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Neuronal PAS Domain Proteins 1 and 3 Are Master Regulators of Neuropsychiatric Risk Genes

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Abstract

BACKGROUND: *NPAS3* has been established as a robust genetic risk factor in major mental illness. In mice, loss of neuronal PAS domain protein 3 (NPAS3) impairs postnatal hippocampal neurogenesis, while loss of the related protein NPAS1 promotes it. These and other findings suggest a critical role for NPAS proteins in neuropsychiatric functioning, prompting interest in the molecular pathways under their control.

METHODS: We used RNA sequencing coupled with chromatin immunoprecipitation sequencing to identify genes directly regulated by NPAS1 and NPAS3 in the hippocampus of wild-type, *Npas1*$^{-/-}$, and *Npas3*$^{-/-}$ mice. Computational integration with human genetic and expression data revealed the disease relevance of NPAS-regulated genes and pathways. Specific findings were confirmed at the protein level by Western blot.

RESULTS: This is the first in vivo, transcriptome-scale investigation of genes regulated by NPAS1 and NPAS3. These transcription factors control an ensemble of genes that are themselves also major regulators of neuropsychiatric function. Specifically, *Fmr1* (fragile X syndrome) and *Ube3a* (Angelman syndrome) are transcriptionally regulated by NPAS3, as is the neurogenesis regulator *Notch*. Dysregulation of these pathways was confirmed at the protein level. Furthermore, NPAS1/3 targets show increased human genetic burden for schizophrenia and intellectual disability.

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CONCLUSIONS: Together, these data provide a clear, unbiased view of the full spectrum of genes regulated by NPAS1 and NPAS3 and show that these transcription factors are master regulators of neuropsychiatric function. These findings expose the molecular pathophysiology of NPAS1/3 mutations and provide a striking example of the shared, combinatorial nature of molecular pathways that underlie diagnostically distinct neuropsychiatric conditions.

Keywords

Gene expression; Hippocampus; Neurogenesis; Psychiatric genetics; Transcriptional regulation; Transcription factors

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Neuronal PAS domain protein 1 (NPAS1) and NPAS3 (1,2), members of the neuronal PAS (Per-Arnt-Sim) domain transcription factor (TF) family (3–5), are prominently expressed in the brain with conserved structure for multiligand binding, lending them to physiologic and potentially pharmacologic regulation (6). *NPAS3* aberrations are implicated in many neuropsychiatric conditions, including schizophrenia (7–12), bipolar disorder (9,13,14), major depression (13,15), attention-deficit/hyperactivity disorder (15), and intellectual disability (10,16,17). *NPAS3* is also one of the fastest evolving human genes (18,19), and one of 27 genes exhibiting recurrent double-strand DNA break clusters in neural stem cells, a measure of gene fragility related to neuropsychiatric disorders (20). Preclinical models show that NPAS3 and NPAS1 affect physiologic processes relevant to neuro-psychiatric disease (21–23), including support of postnatal hippocampal neurogenesis by NPAS3 (24) and inhibition by NPAS1 (25). Postnatal hippocampal neurogenesis is altered in many neuropsychiatric conditions (14,24,26–33).

Despite the importance of NPAS1 and NPAS3, little is known about their target genes. Most of the current literature comprises hypothesis-driven, low-throughput studies (21,22,24,25,34) that do not capture the full extent of NPAS1/3-dependent regulation. While there is one published highthroughput (microarray) investigation of gene expression in NPAS3-overexpressing HEK293 cells (35), correlative in vivo testing was not conducted. NPAS-dependent pathophysiology may be caused by a completely novel molecular pathway, or it may represent a combinatorial effect of perturbing known pathways already linked to neuropsychiatric conditions. These possibilities are not mutually exclusive, given the broad role for NPAS1/3 in neuropsychiatric disease. Thus, full understanding of the regulatory programs of these TFs in the brain is critical.

To comprehensively characterize NPAS1/3 regulatory targets, we quantified gene expression by RNA sequencing (RNAseq) in wild-type (WT) and mutant (*Npas1*$^{-/-}$ or *Npas3*$^{-/-}$) hippocampus (Figure S1 in Supplement 1). We also determined genomic sites of regulatory binding of NPAS1/3 by chromatin immunoprecipitation sequencing (ChIP-seq) in mutant and WT hippocampus. The goal was to identify genes showing transcriptional perturbation in mutant animals as well as proximal binding of the respective TF in WT animals and thus establish putative NPAS1/3 regulatory targets that could be validated at the protein level.

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METHODS AND MATERIALS

Details regarding RNA-seq analysis, ChIP-seq analysis, network analysis, human genetic analysis, and Western blotting are provided in Supplement 1.

Animals

For RNA-seq experiments, 12-week-old male ($n = 10$ each) *Npas3*$^{-/-}$, *Npas1*$^{-/-}$, or WT littermate mice on C57BL/6 background were used. All mice were maintained under temperature-controlled (22°C–23°C), light-controlled (12-hour light cycle from 6 AM to 6 PM), and humidity-controlled (40%–60%) conditions with free access to food and water. Animals were sacrificed at the same time during the light period of the light/dark cycle (3 PM). All work was approved by University of Iowa Institutional Animal Care and Use Committee. Before brain dissection, animals were euthanized according to American Veterinary Medical Association guidelines (36).

RNA Preparation and Sequencing

Total RNA was prepared from murine whole hippocampus using the Ambion mirVana miRNA Isolation Kit (AM1560; Invitrogen, Waltham, MA) and quantified using the Agilent 2100 Bioanalyzer nanochip (Agilent Technologies, Santa Clara, CA). Mean RNA integrity number for the samples was 9.2 (SD 0.5). Sixty libraries were prepared using the Illumina TruSeq Stranded Total RNA with Ribo-Zero gold sample preparation kit (Illumina, Inc., San Diego, CA), using 500 ng of input total RNA. Libraries were run on the Agilent 2100 Bioanalyzer High Sensitivity kit (Agilent Technologies) and combined in equimolar concentrations into three pools based on index color balance. Concentration of pools was measured with the KAPA Illumina Library Quantification Kit (KAPA Biosystems, Wilmington, MA). Pools were sequenced on Illumina HiSeq 2000 and 2500 sequencers with 100-bp Paired-End v3 SBS chemistry (Illumina) at the Iowa Institute of Human Genetics.

Chromatin Immunoprecipitation

Chromatin was prepared from murine whole hippocampus (12-week-old WT, 79.3 mg [2 females, 1 male]; *Npas1*$^{-/-}$, 78.6 mg [1 female, 2 males]; *Npas3*$^{-/-}$, 74 mg [3 females]). Figure S7 in Supplement 1 shows that different sex distributions within each group had minimal effect on ChIP-seq peak call rate on sex chromosomes. ChIP was performed using anti-NPAS1 (ab183655; Abcam, Cambridge, MA), anti-NPAS1 (sc-482; Santa Cruz Biotechnology, Santa Cruz, CA), and anti-NPAS3 (PA5–20365; Thermo Scientific, Waltham, MA) antibodies, using 14–15 mg of tissue. Tissue was diced into approximately 1-mm^3 pieces with a razor blade on ice with cold phosphate-buffered saline (supplemented with phenylmethylsulfonyl fluoride), washed in cold phosphate-buffered saline (supplemented with phenylmethylsulfonyl fluoride), and resuspended in cold 1× Covaris fixing buffer A (PN520083; Covaris, Inc., Woburn, MA). Tissue was then fixed in 1% formaldehyde (28906; Thermo Scientific) for 10 minutes, followed by quenching with 0.75 mol/L Tris (pH 7) for 5 minutes at room temperature. Cross-linked tissue was cryofractured twice at setting 5 using a cryoPREP Impactor (CP02; Covaris, Inc.). Chromatin was isolated and sheared according to the Covaris truChIP Tissue protocol (PN 520083; Covaris, Inc.). Cells and tissue were disrupted and extracted in Covaris lysis buffer, washed with Covaris

