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Long noncoding RNA-directed epigenetic regulation of gene expression is associated with anxiety-like behavior in mice

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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 (RNA-seq) was used to identify changes in the experience-dependent expression of long non-coding RNAs (lncRNAs) within the medial prefrontal cortex (mPFC) of adult mice. Transcripts were validated by real-time quantitative PCR 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 mPFC, followed by behavioral training and assessment of fear-related anxiety. lncRNA-directed epigenetic regulation of gene expression was investigated by chromatin and RNA immunoprecipitation assays.

Results—RNA-seq 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 down-regulation 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.

Conclusion—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.

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Keywords

non-coding RNA; Gomafu; anxiety; epigenetics; behavior; Crybb1

Introduction

Once considered vestiges of our evolutionary history associated with “junk” DNA, non-coding transcriptional activation, and post-transcriptional 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- 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 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). lncRNAs have been found to be co-expressed with activity-dependent genes such as *C-fos*, *Arc*, *Nr4a2* and *Bdnf*, suggesting a coordinated network of coding and non-coding 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 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.

Materials and Methods

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 12h 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, 3 groups (n=8 per group) of mice were used: a naïve, age-matched, home-cage control group, a fear-conditioned group that received six pairings of a 2 min 80 dB white noise conditioned stimulus (CS) that co-terminated with a 1 s foot-shock at 0.7 mA as the unconditioned stimulus (US, inter-trial interval of 2 min), and a context only group that was exposed to the CS but not the US. Animals were sacrificed 90 min post-training, 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 600nM of a Gomafu antisense oligonucleotide (ASO), directly into the mPFC, whereas the control group received an infusion of a scrambled control. 3h after infusion, mice were trained using a mild fear acquisition protocol in order to avoid a non-specific response (3 CS-US pairing with foot shocks of 0.4mA), followed by a 2CS test for memory recall in the same context 24h later.

Measures of anxiety

Anxiety tests for the knockdown and control groups were performed 3h after ASO infusion, and 2 weeks after lentiviral infusion respectively, and involved the use of mild stressors (38). This included a 10min exposure to a 27x27x20.3cm open field chamber with 200 lux of light intensity, which was followed by an interval of 30min. Mice were then introduced for 10min 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 10min mice were once more placed in the open field for 10min, at which point time in the center was automatically determined in seconds, while ambulatory time, distance traveled 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 (10mg/kg). Surgeries were performed under stereotaxic guidance and cannulas were implanted bilaterally within the prelimbic region of the PFC (PLPFC) at +1.8mm anteroposterior and -2mm dorsoventral to Bregma. Mice were allowed to recover for a minimum of 7 days before experiments. Animals were transcardially perfused post-training with cold phosphate buffered saline (PBS) (Lonza, Walkersville, MD, USA), followed by 4% paraformaldehyde in PBS. Brains were dissected out, sectioned and the localization of the ASO and shRNA infusion was determined by fluorescence microscopy.

Nuclear enrichment, RNA extraction and reverse transcription

mPFC derived from fear-conditioned mice was homogenized in nuclear buffer (detailed in Supplement 1) followed by RNA extraction using the RNeasy Mini kit (Qiagen, Hilden, Germany). Reverse transcription and cDNA synthesis was performed following the Quantitech Reverse Transcription kit protocol (Qiagen, Hilden, Germany), with the exception of RNA eluted from RNA immunoprecipitation (RIP) assays, which was reverse transcribed using Super Script III First Strand Synthesis (Invitrogen, Carlsbad, CA, USA). The assay was validated both *in vitro* and *in vivo* (Fig S6 A-B in Supplement 1).

RNA sequencing

1µg of nuclear-enriched RNA, with a minimum RNA Integrity (RIN) value of 8, from 6 samples per treatment was used to build a total RNA library using the Illumina TruSeq RNASample Preparation v2 protocol. Each animal was individually indexed, representing a single library per mouse, which was further multiplexed in 2 pools of 9 animals before loading into the Illumina flow cell. Samples with low read alignment were excluded, resulting in 5 animals for Naïve and Context groups and 4 mice for FC. The quality of the RNA library was verified on an Agilent DNA 1000 chip and run on the Bioanalyzer, while quantification was determined by qPCR, after which the samples were sequenced using the Illumina HiSeq v3 platform.

