Molecular and Neural Functions of \textit{Rai1}, the Causal Gene for Smith-Magenis Syndrome

**Highlights**
- \textit{Rai1} preferentially binds active promoters and promotes transcription.
- Pan-neural loss of \textit{Rai1} causes motor function and learning deficits and obesity.
- \textit{Rai1} loss in inhibitory and subcortical excitatory neurons causes learning deficits.
- \textit{Rai1} loss in subcortical excitatory, Sim1$^+$, and SF1$^+$ neurons causes obesity.

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**In Brief**
Huang et al. show that \textit{Rai1}, the causal gene for neurodevelopmental disorder Smith-Magenis syndrome (SMS), binds active promoters and promotes transcription. \textit{Rai1} loss in different neuronal subtypes gives rise to specific SMS-like deficits in motor function, learning, and food intake.
Molecular and Neural Functions of Rai1, the Causal Gene for Smith-Magenis Syndrome

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SUMMARY

Haploinsufficiency of Retinoic Acid Induced 1 (RAI1) causes Smith-Magenis syndrome (SMS), which is associated with diverse neurodevelopmental and behavioral symptoms as well as obesity. RAI1 encodes a nuclear protein but little is known about its molecular function or the cell types responsible for SMS symptoms. Using genetically engineered mice, we found that Rai1 preferentially occupies DNA regions near active promoters and promotes the expression of a group of genes involved in circuit assembly and neuronal communication. Behavioral analyses demonstrated that pan-neural loss of Rai1 causes deficits in motor function, learning, and food intake. These SMS-like phenotypes are produced by loss of Rai1 function in distinct neuronal types: Rai1 loss in inhibitory neurons or subcortical glutamatergic neurons causes learning deficits, while Rai1 loss in Sim1+ or Sf1+ cells causes obesity. By integrating molecular and organismal analyses, our study suggests potential therapeutic avenues for a complex neurodevelopmental disorder.

INTRODUCTION

Copy number variations (CNVs) cause numerous neurodevelopmental and psychiatric disorders (Malhotra and Sebat, 2012; Ramocki and Zoghbi, 2008). It is generally difficult to dissect the etiology and develop effective treatments for disorders associated with large CNVs due to uncertainty about which genes within a deletion or duplication are responsible for the symptoms (Zhang et al., 2009). A prominent CNV disorder is Smith-Magenis syndrome (SMS) (Smith et al., 1993), which affects one in ~15,000 individuals. SMS patients exhibit craniofacial abnormalities, obesity, circadian abnormality, hypotonia, intellectual disabilities, stereotypies, and autistic features (Greenberg et al., 1996; Smith et al., 1993). 70% of SMS patients have an ~3.7 Mb interstitial deletion of chromosome 17p11.2 that contains 76 genes (Elsea and Girirajan, 2008). Importantly, 10% of SMS patients harbor point mutations or small deletions causing haploinsufficiency of a single gene within this region, Retinoic Acid Induced 1 (RAI1) (Dubourg et al., 2014; Slager et al., 2003). Patients with RAI1 mutations exhibit almost all of the core features of SMS, indicating that RAI1 is the dosage-sensitive gene responsible for most symptoms even in patients with large deletions. Furthermore, the reciprocal duplication in 17p11.2 causes Potocki-Lupski syndrome (PTLS), which shares many neuropsychological symptoms with SMS (Potocki et al., 2000, 2007). The smallest region common to PTLS patients with different duplications is a 125-kb region containing only RAI1 (Zhang et al., 2010), suggesting that duplication of RAI1 may also be responsible for the symptoms of PTLS. Therefore, brain development and function is exquisitely sensitive to RAI1 copy number.

RAI1 is a nuclear protein with two predicted protein-interacting domains: an extended plant homeo-domain (ePHD) and a nucleosome-binding domain (NBD) (Darvekar et al., 2012, 2013). In vitro studies revealed that overexpressed RAI1 associates with nuclear structures with high affinity (Darvekar et al., 2013). Overexpressed RAI1 binds to Brain-Derived Growth Factor (BDNF) and Circadian Locomotor Output Cycles Kaput (CLOCK) enhancers in vitro (Burns et al., 2010; Williams et al., 2012). While RAI1 does not possess a known DNA binding domain, when fused with a GAL4 DNA-binding domain, RAI1 shows moderate transcriptional activity in a luciferase assay (Bi et al., 2005). Although these data suggest a role for RAI1 in transcriptional regulation, its in vivo mode of action and targets remain unknown.

Mouse models have been used to study Rai1 function in vivo. Using an Rai1LacZ/+ allele that expresses β-galactosidase from the mouse Rai1 locus, it was found that Rai1 is expressed in many tissues including the developing and adult nervous system (Bi et al., 2005). Most Rai1 null mice die in utero; the few that survive exhibit craniofacial and skeletal abnormalities, motor
dysfunction, and fear-learning deficits (Bi et al., 2007). Rai1 heterozygous mice display mild SMS-like symptoms including obesity, circadian abnormalities, and characteristic craniofacial features (Bi et al., 2005; Lacaria et al., 2013). Overexpression of Rai1 in mice results in growth retardation, hyperactivity, and motor deficits (Girirajan et al., 2008). Given the broad expression pattern of Rai1 and diverse SMS symptoms, it is critical to determine whether specific symptoms are results of Rai1 requirement in specific cell types, in order to understand SMS pathogenesis and develop targeted therapies. Furthermore, while removing one copy of Rai1 better mimics the genetic underpinnings of SMS, deleting both copies in specific cell types may result in more severe phenotypes that reveal biological functions of Rai1.

Here, we have taken an integrative approach to dissect the molecular and neural functions of Rai1. Using a Rai1 conditional allele and an epitope-tagged Rai1 allele, we found that Rai1 preferentially occupies DNA regions near active promoters and enhances the expression of genes that function in cell-cell communication. We identified cell types that require Rai1 for proper motor function, learning, and food intake. Our integrative approach provides mechanistic insights into the etiology of SMS and suggests specific therapeutic strategies.

RESULTS

Rai1 Is Broadly Expressed in Postmitotic Neurons
To characterize Rai1 expression and to analyze its molecular functions, we engineered knockin mice with tandem FLAG and myc peptides (hereafter, Tag) fused to the carboxyl terminus of endogenous Rai1 (Rai1-Tag; Figure 1A). Western blot showed that the anti-FLAG antibody specifically recognized Rai1-Tag but not endogenous Rai1 (Figure S1A). Rai1-Tag was expressed preferentially in regions near active promoters and enhances the expression of genes that function in cell-cell communication. We identified cell types that require Rai1 for proper motor function, learning, and food intake. Our integrative approach provides mechanistic insights into the etiology of SMS and suggests specific therapeutic strategies.
at a similar level as un-tagged Rai1 (Figure S1B), as shown by an anti-Rai1 antibody we developed (and validated by lack of staining in conditional knockout; see below). Immunostaining revealed that Rai1-Tag co-localized with signals detected by the anti-Rai1 antibody in the postnatal day (P) 21 somatosensory cortex (white arrowheads, Figure 1B). 99.6% Rai1+ cells expressed Tag, 98.2% of the Tag+ cells expressed Rai1 (Figure 1B; quantified from 1,432 DAPI+ cells from 9 sections). We conclude that Rai1-Tag faithfully represents the endogenous Rai1 expression and used Rai1-Tag to further characterize Rai1 expression.

Consistent with a previous report (Bi et al., 2005), we observed Rai1-Tag expression in the embryonic day 9.5 (E9.5) branchial arch (Figure S1C) that develops into craniofacial structures. In E18.5 cortex, Rai1-Tag was broadly expressed in the cortical plate enriched in postmitotic neurons but rarely in the Ki67+ actively dividing cells near the ventricular zone (Figure 1C). The enrichment of Rai1 in postmitotic but not in proliferating cells was also observed in the developing dentate gyrus (Figure S1D and Table S1), cerebellum, and olfactory cortex (data not shown). Rai1 was also detected in a small fraction of S100β+ cerebellar Bergmann glia (Figure S1E and Table S1). Consistent with immunostaining, qRT-PCR using mouse cortices indicated that Rai1 mRNA levels increased during prenatal development, peaked around 1 week after birth, and persisted into adulthood (Figure 1D). Rai1-Tag was broadly expressed throughout the adult mouse brain (Figure 1E) and co-localized with 78% of the NeuN+ cortical neurons (Figure S1F and Table S1). Rai1 is expressed in both excitatory and inhibitory neurons in the thalamus (Figures S1G–S1J) and cortex (Figures S1K and S1L). Quantification based on double labeling of Rai1-Tag and in situ hybridization showed that Rai1 is expressed in 75% of excitatory neurons expressing Vglut1 (encoding vesicular glutamate transporter 1) and 57% of inhibitory neurons expressing Gad1 and/or Gad2 (encoding glutamate decarboxylases) in cortex (Figures S1K and S1L and Table S1). In summary, Rai1 is expressed in many cell types in the brain, with an onset that parallels the neuronal differentiation process.

Rai1 Occupies DNA Regions Near Active Promoters In Vivo

Mouse Rai1 and human RA11 share the same protein structure, with 82% overall sequence identity, and 88% and 82% identity in the C-terminal NBD and ePHD, respectively (Figure 2A). Human RA11NB was shown to interact with nucleosomes in vitro (Darvekar et al., 2013). To further characterize molecular functions of Rai1, we purified the recombinant human and mouse NBDs and ePHDs expressed in E. coli and performed a nucleosome pull-down assay. We found that NBDs from both species interact with Hexas nucleosomes, whereas an equal amount of ePHD protein did not (Figures 2B, S2A, and S2B). In a cellular fractionation assay using mouse cortices, Rai1 was present in both nucleoplastic and chromatin-binding fractions (Figure 2C). These experiments suggest that Rai1 interacts with chromatin in vitro and in vivo.

To investigate the genome-wide DNA binding pattern of Rai1 in vivo, we performed chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) using Rai1TAG/- mice. After confirming that FLAG-tagged Rai1 was enriched in anti-FLAG immunoprecipitation (Figure S2C), we performed ChIP-seq using 8-week-old Rai1TAG/+ mouse cortices. We obtained ~35 million paired-end reads, which exhibited a bimodal enrichment pattern along the mouse genome for each of the two replicates (Figure S2D and Table S2). We identified ~15,000 reproducible peaks (irreproducible Discovery Rate < 0.05) as high-confidence Rai1 binding sites (Figure S2E and Table S3). ChIP-qPCR also validated Rai1 binding sites identified by ChIP-seq (Figure S2F). Genome-wide enrichment analysis revealed that Rai1 binding sites were enriched in CpG islands, 5’ UTRs, and promoters (Figure 2D), but not intergenic or repetitive regions (Figure S2G). Normalized Rai1 ChIP-seq read densities on RefSeq gene bodies showed strong enrichment around transcriptional start sites (TSSs) (Figure 2E). These results demonstrate that Rai1 preferentially occupies promoter regions. We then categorized regions of the mouse genome into different states based on combinations of active or repressive chromatin marks (Ernst and Kellis, 2012). The overlay of Rai1 binding sites with different chromatin states further indicated that Rai1 binds active promoter and enhancer regions (Figures 2F and S2H; Table S4).

To determine the specificity of Rai1 binding to different DNA sequences, we applied a de novo motif-discovery algorithm (Heinz et al., 2010) on the high-confidence Rai1 binding sites. Among the significantly over-represented motifs (Figure S2I), an 8-mer motif was found in 45% Rai1 binding sites (Figure 2G; p = 1e–58). A comparison with previously discovered DNA motifs revealed that the 8-mer motif resembles the consensus sequences bound by zinc finger transcription factors ZNF711 and Zfx (Figure 2G). Interestingly, ZNF711 is highly expressed in the brain (Kleine-Kohlbrecher et al., 2010), and truncating mutations of ZNF711 have been identified in X-linked mental retardation (Tarpey et al., 2009). It remains to be experimentally determined whether these similar binding motifs are a consequence of physical interactions between Rai1 and ZNF711/Zfx, or could result in their competition. Comparisons of the positions between Rai1 peaks with existing mouse Zfx ChIP-seq data (Chen et al., 2008) revealed that Zfx binding signals and Rai1 peaks were globally co-localized, along with RNA polymerase 2 (Pol2) binding sites and the permissive transcription mark H3K4me3 (Figure 2H). By ranking the ChIP-seq peaks based on Rai1 peak intensity, we generated a heatmap showing the corresponding ChIP-seq read intensities of H3K4me3, Pol2, Zfx, and a negative control (anti-GFP antibody) (Figure 2I). The heatmap showed a high co-occupancy between Rai1 peaks with H3K4me3, Pol2, and Zfx peaks, but not with the negative control. Together, our genome-wide analysis indicates that Rai1 binds to specific loci in the genome associated with active transcription.

