Archival Report

Biological Psychiatry

Long Noncoding RNA-Directed Epigenetic Regulation of Gene Expression Is Associated With Anxiety-like Behavior in Mice

Paola A. Spadaro, Charlotte R. Flavell, Jocelyn Widagdo, Vikram S. Ratnu, Michael Troup, Chikako Ragan, John S. Mattick, and Timothy W. Bredy

ABSTRACT

BACKGROUND: RNA-directed regulation of epigenetic processes has recently emerged as an important feature of mammalian differentiation and development. Perturbation of this regulatory system in the brain may contribute to the development of neuropsychiatric disorders.

METHODS: RNA sequencing was used to identify changes in the experience-dependent expression of long noncoding RNAs (lncRNAs) within the medial prefrontal cortex of adult mice. Transcripts were validated by real-time quantitative polymerase chain reaction and a candidate lncRNA, Gomafu, was selected for further investigation. The functional role of this schizophrenia-related lncRNA was explored in vivo by antisense oligonucleotide-mediated gene knockdown in the medial prefrontal cortex, followed by behavioral training and assessment of fear-related anxiety. Long noncoding RNA-directed epigenetic regulation of gene expression was investigated by chromatin and RNA immunoprecipitation assays.

RESULTS: RNA sequencing analysis revealed changes in the expression of a significant number of genes related to neural plasticity and stress, as well as the dynamic regulation of lncRNAs. In particular, we detected a significant downregulation of Gomafu lncRNA. Our results revealed that Gomafu plays a role in mediating anxiety-like behavior and suggest that this may occur through an interaction with a key member of the polycomb repressive complex 1, BMI1, which regulates the expression of the schizophrenia-related gene beta crystallin (*Crybb1*). We also demonstrated a novel role for *Crybb1* in mediating fear-induced anxiety-like behavior.

CONCLUSIONS: Experience-dependent expression of lncRNAs plays an important role in the epigenetic regulation of adaptive behavior, and the perturbation of Gomafu may be related to anxiety and the development of neuropsychiatric disorders.

Keywords: Anxiety, Behavior, *Crybb1*, Epigenetics, *Gomafu*, Noncoding RNA

http://dx.doi.org/10.1016/j.biopsych.2015.02.004

Once considered vestiges of our evolutionary history associated with junk DNA, noncoding RNAs (ncRNAs) have emerged as important regulators of epigenetic processes, transcriptional activation, and posttranscriptional gene silencing (1–5). Advances in RNA sequencing (RNA-seq) technology have resulted in the discovery of a large number of long ncRNAs (lncRNAs) (6–11), many of which show features of functionality (12–14). There are at least 50,000 genes specifying lncRNAs scattered throughout the human genome, with many expressed in a highly cell type-specific and developmental stage-specific manner (15–20). Moreover, a significant number of brain-enriched or brain-specific lncRNAs are found adjacent to genes encoding transcriptional regulators and key drivers of neural development (21), including those involved in the regulation of stem cell pluripotency, neuronal differentiation, and synaptogenesis (16,18,22–24).

In agreement with these findings, lncRNAs have been implicated in neurodevelopmental disorders such as Rett syndrome (25), autism (26,27), schizophrenia (SZ) (28,29), and Fragile X syndrome (30). Screening of lncRNA activity has also shown links between lncRNA expression and drug abuse (31,32), suicidal behavior (33), and potentially anxiety disorders. For instance, it has been reported that experimental knockdown (KD) BC1 leads to increased anxiety-like behavior in mice (34,35).

Recent evidence indicates that the expression of lncRNAs can be altered in an activity-dependent manner (29,36). Long ncRNAs have been found to be coexpressed with activity-dependent genes such as *C-fos*, *Arc*, *Nr4a2*, and *Bdnf*, suggesting a coordinated network of coding and noncoding gene expression associated with neuronal plasticity (29,36,37).

Despite these correlative links, little is known about the expression and function of brain lncRNAs, nor the mechanisms by which these transcripts influence protein-coding gene expression within the context of neuropsychiatric disease. Here, we combined high-throughput RNA-seq with

 ISSN: 0006-3223

molecular and behavioral approaches to identify changes in the expression of lncRNAs. We also determined whether these changes contribute to the epigenetic regulation of gene expression underlying the development of anxiety disorders.

METHODS AND MATERIALS

Animals

Naïve 9-week-old C57BL/6 male mice were housed individually in sections of divided cages, with free access to food and water under a 12-hour light/dark cycle in a humidity- and temperature-controlled vivarium. Behavioral tests were conducted during the light cycle, and all procedures were performed with approval from the Animal Ethics Committee of The University of Queensland.

Fear Conditioning

For tissue collection and sequencing library preparation, three groups of mice ($n = 8$ per group) were used: a naïve, age-matched, home-cage control group; a fear-conditioned group that received six pairings of a 2-minute 80 dB white noise conditioned stimulus (CS) that coterminated with a 1-second foot-shock at .7 mA as the unconditioned stimulus (US) (intertrial interval of 2 minutes); and a context only group that was exposed to the CS but not the US. Animals were sacrificed 90 minutes posttraining, followed by medial prefrontal cortex (mPFC) dissection and nuclear-enriched RNA extraction. To determine the effect of Gomafu knockdown on fear conditioning, animals were infused with 600 nmol/L of a Gomafu antisense oligonucleotide (ASO), directly into the mPFC, whereas the control group received an infusion of a scrambled control. Three hours after infusion, mice were trained using a mild fear acquisition protocol to avoid a nonspecific response (three CS-US pairing with foot-shocks of .4 mA), followed by a two-CS test for memory recall in the same context 24 hours later.

Measures of Anxiety

Anxiety tests for the knockdown and control groups were performed 3 hours after ASO infusion and 2 weeks after lentiviral infusion, respectively, and involved the use of mild stressors (38). This included a 10-minute exposure to a 27 × 27 × 20.3 cm open field chamber with 200 lux of light intensity, which was followed by an interval of 30 minutes. Mice were then introduced for 10 minutes into an elevated plus maze (under bright light at 900 lux in the open arms and 200 lux in the closed arms), and the time spent in the open arms was recorded. Following a second interval of 10 minutes, mice were once more placed in the open field for 10 minutes, at which point time spent in the center was automatically determined in seconds, while ambulatory time, distance travelled, and self-grooming time were calculated as a ratio between zone results.

Stereotaxic Surgery and Cannula Implantation

Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Surgeries were performed under stereotaxic guidance and cannulas were implanted bilaterally within the prelimbic region of the PFC (PLPFC) at +1.8 mm anterioposterior and −2 mm dorsoventral to bregma. Mice were allowed to recover for a minimum of 7 days before experiments. Animals were transcardially perfused posttraining with cold phosphate buffered saline (Lonza, Walkersville, Maryland), followed by 4% paraformaldehyde in phosphate buffered saline. Brains were dissected out, sectioned, and the localization of the ASO and short hairpin RNA infusion was determined by fluorescence microscopy.

Nuclear Enrichment, RNA Extraction, and Reverse Transcription

Medial PFC derived from fear-conditioned mice was homogenized in nuclear buffer (detailed in Supplement 1) followed by RNA extraction using the RNAeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription and complementary DNA synthesis was performed following the Quantitech Reverse Transcription kit protocol (Qiagen), with the exception of RNA eluted from RNA immunoprecipitation assays, which was reverse transcribed using Super Script III First Strand Synthesis (Invitrogen, Carslbad, California). The assay was validated both in vitro and in vivo (Figure S6A,B in Supplement 1).

RNA Sequencing

Nuclear-enriched RNA (1 μg), with a minimum RNA Integrity value of 8, from six samples per treatment was used to build a total RNA library using the Illumina TruSeq RNASample Preparation v2 protocol (Illumina Inc., San Diego, California). Each animal was individually indexed, representing a single library per mouse, which was further multiplexed in two pools of nine animals before loading into the Illumina flow cell. Samples with low read alignment were excluded, resulting in five animals for naïve and context groups and four mice for fear conditioning. The quality of the RNA library was verified on an Agilent DNA 1000 chip (Agilent Technologies, Santa Clara, California) and run on the Bioanalyzer (Agilent Technologies), while quantification was determined by quantitative real-time polymerase chain reaction, after which the samples were sequenced using the Illumina HiSeq v3 platform (Illumina Inc.).

Bioinformatic Analysis

Mapping of reads to the mouse genome (mm10) was performed using the Bowtie2 and TopHat 2.0.6 programs (Johns Hopkins University, Baltimore, Maryland) (39). Cufflinks 2.0.2 (BETA) algorithms (Cole Trapnell Lab, University of Washington, Seattle, Washington) were then implemented for assembly of RNA sequencing reads into transcripts and analysis of differential levels of transcript expression among treatment groups as previously described (40,41). For the purpose of this investigation, we used a cutoff of $p < .03$.

Gene Knockdown

ASOs targeting Gomafu lncRNA were designed using the IDT Antisense design software (Integrated DNA Technologies Inc., Commercial Park, Coralville, Iowa) targeting the splicing regulatory regions known as exonic splicing enhancers, as well as sequences with higher G and C content and low potential for RNA secondary structure formation. Primer sequences are detailed in Table S4 in Supplement 1. Short hairpin RNAs (GeneCopoeia Inc., Rockville, Maryland) targeting *Crybb1* messenger RNA (mRNA) were packaged in-house using a third generation lentiviral packaging system.

Primary Neurons Cell Culture and Electroporation

Cortical neuronal cells were dissociated from C57BL/6 mouse embryos at embryonic day 16 and plated onto poly-L-ornithine hydrochloride-coated plates. Cultures were grown in Neurobasal medium (GIBCO Life Technologies, Grand Island, New York) containing 5% fetal bovine serum, 1% penicillin/streptomycin, 1% GlutaMAX, and 2% B27 supplement (GIBCO Life Technologies) and were maintained at 37°C with 5% carbon dioxide. Electroporation of ASOs was performed using the Nucleofector transfection system (Lonza, Basel, Switzerland) according to the manufacturer's instructions. Potassium chloride treatments were performed at 50 mmol/L concentration.