Bioinformatic analysis

Mapping of reads to the mouse genome (mm10) was performed using the Bowtie2 and TopHat 2.0.6 programs (39). Cufflinks 2.0.2 (BETA) algorithms 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 $p < 0.03$.

Gene knockdown

ASOs targeting Gomafu lncRNA were designed using the IDT Antisense design software 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 (Supplement 1). ShRNAs (Genecoepeo) targeting *Crybb1* mRNA were packaged in-house using a 3rd 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, NY, USA) containing 5% fetal bovine serum, 1% penicillin/streptomycin, 1% GlutaMAX, and 2% B27 supplement (GIBCO Life Technologies, NY, USA), and were maintained at 37°C with 5% CO₂. Electroporation of ASOs was performed using the Nucleofector transfection system (Lonza, Basel, Switzerland) according to the manufacturer's instructions. KCl treatments were performed at 50mM concentration.

Technical validation of RNA sequencing gene target selection

Gene expression was measured using the SYBR Green quantitative real-time PCR (qRT-PCR) detection method on a RotorGene 3000 (Qiagen, Hilden, Germany). The 2^{-ΔΔCt} method was applied to estimate differential levels of gene expression. ANOVA and Holm-Sidak's 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 (ChIP)

Dissociated cortical neurons were subjected to electroporation with 200nM ASO for Gomaflu knockdown followed by BMI1 pull-downs 3h post-transfection. For *in vivo* studies, tissues were dissected out and homogenized 1.5h after training. Tissue and cells were processed as previously described (42). Chromatin was immunoprecipitated using 4 µg ChIP-grade antibodies specific to BMI1 (1T21, Abcam, Cambridge, England, United Kingdom), SUZ12 (ab12073, Abcam, Cambridge, England, United Kingdom), and RING1B (D22F2, Cell Signaling, Danvers, Massachusetts, USA), H2AK119 (D2764, Cell Signaling, Danvers, Massachusetts, USA), mouse IgG (103533, Active Motif, Carlsbad, CA, USA) and rabbit IgG (2295402, Millipore, Billerica, Massachusetts, USA). DNA-protein interactions were analyzed by qRT-PCR using primers specific to the binding motifs of the proteins being investigated.

RNA immunoprecipitation (RIP)

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, CA, USA), followed by DNase treatment using the TURBO DNA-free Ambion kit (Ambion, Carlsbad, CA, USA).

Results

Long non-coding 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 to context-only exposed mice (Fig. S1A in Supplement 1). Nuclear RNA was extracted from the mPFC of mice sacrificed 90 min 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 non-coding RNAs (Fig. 1A). A complete list of differentially expressed transcripts identified by RNA-seq is provided in Table S1 in Supplement 2.

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 100kb of the nearest protein-coding gene, consistent with a potential *cis*-regulatory function (21, 43) (Fig. 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

In order 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 (Table S3 in Supplement 1; Fig. 2A and B). *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* (Fig. 2C). Although *Npas4* expression is related to fear learning, a down-regulation 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 (6CS-US pairings of a 0.7mA 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 inter-trial interval (Fig. S1 A and B in Supplement 1).

Concordantly, we also observed an increase in the expression of the stress- and anxiety-related gene, *Hsp901b*, following fear conditioning (Fig. 2D). Although there was no effect of fear conditioning on the expression of the synaptogenesis-related lncRNA Malat1 (Fig. 2E), two intergenic lncRNAs of unknown function exhibited a significant, but non-specific, decrease in expression following behavioral training (Fig. 2F and 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, Gm11762, is antisense to neuronal pentraxin 1 (*Nptx1*), which has been reported to enhance synaptogenesis and glutamate signalling through clustering of AMPA receptors (56). Finally, we observed a significant down-regulation of the neuropsychiatric disease-related lncRNA Gomafu in the mPFC, which occurred following fear conditioning but not after context exposure (Fig. 2H). Importantly, the down-regulation of Gomafu in fear-conditioned animals was also observed in a targeted RNA-seq experiment performed in these mice (Fig. 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

In order 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 (Fig. S3A and B in Supplement 1). The preliminary screening indicated an approximately 50% knockdown at a concentration of 1 μ M, when measured 3h post-administration (Fig. 3A), which decreased further by 6h post-infusion (Fig. S3A and B in Supplement 1).

