Early adolescent Rai1 reactivation reverses transcriptional and social interaction deficits in a mouse model of Smith–Magenis syndrome

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Haploinsufficiency of Retinoic Acid Induced 1 (RAI1) causes Smith–Magenis syndrome (SMS), a syndromic autism spectrum disorder associated with craniofacial abnormalities, intellectual disability, and behavioral problems. There is currently no cure for SMS. Here, we generated a genetic mouse model to determine the reversibility of SMS-like neurobehavioral phenotypes in Rai1 heterozygous mice. We show that normalizing the Rai1 level 3–4 wk after birth corrected the expression of genes related to neural developmental pathways and fully reversed a social interaction deficit caused by Rai1 haploinsufficiency. In contrast, Rai1 reactivation 7–8 wk after birth was not beneficial. We also demonstrated that the correct Rai1 dose is required in both excitatory and inhibitory neurons for proper social interactions. Finally, we found that Rai1 heterozygous mice exhibited a reduction of dendritic spines in the medial prefrontal cortex (mPFC) and that optogenetic activation of mPFC neurons in adults improved the social interaction deficit of Rai1 heterozygous mice. Together, these results suggest the existence of a postnatal temporal window during which restoring Rai1 can improve the transcriptional and social behavioral deficits in a mouse model of SMS. It is possible that circuit-level interventions would be beneficial beyond this critical window.

Significance
Losing one copy of the RAI1 gene causes Smith–Magenis syndrome (SMS), a neurodevelopmental disorder. Using a newly generated SMS mouse model, this study demonstrates that restoring the Rai1 gene dose in an early postnatal window could repair gene expression and social interaction deficits in this SMS model. The SMS mouse model also showed a reduced density of dendritic spines, anatomical correlates of excitatory synapses, in the prefrontal cortex. Artificial activation of prefrontal cortex neurons partially alleviated the behavioral deficits. These findings suggest that, similar to Rett syndrome, SMS is caused by disruption of a chromatin-modifying gene with reversible developmental phenotypes, highlighting the potential treatment windows in childhood or adolescence.
Results
An SMS Mouse Model That Allows Conditional Reactivation. To test reversibility of SMS in mice, we generated a knock-in Rai1 allele (Rai1STOP) with an insertion of a loxp-flanked transscriptional stop cassette before the start codon, which should prevent the expression of any Rai1 protein before Cre-mediated recombination (Fig. 1 and SI Appendix, Fig. S1A). We found that, similar to Rai1−/− mice (13), most Rai1STOPSTOP mice died in utero (91%, n = 175), suggesting that Rai1STOP functions as a null allele in the absence of Cre activity. Quantitative RT-PCR and Western blot confirmed that the stop cassette effectively inhibited the expression of Rai1 (Fig. 1 B and C). We then performed a battery of behavioral assays using 8- to 10-wk-old male Rai1STOP/+ mice and their wild-type (WT) littermates to examine their neural functions.

In the activity chamber, Rai1STOP/+ mice showed an increased stereotypical vertical activity (rearing) (Fig. 1D) while retaining an otherwise normal activity level (Fig. 1E). The time spent in the periphery and center of the activity chamber was not different between Rai1STOP/+ and WT mice (SI Appendix, Fig. S1 B and C), suggesting a normal anxiety level. Rai1STOP/+ mice showed normal motor coordination in the pole test and normal context- and cued- recalls in the fear-conditioning test (SI Appendix, Fig. S1 D and E). Furthermore, they retained normal spatial learning as shown by Y maze and Morris water maze (SI Appendix, Fig. S1 F–H). A hot plate test showed that Rai1STOP/+ mice retained normal pain sensitivity (SI Appendix, Fig. S1I). These results are consistent with previous findings in Rai1−/+ mice (12, 14).

One cardinal feature of ASD is abnormal reciprocal social interaction (15, 16). We used a tube test to measure social interaction by quantifying the encounters between two unfamiliar mice (17). When two mice are simultaneously released from opposing ends of a transparent tube, one mouse will eventually be pushed out or voluntarily retreat from the tube and be declared as the “loser” (SI Appendix, Fig. S1J). When performed on mice that share a cage, the tube test measures social hierarchy and dominance (18, 19). For unfamiliar mice that have not established social hierarchy, as in our case, the tube test reflects the willingness of stranger mice to maintain close physical proximity in a face-to-face social encounter (20). We found that Rai1STOP/+ mice showed a dramatic defect in the tube test, losing ∼90% of the matches to noncageate WT mice (Fig. 1F), similar to a recent report for Rai1+/− mice (21). We previously found that 3-wk-old Rai1 conditional knockout mice exhibit transcriptional misregulation in the brain (12). Accordingly, we tested the social interaction of Rai1STOP/+ mice in the tube test at 3 wk of age and found a significant deficit (Fig. 1G). Importantly, performance in the tube test was independent for the weight or the locomotive activity in the open field (19).