Technical Validation of RNA Sequencing Gene Target Selection

Gene expression was measured using the SYBR Green quantitative reverse-transcription polymerase chain reaction detection method on a RotorGene 3000 (Qiagen). The $2^{-\lambda\lambda Ct}$ method was applied to estimate differential levels of gene expression. Analysis of variance and Holm-Sidak multiple comparison tests were applied to establish gene expression differences among groups. Validation was performed with the original RNA used for RNA-seq.

Chromatin Immunoprecipitation

Dissociated cortical neurons were subjected to electroporation with 200 nmol/L ASO for Gomafu knockdown followed by BMI1 pull-downs 3 hours posttransfection. For in vivo studies, tissues were dissected out and homogenized 1.5 hours after training. Tissue and cells were processed as previously described (42). Chromatin was immunoprecipitated using 4 μg chromatin immunoprecipitation (ChIP)-grade antibodies specific to BMI1 (1T21, Abcam, Cambridge, England, United Kingdom), SUZ12 (ab12073, Abcam), RING1B (D22F2, Cell Signaling, Danvers, Massachusetts), H2AK119 (D2764, Cell Signaling), mouse immunoglobulin G (103533, Active Motif, Carlsbad, California), and rabbit immunoglobulin G (2295402, Millipore, Billerica, Massachusetts). DNA-protein interactions were analyzed by quantitative reverse-transcription polymerase chain reaction using primers specific to the binding motifs of the proteins being investigated.

RNA Immunoprecipitation

Naïve tissues were dissected out and homogenized followed by fixation and cross-linking. Immunoprecipitation with 4 μg of the antibody of interest was performed as previously described (22), and RNA was extracted using the Trizol method (Ambion, Carlsbad, California), followed by DNase treatment using the TURBO DNA-free Ambion kit (Ambion).

RESULTS

Long Noncoding RNAs Are Dynamically Expressed in the mPFC in Response to Behavioral Experience

Fear-conditioned mice exhibited a robust increase in fear-related behavior at the end of training compared with context-only exposed mice (Figure S1A in Supplement 1). Nuclear RNA was extracted from the mPFC of mice sacrificed 90 minutes after behavioral training, and transcriptome-wide profiles were obtained by RNA-seq. In total, we detected more than 400 loci that exhibited differential expression of protein-coding and noncoding RNAs (Figure 1A). A complete list of differentially expressed transcripts identified by RNA-seq is provided in Table S1 in Supplement 1.

Intergenic lncRNAs from 53 loci were identified as being significantly altered in either context-exposed or fear-conditioned mice, with the majority of these transcripts being found within 100 kilobase (kb) of the nearest protein-coding gene, consistent with a potential *cis*-regulatory function (21,43) (Figure 1B–E; Table S2 in Supplement 1). RNA-seq analysis also revealed a cluster of modulated protein-coding genes, some of which have been implicated in behavioral regulation, in mediating the stress response, and in neuropsychiatric disorders (Table S3 in Supplement 1).

Experience-Dependent Alterations in lncRNA Expression Within the mPFC

To validate the RNA-seq findings, candidates were selected for downstream analyses. We first examined the expression of two protein-coding genes, *Bdnf* (exon IV) and *Homer1,* which are involved in neural plasticity and memory (Figure 2A,B; Table S3 in Supplement 1). *Bdnf* has been reviewed as the master regulator of neuronal circuitries driving learning and synaptic plasticity (44). This gene has a complex structure that allows its differential epigenetic response to stimuli (45,46); however, *Bdnf* exon IV has particularly been shown to participate in memory processes within the mammalian PFC (47). There was a significant increase in *Bdnf* exon IV and *Homer1* mRNA expression in context-exposed mice, confirming previous studies demonstrating the activity-dependent nature of these genes in models of experience-dependent plasticity.

We further observed a significant decrease in the expression of the activity-dependent immediate early gene *Npas4* (Figure 2C). Although *Npas4* expression is related to fear learning, a downregulation of this gene is correlated with both exposure to stress and impairments in the formation of fear memory (48–51). Indeed, stressors can also lead to fear incubation or enhanced fear-potentiated startle, which may be related to anxiety rather than learning per se (52–54). In the experiments described here, we used a strong fear conditioning protocol (six CS-US pairings of a .7 mA foot-shock), which we expected could lead to an increase in stress reactivity and anxiety. In this paradigm, fear-conditioned mice showed high levels of freezing, not only during CS onset but also during the intertrial interval (Figure S1A,B in Supplement 1).

Concordantly, we also observed an increase in the expression of the stress- and anxiety-related gene, *Hsp901b*, following fear conditioning (Figure 2D). Although there was no effect of fear conditioning on the expression of the synaptogenesis-related lncRNA Malat1 (Figure 2E), two intergenic lncRNAs of unknown function exhibited a significant, but nonspecific, decrease in expression following behavioral training (Figure 2F,G). The first of these, Gm21781, is upstream of a gene that encodes a DNA binding protein known as zinc finger and BTB domain containing 2 (*Zbtb2*), which has been shown to be a potent epigenetic regulator (55), and the second,

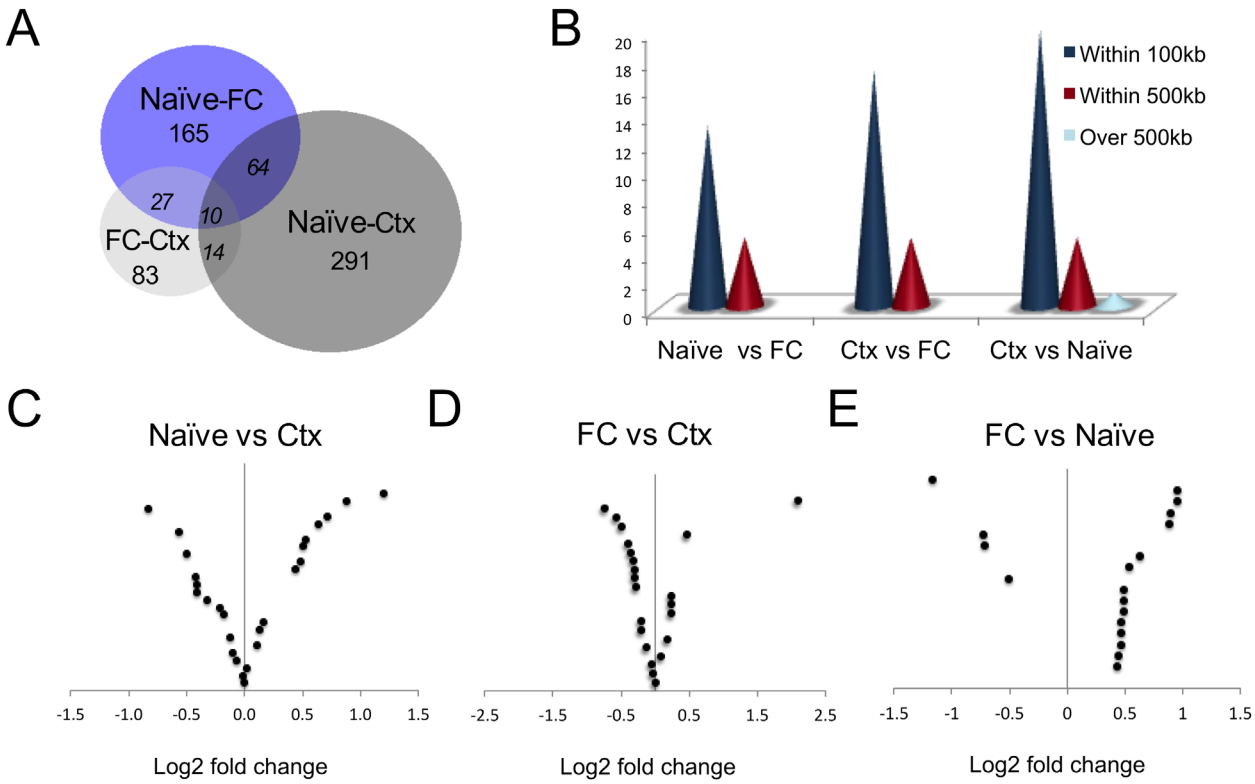

Figure 1. Transcriptome-wide analysis of nuclear RNA expression in the mouse prefrontal cortex reveals dynamic regulation of coding genes as well as long noncoding RNAs (lncRNAs). **(A)** BioVenn graphic of loci detected by nuclear-enriched RNA-sequencing with $p < .03$ corresponding to each pairwise comparison for naïve, context exposure (Ctx), and fear conditioning (FC) groups. **(B)** Distribution analysis of lncRNA loci revealing close proximity to coding genes. **(C–E)** Volcano plot showing log2 fold change magnitude of lncRNAs with $p < .03$ in each pairwise analysis. Upregulation of genes in context **(D, E)** and in naïve **(F)** animals is shown to the right side of the 0 in each figure.