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wash buffer, and sheared in Covaris shearing buffer with a Covaris E220 instrument (Covaris, Inc.). Shearing was performed in microTUBEs (PN520045; Covaris, Inc.) for 10 minutes with 2% acoustic duty factor, 105 W peak incident power, and 200 cycles/burst. The sheared lysate was centrifuged at approximately 21,000 g for 10 minutes at 4°C, and cleared supernatant was used for immunoprecipitation. Pull down was performed in 1.5-mL DNA LoBind tubes (022431021; Eppendorf North America, Hauppauge, NY), using 25 μL of Protein G Dynabeads (1003D; Invitrogen) in radio-immunoprecipitation assay buffer (10 mmol/L Tris-Cl pH 7.6, 1 mmol/L ethylenediamine tetraacetate, 150 mmol/L sodium chloride, 5% glycerol, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100) with 0.6 μg/μL bovine serum albumin overnight. The following day, beads were washed four times using radioimmunoprecipitation assay buffer with 500 mmol/L sodium chloride and stringent lithium chloride buffer (20 mmol/L Tris-Cl pH 8.0, 1 mmol/L ethylenediamine tetraacetate, 500 mmol/L lithium chloride, 1% NP-40 substitute, 1% sodium deoxycholate). Immunoprecipitation material was treated with RNase (EN0531; Thermo Scientific) for 30 minutes at 37°C, followed by liberation of DNA from proteins by heat-reversing cross-linking at 65°C overnight in the presence of 1.6 U Proteinase K (P8107S; New England Biolabs, Inc., Ipswich, MA). ChIP and input DNA were purified using MinElute PCR Purification kit (28004; QIAGEN, Valencia, CA). Adequate shearing (DNA 100–600 bp) was assayed by the BioAnalyzer (Agilent). Libraries were prepared with the Ovation Ultralow system V2 Prep Kit (0344NB; NuGEN, San Carlos, CA).

RESULTS

NPAS3 Regulates Approximately Three Times as Many Genes as NPAS1

There were 443 protein-coding transcripts significantly regulated by loss of NPAS1 (Figure 1A and Table S1 in Supplement 2). Most were downregulated (31% upregulated and 69% downregulated; $p < 2.2 \times 10^{-16}$, binomial test). Loss of NPAS3 resulted in approximately threefold greater transcriptional perturbation (1141 differentially regulated protein-coding transcripts). As with NPAS1, most differentially regulated genes in NPAS3-deficient mice showed decreased expression compared with WT mice, with 60% decreased and 40% increased ($p < 2.2 \times 10^{-16}$, binomial test). Thus, both TFs serve predominantly an activating role in mouse brain, with NPAS3 regulating approximately three times as many genes as NPAS1. In addition, *Npas1* is downregulated in the *Npas3* knockout, while *Npas3* expression remains statistically unchanged in the *Npas1* knockout. This was confirmed by quantitative polymerase chain reaction (Supplement 1).

Reciprocally Regulated NPAS1/3 Genes

Among the most striking aspects of the NPAS1/3 relationship is that loss of NPAS1 promotes postnatal hippocampal neurogenesis, while loss of NPAS3 inhibits it. To explore this reciprocity, we investigated genes regulated by both NPAS1 and NPAS3. There were 93 protein-coding transcripts coregulated by NPAS1 and NPAS3 (odds ratio [OR] = 8.3, $p < 2.2 \times 10^{-16}$) (Figure 1B), with 80% regulated in the same direction. Coregulated genes showed significant preference ($p < 1 \times 10^{-8}$, binomial test with null proportion of 0.5) for regulation in the same direction by NPAS1 and NPAS3, with a large majority (71) being downregulated in the absence of NPAS1 or NPAS3. Transcripts of only three genes were upregulated in

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both mutant strains. Eight transcripts were reciprocally regulated with increased expression in NPAS1-deficient mice and decreased expression in NPAS3-deficient mice, and 11 transcripts were reciprocally regulated in the opposite manner (Figure 1B and Table S2 in Supplement 2).

Both NPAS1- and NPAS3-regulated transcripts showed significant functional enrichment for neuronal and neurogenesis related processes (Figure S2A in Supplement 1, Tables S3 and S4 in Supplement 2), including roles in neuritic projections and synaptic function. Relative to NPAS3-regulated genes, NPAS1-regulated genes were enriched for RNA binding, myelination, and glutamate receptor binding (Figure S2B in Supplement 1). By contrast, NPAS3-regulated genes were enriched for processes related to the extracellular matrix and cardiovascular, skin, and respiratory development (Figure S2B in Supplement 1). NPAS3 and its ortholog in *Drosophila*, trachealess, have been connected to respiratory development (37,38).

We next confirmed protein expression by Western blot (Figure 1C, D). Neurobeachin, cannabinoid receptor 1, neuronal pentraxin receptor, MKL/myocardin-like 2, and microtubule-associated protein 2 were significantly regulated in the expected directions in NPAS1-deficient (up) and NPAS3-deficient (down) hippocampus, and RYR2 trended in the expected direction. Bone morphogenetic protein–binding endothelial regulator did not achieve significance in either condition and did not trend in the expected direction. Finally, interferon induced protein with tetratricopeptide repeats 2 was significantly repressed in both NPAS1- and NPAS3-deficient mice, in contrast to the transcriptional data that indicated upregulation in *Npas3*$^{-/-}$ and downregulation in *Npas1*$^{-/-}$. Thus, for reciprocally regulated genes identified by RNA-seq, there was a high degree of agreement at the protein level. Future examination of reciprocally regulated genes may lend insight into the opposing roles of NPAS1 and NPAS3 in postnatal hippocampal neurogenesis.

Direct Transcriptional Targets of NPAS1/3

ChIP-seq enables identification of genomic binding sites for NPAS1/3. While RNA-seq identified all transcriptional changes that were due to loss of NPAS1/3, ChIP-seq provides the necessary information to infer which of those changes are due to direct transcriptional regulation via DNA binding of the TFs.

ChIP-seq analysis of hippocampal chromatin from all three genotypes (Figure 2A) yielded 7183 NPAS1 peaks (i.e., genomic binding events) and 12,914 NPAS3 peaks (Figure 2B and see Supplement 1 for criteria). Of those, 347 (NPAS1) and 1697 (NPAS3) peaks mapped to transcripts regulated by the respective TF in the RNA-seq data. Specificity of peaks was demonstrated by their absence in the respective NPAS1- or NPAS3-deficient strain (Figure 2A). Complete peak data are presented in Tables S5 and S6 in Supplement 2. NPAS1 and NPAS3 peaks were significantly enriched upstream of regulated transcripts in NPAS1- and NPAS3-deficient mice, respectively (Figure 2D, E), and significantly depleted downstream. NPAS3 peaks were also significantly enriched within the gene bodies of regulated transcripts.

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A direct target (i.e., a gene) of a TF was defined as a gene found by RNA-seq to be regulated by the TF and also found by ChIP-seq to have corresponding binding either proximally or within the body of the gene for that transcript. NPAS3 directly regulated 461 target genes, while NPAS1 directly regulated 140 target genes (Figure 2C). We then determined the distance between transcription start sites of all transcripts and the nearest NPAS1 or NPAS3 peak. ChIP peaks were enriched upstream of regulated transcripts for both TFs, with NPAS3 peaks especially pronounced (Figure 2F, G). Together, these complementary analyses demonstrate enrichment of NPAS1/3 genomic binding proximal to genes dysregulated in their respective TF knockout. This suggests direct transcriptional regulation by DNA binding of these TFs as a major contributing factor to gene expression changes identified by RNA-seq.

NPAS1/3 Regulatory Network Analysis

Two distinct clusters in the NPAS3 regulatory network were observed (Figure S5 in Supplement 1). The first centered around Notch1/2 and Jag1 (Figure 3A), implicating the Notch signaling pathway, and the second centered around the NPAS3 target gene Reelin (Figure 3B). This analysis also implicated *Tbr1, Calb1*, and *Gad2*, all direct targets of NPAS3. Although a number of known neurogenesis genes are also regulated by NPAS1 (Tables S1 and S7 in Supplement 2), only one cluster of neurogenesis genes was identified within the NPAS1 regulatory network, centered around *Sema3a* and connecting to the axon guidance genes *Plxna1, Plxna4, Slit1*, and *Slit3* (Figure 3C). See Supplement 1 for further details.

Comparison of Transcriptional Regulation in Mouse and Human

We compared our results in murine hippocampus with expression data from human hippocampus to explore the degree to which putative regulatory relationships were conserved across species. We used postmortem hippocampal expression data from BrainSpan (www.brainspan.org) from 18 postnatal individuals (age 1–40 years, mean age 18 years) and computed the Pearson correlation of human *NPAS1* and *NPAS3* expression to the human orthologs of their respective regulatory targets identified in our analysis. In the process, we unexpectedly uncovered an apparent transition of NPAS1 from transcriptional repressor in mouse to transcriptional activator in human. Specifically, when examining the directionality of NPAS1 regulation in mouse hippocampus, we found that NPAS1 targets were positively coexpressed (with *Npas1*) with increasing regulatory status (Figure 4A). By contrast, NPAS1 regulation in human hippocampus showed increasingly negative coexpression with increasing regulatory status (Figure 4B), indicating a transcriptional repressor function in humans. In both species, however, we observed consistently positive correlation of directionality of NPAS3-target coexpression (Figure 4C, D), indicating preservation of a transcriptional activator role of NPAS3 in mouse and human.

