders. PLoS ONE 2012; 7: e33224.

67 Desai S, Shukla G, Goyal V, Srivastava A, Srivastava MV, Tripathi M et al. Changes in psychiatric comorbidity during early postsurgical period in patients operated for medically refractory epilepsy—a MINI-based follow-up study. Epilepsy Behav 2014; 32: 29–33.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2017

Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)