To test if these neurobehavioral phenotypes are sexually dimorphic, we also tested female Rai1STOP/+ mice. We found that female Rai1STOP/+ mice showed normal horizontal activity in the activity chamber, increased rearing, abnormal social interaction in the tube test, and increased body weight (SI Appendix, Fig. S2 A–D). Thus, impaired social interaction is a robust and early onset phenotype consistent between male and female Rai1STOP/+ mice.

Several lines of evidence suggest that aggression was not a major contributing factor to social interaction deficit from the tube test. First, both male and female Rai1STOP/+ mice showed a similar social deficit in the tube test; yet it is known that females exhibit a low level of aggression (22). Second, 3-wk-old mice had not yet become aggressive toward their male conspecifics, yet 3-wk-old Rai1STOP/+ mice already had shown a social interaction deficit. Third, we did not observe aggressive behavior for both 3-wk-old and adult mice during the tube test, consistent with previous findings that mice that won the tube test do not show increased aggression in the resident-intruder assay (18). Finally, we used age- and weight-matched mice for the tube test so these factors do not contribute to the results.

Partial Rescue of Transcriptional Deficits. To restore the Rai1 level in a temporally precise manner, we utilized an UbcCreERT2 allele that allows global Cre expression upon tamoxifen (TM) injection (23). To avoid the toxicity associated with consecutive TM injections (24), we established a regime of five TM treatments (100 mg/kg) over 10 d, which restored Rai1 mRNA and protein expression of UbcCreERT2Rai1STOP/+ mice to WT level 10 d after the last injection (Fig. 2 A–C). Injection of vehicle (corn oil) did not result in nonspecific activation of Rai1 (Fig. 2B).

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Loss of *Rai1* affects the mRNA expression of hundreds of genes (12). We tested whether reinstatement of Rai1 after symptom onset is sufficient to repair the transcriptome. Loss of *Rai1* results in behavioral symptoms (Fig. 1G) and transcriptional misregulation (12) at 3 wk of age. Therefore, we treated 3-wk-old WT, *Rai1*STOP+, and *UbcCreERT2;Rai1*STOP+ (hereafter, rescue) mice with TM and performed whole-transcriptome sequencing (RNA-seq) to examine their cortical transcriptomes at 4 mo of age (n = 5 for each genotype). When comparing WT and *Rai1*STOP+ cortices, we detected 792 differentially expressed genes (DEGs) with a false discovery rate adjusted P value < 0.05 and a log2 fold change > 0.5 (SI Appendix, Fig. S3A and Dataset S1). Notably, Rai1 reactivation significantly reduced the number of DEGs between WT and rescue mice (down to 356 genes, a 55% reduction) (SI Appendix, Fig. S3A and Dataset S2). We further performed hierarchical clustering to examine the expression pattern of individual DEGs across groups. Despite an early absence of *Rai1*, the DEG profile of the rescue mice clustered with WT littermates (Fig. 2D). Quantitative RT-PCR confirmed that *Rai1* reactivation normalized several misregulated genes that were also downregulated in our previous RNA-seq experiments (SI Appendix, Fig. S3B) (12). Principal component analysis using DEGs found that WT and *Rai1*STOP+ transcriptomes formed distinct clusters, and the transcriptomes of rescue mice occupied intermediate positions (Fig. 2E), suggesting at least a partial rescue at a genome-wide scale.

Next, we asked if the corrected genes belong to specific functional categories. Gene ontology (GO) analysis comparing WT and *Rai1*STOP+ DEGs indicated that pathways, including in nervous system development and neurogenesis, were significantly misregulated in the *Rai1*STOP+ brain (Fig. 2F and SI Appendix, Fig. S3C), consistent with our previous findings from *Rai1* conditional knockout mice (12). Notably, at 4 mo of age, pathways related to neurodevelopment were corrected by *Rai1* reactivation in the rescue mice (Fig. 2F). GO analysis using the DEGs detected in WT versus rescue mice also showed that genes involved in the cellular metabolic process were misregulated, suggesting that delayed *Rai1* reactivation did not fully rescue the transcriptomic difference between WT and *Rai1*STOP+ cortex. We noted that the 4-mo-old *Rai1*STOP+ cortex showed more pronounced transcriptional misregulation than what we previously found in the 3-wk-old NestinCre;*Rai1*KO cortex (SI Appendix, Fig. S3D), suggesting that the transcriptional deficits worsened with age. Thus, early reversal of the Rai1 level could hold more therapeutic promise. Together, restoration of Rai1 in juvenile symptomatic mice partially reversed the pathogenic transcriptional events in the neurodevelopmental pathways.