Enrichment of Genetic Burden for Schizophrenia and Intellectual Disability Among NPAS1/3 Targets

We next investigated whether human orthologs of NPAS1/3 regulation showed enrichment of genes linked to neuropsychiatric conditions. A positive finding in this analysis would demonstrate that *NPAS1* and *NPAS3* are major genetic risk factors for neuropsychiatric

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disease by mass dysregulation of individual risk factors. We focused on schizophrenia, autism spectrum disorder, and intellectual disability, which are currently the most genetic data–rich neuropsychiatric conditions. Literature analysis of gene cocitation with "schizophrenia," "autism," or "intellectual disability" indicated significant enrichment among NPAS3 target genes, even after accounting for baseline enrichment of "brain genes" (Figure 4E, F and Table S9 in Supplement 2). We followed this with direct testing of genetic burden among regulatory targets of NPAS1/3. Significantly increased burden of predicted damaging variants in probands with schizophrenia compared with control subjects was seen for both NPAS1 and NPAS3 targets (NPAS1 direct targets, OR = 1.36, p = .03; all NPAS1 targets, OR = 1.2, $p = .03$; NPAS3 direct targets, OR = 1.18, $p = 004$; all NPAS3 targets, OR = 1.0, $p = .41$) (Figure 4E). By contrast, no excess burden was found in probands with autism spectrum disorder compared with unaffected siblings (OR = 0.82–0.95, $p = .82–.88$). With respect to intellectual disability, NPAS1 targets were not significantly enriched for nonsilent de novo variants in probands with intellectual disability (all targets, OR = 1.8, $p = .16$; no hits in NPAS1 direct targets) (Figure 4F), while NPAS3 targets were significantly enriched as a whole (OR = 1.95, $p = .03$) and to greater extent when considering direct targets only (OR = 3, $p = .007$). Finally, we assessed NPAS1/3 targets for enrichment of genes implicated in a recent large-scale study of common variation in schizophrenia (39). Notably, nine NPAS1 targets (OR = 4, $p < .001$) and 10 NPAS3 targets (OR = 1.4, p = .2) were implicated as schizophrenia genes in this study. These findings suggest a role for NPAS1/3 regulation in schizophrenia and for NPAS3 regulation in intellectual disability.

NPAS3 Regulates Notch, Fragile X Mental Retardation Protein, and Ubiquitin-Protein Ligase E3A Signaling Pathways

RNA-seq and ChIP-seq analyses identified three prominent regulators of neuronal function as direct NPAS3 transcriptional targets: *Notch1/2, Fmr1*, and *Ube3a*. We therefore examined the extent to which each pathway was dysregulated in the brain as a result of *Npas3* knockout, using both WT and NPAS1-deficient hippocampus as controls.

In NPAS3-deficient mice, we observed increased messenger RNA (mRNA) expression of *Notch1* and *Notch2* but not *Notch3* or *Notch4*. Western blotting confirmed upregulation of both Notch1 and Notch2 receptors in NPAS3-deficient hippocampus, relative to WT and NPAS1-deficient hippocampus (Figure 5A), indicating that NPAS3 directly represses their expression. Coincident with increased Notch1 and Notch2 expression, we also observed increased expression of downstream signal transducer and activator of transcription 6 and hairy and enhancer of split-1 proteins (Figure 5A). Consistent with this observation, comparison of our regulated genes with previously published Notch-regulated genes (40) suggests a broad increase in Notch signaling in *Npas3*$^{-/-}$ mice (OR = 1.6, p = .007).

Western blotting showed approximately 50% reduction of fragile X mental retardation protein (FMRP) in *Npas3*$^{-/-}$, relative to WT and *Npas1*$^{-/-}$ (Figure 5B), confirming RNA-seq observations. Furthermore, FMRP-deficient mice have lower levels of postsynaptic density protein 95 (PSD-95) (41), and we observed lower levels of PSD-95 in NPAS3-deficient hippocampal tissue as well, compatible with decreased levels of FMRP (Figure 5B). FMRP also regulates expression of other postsynaptic density proteins, and FMRP-deficient mice

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express higher levels of SAP90/PSD-95-associated protein 2 and SAP90/PSD-95-associated protein 3 as well as Shank1 and the NR1 and NR2B subunits of *N*-methyl-*D*-aspartate glutamate receptor (42). Examination of protein levels from NPAS3-deficient tissue showed a similar selective increase, relative to WT and NPAS1-deficient tissue (Figure 5B). Finally, FMRP-deficient mice have altered levels of α-calcium-calmodulin-dependent kinase II mRNA but normal protein levels (43), and this same pattern of protein expression was confirmed in *Npas3*$^{-/-}$ (Figure 5B). Taken together, NPAS3 is a transcriptional activator of *Fmr1*, and analysis of downstream events normally mediated by FMRP shows that NPAS3-deficient mice display a similar pattern of changes normally seen with FMRP deficiency. Thus, the FMRP system is under broad regulatory control by NPAS3.

We also identified *Ube3a* as a direct NPAS3 target, and Western blotting demonstrated 50% decrease in ubiquitin-protein ligase E3A (UBE3A) protein expression in NPAS3-deficient hippocampus, relative to WT and NPAS1-deficient hippocampus (Figure 5C). Subsequent Western blotting of hippocampal total protein with anti-ubiquitin antibody showed an approximately 45% decrease in ubiquitination across the proteome of *Npas3*$^{-/-}$ hippocampus relative to WT and *Npas1*$^{-/-}$ (Figure 5C). The neuropathology of Angelman syndrome is related to UBE3A regulation of the development of excitatory synapses, largely by controlling degradation of both activity-regulated cytoskeleton-associated protein (44) and Ephexin-5 (45). Disruption of UBE3A function in neurons leads to increased activity-regulated cytoskeleton-associated protein expression, precipitating dysregulation of alpha-amino3-hydroxy-5-methyl-4-isoxazole propionic acid receptor expression at excitatory synapses (44), and Ephexin-5 must also be degraded for proper balancing of excitatory synapse development in the maturing brain (45). Here, investigation of activity-regulated cytoskeleton-associated protein and Ephexin-5 proteins by Western blot showed an almost twofold increase in NPAS3-deficient hippocampus relative to WT and NPAS1-deficient hippocampus (Figure 5C), as expected with impaired ubiquitination. Thus, NPAS3 directly regulates *Ube3a* and its downstream effects in ways that are likely to influence intellectual capacity.

DISCUSSION

Although NPAS1 and NPAS3 are important TFs linked to normal and pathologic functioning of the brain, their gene and pathway regulatory targets have been unclear. We therefore conducted an unbiased, in vivo investigation of the NPAS1/3 regulatory program through RNA-seq, ChIP-seq, and network analysis of NPAS1- and NPAS3-deficient hippocampus. We showed that regulatory targets of NPAS1/3 are significantly enriched for genes related to neuronal processes, and we confirmed regulation at the protein level of key genes related to postnatal hippocampal neurogenesis that are reciprocally regulated by NPAS3 and NPAS1. We also identified NPAS1/3 DNA binding sites that are proximally enriched to their respective differentially expressed genes, demonstrating an important correlation between RNA-seq and ChIP-seq data. A vast majority of the regulated genes we found are novel observations, though we also confirm known regulatory relationships, such as downregulation of *Reln* in NPAS3-deficient hippocampus (22).