Rai1 Positively Regulates Steady-State Transcription

Given that Rai1 binds to promoter regions, we next examined how loss of Rai1 impacts the transcriptome. To circumvent embryonic lethality of Rai1 null mice, we generated a conditional knockout (CKO) allele by flanking exon 3, which encodes 97% of the Rai1 open reading frame including the translational start, with loxP sites (Figures S3A and S3B). Rai1flox/lox mice were born at the expected Mendelian ratio without apparent abnormalities. We then conditionally deleted Rai1 in the nervous
Figure 2. Rai1 Preferentially Occupies the Promoter Regions of Active Chromatin
(A) Schematic representation of predicted Rai1 protein domains. The nucleosome binding domain (NBD, blue) and extended plant homeo-domain (ePHD, brown) of human RAI1 and mouse Rai1 show a high degree of sequence conservation (identical amino acids are indicated as vertical green lines at bottom). In the magnified C termini of both proteins, numbers indicate amino acids in the primary sequence, and thin lines indicate gaps in alignment.

(B) In vitro nucleosome interaction assay showing that both human and mouse NBDs, but not ePHDs, bind purified HeLa nucleosomes.

(C) Cellular fractionation assay using mouse cortex showing that endogenous Rai1 co-fractionated with both histone H3 (chromatin fraction) and HDAC2 (nucleoplasmic fraction).

(D) Genome-wide annotation showing Rai1 binding sites identified by ChIP-seq are highly enriched at CpG island, 5' UTR, and promoter regions. ncRNA, non-coding RNA; TES, transcription end site.

(legend continued on next page)
system using a pan-neural Nestin\textsuperscript{Cre} line (Tronche et al., 1999). Western blot analysis using an anti-Rai1 antibody confirmed that Rai1 protein was undetectable in Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} brains (Figure S3C). As Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice exhibited postnatal lethality at increasing frequency with age (see below), we performed RNA sequencing (RNA-seq) using cortices from 3-week-old Rai1\textsuperscript{flo/flo} (control) and Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice. RNA-seq (Figure 3A) and qRT-PCR (Figure 3B) confirmed that exon 3 of Rai1 was deleted in the Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} cortices. Unexpectedly, the expression of small exons 3\textsuperscript{0} to the deleted region of Rai1 was upregulated (Figures 3A and 3B), possibly due to

Figure 3. Transcriptomic Changes in Rai1 Mutant Brains
(A) UCSC browser tracks of the cortex RNA-seq data of the Rai1 locus. The expression of floxed exon 3 (red box) is largely absent in the Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} cortex, while exons 4 to 6 are all upregulated compared to control. Coding exons, tall boxes.
(B) qRT-PCR showing Cre-dependent decrease of Rai1 exon 3 mRNA expression, and upregulation of Rai1 exons 4/5. Data are mean ± SEM (*p < 0.05, unpaired t test, n = 3).
(C) Genome-wide gene expression change in 3-week-old Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} cortex compared to control. Red dots show genes with False Discovery Rate (FDR) < 0.05 and brown dots show genes with 0.05 < FDR < 0.1.
(D) Genes with Rai1 binding sites identified by ChiP-seq in cortex showing a leftward shift in the cumulative distribution plot (red) compared with cumulative distribution of all genes (black), suggesting a global downregulation of Rai1-bound genes in the Rai1 mutant cortex.
(E) Downregulated genes in Gad2\textsuperscript{Cre};Rai1\textsuperscript{CKO} striatum at 3 weeks are still downregulated at 12 weeks (red dots in the bottom left but not top left quadrants). However, several 3-week upregulated genes become significantly downregulated at 12-weeks (red dots in the bottom right quadrant).
(F) Gene ontology analysis of downregulated genes in cortex and striatum showing enrichment in cell adhesion and morphogenesis-related terms. Gene ontology terms are ranked by FDR. See also Figure S3.
enhanced splicing efficiency after Cre-mediated deletion of the preceding exon 3. Globally, RNA-seq showed that loss of Rai1 caused changes in the expression levels of a small number of genes (Figure 3C, a subset of which were independently verified with qRT-PCR (Figure S3D). To further understand how Rai1 regulates transcription, we combined the ChIP-seq and RNA-seq data obtained from the cortex. We found that Rai1-bound genes were globally downregulated in the NestinCre,Rai1CKO brains; the fold changes were small but highly significant (Figure 3D). Therefore, Rai1 appears to positively regulate expression of its direct targets. Genes upregulated in NestinCre,Rai1CKO cortices are likely due to indirect effect of Rai1 loss.

We further investigated how Rai1 regulates transcription across developmental stages. The transcriptome of young mice may more closely reflect primary transcriptional changes due to loss of Rai1, whereas the adult tissue reflects a transcriptional state after prolonged Rai1 dysfunction. We chose GABAergic neurons for this comparison as GABAergic spiny projection neurons represent 95% of all neurons within dorsal striatum, allowing us to compare Rai1 in a relatively homogenous population. qRT-PCR showed that Rai1 exon 3 mRNA level in the Gad2Cre,Rai1CKO dorsal striatum decreased by 80% in young (3-week-old) mice and 93% in adult (12-week-old) mice compared to sex- and age-matched control littermates (Figure S3E). We performed RNA-seq using dorsal striatum collected from 3- and 12-week-old control and Gad2Cre,Rai1CKO mice. We found that most of the downregulated genes in the young mice remained downregulated in the adult stage, whereas many upregulated genes in the young mice became downregulated in the adult stage (Figure 3E). We also confirmed these expression patterns at both time points using qRT-PCR (Figures S3E and S3F). Therefore, genes are progressively downregulated in the Rai1 mutant brain with age progression, supporting a general role for Rai1 in positively regulating steady-state gene expression.

To gain insight into the biological processes regulated by Rai1, we compiled the downregulated genes identified from cortex and striatum (both young and adult) RNA-seq data (Table S5) and performed a gene ontology enrichment test. Functional annotation showed that Rai1 regulates genes involved in cell adhesion, axon guidance, and neuronal morphogenesis (Figure 3F and Table S6), such as Cdh7, Cdh8, Cdh9, EphA7, Pcdh20 (Figure S3G, left), and Sema3a (Figure S3G, right), suggesting that Rai1 may help assemble and maintain neural circuits.

Pan-neural Knockout of Rai1 Leads to Severe SMS-like Phenotypes
To explore the neural functions of Rai1, we next performed a battery of quantitative behavioral assays using the NestinCre,Rai1CKO mice and their control littermates. It should be noted that although NestinCre is expressed in progenitors that give rise to most or all neurons and glia in the CNS, it is also expressed in other organs (Harno et al., 2013; Tronche et al., 1999). The birth rate of NestinCre,Rai1CKO mice conformed to a Mendelian ratio, suggesting that the embryonic lethality exhibited by Rai1 null mice originates from NestinCre-negative tissues. Due to occasional germline activity of NestinCre, we also included mice produced in our breeding regime that lacked one Rai1 allele in their entire body (Rai1+/– or Rai1+/flox), these are the closest mouse models of SMS.

NestinCre,Rai1CKO mice were smaller than littermates prior to weaning and showed prominent hindlimb clasping (Figure S4A). More than 80% of NestinCre,Rai1CKO mice died before 25 weeks of age (Figure 4A). Most NestinCre,Rai1CKO mice that died prior to 10 weeks of age were gaunt, exhibiting prominent kyphosis and demonstrated weight loss in the 2 weeks preceding death (Figure S4B). It has previously been reported that both SMS patients and Rai1+/- mice are obese (Burns et al., 2010; Lacaria et al., 2012) and that female patients are more likely to exhibit food-seeking behavior than males (Edelman et al., 2007). In our experiments, only female NestinCre,Rai1CKO mice became significantly overweight beginning at 5 weeks of age, while male mice were not affected (Figure 4B). The cause for the sexually dimorphic phenotype is unclear, and the interpretation could be confounded by weight loss prior to lethality in some mice. At 20 weeks of age, female NestinCre,Rai1flox/+ and NestinCre,Rai1CKO mice became 35% and 101% heavier than control mice, respectively (Figure 4B). We thus used male NestinCre,Rai1CKO mice for behavioral studies, so that behavioral assays would not be confounded by different body weights (Figure S4C).

First, we tested the motor function of NestinCre,Rai1CKO mice. These mice displayed normal gait in the Catwalk assay (Figures S4D and S4E). In the activity chamber, the distance (Figures 4C and S4F) and zones (Figure S4G) traveled by NestinCre,Rai1CKO mice were statistically indistinguishable from controls. The mean velocity and vertical activity (Figures S4H and S4I) was also normal. In the pole test, NestinCre,Rai1CKO mice fell, slipped from the pole, or climbed down in a slow and uncoordinated fashion (Figure 4D). NestinCre,Rai1CKO mice also showed decreased latency to fall in a wire hang test (Figure 4E). Interestingly, Rai1+/- mice showed a decreased latency to fall, suggesting that performance in the wire hang test is more sensitive to Rai1 dosage than the pole test.

Next, we examined whether the mice had deficits in tests purported to assess anxiety, sociability, and cognition. We measured anxiety-like behavior using the elevated plus maze and found that the time NestinCre,Rai1CKO mice spent in open and closed arms was not statistically different from their control littermates (Figures S4J and S4K). Consistently NestinCre,Rai1CKO and control mice exhibited no difference in exploring the periphery versus center in open field test (Figure S4G). NestinCre,Rai1CKO mice also appeared normal in sociability and social discrimination assays (Figures S4L and S4M). However, NestinCre,Rai1CKO mice exhibited a significantly reduced tendency to investigate the new arm in the Y-maze test (Figure S4N), suggesting a spatial working memory deficit.

Finally, in a Pavlovian fear-conditioning task that tests learning and memory, control mice progressively developed a freezing response to a tone followed by a shock, whereas NestinCre,Rai1CKO mice had low freezing behavior after repeated tone-shock pairings during training (Figure 4F). As a result, these mice had reduced total freezing on the training day compared to littermates of other genotypes (Figure 4G). It is unlikely that these defects were caused by deficits in audition or pain sensation, as NestinCre,Rai1CKO mice exhibited normal pain responses in the hot plate assay and normal auditory startle responses.
Figure 4. Pan-neural Loss of Rai1 Causes SMS-like Phenotypes in Mice

(A) Survival curves for male and female control (Rai1WT/+, Rai1flox/flox, or NestinCre−/−, n = 26, black line), whole-body heterozygous knockout (Rai1Δ+/ or Rai1Δ/Δ, n = 27, gray line, overlaying the controls), whole-body heterozygous knockout plus NestinCre (NestinCre; Rai1Δ+/, n = 5, blue line), heterozygous NestinCre knockout (NestinCre; Rai1Δ/Δ, n = 12, pink line, overlapped with controls), and homozygous NestinCre knockout (NestinCre; Rai1CKO, n = 12, red line) mice. Most NestinCre; Rai1CKO mice die in early-to-mid adulthood, significantly younger than mice in all other groups (Log-rank test, *p < 0.0001).