ASO-mediated Gomafu KD also produced a high rate of neuronal transfection when infused directly into the PLPFC *in vivo* (Fig. 3B) prior to fear conditioning, and was preliminarily verified by gene expression assay compared to a saline and a scrambled control group (Fig. S4A and 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 (Fig. 3C). However, 24h later these mice did not demonstrate a difference in fear recall of the previous conditioning episode, when compared to the control group, indicating there was no effect of Gomafu on long-term memory *per se* (Fig. 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 (Fig. 4A and E), which was accompanied by a significant increase in distance traveled (Fig. 4B), ambulatory time (Fig. 4C), and stereotypic grooming behavior (Fig. 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

lncRNAs 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 100kb 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 down-regulated in the mPFC following behavioral 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 (Fig. 5A), with no change being observed with its close homolog, *Cryba4*, which is located on the same strand as Gomafu (Fig. S2 in Supplement 1).

In order 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 (Fig. 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 2kb of the transcription start site (TSS) of *Crybb1*, and within the gene body of Gomafu (Fig. 5C). RNA immunoprecipitation using an antibody specific to BMI1 revealed an interaction with Gomafu lncRNA (Fig. 5D). In contrast, there was no significant interaction between Gomafu and the PRC2 components SUZ12 and RING1B (Fig. 5E). In addition, no interaction was observed between a U6 negative control and these proteins (Fig. 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 KCl-induced depolarization (Fig. 5F). Although binding of the PRC2 component SUZ12 was observed, the interaction was unresponsive to stimulation (Fig. 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 (Fig. 5G). However, importantly, there was no effect of the knockdown on SUZ12 occupancy (Fig. 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 which 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 to naïve animals (Fig. 5H). Relative to input, there was little evidence of BMI1 occupancy in a region of the *Crybb1* promoter distal to the BMI1 binding motif (Fig. 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 (*Crybb1* KD) (Fig. S5 in Supplement 1). Consistent with our Gomafu findings, knockdown of *Crybb1* decreased anxiety-like behaviour in the activity-monitoring chamber (Fig. 6A and 6B). Interestingly, in the pre-training test used to induce stress in these subjects, mice treated with *Crybb1* KD spent significantly more time in the open arms, also supporting the idea that decreased *Crybb1* led to a decrease in anxiety (Fig. 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 non-overlapping 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 *post mortem* 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 sub-nuclear 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 4-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 down-regulation of Gomafu, and its subsequent dissociation from BMI1, relieves its repressive control over the *Crybb1* promoter, leading to increased *Crybb1* gene expression (Fig. 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).

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 down-regulation 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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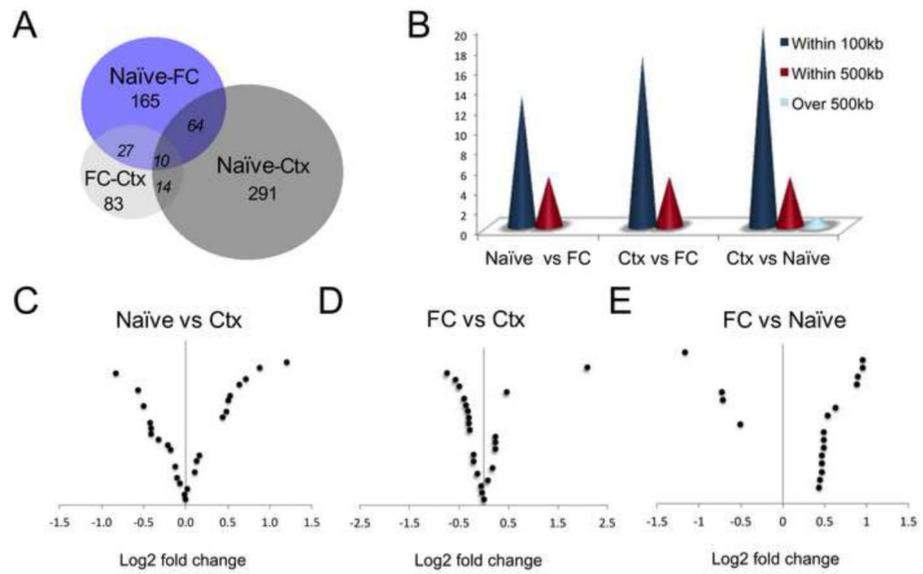