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In this rich data set, several notable patterns emerge. First, we have established conservation of regulatory relationships among orthologous target genes in the human hippocampus, underscoring the utility of mouse models of neuropsychiatric disease related to these TFs. Previous in vitro work has described NPAS1 as a transcriptional repressor (34), and our finding that most of the direct targets of NPAS1 in the mouse brain were actually downregulated in NPAS1-deficient mouse hippocampus seems to contradict that finding. However, the previous in vitro study (34) used a combination of human and murine cell lines, and most of the expression evidence was derived from experiments conducted with human cells. This is important, as we have now identified discordance between mouse and human in terms of the direction of coexpression of NPAS1 and its targets. In mouse brain, *Npas1* is positively coexpressed with its regulated genes, whereas this trend in reversed in the human brain, with *NPAS1* showing negative coexpression with orthologous targets. This suggests that somewhere between mouse and human lineage, NPAS1 maintained its regulatory targets as it transitioned from transcriptional activator to repressor. By contrast, *Npas3* shows positive coexpression with its targets in both mouse and human. These findings speak to the evolution of these TFs and provide important considerations for how these mouse models relate to the human condition. The divergence of NPAS1 between mouse and human as well as its regulatory and phenotypic consequences will be the subject of further study. Indeed, it is important to note that the results presented here represent only a single snapshot in time of what NPAS1 and NPAS3 are doing in a single region of the brain. Many TFs act as both a repressor and an activator under different conditions at different promoters, and as such the function of NPAS1 and NPAS3 under different conditions throughout the brain will be an important area of future investigation.

Network analysis revealed key neurobiologic pathways directly regulated by NPAS1/3. One of the most salient findings is that NPAS3 represses expression of *Notch1* and *Notch2*. Notch signaling inhibits hippocampal neurogenesis (46), and suppression of Notch signaling initiates latent neurogenic programs in astrocytes (47). The NPAS3 regulatory network is particularly densely connected around *Notch1* and *Notch2*, with many of the neighboring genes previously implicated in neurogenesis. The Notch ligand *Jag1* is also transcriptionally repressed by NPAS3, while the Notch ligand *Cntn1* (48) is transcriptionally activated by NPAS3. Furthermore, *Mib1*, which promotes Notch endocytosis, and *Adam10*, which cleaves Notch, are both transcriptionally activated by NPAS3.

The *Fmr1* pathway is also regulated at multiple levels by NPAS3. *Fmr1*, which itself regulates many neuronal genes through its protein product FMRP, is a direct transcriptional target of NPAS3 and thereby downregulated by its loss. There is significant overlap of genes directly regulated by both NPAS3 and FMRP (OR = 3.6, $p = 7 \times 10^{-15}$, 60 genes) (Table S10 in Supplement 2). NPAS3+FMRP-regulated genes are especially vulnerable to loss of NPAS3 through a multiple-hit mechanism that disrupts transcriptional regulation (via loss of NPAS3), mRNA stability and localization (via downregulation of *Fmr1*), and translational regulation (via downregulation of *Fmr1*). While *Fmr1* and its protein product FMRP have traditionally been associated with translational regulation, previous work has also demonstrated that FMRP affects stability of its mRNA targets (41). This may account for some of the apparent transcriptional repression we observe of genes that are known targets of FMRP but not direct targets of NPAS3 ($n = 52$). Genes involved in synaptogenesis and

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synapse maintenance, including *Nrxn1, Shank2, Syn1*, and *Syt1*, are also disproportionately affected by disruption of the NPAS3/FMRP regulatory axis (NPAS3+FMRP targets are enriched for synaptic localization; OR = 12, $p = 6.8 \times 10^{-22}$). Table S10 in Supplement 2 contains a complete list of target genes coregulated by NPAS3 and FMRP.

The Angelman syndrome gene, *Ube3a*, was also found to be a direct target of NPAS3, suggesting a further role for NPAS3 in synapse development and maintenance. We observed that loss of NPAS3 results in downregulation of *Ube3a* and that *Arc*, a target of *Ube3a* at transcriptional (49) and protein levels (44), is upregulated by loss of NPAS3. As *Arc* was found to be an indirect target of NPAS3, its observed upregulation may result from attenuated UBE3A levels. Indeed, the direction of regulation is consistent with previous work showing that loss of UBE3A results in increased *Arc* expression (49).

Examination of orthologous human genes showed a role for NPAS1 and NPAS3 regulation in schizophrenia and for NPAS3 in intellectual disability. While *NPAS3* has been repeatedly demonstrated to be a risk gene in major mental illness, comparable evidence for disease associations with *NPAS1* is lacking. This may be partly explained by the fact that *NPAS3* is over an order of magnitude longer than *NPAS1* (865 kb vs. 25 kb) and thus has greater opportunity for sustaining mutations. However, our findings suggest that the more pronounced disease relevance of NPAS3 may also be due to its more extensive regulation of transcription compared with NPAS1, as loss of NPAS3 perturbs approximately threefold more genes than loss of NPAS1. Furthermore, we observed nearly twice as many NPAS3 genomic binding events than for NPAS1. Although NPAS1 and NPAS3 regulate many of the same processes, NPAS3 has a substantially broader reach in the adult hippocampus in regulation of transcriptional pathways known to affect brain development and function. Consequently, loss of NPAS3 would be predicted to have far more drastic consequences than loss of NPAS1.

Finally, a large-scale human genetic study found an association between educational attainment, a proxy for cognitive ability, and three NPAS1/3 targets: *Tbr1, Nbea*, and *Mef2c* (50). This study put forth only 15 genes as the most robust candidates, so the occurrence of these three NPAS3-regulated genes, two of which are also NPAS1 targets (*Nbea and Mef2c*), is remarkable (NPAS3 targets, OR = 3.6, $p = .07$; NPAS1 targets, OR = 6, $p = .05$). Altogether, our results provide fundamental insight into how *NPAS3*, one of the most rapidly evolving human genes, and the related gene *NPAS1* shape brain development and function by controlling key neuropsychiatric pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Zhou YD, Barnard M, Tian H, Li X, Ring HZ, Francke U, et al. (1997): Molecular characterization of two mammalian bHLH-PAS domain proteins selectively expressed in the central nervous system. Proc Natl Acad Sci U S A 94:713–718. [PubMed: 9012850]
2. Brunskill EW, Witte DP, Shreiner AB, Potter SS (1999): Characterization of npas3, a novel basic helix-loop-helix PAS gene expressed in the developing mouse nervous system. Mech Dev 88:237–241. [PubMed: 10534623]
3. Rutter J, Reick M, Wu LC, McKnight SL (2001): Regulation of clock and NPAS2 DNA binding by the redox state of NAD cofactors. Science 293:510–514. [PubMed: 11441146]
4. Dudley CA, Erbel-Sieler C, Estill SJ, Reick M, Franken P, Pitts S, et al. (2003): Altered patterns of sleep and behavioral adaptability in NPAS2deficient mice. Science 301:379–383. [PubMed: 12843397]
5. Taylor BL, Zhulin IB (1999): PAS domains: Internal sensors of oxygen, redox potential, and light. Microbiol Mol Biol Rev 63:479–506. [PubMed: 10357859]
6. Wu D, Su X, Potluri N, Kim Y, Rastinejad F (2017): NPAS1-ARNT and NPAS3-ARNT crystal structures implicate the bHLH-PAS family as multi-ligand binding transcription factors. eLife 5:e18790.
7. Gonzalez-Penas J, Arrojo M, Paz E, Brenlla J, Paramo M, Costas J (2015): Cumulative role of rare and common putative functional genetic variants at NPAS3 in schizophrenia susceptibility. Am J Med Genet B Neuropsychiatr Genet 168:528–535. [PubMed: 25982957]
8. Macintyre G, Alford T, Xiong L, Rouleau GA, Tibbo PG, Cox DW (2010): Association of NPAS3 exonic variation with schizophrenia. Schizophr Res 120:143–149. [PubMed: 20466522]
9. Pickard BS, Christoforou A, Thomson PA, Fawkes A, Evans KL, Morris SW, et al. (2009): Interacting haplotypes at the NPAS3 locus alter risk of schizophrenia and bipolar disorder. Mol Psychiatry 14: 874–884. [PubMed: 18317462]
10. Pickard BS, Malloy MP, Porteous DJ, Blackwood DH, Muir WJ (2005): Disruption of a brain transcription factor, NPAS3, is associated with schizophrenia and learning disability. Am J Med Genet B Neuro-psychiatr Genet 136B:26–32.
11. Kamnasaran D, Muir WJ, Ferguson-Smith MA, Cox DW (2003): Disruption of the neuronal PAS3 gene in a family affected with schizophrenia. J Med Genet 40:325–332. [PubMed: 12746393]
12. Pickard BS, Pieper AA, Porteous DJ, Blackwood DH, Muir WJ (2006): The NPAS3 gene—emerging evidence for a role in psychiatric illness. Ann Med 38:439–448. [PubMed: 17008307]
13. Huang J, Perlis RH, Lee PH, Rush AJ, Fava M, Sachs GS, et al. (2010): Cross-disorder genomewide analysis of schizophrenia, bipolar disorder, and depression. Am J Psychiatry 167:1254–1263. [PubMed: 20713499]
14. Nurnberger JI Jr, Koller DL, Jung J, Edenberg HJ, Foroud T, Guella I, et al. (2014): Identification of pathways for bipolar disorder: A metaanalysis. JAMA Psychiatry 71:657–664. [PubMed: 24718920]
15. Weber H, Kittel-Schneider S, Gessner A, Domschke K, Neuner M, Jacob CP, et al. (2011): Cross-disorder analysis of bipolar risk genes: Further evidence of DGKH as a risk gene for bipolar disorder, but also unipolar depression and adult ADHD. Neuropsychopharmacology 36:2076–2085. [PubMed: 21654738]
16. Visser R, Gijsbers A, Ruivenkamp C, Karperien M, Reeser HM, Breuning MH, et al. (2010): Genome-wide SNP array analysis in patients with features of Sotos syndrome. Horm Res Paediatr 73: 265–274. [PubMed: 20215773]
17. Phelps R, Tsai A, Hagen A, Pinter J, Smith R, Stein MT (2016): The curse of the dolphins: Cognitive decline and psychosis. J Dev Behav Pediatr 37:343–345. [PubMed: 27096574]
18. Kamm GB, Pisciottano F, Kliger R, Franchini LF (2013): The developmental brain gene NPAS3 contains the largest number of accelerated regulatory sequences in the human genome. Mol Biol Evol 30: 1088–1102. [PubMed: 23408798]
19. Kamm GB, Lopez-Leal R, Lorenzo JR, Franchini LF (2013): A fastevolving human NPAS3 enhancer gained reporter expression in the developing forebrain of transgenic mice. Philos Trans R Soc Lond B Biol Sci 368:20130019.