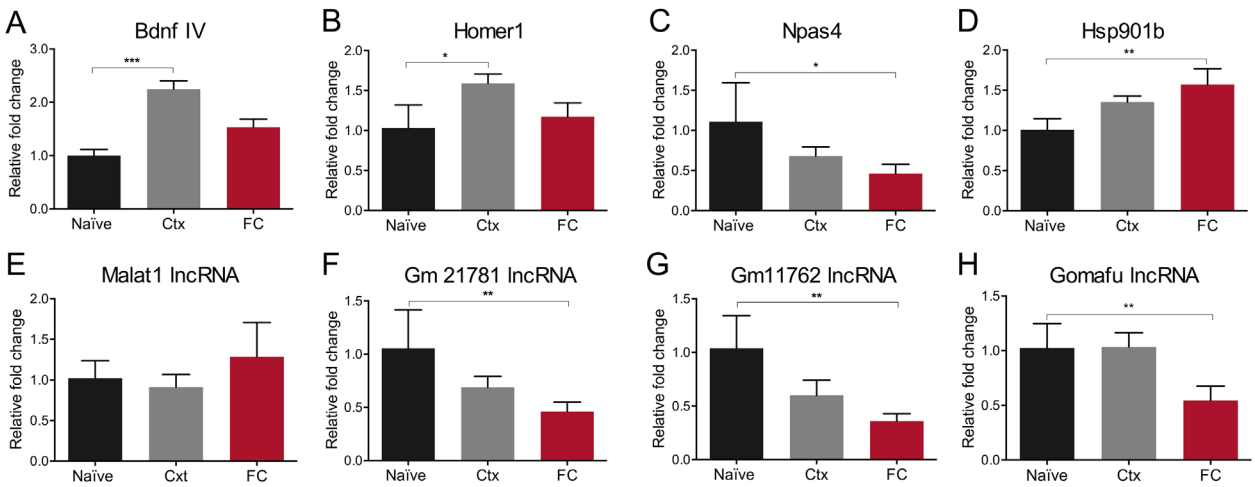

Figure 2. Quantitative reverse-transcription polymerase chain reaction validation of plasticity-related genes and noncoding RNA transcripts. **(A-D)** Analyses of protein coding genes confirm upregulation of *Bdnf* exon IV messenger RNA (naïve vs. context exposure [Ctx], $^{***}p < .001$, Kruskal-Wallis test statistics 16.58), upregulation of *Homer1* (naïve vs. Ctx, $^{*}p < .05$, analysis of variance [ANOVA], $F_{2,20} = 5.119$), downregulation of *Npas4* (naïve vs. fear conditioning [FC], $^{*}p < .05$, ANOVA, $F_{2,20} = 5.5$), and upregulation of *Hsp901b* (naïve vs. FC, $^{**}p < .01$, Kruskal-Wallis test statistics 9.459). **(E-G)** Analyses of long noncoding RNAs (lncRNAs) demonstrate a nonsignificant slight upregulation of Malat1, while Gm21781 lncRNA and Gm11762 lncRNA were downregulated following fear conditioning relative to naïve mice (ANOVA, $^{**}p < .01$, $F_{2,20} = 7.308$ and ANOVA, $^{**}p < .01$, $F_{2,20} = 5.892$, respectively). **(H)** Specific downregulation of Gomafu lncRNA in fear-conditioned mice (ANOVA, $^{**}p < .01$, $F_{2,20} = 5.953$ naïve vs. FC and Ctx vs. FC). $n = 8$ per group.

Gm11762, is antisense to neuronal pentraxin 1 (*Nptx1*), which has been reported to enhance synaptogenesis and glutamate signaling through clustering of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (56). Finally, we observed a significant downregulation of the neuropsychiatric disease-related lncRNA Gomafu in the mPFC, which occurred following fear conditioning but not after context exposure (Figure 2H). Importantly, the downregulation of Gomafu in fear-conditioned animals was also observed in a targeted RNA-seq experiment performed in these mice (Figure S2A in Supplement 1) using a recently developed highly sensitive RNA capture protocol (57), thereby confirming the previously reported activity-dependent nature of the expression of this lncRNA (29). Together, these data suggest that experience-induced regulation of lncRNAs may play a role in regulating neuronal plasticity and cognitive processes in the adult brain.

ASO-Mediated Knockdown of Gomafu Within the mPFC Induces Anxiety-like Behavior

To determine whether Gomafu regulates adaptive behavior, we employed an ASO-mediated knockdown approach. The efficacy of an ASO designed to specifically target Gomafu (Gomafu KD) was first tested in cultured primary cortical neurons at different time points (Figure S3A,B in Supplement 1). The preliminary screening indicated an approximately 50% knockdown at a concentration of 1 μmol/L, when measured 3 hours postadministration (Figure 3A), which decreased further by 6 hours postinfusion (Figure S3A,B in Supplement 1).

ASO-mediated Gomafu KD also produced a high rate of neuronal transfection when infused directly into the PLPFC in vivo (Figure 3B) before fear conditioning and was preliminarily verified by gene expression assay compared with a saline and a scrambled control group (Figure S4A,B in Supplement 1). Following a mild fear-conditioning protocol, mice infused with ASO-mediated Gomafu KD exhibited a moderate but significant enhancement in freezing behavior (Figure 3C). However, 24 hours later, these mice did not demonstrate a difference in fear recall of the previous conditioning episode when compared with the control group, indicating there was no effect of Gomafu on long-term memory per se (Figure 3D).

Given that stress-induced anxiety has been shown to enhance fear responding and interfere with memory retrieval events (58–61), we also examined the effect of Gomafu KD on anxiety-like behavior in an independent cohort of mice. Infusion of ASO-mediated Gomafu KD into the PLPFC led to a decrease in the amount of time spent in the center of an open field (Figure 4A,E), which was accompanied by a significant increase in distance traveled (Figure 4B), ambulatory time (Figure 4C), and stereotypic grooming behavior (Figure 4D), all of which are characteristic features of increased anxiety in mice (62). These data suggest that a reduction in the expression of the lncRNA Gomafu in the mPFC promotes behaviors that have been implicated in the development of anxiety disorders.

A Dual Role for the Neuropsychiatric-Disease Related lncRNA Gomafu

Long ncRNAs have been shown to exert dual control over gene expression through both *cis*- and *trans*-mediated mechanisms (63–65). As indicated above, the majority of lncRNAs that were differentially expressed in an experience-dependent manner were encoded within 100 kb of the nearest protein-coding gene, suggesting that these experience-dependent lncRNAs may control gene expression via a *cis*-mediated mechanism. Given that the activity-dependent lncRNA Gomafu was downregulated in the mPFC following behavioral

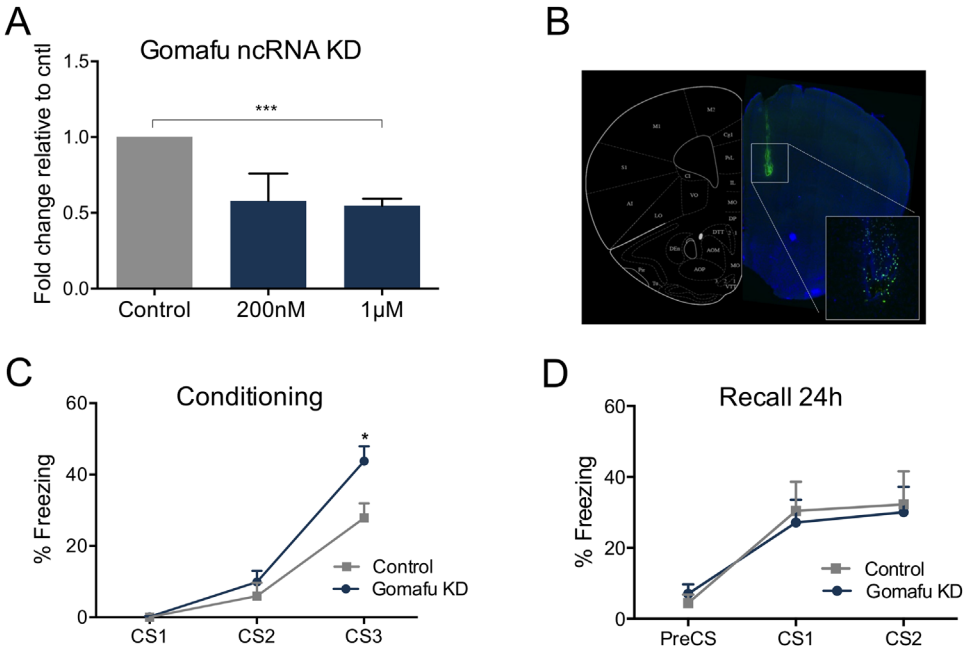

Figure 3. Knockdown (KD) of Gomafu long noncoding RNAs (ncRNA) in the prelimbic region of the prefrontal cortex enhances fear response during behavioral training. **(A)** Knockdown level by Gomafu antisense oligonucleotide (ASO) was assessed in cortical neurons 3 hours posttransfection (***$p < .001$, $t = 11.59$, $df = 4$, $n = 3$ per group) relative to control (cntl). **(B)** Coronal section of mouse brain infused with 6-Fam fluorescein-labeled Gomafu ASO (600 nmol/L) and co-stained with DAPI (blue). **(C)** Fear acquisition profile of animals trained at three conditioned stimulus (CS)-unconditioned stimulus, 3 hours post-infusion (CS3: *$p < .05$, $t = 2.672$, $df = 12$, $n = 6$ in cntl and $n = 8$ in ASO-induced Gomafu KD). **(D)** Recall test performed in mice 24 hours after the initial fear conditioning.