(B) Mean (±SEM) body weights over time of male (left) and female (right) control (n = 12 males and 16 females), NestinCre; Rai1CKO (n = 5 males and 0 females), NestinCre; Rai1flox/+, and NestinCre; Rai1flox/flox, n = 27, gray line, overlaying the controls), whole-body heterozygous knockout plus NestinCre (NestinCre; Rai1Δ+/, n = 5, blue line), heterozygous NestinCre knockout (NestinCre; Rai1Δ/Δ, n = 12, pink line, overlapped with controls), and homozygous NestinCre knockout (NestinCre; Rai1CKO, n = 12, red line) mice. Female NestinCre; Rai1CKO mice exhibited normal locomotor activity (data not shown). Also, the severe reduction of freezing behavior on the training day is unlikely to be caused by hyperactivity, as the NestinCre; Rai1CKO mice exhibited normal locomotor activity (Figures S4F–S4I). When the tone was presented by itself in a different context on the subsequent day (cued recall), NestinCre; Rai1CKO mice exhibited more freezing compared to the training
day but still reduced compared to controls (Figure 4G). They also showed reduced freezing in the conditioning context without the tone (context recall) (Figure 4G). Therefore, despite modest steady-state transcriptomic changes, *Nestin<sup>Cre</sup>;Ra<sup>1CKO</sup> mice showed phenotypes that mimic many SMS symptoms, including deficits in body weight homeostasis, motor skills, and associative learning and memory.

**Rai1 Loss in Subcortical Excitatory Neurons Causes Motor Dysfunction**

As Rai1 is widely expressed in the brain (Figure 1E), it is possible that (1) each phenotype is caused by loss of Rai1 in a distinct group of cells (Figure 5A); (2) each cell type partially contributes to many phenotypes (Figure 5B); (3) most phenotypes are caused by loss of Rai1 in one critical group of cells (Figure 5C); or a combination of the above. To distinguish between these possibilities, we selected three SMS-like phenotypes (motor, learning, and obesity) exhibited by *Nestin<sup>Cre</sup>;Ra<sup>1CKO</sup> mice and conducted a cell-type screen to determine whether loss of Rai1 from specific cell populations would recapitulate these deficits. Specifically, one or both copies of *Rai1* was removed using (1) *Gad2Cre* that targets most GABAergic inhibitory neurons (Taniguchi et al., 2011), (2) *Emx1Cre* that targets cortical and hippocampal excitatory neurons and glia (Gorski et al., 2002), (3) *mGfapCre* that targets astrocytes and subsets of adult neural progenitors (Garcia et al., 2004), and (4) *Vglut2Cre* that targets subcortical excitatory neurons (Vong et al., 2011). Immunostaining confirmed that Rai1 was selectively deleted in Cre-expressing cells (Figures S5A–S5E; see Figure S6 for phenotypes other than motor, learning, and obesity analyzed in these conditional knockouts).

We first tested which cell types were responsible for the motor phenotypes seen in *Nestin<sup>Cre</sup>;Ra<sup>1CKO</sup> mice. All conditional mutants were similar in weight to their control littermates at the time of behavioral assays (Figure S5F). In the pole test, the time required to descend was increased only in *Vglut2Cre;Ra<sup>1CKO</sup>* mice (Figures 5D–5G). In the wire hang test, *Gad2Cre;Ra<sup>1CKO</sup>* mice performed slightly better than controls (Figure 5H), while removing one or both copies of *Rai1* from *Emx1* or *mGfap* cells did not interfere with performance (Figures 5I and 5J). By contrast, *Vglut2Cre;Ra<sup>1CKO</sup>* mice developed poor motor functions, as exemplified by a decreased latency to fall in the wire hang test (Figure 5K). Removing one copy of *Rai1* from the *Vglut2* neurons caused an intermediate phenotype in the wire hang test (Figure 5K). Thus, *Rai1* function in *Vglut2* excitatory neurons is essential for proper motor function and is dosage sensitive in a subset of the assays.

**Rai1 Loss in Either GABAergic Neurons or Subcortical Excitatory Neurons Disrupts Fear Conditioning**

Fear conditioning is regulated by distributed networks in the brain involving excitatory and inhibitory neurons (Letzkus et al., 2015). We found that *Emx1Cre;Ra<sup>1CKO</sup>* and *mGfapCre;Ra<sup>1CKO</sup>* mice did not show learning deficits (Figures 5M and 5N). However, both *Gad2Cre;Ra<sup>1CKO</sup>* and *Vglut2Cre;Ra<sup>1CKO</sup>* mice exhibited reduced freezing during training (Figures 5L and 5O). All conditional mutants and controls responded to shocks during training with increased locomotion or jumping, similar to control mice (data not shown). Both *Gad2Cre;Ra<sup>1CKO</sup>* and *Vglut2Cre;Ra<sup>1CKO</sup>* mice exhibited normal pain sensitivity in a hot plate assay (Figure S5G), suggesting that the fear conditioning phenotypes were due to learning deficits rather than secondary consequences of defective pain sensitivity. Additionally, *Vglut2Cre;Ra<sup>1CKO</sup>* and *Gad2Cre;Ra<sup>1CKO</sup>* mice both showed reduced cue- and contextual-memory recall (Figures 5L and 5O), mimicking the *Nestin<sup>Cre</sup>;Ra<sup>1CKO</sup>* phenotype. Together, these results indicate that *Rai1* is required in both Gad2* inhibitory and Vglut2* subcortical excitatory neurons for associative learning and memory.

**Rai1 Loss in Subcortical Excitatory Neurons, Sim1* Cells, or SF1* Cells Causes Obesity**

A prominent feature of SMS is truncal obesity, which develops in the young adolescent stage (Burns et al., 2010). Our analysis of *Nestin<sup>Cre</sup>;Ra<sup>1CKO</sup>* mice suggested that *Rai1* levels in the nervous system significantly contributed to this deficiency, at least in females (Figure 4B). To identify the cell types responsible for obesity in *Rai1* mutant mice, we deleted *Rai1* using *Gad2Cre*, *Emx1Cre*, *mGfapCre*, and *Vglut2Cre* lines. Only *Vglut2Cre;Ra<sup>1CKO</sup>* mice showed increased body weight when compared to control littermates in both females (Figures 6A–6D) and males (Figures S7A–S7D). Unlike *Nestin<sup>Cre</sup>;Ra<sup>1CKO</sup>* mice, *Vglut2Cre;Ra<sup>1CKO</sup>* mice did not show premature lethality, and thus weight analysis did not suffer from the complication of weight loss prior to death, as in *Nestin<sup>Cre</sup>;Ra<sup>1CKO</sup>* mice. Male and female *Vglut2Cre;Ra<sup>1CKO</sup>* mice became obese at 9 and 7 weeks of age, respectively (Figures 6D and S7D) and were 56% (males) and 116% (females) heavier than control littermates at 20 weeks of age. Mice losing one copy of *Rai1* in the Vglut2* neurons were moderately overweight (males: 16% overweight, female: 29% overweight). By contrast, the body weight of *Emx1Cre;Ra<sup>1CKO</sup>* and *mGfapCre*;Ra<sup>1CKO</sup>* mice was not significantly different from their control littermates, highlighting that *Rai1* is specifically required for Vglut2 subcortical excitatory neurons to regulate energy homeostasis.

Next, we asked which subtypes of Vglut2* neurons regulate body weight. The hypothalamus is an important brain center for controlling appetite and energy expenditure (Saper and Lowell, 2014). Within the hypothalamus, *Vglut2Cre* is expressed in the posterior hypothalamus, dorsal nucleus of hypothalamus, lateral hypothalamus, paraventricular nucleus of hypothalamus (PVH), ventromedial nucleus of hypothalamus (VMH), and a subset of POMC neurons in the arcuate nucleus (Vong et al., 2011). The VMH and PVH have emerged as critical hypothalamic nuclei that control feeding (Dhillon et al., 2006; Krashes et al., 2014). Therefore, we tested whether *Rai1* loss in PVH and/or the VMH would result in obesity.

In the hypothalamus, *Sim1Cre* targets PVH (but not VMH) neurons, the majority of which are Vglut2* (Balthasar et al., 2005; Xu et al., 2013). *SF1Cre* targets VMH (but not PVH) neurons, and the majority of VMH neurons targeted by *SF1Cre* are also Vglut2* (Dhillon et al., 2006; Tong et al., 2007). We generated female *Sim1Cre;Ra<sup>1CKO</sup>* and *SF1Cre;Ra<sup>1CKO</sup>* mice, in which *Rai1* was preferentially deleted in the PVH or VMH, respectively, whereas in *Vglut2Cre;Ra<sup>1CKO</sup>* mice *Rai1* was deleted from both nuclei (Figures S7E and S7F). Notably, *Sim1Cre;Ra<sup>1CKO</sup>* mice were 57% heavier than their control littermates at 20 weeks of age.
Figure 5. Rai1 Is Required in Specific Neuronal Types for Motor Functions and Learning

(A–C) Three models for the relationship between Rai1’s function in specific cell types and its organismal functions. See text for details.

(D–K) Latency to descend in the pole test (D–G) and fall in the wire hang test (H–K) for mice in which Rai1 was deleted in specific cell types as indicated. Data are mean ± SEM.

(L–O) Removal of Rai1 in either Gad2+ (L) or Vglut2+ (O) neurons, but not in Emx1+ (M) or mGfap+ (N) cells, impairs performance in a fear-conditioning task. Data are mean ± SEM.

Statistics: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, Tukey’s post hoc test following a significant ANOVA. See also Figures S5 and S6.
SF1Cre;Rai1CKO mice were 27% heavier than controls at the same age (Figure 6F). Removing one copy of Rai1 from the Sim1+ cells generated an intermediate overweight phenotype (12.7% heavier than controls at 20 weeks). Mice losing one copy of Rai1 from the SF1+ cells were equally obese as SF1Cre;Rai1CKO mice, suggesting that Sim1+ and SF1+ cells are differentially sensitive to Rai1 dosage. Therefore, both Sim1+ and SF1+ cells contribute to the obesity phenotype observed in Vglut2Cre;Rai1CKO mice, likely due to loss of Rai1 in PVH and VMH neurons, respectively, although we cannot rule out the contribution of Sim1+ or SF1+ cells outside the PVH or VMH.

Given that Sim1+ cells played a more dominant role than SF1+ cells in obesity due to Rai1 loss, we next aimed to identify the source of increased body weight in Sim1Cre;Rai1CKO mice. We quantified body composition with dual-energy X-ray absorptiometry (DEXA) and found that Sim1Cre;Rai1CKO mice showed significantly increased lean and fat mass (Figures 6G and 6H). Consistent with DEXA results, the weight of each dissected fat pad, including brown adipose tissue (BAT), was greater in Sim1Cre;Rai1CKO mice (Figure 6I). These data indicate that Sim1Cre;Rai1CKO mice developed increased adiposity. In addition to obesity, SMS patients show higher levels of total cholesterol, HDL, LDL, and triglycerides (Smith et al., 2002). Consistent with

Figure 6. Rai1 in Vglut2+ Neurons Controls Energy Homeostasis

(A–D) Mean (±SEM) body weights over time of female mice missing one or both copies of Rai1 in the Gad2 (A, n = 10–20 for each genotype), Emx1 (B, n = 6–8 for each genotype), mGfap (C, n = 6–10 for each genotype), and Vglut2 (D, n = 12–19 for each genotype)-Cre lineages. Female Vglut2Cre;Rai1lox/lox and Vglut2Cre;Rai1lox/CKO mice become significantly obese beginning at 9 and 7 weeks of age, respectively. For Vglut2Cre;Rai1lox/lox mice: * at 9 weeks, ** at 10 weeks, *** at 11 and 12 weeks, **** at 13–25 weeks.

(E and F) Mean (±SEM) body weights over time of female mice missing one or both copies of Rai1 in the Sim1+ (E, n = 10–13 for each genotype) or SF1+ (F, n = 8–16 for each genotype) neurons.

(G and H) Body composition (G, mean ± SEM) and representative images (H) of 30-week-old female Rai1lox/lox and Sim1Cre;Rai1CKO mice as measured with dual-energy X-ray absorptiometry (n = 8 for each genotype).

(I) Fat pad weight (mean ± SEM) of 30-week-old female Rai1lox/lox and Sim1Cre;Rai1CKO mice (n = 8 for each genotype).