**Rescue of Social Interaction Deficits by Restoring the Rai1 Level at Postnatal 3 WK but Not 7 WK.** To test if transcriptional repairs are accompanied by functional rescue after reactivating *Rai1* at 3 wk of age, we performed behavioral assays at 8 wk of age. In the activity chamber, the rescue mice exhibited an intermediate level of activity compared with WT and *Rai1*STOP+ mice, although statistically indistinguishable from *Rai1*STOP+ mice (Fig. 3A). Other aspects of the locomotor and exploratory behaviors remained comparable between genotypes (SI Appendix, Fig. S4A–C). Remarkably, while *Rai1*STOP+ mice consistently lose ~90% of the matches against WT mice in the tube test, the rescue mice showed an equal winning rate against WT mice (Fig. 3B). Furthermore, the rescue mice won 86% of the matches against the *Rai1*STOP+ mice (Fig. 3B).

To investigate the nature of the social interaction deficit, we quantified the pattern of mouse interaction in the tube test by frame-to-frame scoring of the video blind to genotypes. We found that, in the WT vs. *Rai1*STOP+ social encounters, *Rai1*STOP+ mice voluntarily retreated more frequently than WT mice (SI Appendix, Fig. S4D). Rescue mice showed a retreat number similar to WT mice. When encountering *Rai1*STOP+ mice, rescue mice also showed a significantly lower retreat number (SI Appendix, Fig. S4D). Finally, WT, *Rai1*STOP+, and rescue mice all showed similar social preference for intruder mice over a novel object in a home cage social discrimination test (SI Appendix, Fig. S4E), suggesting that the social deficit was not due to the lack of social motivation. In summary, correlating with transcriptional repair, restoring the Rai1 level in symptomatic juvenile mice fully reversed the social interaction deficit in the tube test.

Prompted by the full reversal of the social interaction deficit in the *Rai1*STOP+ juvenile mice, we next normalized the Rai1 level by treating 7-wk-old male mice with TM and subjecting them to behavioral assays at 4 mo of age. We found that neither rearing (Fig. 3C) nor social interaction (Fig. 3D) were rescued, indicating that restoration of the Rai1 level in adult mice was insufficient to reverse the social interaction deficits of *Rai1*STOP+ mice.

**Proper Social Interaction Requires a Correct Rai1 Dose in both Excitatory and Inhibitory Neurons.** *Rai1* is expressed in a multitude of cell types in the brain, including excitatory and inhibitory...
neurons (12). To test if restoring the Rai1 level in excitatory or inhibitory neurons alone is sufficient to correct the abnormal social interaction, we crossed Rai1STOP/+ mice with Vglut2Cre or VgatCre mice and selectively normalized Rai1 expression in excitatory or inhibitory neurons, respectively (SI Appendix, Fig. S5 A and B) (25). We found that both Vglut2-Rescue and Vgat-Rescue mice behaved similar to Rai1STOP/+ mice (SI Appendix, Fig. SSC), suggesting that restoring Rai1 level in excitatory or inhibitory neurons alone was not sufficient to normalize social interaction.

In a complementary set of experiments, we deleted one copy of Rai1 in excitatory and inhibitory neurons by crossing Rai1floxEgfp mice (12) with Vglut2Cre and VgatCre mice and generated Vglut2Cre; Rai1floxEgfp and VgatCre; Rai1floxEgfp mice. We confirmed reduced Rai1 expression in brain regions enriched with Cre-expressing neurons (SI Appendix, Fig. SSD). Similar to Rai1+/− mice, mice losing one copy of Rai1 in Vglut2Cre or VgatCre-expressing neurons showed a defective social interaction when encountering unfamiliar WT mice (SI Appendix, Fig. SSE). Interestingly, both Vglut2Cre; Rai1floxEgfp and VgatCre; Rai1floxEgfp mice won more matches when encountering Rai1+/− mice, suggesting that proper levels of Rai1 expression in Vglut2Cre or VgatCre-negative cells confer an intermediate phenotype (SI Appendix, Fig. SSE). Together, these data indicate that a proper dose of Rai1 is required in both excitatory and inhibitory neurons to control social interactions. As a result, global (Fig. 3A) rather than restricted (SI Appendix, Fig. SSC) re-expression of Rai1 is necessary to normalize social interaction abnormalities caused by Rai1 haploinsufficiency.