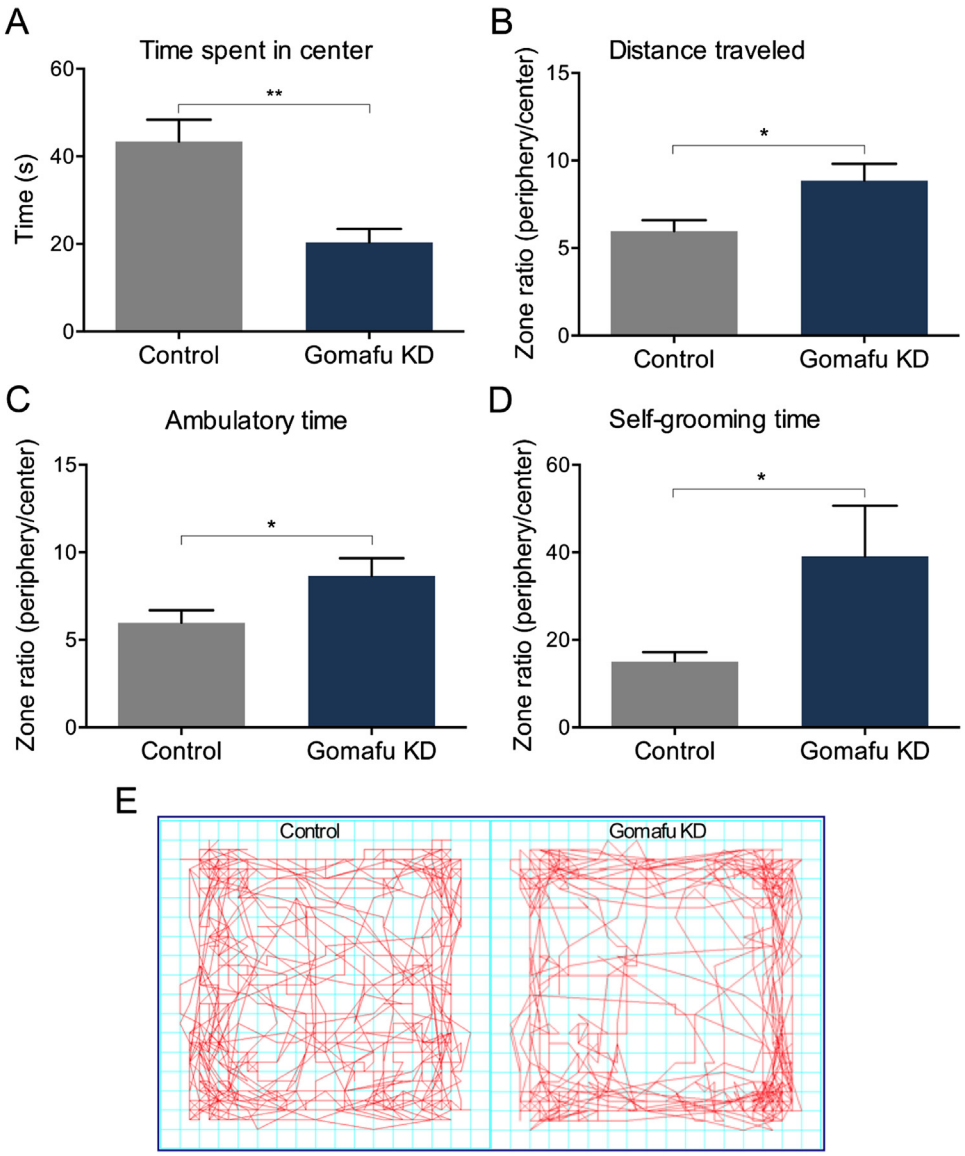

Figure 4. Knockdown (KD) of Gomafu long noncoding RNAs induces anxiety-like behavior. **(A,E)** A mild anxiety test performed on Gomafu knockdown animals in an activity monitoring chamber demonstrated that significantly less time was spent in the center of the field (**$p < .01$, $t = 3.922$, $df = 16$, $n = 9$ per group). **(B–D)** Gomafu knockdown mice also exhibited a significant tendency to travel longer distances outside the center of the field, as well as having an increased ambulatory and stereotypic grooming time compared with control animals (*$p < .05$, $t = 2.523$, $df = 16$, $n = 9$ per group; *$p < .05$, $t = 2.158$, $df = 16$, $n = 9$ per group; and *$p < .05$, Mann-Whitney U score $= 16$, $n = 9$ per group, respectively).

training and that it has previously been shown to regulate alternative splicing associated with neuropsychiatric disease through a *trans*-mediated mechanism (29), we examined whether this lncRNA also regulates gene expression associated with fear-induced anxiety.

Crystallin clusters are molecular chaperone proteins belonging to the heat shock family of genes and are known to play a major role in maintaining cellular homeostasis in response to stress (66–68). While the alpha-beta cluster has been associated with neurodegenerative diseases (69,70), a beta family member, *Crybb1,* has been reported to exert a major effect over these crystallin clusters by acting as an intermolecular chaperone that regulates their correct folding and misfolding (71). Furthermore, *Crybb1* has previously been associated with SZ and stress (72–75). In contrast to Gomafu, fear conditioning led to a significant and specific increase in the level of *Crybb1* mRNA (Figure 5A), with no change being observed with its close homolog, *Cryba4*, which is located on the same strand as Gomafu (Figure S2 in Supplement 1).

To test the hypothesis that Gomafu exerts its influence on proximal gene expression via a *cis*-mediated mechanism, we employed a model of primary cortical neurons in vitro. As expected, infusion of ASO-mediated Gomafu KD in primary cortical neurons led to a significant increase in *Crybb1* mRNA expression (Figure 5B). Next, considering that members of the polycomb repressive complex (PRC) have been shown to interact with lncRNAs to affect gene expression (22,76–80), we explored the relationship between Gomafu and major components of PRC1 and PRC2, namely BMI1 and RING1B and SUZ12, respectively. The binding motifs for these molecules have been identified (81,82) and were present within 2 kb of the transcription start site of *Crybb1* and within the gene body of Gomafu (Figure 5C). RNA immunoprecipitation using an antibody specific to BMI1 revealed an interaction with Gomafu