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20. Wei PC, Chang AN, Kao J, Du Z, Meyers RM, Alt FW, et al. (2016): Long neural genes harbor recurrent DNA break clusters in neural stem/progenitor cells. Cell 164:644–655. [PubMed: 26871630]
21. Brunskill EW, Ehrman LA, Williams MT, Klanke J, Hammer D, Schaefer TL, et al. (2005): Abnormal neurodevelopment, neuro-signaling and behaviour in Npas3-deficient mice. Eur J Neurosci 22:1265–1276. [PubMed: 16190882]
22. Erbel-Sieler C, Dudley C, Zhou Y, Wu X, Estill SJ, Han T, et al. (2004): Behavioral and regulatory abnormalities in mice deficient in the NPAS1 and NPAS3 transcription factors. Proc Natl Acad Sci U S A 101:13648–13653. [PubMed: 15347806]
23. Yang D, Zhang W, Padhiar A, Yue Y, Shi Y, Zheng T, et al. (2016): NPAS3 regulates transcription and expression of VGF: Implications for neurogenesis and psychiatric disorders. Front Mol Neurosci 9:109. [PubMed: 27877109]
24. Pieper AA, Wu X, Han TW, Estill SJ, Dang Q, Wu LC, et al. (2005): The neuronal PAS domain protein 3 transcription factor controls FGF-mediated adult hippocampal neurogenesis in mice. Proc Natl Acad Sci U S A 102:14052–14057.
25. Stanco A, Pla R, Vogt D, Chen Y, Mandal S, Walker J, et al. (2014): NPAS1 represses the generation of specific subtypes of cortical interneurons. Neuron 84:940–953. [PubMed: 25467980]
26. Kempermann G, Krebs J, Fabel K (2008): The contribution of failing adult hippocampal neurogenesis to psychiatric disorders. Curr Opin Psychiatry 21:290–295. [PubMed: 18382230]
27. Lee AS, De Jesus-Cortes H, Kabir ZD, Knobbe W, Orr M, Burgdorf C, et al. (2016): The neuropsychiatric disease-associated gene cacna1c mediates survival of young hippocampal neurons. eNeuro 3(2).
28. Walker AK, Rivera PD, Wang Q, Chuang JC, Tran S, Osborne-Lawrence S, et al. (2015): The P7C3 class of neuroprotective compounds exerts antidepressant efficacy in mice by increasing hippocampal neurogenesis. Mol Psychiatry 20:500–508. [PubMed: 24751964]
29. Serafini G, Hayley S, Pompili M, Dwivedi Y, Brahmachari G, Girardi P, et al. (2014): Hippocampal neurogenesis, neurotrophic factors and depression: Possible therapeutic targets? CNS Neurol Disord Drug Targets 13:1708–1721. [PubMed: 25470403]
30. Le Strat Y, Ramoz N, Gorwood P (2009): The role of genes involved in neuroplasticity and neurogenesis in the observation of a gene-environment interaction (GxE) in schizophrenia. Curr Mol Med 9: 506–518. [PubMed: 19519407]
31. Knight HM, Walker R, James R, Porteous DJ, Muir WJ, Blackwood DH, et al. (2012): GRIK4/KA1 protein expression in human brain and correlation with bipolar disorder risk variant status. Am J Med Genet B Neuropsychiatr Genet 159B:21–29. [PubMed: 22052594]
32. Amiri A, Cho W, Zhou J, Birnbaum SG, Sinton CM, McKay RM, et al.(2012): Pten deletion in adult hippocampal neural stem/progenitor cells causes cellular abnormalities and alters neurogenesis. J Neurosci 32:5880–5890. [PubMed: 22539849]
33. Jolly LA, Homan CC, Jacob R, Barry S, Gecz J (2013): The UPF3B gene, implicated in intellectual disability, autism, ADHD and childhood onset schizophrenia regulates neural progenitor cell behaviour and neuronal outgrowth. Hum Mol Genet 22:4673–4687. [PubMed: 23821644]
34. Teh CH, Lam KK, Loh CC, Loo JM, Yan T, Lim TM (2006): Neuronal PAS domain protein 1 is a transcriptional repressor and requires arylhydrocarbon nuclear translocator for its nuclear localization. J Biol Chem 281:34617–34629.
35. Sha L, MacIntyre L, Machell JA, Kelly MP, Porteous DJ, Brandon NJ, et al. (2012): Transcriptional regulation of neurodevelopmental and metabolic pathways by NPAS3. Mol Psychiatry 17:267–279. [PubMed: 21709683]
36. Leary S, Underwood W, Anthony R, Cartner S, Corey D, Grandin T, et al. (2013): AVMA Guidelines for the Euthanasia of Animals. Schaumburg: American Veterinary Medical Association.
37. Zhou S, Degan S, Potts EN, Foster WM, Sunday ME (2009): NPAS3 is a trachealess homolog critical for lung development and homeostasis. Proc Natl Acad Sci U S A 106:11691–11696.

38. Pereira-Terra P, Kholdebarin R, Higgins M, Iwasiow BM, Correia-Pinto J, Keijzer R (2015): Lower NPAS3 expression during the later stages of abnormal lung development in rat congenital diaphragmatic hernia. Pediatr Surg Int 31:659–663. [PubMed: 25862168]
39. Schizophrenia Working Group of the Psychiatric Genomics Consortium (2014): Biological insights from 108 schizophrenia-associated genetic loci. Nature 511:421–427. [PubMed: 25056061]
40. Guichet PO, Guelfi S, Teigell M, Hoppe L, Bakalara N, Bauchet L, et al. (2015): Notch1 stimulation induces a vascularization switch with pericyte-like cell differentiation of glioblastoma stem cells. Stem Cells 33:21–34. [PubMed: 24898819]
41. Zalfa F, Eleuteri B, Dickson KS, Mercaldo V, De Rubeis S, di Penta A, et al. (2007): A new function for the fragile X mental retardation protein in regulation of PSD-95 mRNA stability. Nat Neurosci 10: 578–587. [PubMed: 17417632]
42. Schutt J, Falley K, Richter D, Kreienkamp HJ, Kindler S (2009): Fragile X mental retardation protein regulates the levels of scaffold proteins and glutamate receptors in postsynaptic densities. J Biol Chem 284:25479–25487.
43. Zalfa F, Giorgi M, Primerano B, Moro A, Di Penta A, Reis S, et al. (2003): The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. Cell 112:317–327. [PubMed: 12581522]
44. Greer PL, Hanayama R, Bloodgood BL, Mardinly AR, Lipton DM, Flavell SW, et al. (2010): The Angelman syndrome protein Ube3A regulates synapse development by ubiquitinating arc. Cell 140: 704–716. [PubMed: 20211139]
45. Margolis SS, Salogiannis J, Lipton DM, Mandel-Brehm C, Wills ZP, Mardinly AR, et al. (2010): EphB-mediated degradation of the RhoA GEF Ephexin5 relieves a developmental brake on excitatory synapse formation. Cell 143:442–455. [PubMed: 21029865]
46. Wilhelmsson U, Faiz M, de Pablo Y, Sjoqvist M, Andersson D, Widestrand A, et al. (2012): Astrocytes negatively regulate neurogenesis through the Jagged1-mediated Notch pathway. Stem Cells 30:2320–2329. [PubMed: 22887872]
47. Magnusson JP, Goritz C, Tatarishvili J, Dias DO, Smith EM, Lindvall O, et al. (2014): A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse. Science 346:237–241. [PubMed: 25301628]
48. Hu QD, Ang BT, Karsak M, Hu WP, Cui XY, Duka T, et al. (2003): F3/contactin acts as a functional ligand for Notch during oligodendrocyte maturation. Cell 115:163–175. [PubMed: 14567914]
49. Kuhnle S, Mothes B, Matentzoglu K, Scheffner M (2013): Role of the ubiquitin ligase E6AP/UBE3A in controlling levels of the synaptic protein Arc. Proc Natl Acad Sci U S A 110:8888–8893. [PubMed: 23671107]
50. Okbay A, Beauchamp JP, Fontana MA, Lee JJ, Pers TH, Rietveld CA, et al. (2016): Genome-wide association study identifies 74 loci associated with educational attainment. Nature 533:539–542. [PubMed: 27225129]