(J–M) Serum levels (mean ± SEM) of total cholesterol (J), high-density lipoprotein (HDL) (K), low-density lipoprotein and very low-density lipoprotein (LDL and VLDL) (L), and triglyceride (M) in 30-week-old female Rai1lox/lox and Sim1Cre;Rai1CKO mice (n = 8 for each genotype).
Figure 7. Mechanisms Underlying Hypothalamic Dysfunction upon Rai1 Deletion

(A) Daily food intake of normal chow-fed mice (averaged over 7 days, mean ± SEM). Sim1Cre;Rai1CKO mice show significantly increased daily food intake (n = 8 for each genotype).

(B) Beam-break counts (mean ± SEM) indicating a decrease of horizontal locomotor activity in 30-week-old Sim1Cre;Rai1CKO mice (n = 8 for each genotype).

(C) Respiratory exchange rate (mean ± SEM) as calculated by VCO2/VO2 is not significantly different among groups (n = 8 for each genotype).

(D) Expression of Rai1 exon 3 and Bdnf are downregulated in the Vglut2Cre;Rai1CKO hypothalamus, as revealed by qRT-PCR. Data are mean ± SEM (n = 3).

(E) Volcano plot of RNA-seq results of control and Vglut2Cre;Rai1CKO hypothalamus. Red dots indicate genes that were further validated.

(F) qRT-PCR showing that the mRNA expression of Pcdh20, Htr2c, and Sema3a are commonly downregulated in the NestinCre;Rai1CKO cortex, Gad2Cre;Rai1CKO striatum (both 3 weeks and 12 weeks), and Vglut2Cre;Rai1CKO hypothalamus. Data are mean ± SEM (n = 3).

(G) UCSC browser tracks of mouse Bdnf (left) and Htr2c (right) genomic loci annotated with Rai1 ChIP-seq and input signals (orange, our data from cortex), histone marks (gray, ENCODE cortex data), and our RNA-seq signals from the control (light blue) and Vglut2Cre;Rai1CKO (dark blue) hypothalamus. Red arrows

(legend continued on next page)
that were downregulated not only in obesity (Kernie et al., 2000; Tecott et al., 1995), and Rai1 binds independently by qRT-PCR (Figure 7F). Interestingly, both our hypothalamus RNA-seq data suggest that decreased expression due to Rai1 loss may contribute to obesity.

**Potential Mechanisms Underlying Obesity in Rai1 Mutants**

Obesity can be caused by increased food intake, decreased energy expenditure, or their combination. To distinguish among these possibilities in Sim1<Cre;Rai1<CKO mice, we monitored their home cage food intake for a week. We found that they displayed hyperphagia, with a 47% increase in average daily food intake compared to control littermates (Figure 7A), and decreased horizontal locomotor activity over a 48 hr period (Figure 7B). Energy expenditure after normalization to lean body mass (Figures S7H–S7J) and respiratory exchange rate (Figure 7C) was not significantly affected. Together, these data suggest that hyperphagia may underlie the severe obesity in Sim1<Cre;Rai1<CKO mice.

To identify the misregulated genes that may explain Rai1-associated obesity and increased food intake, we performed RNA-seq using hypothalami isolated from Vglut2<Cre,Rai1<CKO and control littermates at 8 weeks of age, before the conditional mutants became obese. Rai1 mRNA was downregulated by ~50% in the Vglut2<Cre,Rai1<CKO hypothalamus (Figure 7D), consistent with the fact that Vglut2+ excitatory neurons only account for a subset of hypothalamic cells. Due to this dilution effect, and potential gene expression heterogeneity in Vglut2+ neurons in different hypothalamic nuclei, the magnitude and number of differentially expressed genes in Vglut2+ neurons are likely to be an underestimation. Still, we detected by RNA-seq (Figure 7E) and confirmed by qRT-PCR (Figure 7D) a previous observation that Bdnf is downregulated in Rai1 heterozygous mice (Burns et al., 2010). In addition, we identified several genes involved in cell-cell communication, such as Htr2c (encoding the serotonin receptor 2c), Pcdh20 (encoding a cell adhesion molecule, protocadherin), and Sema3a (encoding an axon guidance protein), that were downregulated not only in Vglut2<Cre,Rai1<CKO hypothalamus (Figure 7E), but also in the Rai1 mutant cortex and striatum (Figure S3G and Table S5). We confirmed these downregulations independently by qRT-PCR (Figure 7F). Interestingly, both Bdnf and Htr2c mutant mice exhibit over-eating behaviors that lead to obesity (Kenne et al., 2000; Tecott et al., 1995), and Rai1 binds to the promoters of both Bdnf and Htr2c (Figure 7G). Together, our hypothalamic RNA-seq data suggest that decreased Bdnf and Htr2c expression due to Rai1 loss may contribute to obesity.

**DISCUSSION**

Designing treatment strategies for neurodevelopmental disorders associated with genetic mutations requires a comprehensive understanding of their genetic causes, molecular functions of the affected genes, and the cell types that underlie different symptoms (Kaiser and Feng, 2015; Mullins et al., 2016; Wells et al., 2016; Zoghbi and Bear, 2012). In this study, we present an extensive functional analysis of Rai1—a causal gene for two syndromic neurodevelopmental disorders (SMS and PTLS)—at molecular and behavioral levels. Our data show that Rai1 is broadly expressed in postmitotic neurons and binds to promoter regions to positively regulate the expression of target genes, many of which function in circuit assembly and neuronal communication. Each SMS phenotype may be caused by loss of Rai1 in multiple non-overlapping cell types (Figure 8). Our finding that Vglut2+ subcortical excitatory neurons are major contributors of most phenotypes further highlights the importance of targeting these neurons for therapeutic intervention.

Our systematic conditional knockout analyses provide new insights into the neural functions of Rai1. First, different cell types are differentially sensitive to loss of Rai1. For example, we did not detect any phenotypes in fear conditioning (Figure 5) or social interactions (Figure S6) resulting from removal of Rai1 from the Emx1+ cells, which includes all excitatory neurons in the cortex and hippocampus, despite the prominent Rai1 expression in those cells. This is not because Emx1+ cells are not required in these behavioral tasks. For instance, it is well established that hippocampal function is required in contextual fear conditioning (Tovote et al., 2015), and social interactions engage frontal cortex (Barak and Feng, 2016). These data suggest that Rai1 does not have a general, house-keeping function that is required for every cell type. Rather, Rai1 has more specific roles in certain cell types.

Second, dosage sensitivity of Rai1 differs for cell types and phenotypic assays. For example, for obesity, Sim1+ cells exhibit at most a mild heterozygous phenotype, SF1+ cells exhibit a heterozygous phenotype that is as severe as homozygous knockout, and Vglut2+ neurons—which include both Sim1+ and SF1+ cells—exhibit an intermediate heterozygous phenotype. Within Vglut2+ neurons, losing one copy of Rai1 causes a motor defect in the wire hang test but not pole test. Further, Vglut2+ and Gad2+ neurons necessary for learning are only dysfunctional when both copies of Rai1 were deleted. The lack of some phenotypes after losing one copy of Rai1, which more closely mimics the human syndrome, may be due to differences in physiology of mice and humans or due to insufficient sensitivity of assays in mice to mimic human conditions.

Third, Rai1 function in multiple cell types contributes to SMS-like phenotypes in mice (Figure 8). Learning deficits originate from loss of Rai1 in either Vglut2+ or Gad2+ neurons, and obesity likely results from a combination of Rai1 deficiency in hypothalamic PVH and VMH neurons. Indeed, loss of Rai1 in Sim1+ and SF1+ cells alone results in less severe weight gain than if Rai1 is removed from all Vglut2+ neurons, suggesting an involvement of additional cell types. Rai1 loss in Vglut2+ neurons causes
milder phenotypes than pan-neural Rai1 loss in pole test for motor function, suggesting the involvement of additional cell types. Since removing Rai1 from the subcortical Vglut2* but not cortical Emx1* glutamatergic neurons at least partially recapitulates many phenotypes seen in pan-neural Rai1 knockout, our study uncovers a previously unappreciated involvement of subcortical excitatory neurons in SMS pathogenesis. Given that Rai1 regulates a set of genes responsible for circuit assembly and neurotransmitter signaling, it will be interesting in the future to determine how loss of Rai1 affects morphology and functions of subcortical excitatory neurons.

Monogenic models of neurodevelopmental disorders display a wide range of anatomical bases that account for their phenotypes. In tuberous sclerosis, conditional deletion of Tsc1 in astrocytes, excitatory neurons, or inhibitory neurons all results in seizure in mice (Bateup et al., 2013; Fu et al., 2012; Uhlmann et al., 2002). In Rett syndrome, GABAergic neurons alone account for the majority of behavioral symptoms (Chao et al., 2010), with somatostatin- and parvalbumin-expressing interneurons each mediating non-overlapping Rett-like phenotypes (Ito-Ishida et al., 2015). Disrupted GABAergic signaling has been consistently demonstrated in neurodevelopmental disorders such as Rett syndrome, fragile X syndrome, MECP2 duplication syndrome, and Dravet syndrome (Braat and Kooi, 2015). By comparison, the involvement of the subcortical glutamatergic system has not been as thoroughly studied. Our systematic analyses highlight the complex relationships between cell types and symptoms in SMS (Figure 8).

Given the strong phenotypes of Rai1 mutant mice and the large number of Rai1 binding sites in the genome revealed by our ChIP-seq analysis, loss of Rai1 caused a surprisingly modest change in the transcriptome both in the number of genes and the magnitude of mRNAs levels. One possible explanation is that besides moderately promoting gene expression, Rai1 may regulate transcription in response to specific stimuli or changes in neuronal activity, which is not easily captured by sampling whole-tissue steady-state level of mRNAs. For example, several chromatin modifications have more important roles during dynamic gene activation and repression than steady-state expression (Weiner et al., 2012). Indeed, Rai1’s role in context-dependent transcription will be an interesting topic of future research. Another possible explanation is that misregulation of a small number of key Rai1 target genes is sufficient to account for SMS symptoms. For example, we detected downregulation of Bdnf and Htr2c expression in Vglut2Cre;Rai1CKO hypothalamic. Given the important roles for hypothalamic Bdnf (An et al., 2015; Xu and Xie, 2016) and Htr2c (Nonogaki et al., 1998; Tecott et al., 1995) in regulating energy homeostasis, the severe obesity observed in Rai1 mutants may result from downregulation of one or both signaling pathways. In this regard, our study suggests an avenue for therapeutic intervention, at least for obesity, by restoring these signaling pathways. Given that there is an FDA-approved Htr2c agonist for treating obesity (Colman et al., 2012), this strategy can be tested experimentally.

EXPERIMENTAL PROCEDURES

Mouse Behavioral Assays

Male mice were housed in groups on an inverted 12/12 hr light/dark cycle with ad libitum access to food and water and were tested between 6 and 10 weeks of age. Behavioral testing was conducted during the mouse’s subjective night, except the hot plate assay, which was performed during the subjective day. Mice were habituated to handling for 3 days prior to the onset of the first behavioral tests. Behavioral tests were conducted in multiple cohorts of mice; each cohort followed the same sequence of behavioral tests as listed in Supplemental Experimental Procedures. Experimenters were blind to mouse genotype during testing.

Detailed description of mouse behavioral assays, as well as additional methods are described in Supplemental Experimental Procedures, including mouse husbandry and handling, generation of the Rai1-Tag mice and Rai1-flox mice, mouse genotyping, protein expression and purification, in vitro binding assay, generation of Rai1 antibody, in situ hybridization followed by immunostaining, immunostaining and antibodies, chromatin immunoprecipitation-sequencing (ChIP-seq) and ChiP-qPCR, RNA-seq and qRT-PCR, data analysis for ChiP-seq and RNA-seq, energy homeostasis analyses, and reagents and data sharing.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and nine tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.09.019.

AUTHOR CONTRIBUTIONS

helped design and provided reagents for the in vitro chromatin assay. W.-H.H. and L.L. wrote the paper, with contributions from all authors.