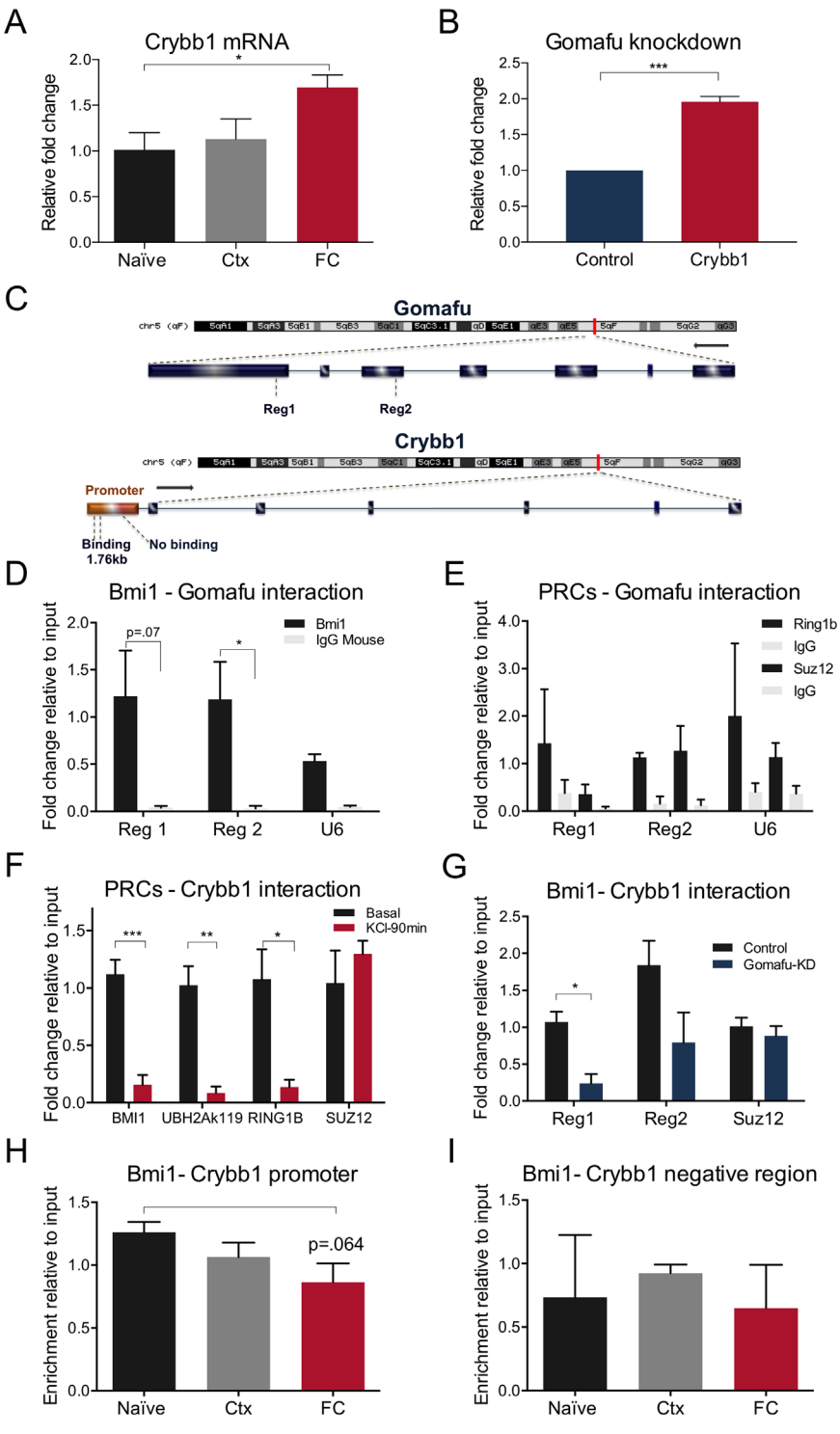

Figure 5. Interaction of Gomafu with the polycomb repressive complex (PRC)1 complex in the Crybb1 promoter region leads to transcriptional repression. **(A)**. In vivo quantitative reverse-transcription polymerase chain reaction analysis of *Crybb1* in fear conditioned (FC) and context (Ctx) mice showed a significant upregulation in response to the fear stimulus relative to naïve animals (analysis of variance [ANOVA] $^{*}p < .05$, Kruskal-Wallis statistic 6.455, $n = 8$ per group). **(B)** Significant in vitro upregulation of *Crybb1* resulted from antisense oligonucleotide-mediated Gomafu knockdown (KD) at 1 μmol/L concentration in cortical neurons ($^{***}p < .001$, $t = 12.89$, $df = 4$, $n = 3$ per group). **(C)** Schematic diagram showing Gomafu long noncoding RNA and *Crybb1* genomic structure and loci on chromosome 5, including the promoter region of *Crybb1* containing the BMI1 consensus sequence. **(D)** In vivo RNA immunoprecipitation assay from medial prefrontal cortex tissue revealed a significant binding of BMI1 on Gomafu long noncoding RNA relative to immunoglobulin G (IgG) in exonic region 2 (Reg2, $^{*}p < .05$, $t = 2.916$, $df = 4$, $n = 3$ per group) and a marked binding in the consecutive primer for exonic region 1 (Reg1, $p = .07$, $t = 2.434$, $df = 4$, $n = 3$ per group), as opposed to the negative locus on *U6* RNA. **(E)** RNA immunoprecipitation analysis for the same regions containing RING1B and SUZ12 binding sites showed no significant enrichment compared with the negative control, *U6* ($n = 3$ per group). **(F)** In vitro chromatin immunoprecipitation (ChIP) analysis under basal conditions and potassium chloride (KCl) stimulation exposed a significant dysregulation of BMI1 at the *Crybb1* promoter region, approximately 1.76 kilobase (kb) from transcription start site (TSS) (region 1) in KCl-treated cells ($^{***}p < .001$, $t = 6.369$, $df = 6$, $n = 4$ per group), which was followed by downregulation of the RING1B and UBH2Ak119 repressive marks ($p < .05$, $t = 3.511$, $df = 4$, $n = 3$ per group and $^{**}p < .01$, $t = 5.426$, $df = 4$, $n = 3$ per group, respectively). No significant changes were seen in the same region for SUZ12. **(G)** In vitro ChIP analysis revealed a significant decrease in BMI1 binding at 1.76 kb from the *Crybb1* TSS, using two consecutive primers, Reg1 and Reg2, following antisense oligonucleotide-mediated Gomafu KD in cortical neurons ($^{*}p < .05$, $t = 4.427$, $df = 4$, $n = 3$ per group and at 1.35 kb from TSS $p = .118$, $t = 1.986$, $df = 4$, $n = 3$ per group), whereas no changes were observed in a negative control containing the SUZ12 binding site. **(H)** In vivo ChIP analysis from medial prefrontal cortex tissue of trained mice showed BMI1 binding at approximately 1.76 kb from the *Crybb1* TSS, which markedly decreased after fear conditioning (ANOVA, $p = .116$, $F_{2,6} = 3.143$, $n = 3$ per group; t test between naïve and fear conditioned mice, $p = .064$, $n = 3$ per group). **(I)** ChIP analysis in a negative region about 1 kb from the *Crybb1* TSS did not show similar regulation of BMI1 on *Crybb1* (ANOVA, $p = .755$, $F_{2,6} = .2943$, $n = 3$ per group). mRNA, messenger RNA.

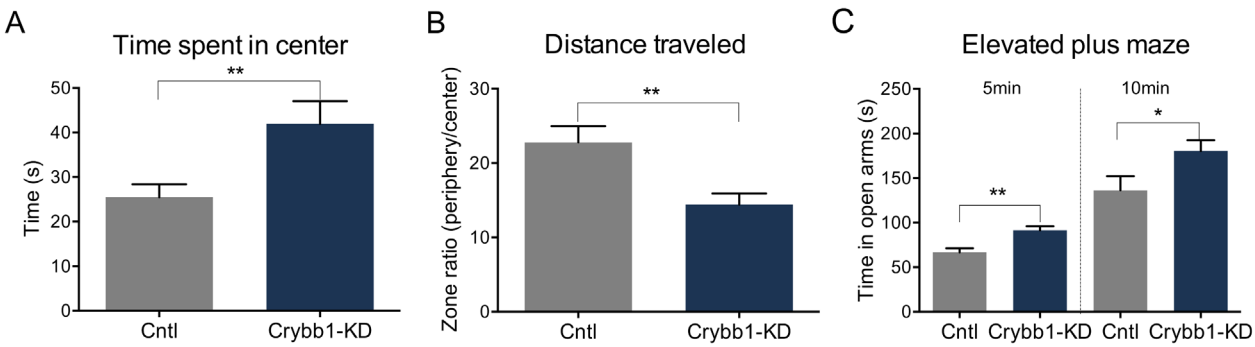

Figure 6. Knockdown (KD) of Crybb1 messenger RNA expression decreases anxiety in mice. **(A, B)** An anxiety test demonstrated that *Crybb1* knockdown animals (Crybb1-KD) spent significantly more time within the center of the activity-monitoring chamber (*$p < .05$, $t = 2.559$, $df = 12$, Control [Cntl] $n = 6$, Crybb1-KD $n = 8$), while travelling significantly shorter distances (**$p < .01$, $t = 3.263$, $df = 12$, Cntl $n = 6$, Crybb1-KD $n = 8$). **(C)** *Crybb1* knockdown mice also spent significantly more time within the open arms of the elevated plus maze (*$p < 0.05$, $t = 2.269$, $df = 12$, Cntl $n = 6$, Crybb1-KD $n = 8$), an effect that was more pronounced within the first 5 minutes of this trial (**$p < .01$, $t = 3.634$, $df = 12$, Cntl $n = 6$, Crybb1-KD $n = 8$).

lncRNA (Figure 5D). In contrast, there was no significant interaction between Gomafu and the PRC2 components SUZ12 and RING1B (Figure 5E). In addition, no interaction was observed between a U6 negative control and these proteins (Figure 5E), further supporting the presence of a specific interaction between Gomafu and BMI1.

We further verified through in vitro ChIP assay an activity-dependent binding of the PRC1 proteins BMI1 and RING1B accompanied by the ubiquitin histone 2A lysine 119 repressive mark within the *Crybb1* promoter, which was released upon potassium chloride-induced depolarization (Figure 5F). Although binding of the PRC2 component SUZ12 was observed, the interaction was unresponsive to stimulation (Figure 5F), confirming the specific activity-dependent recruitment of PRC1 to the *Crybb1* promoter.

To test whether Gomafu is required for the interaction between BMI1 and *Crybb1*, we performed a Gomafu knockdown experiment in cortical neurons, followed by ChIP analysis. After transfection with an ASO targeting Gomafu KD, there was a significant decrease in BMI1 occupancy at the *Crybb1* promoter (Figure 5G). However, importantly, there was no effect of the knockdown on SUZ12 occupancy (Figure 5G). This finding is consistent with the idea that, under basal conditions, BMI1 is maintained at the *Crybb1* promoter through a direct interaction with Gomafu, which serves to repress the transcriptional activity of *Crybb1*.

To investigate the interaction between BMI1 and *Crybb1* in vivo, similar ChIP analyses were performed that revealed BMI1 occupancy at the proximal promoter of *Crybb1* in mPFC tissue obtained from naïve animals. Although not significant, a marked decrease was observed in fear-conditioned compared with naïve animals (Figure 5H). Relative to input, there was little evidence of BMI1 occupancy in a region of the *Crybb1* promoter distal to the BMI1 binding motif (Figure 5I). These results suggest that, upon neuronal stimulation or behavioral training, a decrease in Gomafu releases PRC1 from the *Crybb1* promoter, leading to an activity-dependent increase in *Crybb1* mRNA expression.

A Novel Function for *Crybb1* in the Adult Brain

To test the hypothesis that fear-related anxiety in mice is linked to *Crybb1* expression, we examined the behavioral effect of *Crybb1* knockdown (Figure S5 in Supplement 1). Consistent with our Gomafu findings, knockdown of *Crybb1* decreased anxiety-like behavior in the activity-monitoring chamber (Figure 6A,B). Interestingly, in the pretraining test used to induce stress in these subjects, mice treated with *Crybb1* knockdown spent significantly more time in the open arms, also supporting the idea that decreased *Crybb1* led to a decrease in anxiety (Figure 6C). We therefore show that *Crybb1* directed knockdown within the mPFC has a role in stress-associated responses in mice.

DISCUSSION

The present findings reveal widespread experience-dependent activation of lncRNAs in the mPFC, including differential expression of a significant number of nonoverlapping intergenic lncRNAs. In particular, the expression of the SZ-associated lncRNA, Gomafu, was decreased in the mPFC following fear conditioning, and ASO-mediated knockdown of this lncRNA promoted stress reactivity and anxiety-like behavior with no effect on long-term memory.

Fear is an evolutionary programmed response for self-preservation and survival, but it can also induce pathological behaviors fueled by the persistent memory of adverse events (83). The PFC is a major site mediating stress-associated disorders and can elicit an anxiety response in the fear-conditioning paradigm (84–86). Fear conditioning has further been demonstrated to induce greater anxiety-like behaviors in high fear learning mouse lines, establishing a direct genetic link between fear conditioning and anxiety (87). Concordantly, the PFC is also vulnerable to stress inducing SZ (88) and has been implicated as a likely neurological site for stress and anxiety behavioral responses following fear-related learning (85,86,89).

Consistent with our findings, Gomafu has been reported to show evidence of dysregulation in postmortem cortical tissue of patients who had suffered SZ (29), which is commonly associated with anxiety disorders (90–96). Gomafu is expressed in specific neurons, localized in subnuclear speckle-like structures (97), and has been found to be associated with the splicing factors SRSF1 and QK1 (29), the latter of which has been linked with SZ (98–100). Moreover,

Gomafu knockdown and overexpression affects expression and alternative splicing of genes associated with SZ, such as *Erbb4* and *Disc1* (29).

Here, we have identified a potential pathway of Gomafu regulatory function through recruitment of the PRC1 complex to the site of gene transcription. This suggests a dual function of Gomafu on distal and local genes that guide epigenetic modulators in abnormal behaviors. It is well established that splicing is regulated by epigenetic factors (101,102), possibly via the four-dimensional organization of transcription-splicing complexes (103), which may themselves be built on RNA scaffolds (104). Although the mechanistic basis of the interaction of Gomafu with epigenetic processes has yet to be resolved, these results add to the emerging evidence that epigenetic function of lncRNAs may play a role in behavior and neurological diseases (105,106).