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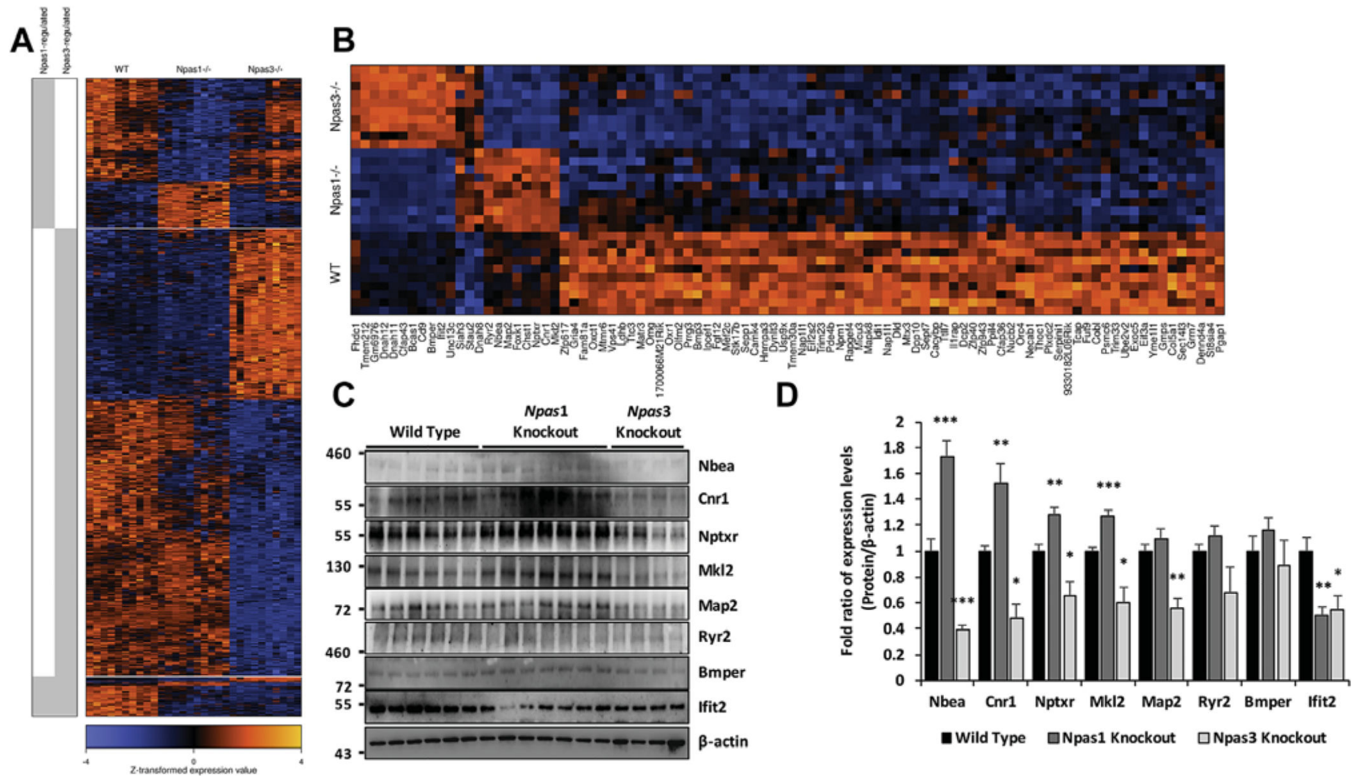

Figure 1.

Neuronal PAS domain protein (NPAS) 1/3–regulated transcripts are enriched for functions related to neurogenesis. **(A)** Hippocampal expression patterns of 1491 significantly regulated transcripts across 30 wild-type (WT), *Npas1*$^{-/-}$, and *Npas3*$^{-/-}$ mice. **(B)** Of 93 transcripts regulated by NPAS1/3, most (71) were downregulated by the absence of these transcription factors. **(C, D)** Western blotting and densitometry analysis showed that expression of neurobeachin (NBEA), cannabinoid receptor 1 (CNR1), neuronal pentraxin receptor (NPTXR), MKL/myocardin-like 2 (MKL2), and microtubule-associated protein 2 (MAP2) is reciprocally regulated by NPAS1/3. Ryanodine receptor 2 (RYR2) did not achieve significance but trended in the expected direction. Bone morphogenetic protein–binding endothelial regulator (BMPER) did not achieve significance in either condition and did not trend in the expected direction. The expression of interferon induced protein with tetratricopeptide repeats 2 (IFIT2) was significantly repressed in both *Npas1*$^{-/-}$and *Npas3*$^{-/-}$. $*p < .05$, $**p < .01$, $***p < .001$ vs. WT. $n = 6$ (WT), 7(*Npas1*$^{-/-}$), and 4 (*Npas3*$^{-/-}$).

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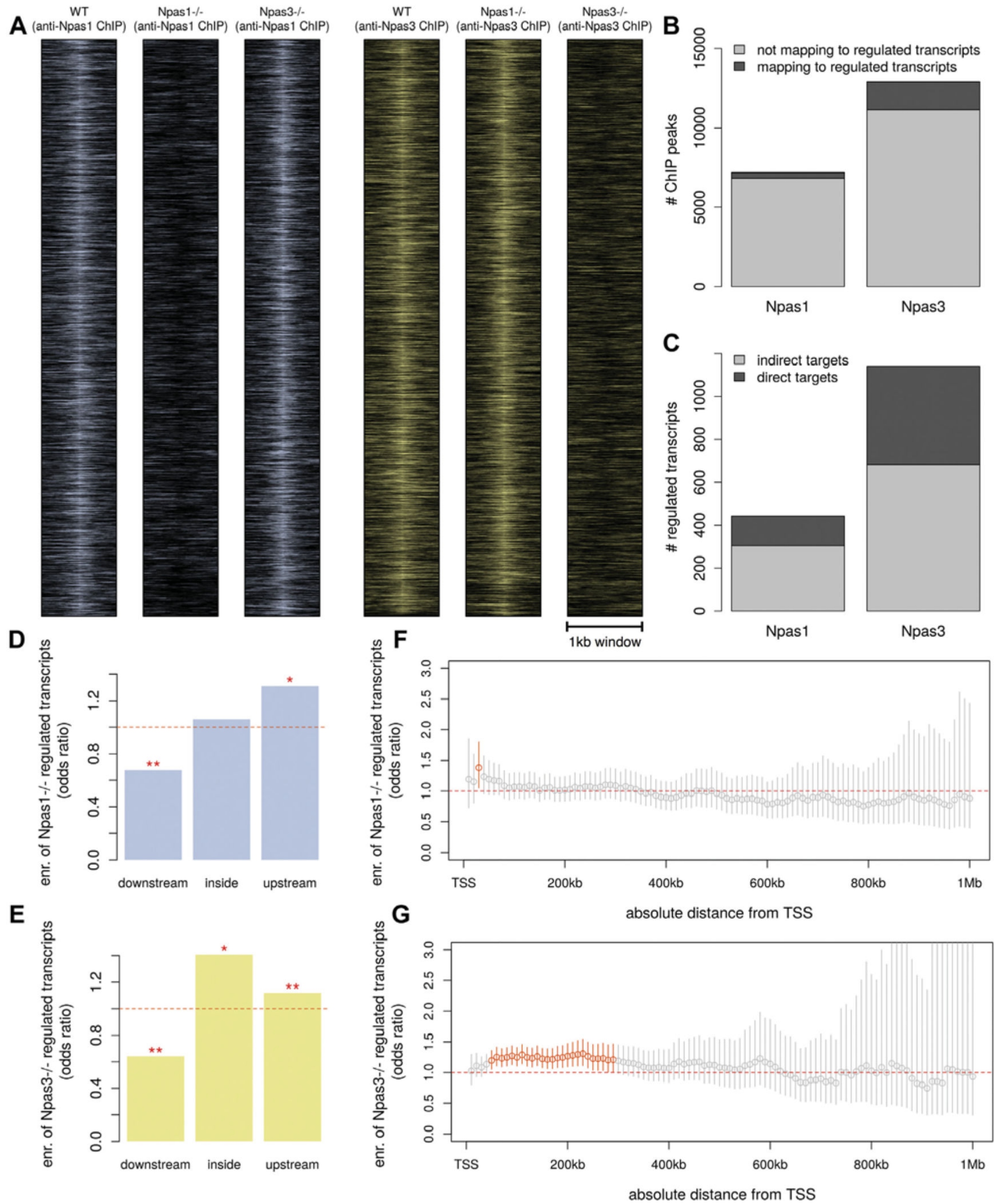