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REFERENCES


Supplemental Information

Molecular and Neural Functions of *Rai1*,
the Causal Gene for Smith-Magenis Syndrome

Wei-Hsiang Huang, Casey J. Guenthner, Jin Xu, Tiffany Nguyen, Lindsay A. Schwarz, Alex W. Wilkinson, Or Gozani, Howard Y. Chang, Mehrdad Shamloo, and Liqun Luo
Figure S1. Characterizing Rai1 Expression Pattern Using Rai1-Tag, Related to Figure 1
(A) Western blot showing that anti-FLAG antibody detects Rai1-Tag fusion protein in Rai1Tag/Tag but not wild-type (Rai1+/+) mouse brains. Anti-histone H3 antibody serves as a loading control.
(B) Western blot using wild-type (Rai1+/+) and Rai1Tag/Tag mouse brains showing that the Tag peptide on the C terminus of Rai1 does not affect the expression of Rai1-Tag fusion protein, detected with an anti-endogenous Rai1 antibody. Anti-histone H3 antibody serves as a loading control.
(C) Sagittal section of an Rai1Tag/+ embryo showing that Tag-expressing cells (green, detected by anti-FLAG antibody) appeared in the first branchial arch (BA1) at E9.5. White box is magnified on the right, with yellow arrowheads pointing to an Rai1-Tag-expressing cell (green) that co-localizes with a DAPI+ nucleus (magenta). Scale bar: 10 μm.
(D) Coronal section of an E18.5 Rai1Tag/+ hippocampus showing the expression pattern of Rai1-Tag (green). White box is magnified on the right, showing that Rai1-Tag and the mitotic marker Ki67 (magenta) do not overlap. Yellow arrowheads indicate Ki67+/Rai1− cells, and white arrowheads indicate Ki67−/Rai1+ cells. Scale bar: 30 μm.
(E) Rai1 is occasionally detected in Bergmann glia in the cerebellar cortex, as Rai1-Tag (green) and a glial marker S100β (magenta) are co-localized. White box is magnified on the right. Scale bar: 25 μm.
(F) Co-staining of anti-myc antibody (magenta) showing that Rai1-Tag co-localizes with a pan-neuronal marker (NeuN, green). Scale bar: 100 μm.
(G) The Rai1Tag allele was designed to express an Rai1-enhanced green fluorescent protein (EGFP) fusion protein following Cre-mediated recombination. After Cre-mediated recombination, the Tag (red) and Neomycin (Neo) cassette were deleted, generating the Rai1EGFP allele encoding an Rai1-EGFP fusion protein.
(H-J) Sagittal section of an E18.5 Vglut2Cre/+;Rai1Tag/+ brain showing the Cre-positive thalamic neurons expressing Rai1-EGFP (detected by an anti-GFP antibody, green) abutting the Cre-negative reticular nucleus expressing Rai1-Tag (H). Dotted line indicates the boundary between the thalamus and reticular nucleus. The Cre-positive neurons express EGFP (green) but not Tag (red, detected by anti-myc antibody), indicating a successful Cre-dependent EGFP-tagging (I). The Cre-negative neurons express Rai1-Tag (red) but not EGFP (green), indicating that Rai1-EGFP expression is strictly Cre-dependent (J). Scale bars: (H): 50 μm, (I-J): 10 μm.
(K-L) In situ hybridization using a probe for Vglut1 (K) and mixed probes for Gad1/2 (L) followed by immunostaining using anti-myc antibody showing the co-localization between Rai1-Tag and neuronal subtype-specific markers. Scale bars: 100 μm.
**Figure A**

Expression levels of various proteins in different cell lines, as indicated by the molecular weight markers (100 KD, 37 KD, 20 KD).

**Figure B**

Anti-GST Western blot showing the presence of GST-fused proteins.

**Figure C**

Anti-FLAG IP experiment results for two different cell lines, demonstrating the presence of the protein of interest at 250 KD and 150 KD.

**Figure D**

Histograms showing the distribution of peak models replicates A (forward tags) and B (reverse tags) with respect to the middle distance.

**Figure E**

Graph plotting DR (effect size) against the number of peaks, indicating a positive correlation.

**Figure F**

Bar chart illustrating the relative fold enrichment for different genes in control and Ra1-Tag conditions.

**Figure G**

Histogram showing log enrichment (observation over expectation) for different gene regions.

**Figure H**

Heatmap representing gene expression levels across various conditions, with color coding indicating different states or treatments.

**Table I**

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Figure S2. Characterization of the Interaction Between Rai1 and Chromatin, Related to Figure 2

(A) SimplyBlue staining showing the protein integrity of HeLa nucleosome and recombinant GST-tagged proteins (*) used in nucleosome interaction assay.

(B) Western blot using an anti-GST antibody as a loading control for nucleosome interaction assay.

(C) Immunoprecipitation performed with anti-FLAG antibody using 8-week-old wild-type (Rai1+/+, left lane) or Rai1Tag/+ cortex (right lane) followed by Western blot using an anti-Rai1 antibody. Rai1 is specifically pulled down from the Rai1Tag/+ but not wild-type cortex.

(D) Paired-end reads from ChIP-seq reveal that DNA fragments are equally likely to be sequenced from both ends. The tag density around a true binding site shows a bimodal enrichment pattern, with forward strand tags enriched upstream of binding and reverse strand tags enriched downstream. The bimodal enrichment was estimated by model-based analysis (MACS2). The two replicas show consistent bandwidth.

(E) Cumulative plot indicates the distribution of IDR (Irreproducible Discovery Rate), similar to False Discovery Rate (FDR), which controls the irreproducibility rate when selecting confidence peaks. The Y-axis indicates the IDR score (small value suggests high confidence in both replicas). The X-axis indicates the number of peaks that passed specific IDR scores on the Y-axis.

(F) ChIP-qPCR of control and Rai1Tag/+ brains using IgG and anti-flag antibodies. Input background was subtracted, and the fold enrichment (calculated as 2−ΔΔCt) of Rai1 was compared to negative control IgG (mean ± SEM, n=3).

(G) Enrichment analysis showing genomic regions with less enriched Rai1 binding sites (orange) when compared to expected value, an extension of Figure 2D. LTR: long terminal repeat, SINE: short interspersed nuclear element, LINE: long interspersed nuclear element.

(H) Emission probability (left) of eight chromatin marks that divide the genome of the mouse cortex into eight chromatin states (right) (based on ChromHMM analysis), an extension of Figure 2F.

(I) Top significant enriched motifs in Rai1 binding regions identified by Homer motif analysis, an extension of Figure 2G. Shown are percentages of binding regions with motifs compared to random background and their respective p-values. The last column shows the best match for transcription factors with known similar motifs.
Figure S3. Generation of an Rai1 Conditional Allele and Rai1 Transcriptome Characterization, Related to Figures 1 and 3–7.

(A) The wild-type Rai1 genomic locus (first row) is shown with yellow boxes indicating the 5’ and 3’ arms for homologous recombination. Blue boxes indicate the Rai1 exons. ES cells were transfected with the Rai1<sup>flox</sup> targeting construct (second row) to produce the post-targeted Rai1 allele (third row). Chimeras were mated with a mouse line that expresses Flp recombinase in the germline to generate the Rai1<sup>flox</sup> allele (fourth row). After Cre-mediated recombination, the largest exon (exon 3) that encodes amino acid 1–1837 of Rai1 is deleted, generating an Rai1<sup>Δ</sup> allele (fifth row). Neomycin (Neo) and diphtheria toxin A (DTA) cassettes were used for ES cell positive and negative selection, respectively. PCR primers A, B, and C were used for genotyping.

(B) Representative genotyping PCR products of the offspring of a Nestin<sup>Cre</sup>;Rai1<sup>flox/+</sup> x Rai1<sup>flox/+</sup> mating. PCRs were performed using primers that distinguish between different Rai1 genotypes (top) and primers that detect Nestin<sup>Cre</sup> (bottom). Each lane represents an individual animal, with the genotype of the animal indicated above. Nestin<sup>Cre</sup> was detected using generic Cre primers that produce a band of ~300-bp in the presence of Cre. Rai1 alleles were distinguished using primers A, B, and C, which produce a 396-bp band (primers A and B) from the wild-type Rai1 allele (Rai1<sup>+</sup>), a 497-bp band (primers A and B) from the floxed Rai1 allele (Rai1<sup>flox</sup>), and a 261-bp band (primers A and C) from the Rai1 null allele (Rai1<sup>Δ</sup>). The product of primers A and C is too long to be efficiently amplified in wild-type or floxed alleles under the PCR conditions used. Nestin<sup>Cre</sup> has occasional germline activity, so matings of Nestin<sup>Cre</sup> and Rai1<sup>flox</sup> mice produce some offspring that are heterozygous for Rai1 throughout their entire bodies (Rai1<sup>Δ/+</sup> and Rai1<sup>Δ/flox</sup>). Nestin<sup>Cre</sup> is also active in at least some tissues in the tail, so the Rai1<sup>Δ</sup> allele can be detected in Rai1<sup>flox/+</sup> and Rai1<sup>flox/flox</sup> mice when Nestin<sup>Cre</sup> is present. As a result, Nestin<sup>Cre</sup>;Rai1<sup>flox/flox</sup> and Nestin<sup>Cre</sup>;Rai1<sup>Δ/flox</sup> mice cannot be distinguished using this genotyping strategy.

(C) Western blot showing that the endogenous Rai1 protein level is decreased in a dose-dependent manner in control (Rai1<sup>flox/flox</sup>), Nestin<sup>Cre</sup>;Rai1<sup>flox/+</sup>, and Nestin<sup>Cre</sup>;Rai1<sup>CKO</sup> cortices. Two mice from each genotype are shown. Histone H3 serves as a loading control.

(D) Quantitative RT-PCR showing differentially expressed genes in the 3-week-old Nestin<sup>Cre</sup>;Rai1<sup>CKO</sup> cortex, confirming RNA-seq results. Data are means ± SEM (*p < 0.05, unpaired t-test, n = 3).

(E) Quantitative RT-PCR of genes consistently down-regulated at different postnatal stages in the Gad2<sup>Cre</sup>;Rai1<sup>CKO</sup> striatum (red bars) compared to controls (black bars). The expression of loxP-flanked Rai1 exon 3 is down-regulated in the conditional mutants, confirming that Gad2<sup>Cre</sup> efficiently removes Rai1 from the dorsal striatum. Data are mean ± SEM (*p < 0.05, unpaired t-test, n = 3).

(F) Quantitative RT-PCR showing genes that are up-regulated in the young Gad2<sup>Cre</sup>;Rai1<sup>CKO</sup> striatum (left) that become down-regulated in the older Gad2<sup>Cre</sup>;Rai1<sup>CKO</sup> striatum (right). Data are means ± SEM (*p < 0.05, unpaired t-test, n = 3).

(G) UCSC genome browser view of Pcdh20 (left) and Sema3a (right) loci. Yellow arrows indicate direction of transcription. Red boxed areas show that the promoter regions of Pcdh20 and Sema3a are enriched with Rai1 binding and the H3K4me3 mark. RNA-seq data for the cortex (3-week-old) and striatum (3- and 12-week-old) are shown below. Pcdh20 and Sema3a expression are decreased in Rai1 deficient tissues.
A Postnatal day 21

B

C

D

E

F

G

H

I

J

K

L

M

N
Figure S4. Behavioral Characterization of the Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} Mice, Related to Figure 4

(A) Representative of the hindlimb clasping phenotype of a Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mouse.

(B) Representative weights of individual males and females (magenta and green lines with different shades, respectively) of Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice prior to death (lines without points) and mean weights (± SEM) of control mice (squares and circles, n=13 for males, n=12 for females). Most Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice gained weight normally immediately following weaning but lost weight in the two weeks prior to death.

(C) The body weights (mean ± SEM) of male Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice are not significantly different from control mice during behavioral analyses. Phenotypes across groups were analyzed by one-way ANOVA unless otherwise stated. n.s., not significantly different.

(D-E) Gait parameters, including step sequence (D) and base of support (E), are normal for Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice in the Catwalk assay (mean with SEM).

(F-I) In the activity chamber, Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice do not differ from controls in the distance moved over time (F), time spent in the periphery versus center of the chamber (G), mean velocity (H), and vertical activity (I) (mean with SEM).