We also showed that the expression of the *Crybb1* gene was increased in the mPFC following fear conditioning and after Gomafu knockdown. Dysregulation of *Crybb1* has been associated with SZ and autism (72) and both Gomafu and *Crybb1* reside in the conserved human locus 22q12.1 (29), which has been related to SZ and generalized anxiety disorders (73–75,107). Although it has been linked to the development of cataracts (68,108,109), the specific function of *Crybb1* in the adult brain has not been determined. Here, in conjunction with lncRNA activity, we have discovered a novel role for *Crybb1* in mediating fear and stress-associated responses, with its repression appearing to reduce anxiety-like behavior in mice.

We suggest that Gomafu, in addition to its role in regulating alternative splicing in *trans* (29), acts in *cis* to direct the epigenetic regulation of *Crybb1*. Under basal conditions, Gomafu maintains the PRC1 at the promoter of *Crybb1*, which serves to repress its expression. In response to neuronal activation or fear conditioning, the activity-dependent downregulation of Gomafu, and its subsequent dissociation from BMI1, relieves its repressive control over the *Crybb1* promoter, leading to increased *Crybb1* gene expression (Figure 7). *Bmi1* has been reported to act as a major regulator of the cell stress response (110–113) and to sustain stem cell self-renewal in both the peripheral and central nervous systems (114–118). It also plays a key role in the defensive response to oxidative stress by affecting neuronal survival through repression of *p53* (119,120).

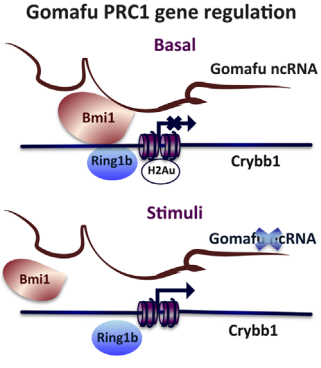

Figure 7. Proposed working model depicting activity-dependent regulation of Gomafu long noncoding RNA (lncRNA) in the mouse prefrontal cortex. Under basal conditions, Gomafu lncRNA associates with the polycomb repressive complex (PRC)1 (BMI1) complex to mediate in *cis* repression of *Crybb1*, potentially through the histone ubiquitination mark. In response to an environmental stimulus, such as that triggered by the fear-conditioning paradigm, Gomafu lncRNA is significantly downregulated, rapidly releasing BMI1 from the *Crybb1* promoter and allowing its transcription, resulting in the upregulation of *Crybb1*.

These results imply a complex relationship between lncRNA activity and transcriptional regulation in the adult brain. The dual function of Gomafu is reminiscent of previous work demonstrating that the lncRNA Malat1 can both function in *trans*, by interacting with hPSF protein (121) and serine/arginine splicing factors to regulate gene expression and cell cycle fate, respectively (63,64), and in *cis* to regulate the expression of adjacent genes (65).

In summary, we have shown that the downregulation of Gomafu lncRNA drives anxiety-like behavior. We further propose a novel role for lncRNAs within the mPFC in directing the epigenetic regulation of gene expression associated with adaptive behavior. Together with its role in governing SZ-related alternative splicing (29), in *trans*, Gomafu also may function in *cis* to control gene expression and complex behavior. These findings suggest that further investigation into lncRNAs as molecular links between epigenetic mechanisms and the development of neuropsychiatric disorders is warranted.

ACKNOWLEDGMENTS AND DISCLOSURES

This work was supported by Grant and fellowship support from the National Health and Medical Research Council of Australia (APP1042051, TWB; Australia Fellowship 631688, JSM) and the Australian Research Council (DP1096148, TWB). PAS is supported by a postgraduate scholarship from the Australian Government and the Queensland Brain Institute, The University of Queensland. TWB is supported by a Grant from the National Institute of Mental Health (1R21MH103812).

We thank Ms. Rowan Tweedale for helpful editing of the manuscript, Ms. Vikki Marshall for performing RNA-Seq, and Miss Marie-Jeanne Kempen and Gabriela Minervini for their assistance with behavioral data revision.

The authors report no biomedical financial interests or potential conflicts of interest.

ARTICLE INFORMATION

From the Queensland Brain Institute (PAS, CRF, JW, VSR, MT, CR, TWB), The University of Queensland Australia, Brisbane, Queensland; and Garvan Institute of Medical Research (JSM), Darlinghurst, and St Vincent's Clinical School, University of New South Wales, Kensington, New South Wales, Australia.

Address correspondence to Timothy W. Bredy, Ph.D., Queensland Brain Institute, The University of Queensland, 79 Upland Rd, St. Lucia, QLD 4072, Australia; E-mail: t.bredy@uq.edu.au.

Received Aug 21, 2014; revised Feb 3, 2015; accepted Feb 4, 2015.

Supplementary material cited in this article is available online at http://dx.doi.org/10.1016/j.biopsych.2015.02.004.

REFERENCES

1. Amaral PP, Dinger ME, Mercer TR, Mattick JS (2008): The eukaryotic genome as an RNA machine. Science 319:1787–1789.
2. Mercer TR, Dinger ME, Mattick JS (2009): Long non-coding RNAs: Insights into functions. Nat Rev Genet, 10, 155–159.
3. Mattick JS, Amaral PP, Dinger ME, Mercer TR, Mehler MF (2009): RNA regulation of epigenetic processes. Bioessays 31:51–59.
4. Koziol MJ, Rinn JL (2010): RNA traffic control of chromatin complexes. Curr Opin Genet Dev 20:142–148.
5. Mercer TR, Mattick JS (2013): Structure and function of long noncoding RNAs in epigenetic regulation. Nat Struct Mol Biol 20: 300–307.

6. Cheng J, Kapranov P, Drenkow J, Dike S, Brubaker S, Patel S, *et al*. (2005): Transcriptional maps of 10 human chromosomes at 5-nucleotide resolution. Science 308:1149–1154.
7. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, *et al*. (2005): The transcriptional landscape of the mammalian genome. Science 309:1559–1563.
8. Katayama S, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nakamura M, *et al*. (2005): Antisense transcription in the mammalian transcriptome. Science 309:1564–1566.
9. Cabili MN, Trapnell C, Goff L, Koziol M, Tazon-Vega B, Regev A, Rinn JL (2011): Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. Genes Dev 25:1915–1927.
10. Amaral PP, Clark MB, Gascoigne DK, Dinger ME, Mattick JS (2011): lncRNAdb: A reference database for long noncoding RNAs. Nucleic Acids Res 39:D146–D151.
11. Bu D, Yu K, Sun S, Xie C, Skogerbo G, Miao R, *et al*. (2012): NONCODE v3.0: Integrative annotation of long noncoding RNAs. Nucleic Acids Res 40:D210–D215.
12. Mattick JS (2004): RNA regulation: A new genetics? Nat Rev Genet 5:316–323.
13. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, *et al*. (2009): Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 458:223–227.
14. Mattick JS (2009): The genetic signatures of noncoding RNAs. PLoS Genet 5:e1000459.
15. Mercer TR, Dinger ME, Sunkin SM, Mehler MF, Mattick JS (2008): Specific expression of long noncoding RNAs in the mouse brain. Proc Natl Acad Sci U S A 105:716–721.
16. Mercer TR, Qureshi IA, Gokhan S, Dinger ME, Li G, Mattick JS, Mehler MF (2010): Long noncoding RNAs in neuronal-glial fate specification and oligodendrocyte lineage maturation. BMC Neurosci 11:14.
17. Chodroff RA, Goodstadt L, Sirey TM, Oliver PL, Davies KE, Green ED, *et al*. (2010): Long noncoding RNA genes: Conservation of sequence and brain expression among diverse amniotes. Genome Biol 11:R72.
18. Lin M, Pedrosa E, Shah A, Hrabovsky A, Maqbool S, Zheng D, Lachman HM (2011): RNA-Seq of human neurons derived from iPS cells reveals candidate long non-coding RNAs involved in neurogenesis and neuropsychiatric disorders. PLoS One 6:e23356.
19. Lv J, Liu H, Huang Z, Su J, He H, Xiu Y, *et al*. (2013): Long noncoding RNA identification over mouse brain development by integrative modeling of chromatin and genomic features. Nucleic Acids Res 41:10044–10061.
20. Lipovich L, Tarca AL, Cai J, Jia H, Chugani HT, Sterner KN, *et al*. (2014): Developmental changes in the transcriptome of human cerebral cortex tissue: Long noncoding RNA transcripts. Cereb Cortex 24:1451–1459.
21. Ponjavic J, Oliver PL, Lunter G, Ponting CP (2009): Genomic and transcriptional co-localization of protein-coding and long non-coding RNA pairs in the developing brain. PLoS Genet 5:e1000617.
22. Ng SY, Johnson R, Stanton LW (2012): Human long non-coding RNAs promote pluripotency and neuronal differentiation by association with chromatin modifiers and transcription factors. EMBO J 31:522–533.
23. Ng SY, Bogu GK, Soh BS, Stanton LW (2013): The long noncoding RNA RMST interacts with SOX2 to regulate neurogenesis. Mol Cell 51:349–359.
24. Bernard D, Prasanth KV, Tripathi V, Colasse S, Nakamura T, Xuan Z, *et al*. (2010): A long nuclear-retained non-coding RNA regulates synaptogenesis by modulating gene expression. EMBO J 29:3082–3093.
25. Petazzi P, Sandoval J, Szczesna K, Jorge OC, Roa L, Sayols S, *et al*. (2013): Dysregulation of the long non-coding RNA transcriptome in a Rett syndrome mouse model. RNA Biol 10:1197–1203.
26. Williams JM, Beck TF, Pearson DM, Proud MB, Cheung SW, Scott DA (2009): A 1q42 deletion involving DISC1, DISC2, and TSNAX in an autism spectrum disorder. Am J Med Genet A 149A:1758–1762.
27. Ziats MN, Rennert OM (2013): Aberrant expression of long noncoding RNAs in autistic brain. J Mol Neurosci 49:589–593.
28. Millar JK, Wilson-Annan JC, Anderson S, Christie S, Taylor MS, Semple CA, *et al*. (2000): Disruption of two novel genes by a translocation co-segregating with schizophrenia. Hum Mol Genet 9:1415–1423.
29. Barry G, Briggs JA, Vanichkina DP, Poth EM, Beveridge NJ, Ratnu VS, *et al*. (2014): The long non-coding RNA Gomafu is acutely regulated in response to neuronal activation and involved in schizophrenia-associated alternative splicing. Mol Psychiatry 19:486–494.
30. Pastori C, Peschansky VJ, Barbouth D, Mehta A, Silva JP, Wahlestedt C (2014): Comprehensive analysis of the transcriptional landscape of the human FMR1 gene reveals two new long noncoding RNAs differentially expressed in Fragile X syndrome and Fragile X-associated tremor/ataxia syndrome. Hum Genet 133:59–67.
31. Michelhaugh SK, Lipovich L, Blythe J, Jia H, Kapatos G, Bannon MJ (2011): Mining Affymetrix microarray data for long non-coding RNAs: Altered expression in the nucleus accumbens of heroin abusers. J Neurochem 116:459–466.
32. Bu Q, Hu Z, Chen F, Zhu R, Deng Y, Shao X, *et al*. (2012): Transcriptome analysis of long non-coding RNAs of the nucleus accumbens in cocaine-conditioned mice. J Neurochem 123:790–799.
33. Punzi G, Ursini G, Shin JH, Kleinman JE, Hyde TM, Weinberger DR (2014): Increased expression of MARCKS in post-mortem brain of violent suicide completers is related to transcription of a long, noncoding, antisense RNA. Mol Psychiatry 19:1057–1059.
34. Lewejohann L, Skryabin BV, Sachser N, Prehn C, Heiduschka P, Thanos S, *et al*. (2004): Role of a neuronal small non-messenger RNA: Behavioural alterations in BC1 RNA-deleted mice. Behav Brain Res 154:273–289.
35. Centonze D, Rossi S, Napoli I, Mercaldo V, Lacoux C, Ferrari F, *et al*. (2007): The brain cytoplasmic RNA BC1 regulates dopamine D2 receptor-mediated transmission in the striatum. J Neurosci 27:8885–8892.
36. Lipovich L, Dachet F, Cai J, Bagla S, Balan K, Jia H, Loeb JA (2012): Activity-dependent human brain coding/noncoding gene regulatory networks. Genetics 192:1133–1148.
37. Kim TK, Hemberg M, Gray JM, Costa AM, Bear DM, Wu J, *et al*. (2010): Widespread transcription at neuronal activity-regulated enhancers. Nature 465:182–187.
38. Ramos A (2008): Animal models of anxiety: Do I need multiple tests? Trends Pharmacol Sci 29:493–498.
39. Trapnell C, Pachter L, Salzberg SL (2009): TopHat: Discovering splice junctions with RNA-Seq. Bioinformatics 25:1105–1111.
40. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, *et al*. (2012): Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 7:562–578.
41. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, *et al*. (2010): Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 28:511–515.
42. Carey MF, Peterson CL, Smale ST (2009): Chromatin immunoprecipitation (ChIP). Cold Spring Harb Protoc 2009:pdb prot5279.
43. Ponting CP, Oliver PL, Reik W (2009): Evolution and functions of long noncoding RNAs. Cell 136:629–641.
44. Bekinschtein P, Cammarota M, Medina JH (2014): BDNF and memory processing. Neuropharmacology 76:677–683.
45. Boulle F, van den Hove DL, Jakob SB, Rutten BP, Hamon M, van Os J, *et al*. (2012): Epigenetic regulation of the BDNF gene: Implications for psychiatric disorders. Mol Psychiatry 17:584–596.
46. Ratnu VS, Wei W, Bredy TW (2014): Activation-induced cytidine deaminase regulates activity-dependent BDNF expression in post-mitotic cortical neurons. Eur J Neurosci 40:3032–3039.
47. Bredy TW, Wu H, Crego C, Zellhoefer J, Sun YE, Barad M (2007): Histone modifications around individual BDNF gene promoters in prefrontal cortex are associated with extinction of conditioned fear. Learn Mem 14:268–276.