Figure 1. Transcriptome-wide analysis of nuclear RNA expression in the mouse prefrontal cortex reveals dynamic regulation of coding genes as well as lncRNAs

(A) BioVenn graphic of loci detected by nuclear-enriched RNA-Seq with p-value (p) < 0.03 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, D, E) Volcano plot showing log₂ fold change magnitude of lncRNAs with p-value < 0.03 in each pairwise analysis. Up-regulation of genes in context (D-E) and in naïve (F) animals is shown to the right side of the 0 in each figure.

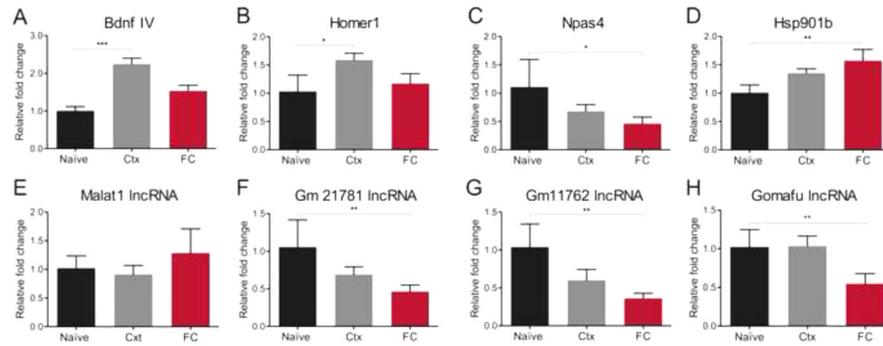


Figure 2. RT-qPCR validation of plasticity-related genes and non-coding RNA transcripts (A-D) Analyses of protein coding genes confirm up-regulation of *Bdnf* exon IV mRNA (naïve vs Ctx *** $p < 0.001$, Kruskal Wallis test statistics 16.58), up-regulation of *Homer1* (naïve vs Ctx, * $p < 0.05$, ANOVA, $F(2, 20) = 5.119$), down-regulation of *Npas4* (naïve vs FC, * $p < 0.05$, ANOVA, $F(2, 20) = 5.5$), and up-regulation of *Hsp901b* (naïve vs FC, ** $p < 0.01$, Kruskal Wallis test statistics 9.459). (E-G) Analyses of lncRNAs demonstrate a non-significant slight up-regulation of Malat1, while Gm21781 lincRNA and Gm11762 lincRNA were down-regulated following fear conditioning relative to naïve mice (ANOVA, ** $p < 0.01$, $F(2, 20) = 7.308$ and ANOVA, ** $p < 0.01$, $F(2, 20) = 5.892$ respectively). (H) Specific down-regulation of Gomafu lincRNA in fear-conditioned mice (ANOVA, ** $p < 0.01$, $F(2, 20) = 5.953$ naïve vs FC and Ctx vs FC). $n = 8$ per group.