(J-K) Time (mean ± SEM) spent in the open (J) and closed (K) arms of an elevated plus maze do not differ between Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice and controls.

(L) Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice, similar to the controls, show a higher interest towards a mouse versus a cup (mean ± SEM, paired t-test), as calculated by discrimination index (see Supplemental Experimental Procedures).

(M) The ability to discriminate a novel versus familiar mouse, as calculated by discrimination index, is statistically indistinguishable between Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} and control mice (mean ± SEM).

(N) Alternation between arms of a Y-Maze is reduced in Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice. Shown are mean ± SEM. One sample t-test to a hypothetical mean of 50% (* p < 0.05).
Figure S5. Characterization of Rai1 Conditional Mutants, Related to Figure 5

(A) Schematic parasagittal adult brain sections showing the regions (in green) targeted by $Emx1^{Cre}$ (middle) and $Gad2^{Cre}$ (right). $Emx1^{Cre}$ predominantly targets excitatory neurons in the cortex and hippocampus, along with a fraction of cells in the olfactory bulb. $Gad2^{Cre}$ targets inhibitory neurons throughout the brain.

(B) Representative images of parasagittal whole brain sections for the above three genotypes stained with an anti-Rai1 antibody (green) and DAPI (magenta). Rai1 signal is greatly reduced in the cortex (arrows) of $Emx1^{Cre};Rai1^{CKO}$ compared with wild-type and $Gad2^{Cre};Rai1^{CKO}$ mice, consistent with most cortical neurons being $Emx1^+$. Rai1 signal is greatly reduced in the striatum (arrowheads) of $Gad2^{Cre};Rai1^{CKO}$ compared with wild-type and $Emx1^{Cre};Rai1^{CKO}$ mice, consistent with most striatal neurons being $Gad2^+$. Scale bars: 2 mm.

(C-E) High magnification images of layer 2/3 of primary somatosensory cortex (C), CA1 region of the hippocampus (D), and the striatum (E) immunostained stained with an anti-Rai1 antibody and DAPI. The left column is anti-Rai1 immunostaining alone (white), and the right column is anti-Rai1 immunostaining (green) merged with DAPI (magenta, co-localization shown in white). In $Emx1^{Cre};Rai1^{CKO}$ mice, Rai1 is absent in most cells of the cortex (C) and hippocampus (D), while in $Gad2^{Cre};Rai1^{CKO}$ mice, Rai1 is absent in most cells of the striatum (E). Scale bars: 100 μm (C), 40 μm (D-E).

(F) Mean body weights (mean ± SEM) of different male $Rai1$ conditional mutant mice are indistinguishable from $Rai1^{flox/flox}$ mice during motor function testing. n.s., not significantly different, one-way ANOVA.

(G) The latency (mean ± SEM) to paw licking or jumping for escape after being placed on either a 50°C (left) or 55°C (right) hot plate do not differ for $Gad2^{Cre};Rai1^{CKO}$ and $Vglut2^{Cre};Rai1^{CKO}$ mice with $Rai1^{flox/flox}$ mice, suggesting a normal analgesic response. n.s., not significantly different, one-way ANOVA.
Figure S6. Behavioral Characterization of Rai1 Conditional Knockout, Related to Figure 5

(A–B) Time spent in the open (A) and closed (B) arms of an elevated plus maze do not differ between Rai1^{flox/flox}, Gad2^{Cre}; Rai1^{CKO}, Emx1^{Cre}; Rai1^{CKO}, and mGfap^{Cre}; Rai1^{CKO} mice. Shown are mean ± SEM. (C) The Gad2^{Cre}; Rai1^{CKO}, Emx1^{Cre}; Rai1^{CKO}, and mGfap^{Cre}; Rai1^{CKO} mice have comparable sociability discrimination index as the Rai1^{flox/flox} mice. Shown are mean ± SEM. (D) Social discrimination index is statistically indistinguishable between Rai1^{flox/flox}, Gad2^{Cre}; Rai1^{CKO}, Emx1^{Cre}; Rai1^{CKO}, and mGfap^{Cre}; Rai1^{CKO} mice. The mGfap^{Cre}; Rai1^{flox/+} group shows a decreased discrimination index due to an outlier mouse that spent most of its time with a familiar mouse. Shown are mean ± SEM. (E) Alternation between arms of a Y-Maze is not different between Rai1^{flox/flox}, Gad2^{Cre}; Rai1^{CKO}, Emx1^{Cre}; Rai1^{CKO}, and mGfap^{Cre}; Rai1^{CKO} mice. Shown are mean ± SEM. Statistics: One-way ANOVA; n.s., not significantly different, * p < 0.05.
Figure S7. Characterization of Obesity \textit{Rai1} Mutants, Related to Figures 6 and 7

(A-D) Mean (± SEM) body weights over time of male mice that lost one or both copies of \textit{Rai1} in the \textit{Gad2} (A, n=15-24 for each genotype), \textit{Emx1} (B, n=15-16 for each genotype), \textit{mGfap} (C, n=8-10 for each genotype), and \textit{Vglut2} (D, n=8-11 for each genotype)-Cre lineages. Male \textit{Vglut2}^{\text{Cre}; \text{Rai1}^{\text{fox/+}}} and \textit{Vglut2}^{\text{Cre}; \text{Rai1}^{\text{CKO}}} mice become significant obese beginning at 14- and 9-weeks of age, respectively. Statistics: * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, Tukey’s post hoc test following significant two-way ANOVA.

(E) \textit{Rai1} expression (green) in the hypothalamic PVH region (white dotted lines circled region), with DAPI staining (magenta, left). \textit{Rai1} staining in the PVH is mostly lost in the \textit{Vglut2}^{\text{Cre}; \text{Rai1}^{\text{CKO}}} and \textit{Sim1}^{\text{Cre}; \text{Rai1}^{\text{CKO}}} mice, but not in the \textit{Rai1}^{\text{fox/fox}} or \textit{SF1}^{\text{Cre}; \text{Rai1}^{\text{CKO}}} mice.

(F) \textit{Rai1} expression (green) in the hypothalamic VMH region (white dotted circles), DAPI staining is shown in magenta. \textit{Rai1} signal in the VMH is mostly lost in the \textit{Vglut2}^{\text{Cre}; \text{Rai1}^{\text{CKO}}} and \textit{SF1}^{\text{Cre}; \text{Rai1}^{\text{CKO}}} mice, but not the \textit{Rai1}^{\text{fox/fox}} or \textit{Sim1}^{\text{Cre}; \text{Rai1}^{\text{CKO}}} mice.

(G) Motor function of \textit{Sim1}^{\text{Cre}; \text{Rai1}^{\text{CKO}}} mice analyzed by the wire hang test. Deletion of \textit{Rai1} in Sim1+ cells does not impair motor skills in either male or female mice. Shown are mean ± SEM. p > 0.05, unpaired t-test.

(H-J) Multiple linear regression analysis (analysis of covariance, ANCOVA) was used to assess the impact of lean mass on energy expenditure. \textit{Rai1}^{\text{fox/fox}} (purple dots) and \textit{Sim1}^{\text{Cre}; \text{Rai1}^{\text{CKO}}} (green dots) mice show similar energy expenditure during total (light + dark) (H, p = 0.2716), the light phase (I, p = 0.1248), and the dark phase (J, p = 0.6489) (n=8 for each genotype).
SUPPLEMENTAL TABLES (Excel files)

Table S1: Summary of Rai1-Tag co-localization with cell type-specific markers, related to Figure 1.
Table S2: Rai1 ChIP-seq sample information, related to Figure 2.
Table S3: Rai1 ChIP-seq quality control, related to Figure 2.
Table S4: Chromatin state discovery and characterization (ChromHMM) analysis, related to Figure 2.
Table S5: Differentially expressed genes of Rai1-deficient cortex and striatum identified by RNA-seq, related to Figure 3.
Table S6: Gene ontology analysis using down-regulated genes in Rai1-deficient cortex and striatum, related to Figure 3.
Table S7: Primers for ChIP-qPCR and quantitative RT-PCR, related to Figures 2 and 3, and Supplemental Experimental Procedures.
Table S8: RNA-seq sample information, related to Figure 3.
Table S9: Differentially expressed genes of Rai1-deficient hypothalamus identified by RNA-seq, related to Figure 7.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse Husbandry and Handling
All animal procedures followed animal care guidelines approved by Stanford University's Administrative Panel on Laboratory Animal Care (APLAC). For behavioral assays, the C57BL/6J:129X1/SvJ F1 hybrid mice were used. Rai1\textsuperscript{flox/+} mice were backcrossed at least four generations onto C57BL/6J or 129X1/SvJ backgrounds; at least one backcross included mating of an inbred C57BL/6J or 129X1/SvJ male with a Rai1\textsuperscript{flox/+} female to set the genetic background of the Y chromosome. Nestin\textsuperscript{Cre} (RRID:IMSR_JAX:003771), Gad2\textsuperscript{Cre} (RRID:IMSR_JAX:010802), Vglut2\textsuperscript{Cre} (RRID:IMSR_JAX:016963), mGfap\textsuperscript{Cre} (RRID:IMSR_JAX:012886), Emx1\textsuperscript{Cre} (RRID:IMSR_JAX:005628), and SF1\textsuperscript{Cre} (RRID:IMSR_JAX:012462) mice were obtained from the Jackson Laboratories. Sim1\textsuperscript{Cre} mice (RRID:IMSR_JAX:006395) were provided by Dr. B. Lowell (Beth Israel Deaconess Medical Center). All Cre mice were backcrossed at least four generations onto a C57BL/6J background.

Generation of the Rai1-Tag Mice
The targeting construct for producing the Rai1-Tag allele was generated by first cloning the 5' homology arm including the last three Rai1 exons and a \textit{loxP} site (with the stop codon of the last Rai1 exon removed) into a pCR2.1-TOPO vector (Invitrogen) as backbone. Then, the following three partially overlapping PCR fragments were linked by In-Fusion cloning (Clontech): Fragment 1: a partial \textit{loxP} site, 3X FLAG, and 5X myc tags which were designed to be in frame with Rai1 ORF, followed by triple translational stop codons in tandem; Fragment 2: partial myc tag and triple stop codons, FRT-pSV40-Neo-pA, and a partial \textit{loxP} site; Fragment 3: an intact \textit{loxP} site and an EGFP sequence designed to be in frame with Rai1 ORF after Cre-mediated recombination. The resulting fragment was subsequently cloned into an EcoRV site of the pCR2.1-TOPO vector containing the 5’ homology arm previously described. The DTA cassette for negative selection was inserted with HindIII/KpnI sites, and the 3’ homology arm was inserted with NotI site. The final construct was linearized with PvuI restriction enzyme and electroporated into 129Sv/SvJ ES cells. The correctly targeted clones were identified by long-range PCR (LA Taq, TaKaRa) and DNA sequencing. Targeted ES cells were microinjected into BL/6 blastocysts, and chimeras with successful germ line transmission were identified by PCR and were used to expand the colony. The proper fusion of FLAG/myc tags and the Cre-dependent fusion of EGFP tag to the C terminal of Rai1 cDNA was verified by extracting mRNA from the brain of Rai1-Tag mice with or without Cre expression, followed by cloning and sequencing (data not shown). ES cell manipulations and blastocyst injections were performed by the Stanford Transgenic Research Facility.