48. Furukawa-Hibi Y, Yun J, Nagai T, Yamada K (2012): Transcriptional suppression of the neuronal PAS domain 4 (Npas4) gene by stress via the binding of agonist-bound glucocorticoid receptor to its promoter. J Neurochem 123:866–875.
49. Yun J, Koike H, Ibi D, Toth E, Mizoguchi H, Nitta A, *et al*. (2010): Chronic restraint stress impairs neurogenesis and hippocampus-dependent fear memory in mice: Possible involvement of a brain-specific transcription factor Npas4. J Neurochem 114:1840–1851.
50. Maya-Vetencourt JF (2013): Activity-dependent NPAS4 expression and the regulation of gene programs underlying plasticity in the central nervous system. Neural Plast 2013:683909.
51. Ploski JE, Monsey MS, Nguyen T, DiLeone RJ, Schafe GE (2011): The neuronal PAS domain protein 4 (Npas4) is required for new and reactivated fear memories. PloS One 6:e23760.
52. Schreurs BG, Smith-Bell CA, Burhans LB (2011): Incubation of conditioning-specific reflex modification: Implications for post traumatic stress disorder. J Psychiatr Res 45:1535–1541.
53. Siegmund A, Wotjak CT (2007): A mouse model of posttraumatic stress disorder that distinguishes between conditioned and sensitised fear. J Psychiatr Res 41:848–860.
54. Kamprath K, Wotjak CT (2004): Nonassociative learning processes determine expression and extinction of conditioned fear in mice. Learn Mem 11:770–786.
55. Lafaye C, Barbier E, Miscioscia A, Saint-Pierre C, Kraut A, Coute Y, *et al*. (2014): DNA binding of the p21 repressor ZBTB2 is inhibited by cytosine hydroxymethylation. Biochem Biophys Res Commun 446: 341–346.
56. Koch SM, Ullian EM (2010): Neuronal pentraxins mediate silent synapse conversion in the developing visual system. J Neurosci 30: 5404–5414.
57. Mercer TR, Gerhardt DJ, Dinger ME, Crawford J, Trapnell C, Jeddeloh JA, *et al*. (2012): Targeted RNA sequencing reveals the deep complexity of the human transcriptome. Nat Biotechnol 30: 99–104.
58. Milad MR, Pitman RK, Ellis CB, Gold AL, Shin LM, Lasko NB, *et al*. (2009): Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. Biol Psychiatry 66:1075–1082.
59. Cai WH, Blundell J, Han J, Greene RW, Powell CM (2006): Postreactivation glucocorticoids impair recall of established fear memory. J Neurosci 26:9560–9566.
60. Miracle AD, Brace MF, Huyck KD, Singler SA, Wellman CL (2006): Chronic stress impairs recall of extinction of conditioned fear. Neurobiol Learn Mem 85:213–218.
61. Wilber AA, Walker AG, Southwood CJ, Farrell MR, Lin GL, Rebec GV, Wellman CL (2011): Chronic stress alters neural activity in medial prefrontal cortex during retrieval of extinction. Neuroscience 174:115–131.
62. Bailey KR, Crawley JN (2009): Anxiety-related behaviors in mice. In: Buccafusco JJ, editor. Methods of Behavior Analysis in Neuroscience, 2nd ed. Boca Raton, FL: CRC Press.
63. Tripathi V, Ellis JD, Shen Z, Song DY, Pan Q, Watt AT, *et al*. (2010): The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. Mol Cell 39:925–938.
64. Tripathi V, Shen Z, Chakraborty A, Giri S, Freier SM, Wu X, *et al*. (2013): Long noncoding RNA MALAT1 controls cell cycle progression by regulating the expression of oncogenic transcription factor B-MYB. PLoS Genet 9:e1003368.
65. Zhang B, Arun G, Mao YS, Lazar Z, Hung G, Bhattacharjee G, *et al*. (2012): The lncRNA Malat1 is dispensable for mouse development but its transcription plays a *cis*-regulatory role in the adult. Cell Rep 2:111–123.
66. Stetler RA, Gan Y, Zhang W, Liou AK, Gao Y, Cao G, Chen J (2010): Heat shock proteins: Cellular and molecular mechanisms in the central nervous system. Prog Neurobiol 92:184–211.
67. Kalmar B, Greensmith L (2009): Induction of heat shock proteins for protection against oxidative stress. Adv Drug Deliv Rev 61:310–318.
68. Wistow G (2012): The human crystallin gene families. Human Genomics 6:26.
69. Lopez-Gonzalez I, Carmona M, Arregui L, Kovacs GG, Ferrer I (2014): alphaB-crystallin and HSP27 in glial cells in tauopathies. Neuropathology 34:517–526.
70. Chang LY, Lowe J, Ardiles A, Lim J, Grey AC, Robertson K, *et al*. (2014): Alzheimer's disease in the human eye. Clinical tests that identify ocular and visual information processing deficit as biomarkers. Alzheimers Dement 10:251–261.
71. Leng XY, Wang S, Cao NQ, Qi LB, Yan YB (2014): The N-terminal extension of betaB1-crystallin chaperones beta-crystallin folding and cooperates with alphaA-crystallin. Biochemistry 53:2464–2473.
72. Garbett KA, Hsiao EY, Kalman S, Patterson PH, Mirnics K (2012): Effects of maternal immune activation on gene expression patterns in the fetal brain. Transl Psychiatry 2:e98.
73. Gill M, Vallada H, Collier D, Sham P, Holmans P, Murray R, *et al*. (1996): A combined analysis of D22S278 marker alleles in affected sib-pairs: Support for a susceptibility locus for schizophrenia at chromosome 22q12. Schizophrenia Collaborative Linkage Group (Chromosome 22). Am J Med Genet 67:40–45.
74. Takahashi S, Ohtsuki T, Yu SY, Tanabe E, Yara K, Kamioka M, *et al*. (2003): Significant linkage to chromosome 22q for exploratory eye movement dysfunction in schizophrenia. Am J Med Genet B Neuropsychiatr Genet 123B:27–32.
75. Pulver AE, Karayiorgou M, Wolyniec PS, Lasseter VK, Kasch L, Nestadt G, *et al*. (1994): Sequential strategy to identify a susceptibility gene for schizophrenia: Report of potential linkage on chromosome 22q12-q13.1. Am J Med Genet 54:36–43.
76. Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, *et al*. (2009): Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc Natl Acad Sci U S A 106:11667–11672.
77. Marin-Bejar O, Marchese FP, Athie A, Sanchez Y, Gonzalez J, Segura V, *et al*. (2013): Pint lincRNA connects the p53 pathway with epigenetic silencing by the Polycomb repressive complex 2. Genome Biol 14:R104.
78. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, *et al*. (2007): Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 129:1311–1323.
79. Kotake Y, Nakagawa T, Kitagawa K, Suzuki S, Liu N, Kitagawa M, *et al*. (2011): Long non-coding RNA ANRIL is required for the PRC2 recruitment to and silencing of p15(INK4B) tumor suppressor gene. Oncogene 30:1956–1962.
80. Yap KL, Li S, Munoz-Cabello AM, Raguz S, Zeng L, Mujtaba S, *et al*. (2010): Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell 38:662–674.
81. Tsai MC, Manor O, Wan Y, Mosammaparast N, Wang JK, Lan F, *et al*. (2010): Long noncoding RNA as modular scaffold of histone modification complexes. Science 329:689–693.
82. Meng S, Luo M, Sun H, Yu X, Shen M, Zhang Q, *et al*. (2010): Identification and characterization of Bmi-1-responding element within the human p16 promoter. J Biol Chem 285:33219–33229.
83. Orsini CA, Maren S (2012): Neural and cellular mechanisms of fear and extinction memory formation. Neurosci Biobehav Rev 36: 1773–1802.
84. Berkowitz RL, Coplan JD, Reddy DP, Gorman JM (2007): The human dimension: How the prefrontal cortex modulates the subcortical fear response. Rev Neurosci 18:191–207.
85. Indovina I, Robbins TW, Nunez-Elizalde AO, Dunn BD, Bishop SJ (2011): Fear-conditioning mechanisms associated with trait vulnerability to anxiety in humans. Neuron 69:563–571.
86. Yuen EY, Wei J, Liu W, Zhong P, Li X, Yan Z (2012): Repeated stress causes cognitive impairment by suppressing glutamate receptor expression and function in prefrontal cortex. Neuron 73:962–977.
87. Ponder CA, Kliethermes CL, Drew MR, Muller J, Das K, Risbrough VB, *et al*. (2007): Selection for contextual fear conditioning affects anxiety-like behaviors and gene expression. Genes Brain Behav 6:736–749.
88. Arnsten AF (2011): Prefrontal cortical network connections: Key site of vulnerability in stress and schizophrenia. Int J Dev Neurosci 29: 215–223.