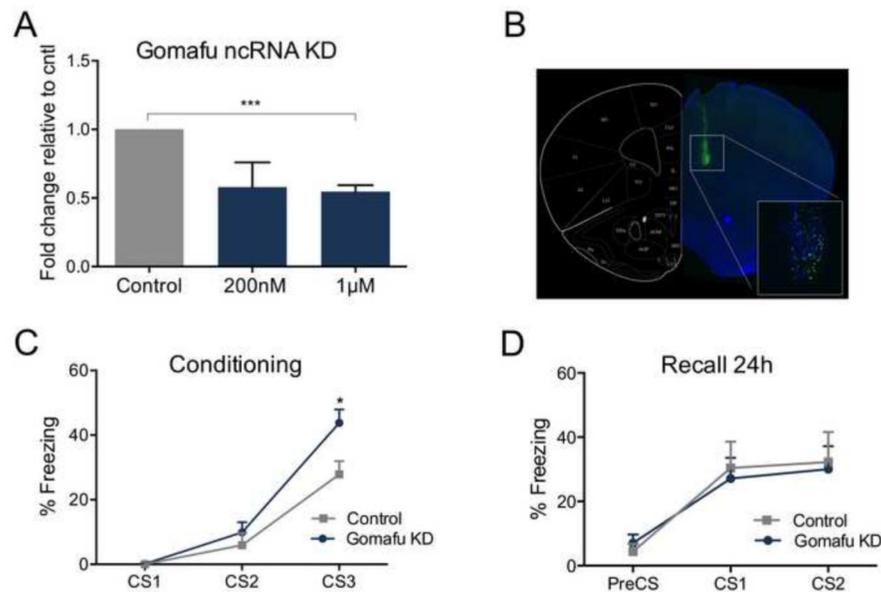


Figure 3. Knockdown of Gomafu lncRNA in the PLPFC enhances fear response during behavioral training

(A) Knockdown level by Gomafu ASO was assessed in cortical neurons 3h post-transfection (** $p < 0.001$, $t = 11.59$ $df = 4$, $n = 3$ per group) relative to control (cntl). (B) Coronal section of mouse brain infused with 6-Fam fluorescein-labeled Gomafu ASO (600nM) and co-stained with DAPI (blue). (C) Fear acquisition profile of animals trained at 3CS-US, 3h post infusion (CS3: $*p < 0.05$, $t = 2.672$ $df = 12$, $n = 6$ in cntl and $n = 8$ in ASO-induced Gomafu KD). (D) Recall test performed in mice 24h after the initial fear conditioning.

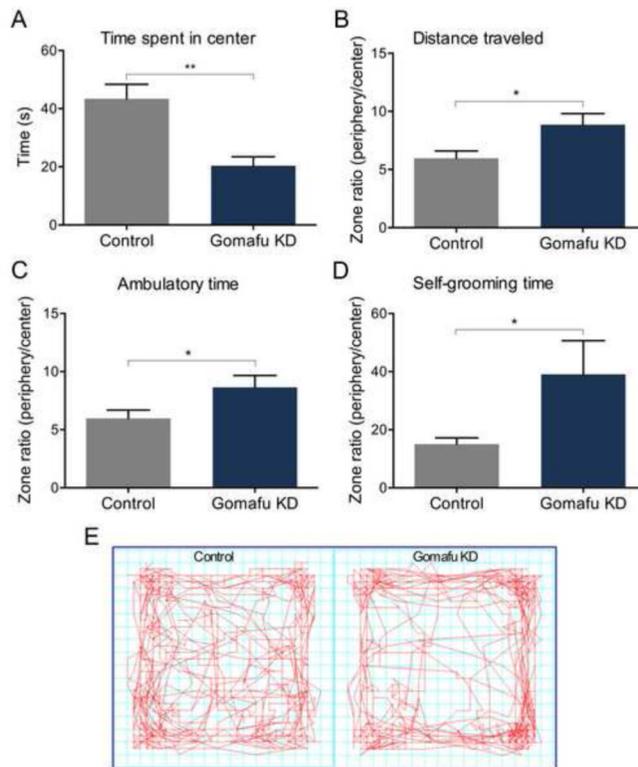


Figure 4. Knockdown of Gomafu lncRNA induces anxiety-like behavior

(**A and 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 (** $p < 0.01$, $t = 3.922$ $df = 16$, $n = 9$ per group). (**B, C, 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 to control animals ($*p < 0.05$, $t = 2.523$ $df = 16$, $n = 9$ per group; $*p < 0.05$, $t = 2.158$ $df = 16$, $n = 9$ per group and $*p < 0.05$, Mann-Witney U 16, $n = 9$ per group respectively).