Generation of the Rai1-flox Mice
The targeting construct for production of the conditional Rai1 allele was generated using conventional cloning approaches with a FRT5-pSV40-Neo-pA-FRT5 cassette and homology arms amplified by high-fidelity PCR (Phusion, Thermo Fisher Scientific) from 129X1/SvJ genomic DNA (Jackson Labs). Growth of the full targeting construct in bacteria at high copy number led to loss of the DNA segment between \textit{loxP} sites, presumably due to the actions of endogenous bacterial recombinases. To circumvent this issue, the construct was sub-cloned into a bacterial artificial chromosome (BAC) vector prior to the final cloning steps, and the construct was maintained at 1-2 copy numbers per bacterial cell. After verification by sequencing, the construct was electroporated into 129Sv/SvJ ES cells. The correctly targeted clones were identified by long-range PCR (LA Taq, TaKaRa) and DNA sequencing. Targeted ES cells were microinjected into BL/6 blastocysts, and chimeras were mated to a germline-active Flp transgenic line to remove the neomycin resistance cassette. Pups lacking the Neo cassette were identified by PCR and were used to expand the colony. ES cell manipulations and blastocyst injections were performed by the Stanford Transgenic Research Facility.
Mouse Genotyping
The presence of an Rai1Tag allele was detected using primers: forward 5'-ATATCATGGCGCAGACAGAGA-3' and reverse 5'-TGGGCGCAGCTTTTCTCTC-3' that detects a 326-bp band. And the wild-type Rai1 allele can be detected using primers: forward 5'-CTAGGGTGATTGCACAGTTCTG-3' and reverse 5'-TGGGCGCAGCTTTTCTCTC-3' that detects a 138-bp band. For the Rai1-flox mice, the Rai1flox and Rai1Δ (null) alleles were identified using primers A-C: 5'-CAGAGTCCAGATGGCACTACAGGGG-3' (A, common forward), 5'-GTGAGCTCCCGCTGAAATGGACAGT-3' (B, wild-type and floxed reverse), and 5'-GGAGGTCTGCGCTTCAGGGCTTAAT-3' (C, Δ reverse). With these primers, Rai1 + produces a 396-bp band, Rai1flox produces a 497-bp band, and Rai1Δ produces a 261-bp band. The NestinCre, Gad2Cre, Vglut2Cre, mGfapCre, and Emx1Cre mice were genotyped with primers Cre -A 5'-CACCCTGTTACGTATAGCCG-3' and Cre-B 5'-GAGTCATCCTTAGCGCCGTA-3' for a 300-bp Cre band, and primers IC 5'-CCAATCTGCTCACACAGGATAGAGCGGAGG-3' and IC- 5'-CCTTGAGGCTGTCCAAGTGATTCAGGCCATCG-3' for a 500-bp internal control band. The SF1Cre mice were genotyped with primers oIMR6243 5'-CTGAGCTGCAGCGCAGGGACAT-3' and oIMR6244 5'-TGCGAACCTCATCACTCGTTGCAT-3' for a 250-bp Cre band, and primers oIMR8744 5'-CAAATGTTGCTTGTCTGGTG-3' and oIMR8745 5'-GTCAGTCGAGTGCACAGTTT-3' for a 200-bp internal control band. The Sim1Cre mice were genotyped with primers Sim1-Cre-1 5'-CACGACCGCAAACGGCAGAA-3', Sim1-Cre-2 5'-TGGGATTAGCGTGTTTCAACTGAGC-3', and Sim1-Cre-3 5'-TTTTGGTTTTGGATGAGTCTGTGGAG-3' for an internal control (600-bp) and a Cre (250-bp) band.

Protein Expression and Purification
GST-tagged protein was expressed in Rosetta (DE3) pLysS cells (Novagen). The cells were grown in LB medium at 37°C and then induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25°C for overnight culture growth. The cells were lysed with lysis buffer (300 mM NaCl, 0.1% NP-40), incubated on ice with 0.25 mg/ml lysozyme for 1 hour, and then sonicated with Branson Digital Sonifier (18% output for 10 seconds, 1 second sonication with 1 second break). Lysates were spun in 12,000 rpm at 4°C for 20 minutes, and then incubated with glutathione beads (Amersham) at 4°C for overnight. The beads were washed three times with lysis buffer, and the GST-tagged fusion protein was eluted with 10 mM Glutathione (Sigma). Finally, 20 mM Dithiothreitol (DTT) was added, and protein was aliquoted and stored in –80°C.

In Vitro Binding Assay
For nucleosome pull-down/Western assay, 1.5 μg of mononucleosomes purified from HeLa cells were incubated with 15 μg of GST-fused protein in binding buffer (300 mM NaCl, 0.1% NP-40, 50 mM Tris-HCl pH 7.5, 10% glycerol) at 4°C for overnight. 30 μl of washed glutathione beads were then added to the binding solution and incubated for one hour. Washed beads were denatured and subjected to 12% Bis-Tris gel electrophoresis and detected with an anti-H3 antibody (ab1791, Abcam, RRID:AB_1079). The purity and integrity of purified proteins were verified with SimplyBlue SafeStain (Thermo Fisher Scientific), the loading controls were blotted with an anti-GST-HRP antibody (GE Healthcare, RRID:AB_771429) using Western blot analysis.

Generation of Rai1 Antibody
Antibodies against the following Rai1 peptides were raised in rabbits by Thermo/Pierce custom antibody services: DKYHRGSKSLQGRPAFPSY (Rai1-76:94), RPDGPADPAKQGPLRTSAR (Rai1-1738:1756). Antisera from six rabbits injected with either Rai1-76:94 or Rai1-1738:1756 were tested by immunohistochemistry, and antisera from two rabbits that produced the highest signal-to-background ratios were affinity purified using the immunizing peptides. The two resulting affinity purified antibodies, ab15 (against Rai1-76:94) and ab20 (against Rai1-1738:1756), were further tested by immunohistochemistry.
In Situ Hybridization Followed by Immunostaining

ISH probes were generated as previously described (Weissbourd et al., 2014). 50 μm coronal sections containing the cortex were collected into a 24-well plate containing PBS. The sections were fixed for 15 minutes in 4% paraformaldehyde in PBS at room temperature, rinsed with PBS, and incubated for 15 minutes with 3% hydrogen peroxide (Sigma, 216763) in PBS. Sections were rinsed with PBS and incubated with 7 μg/ml Proteinase K (Life Technologies, 25530-049) in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA for 10 minutes at 37°C. After fixing again with 4% paraformaldehyde in PBS for 10 minutes and rinsing with PBS, the sections were incubated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, for 15 minutes at room temperature and washed with PBS. Probes were diluted (~1:1000) with the hybridization buffer (50% formamide, 10 mM Tris-HCl pH 8.0, 200 μg/ml tRNA, 10% Dextran Sulfate, 1x Denhardt’s solution, 600 mM NaCl, 0.25% SDS), mixed well, preheated at 85°C for 5 minutes, and applied to sections (300–500 μl/well). After 16-20 hours of incubation at 60°C, the sections were washed, first with 2× SSC-50% formamide, then with 2× SSC, and finally with 0.2× SSC twice for 20 minutes at 65°C. Sections were equilibrated in Tris Buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 5 minutes at room temperature, then blocked in Tris Buffer + 0.5% Blocking Reagent (Perkin Elmer, cat# 11096176001) for 30 minutes at room temperature. Then, sections were incubated with anti-DIG-POD Fab fragments (Roche, cat# 11633716001, AB_514499, lyophilized and diluted in 1 ml of water) at 1:2000 in Tris Buffer + 0.5% Blocking Reagent for 30 minutes at room temperature. Sections were washed with Tris Buffer + 0.05% Tween20 (Sigma, P9416). Next, a TSA Plus Cyanine 3 Kit (Perkin Elmer, NEL744001KT) was used to detect in situ signal. Briefly, Cy3-Tyramide was diluted 1:50 in amplification reagent and applied to the sections for 5–10 minutes at room temperature. Sections were then washed in Tris Buffer + 0.05% Tween20, and incubated with Tris Buffer + 0.05% Tween20 + anti-myc antibody (1:1000, Novus Biologicals, NB600-338, RRID:AB_10001879, for 3 days at 4°C). Sections were washed with Tris Buffer + 0.05% Tween20, then incubated with FITC conjugated donkey anti-goat antibodies (1:500; Jackson ImmunoResearch, RRID:AB_2340400) for an additional 1–2 hours, and washed with PBS three times for 10 minutes. Finally the sections were treated with PBS containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich, D8417) for 20 minutes and mounted with cover glass using Fluorogel (Electron Microscopy Sciences, Cat#17985-10).

PCR Primers used to Prepare Templates for ISH Probes: T3 polymerase recognition site is indicated by underline.

Vglut1
5’-CTGGCAGTGACGAAAGTGAA;
5’- AATTAACCCTCACTAAAGGGACACAACAAATGGCCACTGA

Gad1
5’-CACAAACTCAGCGGCATAGA;
5’- AATTAACCCTCACTAAAGGGACGAGCAACATGCTATGG

Gad2
5’-GGGATGTCAACTACGCGTTT;
5’- AATTAACCCTCACTAAAGGGGTCATCATCCCTCCTCTCTC

Immunostaining and Antibodies

For immunostainings that involve anti-Rai1 antibody, mice were euthanized with isoflurane, and their brains were rapidly dissected, immersed in Optimal Cutting Temperature (OCT) compound (Tissue-Tek, Sakura), and frozen by immersion in a dry ice/ethanol bath. 8 μm thick sections were mounted to Superfrost plus slides, and washed three times in phosphate buffered saline (PBS), incubated 10 minutes at ~20°C in pre-cooled acetone, washed four times in PBS, and then blocked for 2 hours at room temperature in 10% normal donkey serum (NDS) in PBS. Slides were further incubated overnight at 4°C with primary antibodies in 10% NDS in PBS, washed 4 times for 5 minutes in PBS, incubated for 2–3 hours at room temperature with secondary antibodies in 10% NDS in PBS, washed 4 times for 5 minutes in PBS, incubated for ~10 minutes in ~1:30000 dilution of 5 mg/mL DAPI in PBS, washed
once for 5 minutes in PBS, and coverslipped in Fluoromount-G (SouthernBiotech). Immunostainings that do not involve anti-Rai1 antibody followed previously described protocols (Guenthner et al., 2013). The following antibodies were used: mouse anti-FLAG M2 (1:1000, Sigma, RRID:AB_439685), mouse anti-HDAC2 antibody (1:1500, Abcam, RRID:AB_2118547), mouse anti-S100β (1:1000, Sigma, RRID:AB_477499), mouse anti-NeuN (1:1000, EMD Millipore, RRID:AB_177621), rabbit anti-Ki67 (1:1000, Abcam, AB_302459), rabbit anti-H3 (1:2000, Abcam, RRID:AB_302613), rabbit anti-Rai1 (1:250, custom made), goat anti-myc (1:1000, Novus Biologicals, RRID:AB_10001879), chicken anti-GFP (1:2500, Aves Labs, RRID:AB_10000240). Secondary antibodies conjugated to 488 (Donkey anti-chicken, RRID:AB_2340375; Donkey anti-rabbit, RRID:AB_2313584; Donkey-anti-mouse, RRID:AB_2340846; Donkey-anti-goat, RRID:AB_2340428) or Cy3 (Donkey anti-rabbit, RRID:AB_2307437; Donkey-anti-mouse, RRID:AB_2315777; Donkey-anti-goat, RRID:AB_2340411)(Jackson Immunoresearch) were diluted 1:500~1:2000 from 50% glycerol stocks.

Chromatin Immunoprecipitation-sequencing (ChiP-seq) and ChiP-qPCR
8-week-old cortices of male Rai1Tag/+ mice were used for ChiP-seq experiment. Cortical tissues were cross-linked with 1% formaldehyde for 10 minutes at room temperature, followed by quenching with 0.125 M glycine and washed twice with cold PBS containing 0.5 mM PMSF. Chromatin was sonicated to an average size of 0.3–1 kb using Bioruptor (Diagenode). A total of 10 μg of anti-FLAG M2 antibody (Sigma, RRID:AB_439685) was added to the sonicated chromatin and incubated overnight at 4°C. Subsequently, 50 μl of protein G Dynal magnetic beads were added to the ChIP reactions and incubated for 6 hours at 4°C. Magnetic beads were washed and chromatin eluted, followed by reversal of crosslinks and DNA purification (ChIP DNA clean & Concentrator kit, ZYMO research). ChiP-qPCR was performed with independently isolated samples to confirm ChiP-seq results. Chromatin was pulled-down with IgG (negative control) or anti-FLAG antibody, and then amplified with sequence specific primers. Quantitative PCR reactions were conducted using SsoFast EvaGreen Supermix (Bio-Rad) on a StepOnePlus Real-Time PCR System (Thermo-Fisher Scientific).