Figure 2.

Chromatin immunoprecipitation (ChIP) sequencing peaks are associated with differentially regulated transcription. **(A)** Sequencing coverage of neuronal PAS domain protein (NPAS) 1 (blue) and NPAS3 (gold) ChIP sequencing peaks that pass criteria (see Methods and Materials) is shown within 1-kb windows centered around the peak summit. The peaks disappear only when the respective transcription factor is knocked out, emphasizing the specificity of these binding events. **(B)** We discovered 7183 NPAS1 peaks and 12,914 NPAS3 peaks, and of those, 347 and 1697 tagged the respective significantly differentially

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regulated transcripts as the closest genome feature. **(C)** NPAS3 regulated 1141 transcripts (461 of them direct targets with proximal ChIP sequencing peaks upstream or within the gene body), nearly three times the number of NPAS1-regulated transcripts (443—140 of those direct NPAS1 targets). **(D)** NPAS1 peaks were significantly enriched upstream of *Npas1*$^{-/-}$-regulated transcripts and significantly depleted downstream. **(E)** Similarly, NPAS3 peaks were significantly enriched upstream of *Npas3*$^{-/-}$-regulated transcripts and depleted downstream. In addition, NPAS3 peaks were significantly enriched within the gene bodies of regulated transcripts. **(F)** When considering the distance between the transcription start sites (TSS) of all transcripts and the nearest NPAS1 peak (using a transcript-first approach, in contrast to the peak-first analysis in **D** and **E**), we observed modest enrichment of regulated transcripts at ,<30 kb (orange points and bars indicate significance at $p < .05$ by Fisher's exact test). **(G)** In contrast, we see broad enrichment of *Npas3*$^{-/-}$-regulated transcripts for NPAS3 peaks up to 290 kb from the TSS. enr., enrichment.

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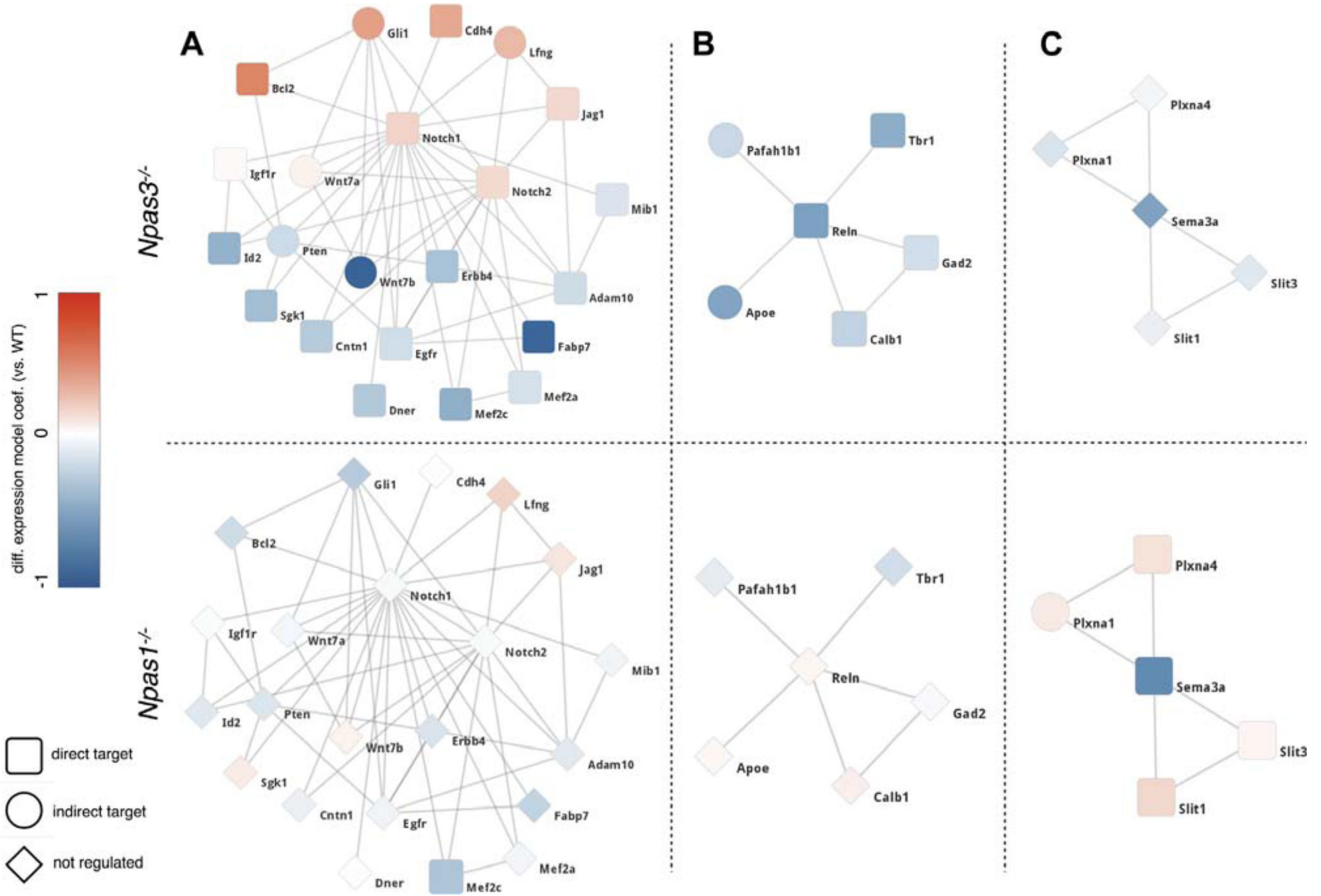

Figure 3.

Regulatory network hubs are related to neurogenesis. **(A)** The most prominent neuronal PAS domain protein (NPAS) 3 network module centered around *Notch1*/2 and *Jag1*, implicating the Notch signaling pathway. **(B)** A distinct NPAS3 network module, centered around reelin. **(C)** The most significant concentration of neurogenesis-related genes in the NPAS1 network centered around *Sema3a* and implicated axon guidance as being under the control of NPAS1. coef., coefficient; diff., differential; WT, wild-type.