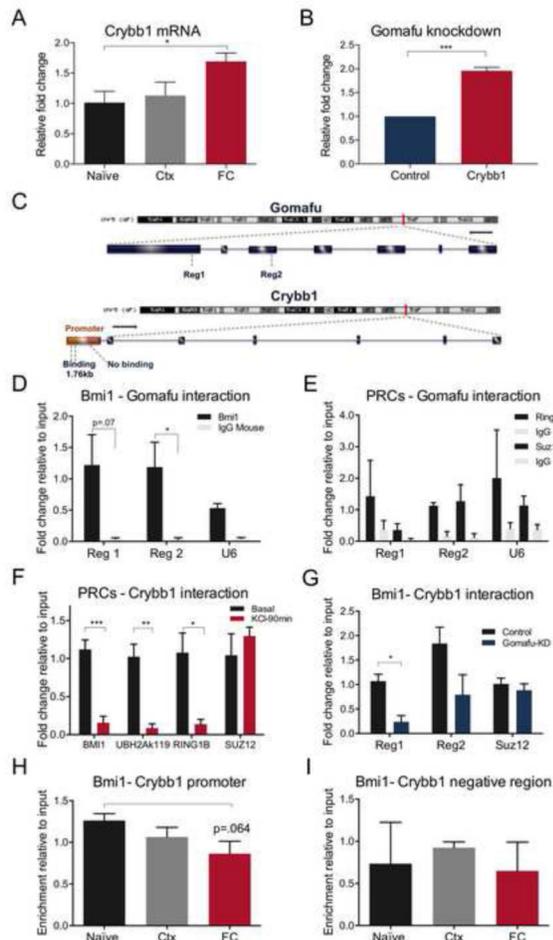


Figure 5. Interaction of Gomafu with the PRC1 complex in the Crybb1 promoter region leads to transcriptional repression

(A). *In vivo* RT-qPCR analysis of *Crybb1* in fear conditioned (FC) and context (Ctx) mice showed a significant up-regulation in response to the fear stimulus relative to naïve animals (ANOVA * $p < 0.05$, Kruskal-Wallis statistic 6.455, $n = 8$ per group). (B) Significant *in vitro* up-regulation of *Crybb1* resulted from ASO-mediated Gomafu KD at $1 \mu\text{M}$ concentration in cortical neurons (** $p < 0.001$, $t = 12.89$ $df = 4$, $n = 3$ per group). (C) Schematic diagram showing Gomafu lncRNA and *Crybb1* genomic structure and loci on chromosome 5, including the promoter region of *Crybb1* containing the BMI1 consensus sequence. (D) *In vivo* RIP assay from mPFC tissue revealed a significant binding of BMI1 on Gomafu lncRNA relative to IgG in exonic region 2 (Reg 2, * $p < 0.05$, $t = 2.916$ $df = 4$, $n = 3$ per group) and a marked binding in the consecutive primer for exonic region 1 (Reg 1, $p = 0.07$, $t = 2.434$ $df = 4$, $n = 3$ per group), as opposed to the negative locus on *U6* RNA. (E) RIP analysis for the same regions containing RING1B and SUZ12 binding sites showed no significant enrichment compared to the negative control, *U6* ($n = 3$ per group). (F) *In vitro* ChIP analysis under basal conditions and KCl stimulation exposed a significant dysregulation of BMI1 at the *Crybb1* promoter region, approximately 1.76kb from TSS (region 1) in KCl-treated cells (** $p < 0.001$, $t = 6.369$ $df = 6$, $n = 4$ per group) which was followed by down-regulation of the RING1B and UBH2Ak119 repressive marks (** $p < 0.01$, $t = 3.511$ $df = 4$, $n = 3$ per group and