Data Analysis for ChiP-seq
The libraries were sequenced using HiSeq 2500 sequencing system (Illumina), yielding 32-39 million of 2 X 50 base paired-end reads for both ChIP and control (input) libraries. The paired-end reads were mapped to the mouse reference genome (mm9) using bowtie2 (version 2.2.3) (Langmead and Salzberg, 2012) with “--very-sensitive” and default parameters. Read pairs, which aligned concordantly to the genome and had a mapping quality more than 10, were kept for following analyses. Read pairs mapped to mitochondrial DNA were discarded. Redundancy read pairs from PCR duplication were also removed afterward using Picard tools (version 1.134, http://broadinstitute.github.io/picard). Peak regions were identified by MACS2 (Version 2.1.0) with the parameters “-g mm -p 0.001 --fix-bimodal ” for each ChIP and Input replicate pair. Consistency analysis of the peaks identified from replicas was done by IDR package following the guideline (Li et al., 2011). Consistency peaks with IDR score lower than 0.05 were selected as high-confidence peaks and kept for the following genome-wide analyses. The final bam file from replicas were merged and then converted into normalized BigWig files using bedtools (v2.17.0) (Quinlan and Hall, 2010) for visualization in the UCSC genome browser. The high-confidence peaks were annotated to different genome features and enrichment score were calculated for each feature using Homer (Heinz et al., 2010). Alignment file of histone modification data were downloaded from ENCODE ftp sites (ftp://hgdownload.cse.ucsc.edu/goldenPath/mm9/encodeDCC/wgEncodeLicrHistone). Six histone modification markers as well as Pol2 and CTCF ChiP-seq data were collected. H3K27ac, H3K4me1, H3K4me3, Pol2 and CTCF were derived from 8-week-old cortex and H3K27me3, H3K36me3 and H3K9me3 were derived from the whole brain at E14.5. The alignment files (in bam format) were first converted into bed file and then analyzed for chromatin state discovery and characterization following the guideline of ChromHMM (Ernst and Kellis, 2012). To identify the potential binding consensus sequence of Rai1, the top 20% of high-confidence peaks were
used to perform a de novo motif search using Homer with the parameters “-size given -mask”. Normalized read intensity of Rai1 ChIP-seq and other ChIP-seq data around gene body or Rai1 binding regions were plotted by ngsplot (Shen et al., 2014). The same data sets of H3K4me3 and Pol2 from ENCODE were used. The ChIP-seq data of Zfx and GFP were downloaded from GEO database (accession numbers GSM288352 and GSM288358). The raw data were mapped with bowtie2 and convert into bam files using samtools (Version 0.1.19).

RNA-seq and qRT-PCR
Cortex of male Nestin\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice, striatum of male Gad2\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice, and hypothalamus of male Vglut2\textsuperscript{Cre};Rai1\textsuperscript{CKO} mice, as well as their Rai1\textsuperscript{flox/flox} littermates (control) were used for RNA-seq experiments. Total RNA was extracted by TRizol reagent (Life Technologies) and phenol-chloroform-isoamyl alcohol (Life Technologies). The residual DNA was removed with on-column DNase digestion (Qiagen) for 30 minutes and RNA was further purified using RNeasy Kit (Qiagen). Total RNA from 2 biological replicas of each genotype was used. Ribosomal RNA was depleted with Ribo-Zero rRNA removal kits (Illumina). The barcoded libraries were prepared with illumine RNA preparation kit according to manufacturer’s instruction. Quantitative RT-PCR (qRT-PCR) was performed with independently isolated samples to confirm RNA-seq results. After isolation of total RNA, mRNA was reverse-transcribed with SuperScript III First-Strand Synthesis System (Thermo-Fisher Scientific). Quantitative PCR reactions were conducted using SsoFast EvaGreen Supermix (Bio-Rad) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific).

Data Analysis for RNA-seq
The libraries were sequenced using HiSeq 2500 sequencing system (Illumina), yielding 30–60 million of 2 X 100-bp paired-end reads per sample. The adaptor sequences were first scanned and removed for the raw reads by cutadapt (version 1.6) (Martin, 2011). The trimmed sequences were mapped to mouse reference genome (mm9) using TopHat2 with parameters “-I 1000000 --max-coverage-intron 1000000 --max-segment-intron 1000000 --no-coverage-search --b2-very-sensitive --microexon-search”. Read pairs, which were aligned concordantly to the genome, were counted according to the RefGene annotation from UCSC using DESeq (v0.50.1). The longest isoform were selected when there are multiple isoforms. Differentially expressed genes between conditional knockout and control for each cell type were analyzed by DESeq2 (Love et al., 2014). Genes with FDR < 0.1 were selected as differential expression genes for following analysis. Gene ontologies enrichment was tested by merging down-regulated gene in either cortex or striatum using DAVID (Huang da et al., 2009). For list of differentially expressed genes identified in Rai1-deficient cortex and striatum, see Table S5. For details of RNA-seq samples, see Table S8. For list of differentially expressed genes identified in Rai1-deficient hypothalamus, see Table S9. Due to potential incomplete deletion of Rai1 exon 3 in Gad2\textsuperscript{Cre};Rai1\textsuperscript{CKO} striatum and Vglut2\textsuperscript{Cre};Rai1\textsuperscript{CKO} hypothalamus, and the up-regulation of Rai1 exons 4–6 in conditional mutants, Rai1 was identified as an up-regulated gene in these conditional mutants. Our qRT-PCR experiments (Figures 3B, 7D, and S3E) have confirmed that Rai1 exon3 was indeed down-regulated in these samples. Western blot also confirmed loss of Rai1 protein in conditional mutants (Figure S3C). RNA splicing algorithm (data not shown) independently confirmed the deletion of Rai1 exon 3 in Gad2\textsuperscript{Cre};Rai1\textsuperscript{CKO} striatum and Vglut2\textsuperscript{Cre};Rai1\textsuperscript{CKO} hypothalamus. Therefore, we removed Rai1 from the DEG list to prevent confusion.

Mouse Behavioral Assays
Activity Chamber
Mice were placed for 10 minutes in the center of a 43.2 x 43.2 cm square arena in a 66 x 55.9 x 55.9 cm sound-attenuating chamber under dim red light. Mice were allowed to move freely in the chamber, and the time spent in the center versus periphery of the arena, distance moved, average velocity, and vertical movement were determined from infrared sensors using an automated system. At the end of each trial, the surface of the arena was cleaned with 1% Virkon.
Y maze
Mice were placed in a Y-shaped maze consisting of white plastic arms positioned 120° apart radially. All arms were 12.7 cm high and 7.62 cm wide; two arms were 15.24 cm in length, and one arm was 20.32 cm in length. Each mouse was allowed to explore the maze for 5 minutes, and the order of arm entries was recorded. The sequence of arm entries was broken into overlapping, consecutive triads (total number of triads = total number of arm entries – 2). The percent alternation was defined as: (number of triads containing entry into all three arms / total number of triads) X 100.

Elevated Plus Maze
Mice were placed in the center of an elevated + shaped maze with two open arms (i.e., without walls) and two closed arms (i.e., with walls). Mice were allowed to explore for 5 minutes, and their positions were monitored using an automated tracking system (EthoVision, Noldus). The time spent in open versus closed arms and the number of entries into each arm, along with other parameters, were calculated. The surfaces and walls of the maze are cleaned with 1% Virkon to eliminate odor.

Cat Walk
Mice were trained to traverse a linear track (CatWalk, Noldus) by placing their homecage at the opposite end of the track. Multiple trials were performed until each mouse had completed three trials in which they traversed the track continuously without stopping. The positioning of the paws during each step was automatically determined using a video-based system, and a variety of gait parameters, including distances between paws, step patterns, and limb swing speeds were determined.

Vertical Pole Descent Test
Mice were placed at the top of a coarse, vertical wooden pole (diameter: 1 cm; height: 55 cm), and the time required to descent was recorded. After a practice trial, 4 test trials were conducted with an inter-trial interval of ~2 minutes. If the mouse did not descend or dropped or slipped down the pole without climbing, a descent time of 60 seconds was recorded.

Wire Hang
Mice were placed on a wire grid, which was inverted such that the mice were hanging upside down by all four paws approximately 50 cm above a cage with bedding. The latency to fall was recorded, and each trial was terminated after 150 seconds. Mice were given three trials per day.

Sociability and Social Discrimination
Mice were placed in a testing cage containing two empty inverted cups and were allowed to habituate for 20 minutes. An unfamiliar mouse (C57BL/6 juvenile) was then placed under one of the cups, and the time spent by the subject mouse investigating the two cups was quantified (sociability). Sociability was calculated by discrimination Index= (M – C) / (M + C). M= time spent sniffing a mouse, C= time spent sniffing a cup. A second unfamiliar juvenile was then placed under the cup in the position of the 1st mouse when introduced to the cage and the familiar mouse was moved to where the empty cup had previously been located (social discrimination). In this task the time spent sniffing novel and familiar mice was quantified. Social discrimination was calculated by discrimination Index= (N – F) / (N + F). N= time spent sniffing novel mouse, F= time spent sniffing familiar mouse. Discrimination index was then compared across genotypes using one-way ANOVA.

Fear Conditioning
On Day 1 (training), mice were placed in Context A and after 3 minutes, they were presented with a tone (75 dB, 2 kHz, 20 sec) followed 18 seconds later by a footshock (0.5 mA, 2 seconds); mice received a total of five tone-shock pairings with an inter-tone interval (from the end of one tone to the start of the next tone) of 80 seconds. On Day 2 (cued recall) mice were placed for 3 minutes in Context B, which had different olfactory, somatosensory, and visual cues from Context A. They were
subsequently presented with three tone presentations (80 seconds inter-tone interval) without any
shocks. On Day 3 (contextual recall), mice were placed in Context A for 5 minutes without any tone
presentation. Freezing, defined as complete lack of motion for at least 0.75 seconds, was quantified on
all three days by an automated video scoring system (FreezeFrame, Actimetrics).

Hot Plate Assay
Mice were placed on a hot plate (Model 39, IITC) heated to either 50°C or 55°C, and the latency to
exhibit pain behavior (paw lifting, paw licking, or jumping) was recorded. Mice were tested in three
trials per temperature.

Energy Homeostasis Analyses

Home Cage Food Intake
Manual food intake measurements were made every 2–3 days for 1 week. Food was weighed prior to
placement in the cage. After 2–3 days, food remaining in the hopper and any significant spillage inside
the cage were combined and weighed. The difference is the amount of food intake during the specific
timeframe.

Adiposity
Body composition (lean and fat mass) was measured by Dual Energy X-ray absorptiometry (DEXA)
(Lunar PIXImus Densitometer, GE Medical Systems). Brown and white adipose tissues from different
depots (gonadal, subcutaneous, retroperitoneal, and mesenteric) were dissected and weighed. Tissue
weights were recorded.

Indirect Calorimetry
Energy expenditure and physical activity were evaluated in age matched (30-32 weeks) female
Rai1^flox/flox and Sim1^Cre;Rai1^CKO mice by indirect calorimetry in the CLAMS (Comprehensive Lab Animal
Monitoring System, Columbus Instruments). Animals were acclimated to the facility for at least 1 week,
and then acclimated to the CLAMS cages and powdered diet (2018 Teklad Global 18% Protein Rodent
Diet) for 48 hours and to the light and temperature-controlled chamber for 24 hours prior to testing.
Analyzed data constitutes data collected from 48 hours of continuous measurement (2 light/2 dark
cycles). Oxygen consumption and carbon dioxide production were measured and used to calculate
energy expenditure (or heat production, kilocalories (kcal)) and respiratory exchange ratio (RER: VCO2
/ VO2). Cage-mounted sensors detect and record measurements of physical activity (beam breaks).
Multiple linear regression analysis (analysis of covariance, ANCOVA) was used to assess the impact
of covariate (lean mass) on energy expenditure.

Reagents and Data Sharing
The following mouse lines will be available from The Jackson Laboratory: Rai1-Tag (JAX#029101) and
Rai1-flox (JAX#029103). Plasmids are deposited to Addgene. Sequence data are deposited at NCBI
GEO GSE81207.
SUPPLEMENTAL REFERENCES