89. Hains AB, Arnsten AF (2008): Molecular mechanisms of stress-induced prefrontal cortical impairment: Implications for mental illness. Learn Mem 15:551–564.
90. Muller JE, Koen L, Soraya S, Emsley RA, Stein DJ (2004): Anxiety disorders and schizophrenia. Curr Psychiatry Rep 6:255–261.
91. Nebioglu M, Altindag A (2009): The prevalence of comorbid anxiety disorders in outpatients with schizophrenia. Int J Psychiatry Clin Pract 13:312–317.
92. Lysaker PH, Salyers MP (2007): Anxiety symptoms in schizophrenia spectrum disorders: Associations with social function, positive and negative symptoms, hope and trauma history. Acta Psychiatr Scand 116:290–298.
93. Achim AM, Maziade M, Raymond E, Olivier D, Merette C, Roy MA (2011): How prevalent are anxiety disorders in schizophrenia? A meta-analysis and critical review on a significant association. Schizophr Bull 37:811–821.
94. Young S, Pfaff D, Lewandowski KE, Ravichandran C, Cohen BM, Ongur D (2013): Anxiety disorder comorbidity in bipolar disorder, schizophrenia and schizoaffective disorder. Psychopathology 46:176–185.
95. Pallanti S, Cantisani A, Grassi G (2013): Anxiety as a core aspect of schizophrenia. Curr Psychiatry Rep 15:354.
96. Braga RJ, Reynolds GP, Siris SG (2013): Anxiety comorbidity in schizophrenia. Psychiatry Res 210:1–7.
97. Sone M, Hayashi T, Tarui H, Agata K, Takeichi M, Nakagawa S (2007): The mRNA-like noncoding RNA Gomafu constitutes a novel nuclear domain in a subset of neurons. J Cell Sci 120:2498–2506.
98. Aberg K, Saetre P, Jareborg N, Jazin E (2006): Human QKI, a potential regulator of mRNA expression of human oligodendrocyte-related genes involved in schizophrenia. Proc Natl Acad Sci U S A 103:7482–7487.
99. Aberg K, Saetre P, Lindholm E, Ekholm B, Pettersson U, Adolfsson R, Jazin E (2006): Human QKI, a new candidate gene for schizophrenia involved in myelination. Am J Med Genet B Neuropsychiatr Genet 141B:84–90.
100. Haroutunian V, Katsel P, Dracheva S, Davis KL (2006): The human homolog of the QKI gene affected in the severe dysmyelination "quaking" mouse phenotype: Downregulated in multiple brain regions in schizophrenia. Am J Psychiatry 163:1834–1837.
101. Luco RF, Allo M, Schor IE, Kornblihtt AR, Misteli T (2011): Epigenetics in alternative pre-mRNA splicing. Cell 144:16–26.
102. Zhou YP, Lu YL, Tian WD (2012): Epigenetic features are significantly associated with alternative splicing. BMC Genomics 13:123.
103. Mercer TR, Edwards SL, Clark MB, Neph SJ, Wang H, Stergachis AB, *et al*. (2013): DNase I-hypersensitive exons colocalize with promoters and distal regulatory elements. Nat Genet 45:852–859.
104. Mercer TR, Mattick JS (2013): Understanding the regulatory and transcriptional complexity of the genome through structure. Genome Res 23:1081–1088.
105. Khan DH, Jahan S, Davie JR (2012): Pre-mRNA splicing: Role of epigenetics and implications in disease. Adv Biol Regul 52:377–388.
106. Mehler MF, Mattick JS (2007): Noncoding RNAs and RNA editing in brain development, functional diversification, and neurological disease. Physiol Rev 87:799–823.
107. DeLisi LE, Shaw SH, Crow TJ, Shields G, Smith AB, Larach VW, *et al*. (2002): A genome-wide scan for linkage to chromosomal regions in 382 sibling pairs with schizophrenia or schizoaffective disorder. Am J Psychiatry 159:803–812.
108. Meyer E, Rahman F, Owens J, Pasha S, Morgan NV, Trembath RC, *et al*. (2009): Initiation codon mutation in betaB1-crystallin (CRYBB1) associated with autosomal recessive nuclear pulverulent cataract. Mol Vis 15:1014–1019.
109. Deng H, Yuan L (2014): Molecular genetics of congenital nuclear cataract. Eur J Med Genet 57:113–122.
110. Bracken AP, Kleine-Kohlbrecher D, Dietrich N, Pasini D, Gargiulo G, Beekman C, *et al*. (2007): The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes Dev 21:525–530.
111. Gargiulo G, Cesaroni M, Serresi M, de Vries N, Hulsman D, Bruggeman SW, *et al*. (2013): In vivo RNAi screen for BMI1 targets identifies TGF-beta/BMP-ER stress pathways as key regulators of neural- and malignant glioma-stem cell homeostasis. Cancer Cell 23:660–676.
112. Nakamura S, Oshima M, Yuan J, Saraya A, Miyagi S, Konuma T, *et al*. (2012): Bmi1 confers resistance to oxidative stress on hematopoietic stem cells. PloS One 7:e36209.
113. Calao M, Sekyere EO, Cui HJ, Cheung BB, Thomas WD, Keating J, *et al*. (2013): Direct effects of Bmi1 on p53 protein stability inactivates oncoprotein stress responses in embryonal cancer precursor cells at tumor initiation. Oncogene 32:3616–3626.
114. Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, Morrison SJ (2003): Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature 425:962–967.
115. Molofsky AV, He S, Bydon M, Morrison SJ, Pardal R (2005): Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. Genes Dev 19:1432–1437.
116. Fasano CA, Dimos JT, Ivanova NB, Lowry N, Lemischka IR, Temple S (2007): shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development. Cell Stem Cell 1:87–99.
117. Fasano CA, Phoenix TN, Kokovay E, Lowry N, Elkabetz Y, Dimos JT, *et al*. (2009): Bmi-1 cooperates with Foxg1 to maintain neural stem cell self-renewal in the forebrain. Genes Dev 23:561–574.
118. He S, Iwashita T, Buchstaller J, Molofsky AV, Thomas D, Morrison SJ (2009): Bmi-1 over-expression in neural stem/progenitor cells increases proliferation and neurogenesis in culture but has little effect on these functions in vivo. Dev Biol 328:257–272.
119. Chatoo W, Abdouh M, David J, Champagne MP, Ferreira J, Rodier F, Bernier G (2009): The polycomb group gene Bmi1 regulates antioxidant defenses in neurons by repressing p53 pro-oxidant activity. J Neurosci 29:529–542.
120. Abdouh M, Chatoo W, El Hajjar J, David J, Ferreira J, Bernier G (2012): Bmi1 is down-regulated in the aging brain and displays antioxidant and protective activities in neurons. PloS One 7:e31870.
121. Li L, Feng T, Lian Y, Zhang G, Garen A, Song X (2009): Role of human noncoding RNAs in the control of tumorigenesis. Proc Natl Acad Sci U S A 106:12956–12961.