** $p < 0.01$, $t = 5.426$ $df = 4$, $n = 3$ 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 1.76kb from the *Crybb1* TSS, using two consecutive primers, Reg1 and Reg2, following ASO-mediated Gomaflu KD in cortical neurons (* $p < 0.05$, $t = 4.427$ $df = 4$, $n = 3$ per group and at 1.35kb from TSS $p = 0.118$, $t = 1.986$ $df = 4$, $n = 3$ per group), whereas no changes were observed in a negative control containing the SUZ12 binding site. **(H)** *In vivo* ChIP analysis from mPFC tissue of trained mice showed BMI1 binding at approximately 1.76kb from the *Crybb1* TSS, which markedly decreased after fear conditioning (ANOVA, $p = 0.116$, $F(2,6) = 3.143$, $n = 3$ per group; t-test between naïve and fear conditioned mice, $p = 0.064$, $n = 3$ per group). **(I)** ChIP analysis in a negative region about 1kb from the *Crybb1* TSS did not show similar regulation of BMI1 on *Crybb1* (ANOVA, $p = 0.755$, $F(2,6) = 0.2943$, $n = 3$ per group).

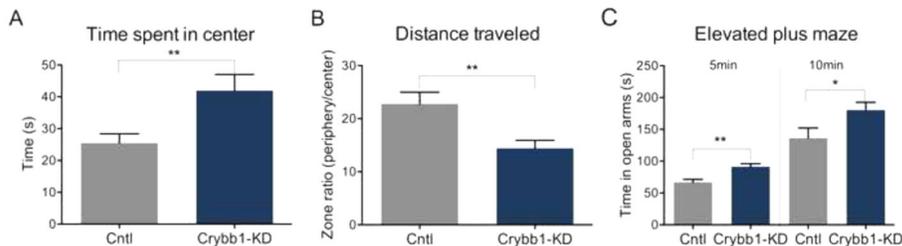


Figure 6. Knockdown of *Crybb1* mRNA expression decreases anxiety in mice
(A-B) An anxiety test demonstrated that *Crybb1* knockdown animals (Crybb1-KD) spent significantly more time within the center of the activity-monitoring chamber (*p<0.05, t= 2.559 df=12, cntl n=6, Crybb1-KD n=8) while travelling significantly shorter distances (**p<0.01, 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 min of this trial (**p<0.01, t= 3.634 df=12, cntl n=6, Crybb1-KD n=8).

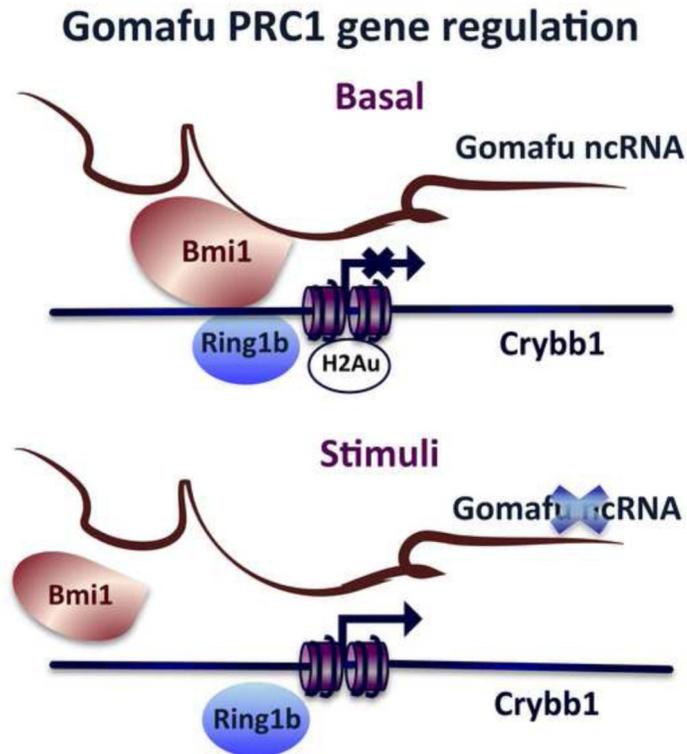


Figure 7. Proposed working model depicting activity-dependent regulation of Gomafu lncRNA in the mouse prefrontal cortex

Under basal conditions, Gomafu lncRNA associates with the PRC1 (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 down-regulated, rapidly releasing BMI1 from the *Crybb1* promoter and allowing its transcription, resulting in the up-regulation of *Crybb1*.