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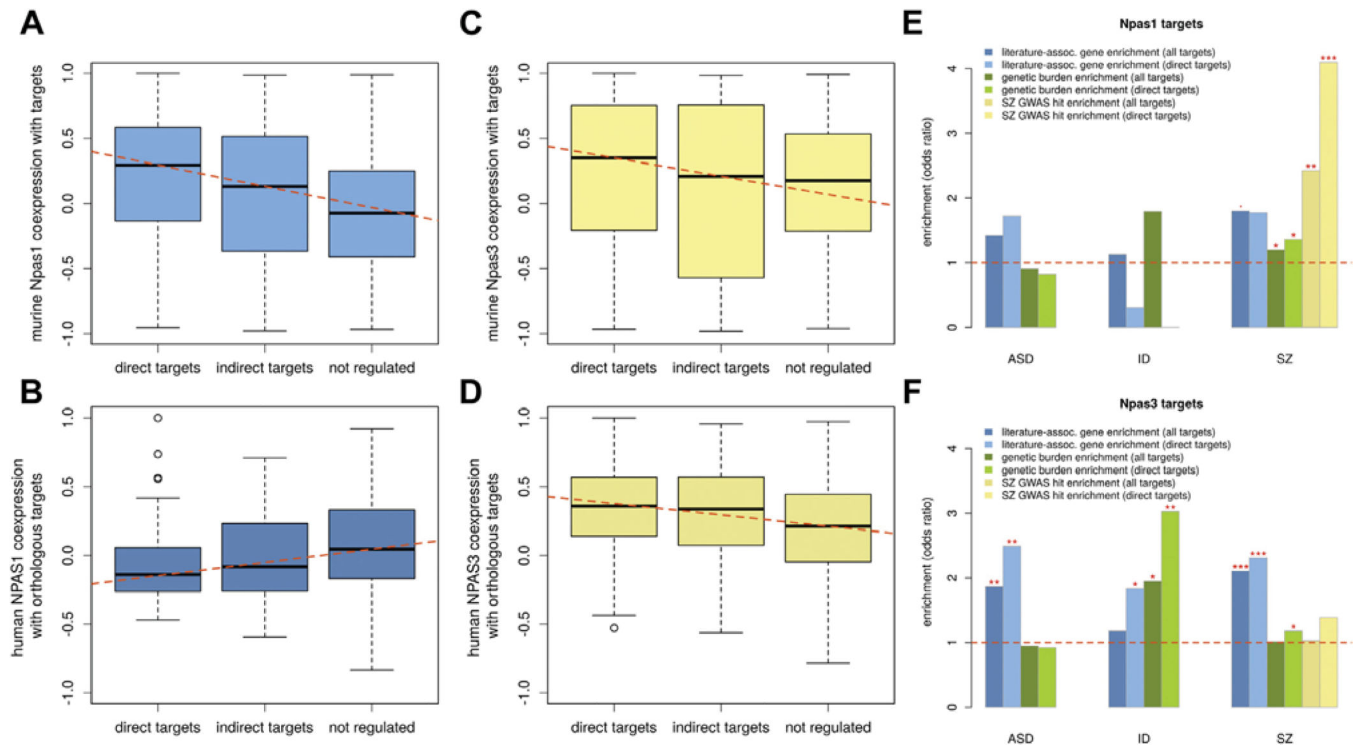

Figure 4.

Conservation and divergence of regulatory relationships between mouse and human and relevance of regulatory targets to human mental illness. **(A)** Neuronal PAS domain protein (NPAS) 1 targets showed increasing coexpression with *Npas1* in agreement with regulatory status, suggesting an activating relationship in mouse. **(B)** Human postmortem hippocampal expression data showed increasingly negative coexpression with *NPAS1* with increasing regulatory status, suggesting that NPAS1 acts predominantly as a repressor in humans. **(C, D)** NPAS3 showed conservation of its role as a transcriptional activator in mouse and human, with targets showing increasing coexpression. **(E)** Enrichment of genes implicated by literature or genetic association in autism spectrum disorder (ASD), intellectual disability (ID), or schizophrenia (SZ) among NPAS1 targets. **(F)** Enrichment of genes implicated by literature or genetic association in ASD, ID, or SZ among NPAS3 targets. ■$p < .1$; *$p < .05$; **$p < .01$; ***$p < .001$. GWAS, genome-wide association study.

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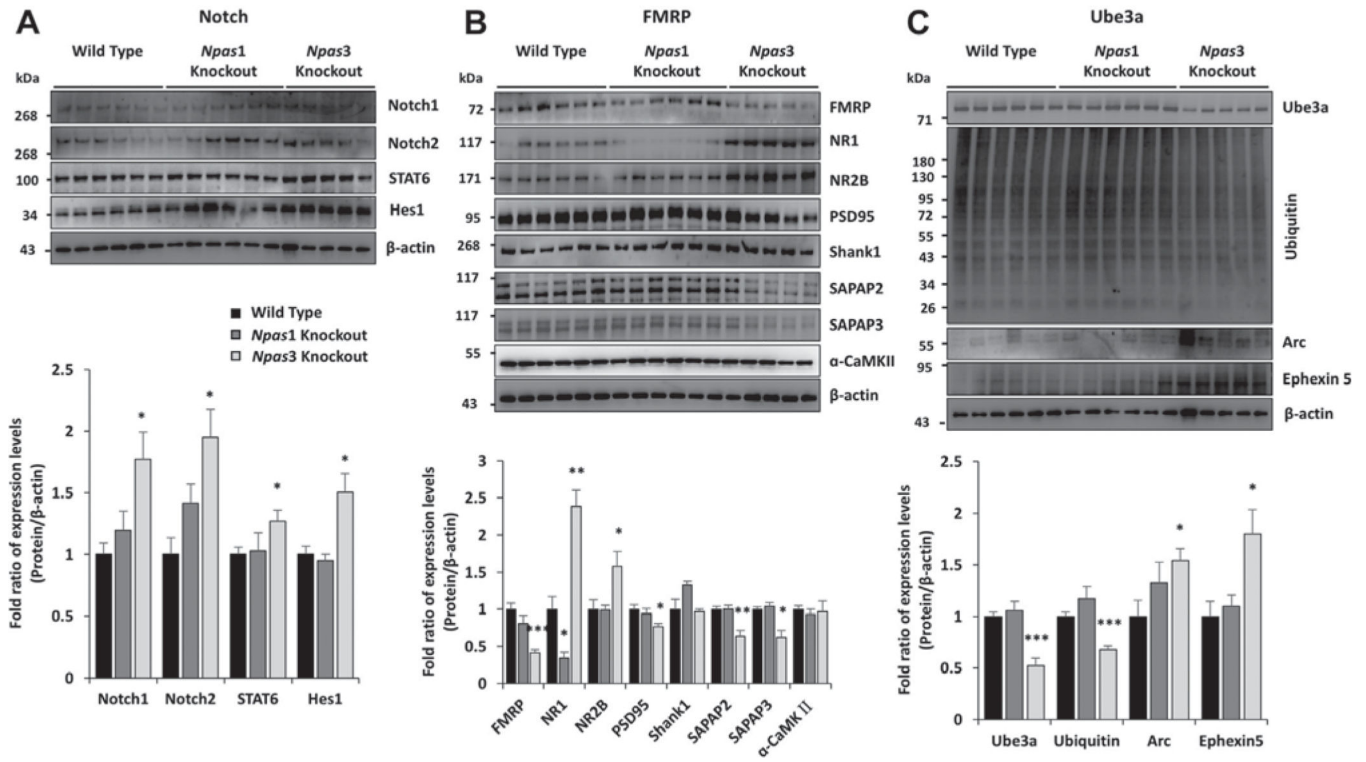

Figure 5.

Neuronal PAS domain protein (NPAS) 3 regulates the major neuronal molecular networks of Notch, fragile X mental retardation 1, and ubiquitin-protein ligase E3a. **(A)** NOTCH1/2 receptors and their downstream targets such as signal transducer and activator of transcription 6 (STAT6) and hairy and enhancer of split-1 (HES1) are upregulated in the hippocampus of NPAS3-deficient mice relative to wild-type and NPAS1-deficient mice. **(B)** NPAS3-deficient mice have lower levels of fragile X mental retardation protein (FMRP) and show altered expression of postsynaptic density protein 95 (PSD-95), NR1, and NR2B, consistent with expression pattern of FMRP-deficient mice. **(C)** Ubiquitin-protein ligase E3A (UBE3A), direct target of NPAS3, is decreased in NPAS3-deficient mice, whereas its target proteins, including activity-regulated cytoskeleton-associated protein (ARC) and Ephexin-5, are upregulated in the hippocampus of NPAS3-deficient mice relative to wild-type and NPAS1-deficient mice. Total ubiquitination is reduced in the proteome of NPAS3-deficient mice. $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$ vs. wild-type. $n = 6$ (wild-type), 6 (*Npas1* knockout), and 5 (*Npas3* knockout). α-CamKII, α-calcium-calmodulin-dependent kinase II; SAPAP2, SAP90/PSD-95-associated protein 2; SAPAP3, SAP90/PSD-95-associated protein 3.