**Reduced Spine Density in Prefrontal Cortex Neurons.** The medial prefrontal cortex (mPFC) is a critical region that controls social cognition in humans and mice (26). Mediodorsal thalamic input to the mPFC regulates social interaction in the tube test (18). In the rodents, the mediodorsal thalamic inputs predominantly synapse onto the apical dendritic trunk of layer V pyramidal cells located within layers II/III (27). To investigate whether Rai1 haploinsufficiency results in structural changes of mPFC, we crossed Rai1STOP/+ with Thy1EGFP (28) mice and quantified the spine density of layer V pyramidal neurons within layers II/III. We observed a significant reduction (~22.8%) of spine density in 1-mo-old Rai1STOP/+; Thy1EGFP mice compared with age-matched Rai1+/−; Thy1EGFP control littermates (Fig. 4A and B). Subregions of the mPFC, including the anterior cingulate cortex (ACC) and the prelimbic cortex (PL), showed a consistent reduction of dendritic spines (Fig. 4C). The spine density phenotype was also more prominent in the anterior (~24% decrease for Bregma +2.2 to +2.8 mm) than in the posterior (~14% decrease for Bregma +1.9 to +2.2 mm) mPFC (Fig. 4D).

**Optogenetic Activation of mPFC Neurons Partially Rescues Social Interaction Deficits.** Optogenetic stimulation of the mPFC excitatory neurons has been shown to improve winning in the tube test (18). We therefore tested if activating mPFC can correct the...
social deficit in adult Rai1STOP+ mice (8–10 wk old). We stereotactically injected adeno-associated virus (AAV) containing humanized channelrhodopsin-2 under the control of the human synapsin promoter (AAV2-hSyn-hChR2(H134R)-EYFP) into the right mPFC of adult Rai1STOP+ mice and implanted an optic fiber directly above the injection site (SI Appendix, Figs. S6A and B). An AAV-encoding enhanced yellow fluorescent protein (EYFP) driven by the same promoter (AAV2-hSyn-EYFP) served as a control. We adopted two photostimulation protocols that have been shown to induce winning in the tube test: a 100-Hz phasic and a 5-Hz tonic protocol (SI Appendix, Figs. S6 C and D) (18). We confirmed that photostimulation significantly increased Fos expression in the channelrhodopsin-injected side (SI Appendix, Fig. S6E). We then performed a tube test by photostimulating both unfamiliar mice to rule out the potential visual effect of laser stimulation (Fig. 5A).

Under the laser-off control condition, WT-EYFP mice won more matches against both Rai1STOP+-EYFP (Fig. 5B) and Rai1STOP+ChR2-EYFP mice (Fig. 5C), while Rai1STOP+-EYFP and Rai1STOP+ChR2-EYFP mice behaved similarly (Fig. 5D). We then delivered 100-Hz phasic light to activate mPFC immediately before the mice entered the tube and throughout the match, which on average lasted 31.47 ± 7.26 s (n = 192 trials). Interestingly, photostimulation significantly increased winning of Rai1STOP+ChR2-EYFP mice when encountering Rai1STOP+-EYFP mice (74.3 ± 6.5%, n = 8 mice for each genotype) (Fig. 5D). However, photostimulation did not affect encounters between Rai1STOP+ChR2-EYFP and WT-EYFP mice (Fig. 5C), suggesting that activating mPFC alone does not restore all circuit deficits. This is consistent with Rai1STOP+ChR2-EYFP mice showing an intermediate phenotype in between WT-EYFP and Rai1STOP+-EYFP mice. The 5-Hz tonic protocol had no impact on tube test performance in Rai1STOP+ChR2-EYFP mice (SI Appendix, Figs. S6 F–H).

Finally, we quantified social encounters between Rai1STOP+-ChR2-EYFP and Rai1STOP+-EYFP mice during laser on-and-off paradigms. We found that mPFC activation reduced the average match duration (SI Appendix, Fig. S6) and decreased the retreat number of Rai1STOP+-ChR2-EYFP mice (SI Appendix, Fig. S6F), suggesting an increased propensity to engage in social encounters. Collectively, these results provide evidence that circuit-level intervention can partially improve social interaction in adult Rai1STOP+ mice.

**Discussion**

A key question in neurodevelopmental disorders such as ASDs is whether the symptoms are caused by early and irreversible defects during neural development or by disruption of potentially reversible defects in adult function (29). On the one hand, ASDs are typically diagnosed before 3 y of age (15), suggesting that ASD-causing genes regulate neural development. On the other hand, some ASD-causing genes have a continuous function throughout life. For example, syndromic autism genes such as MeCP2 are required for adult neural functions (30), suggesting that the temporal window for treatment may extend well beyond early development. Studies using mouse models of syndromic ASDs have shown a range of reversibility. For example, adult restoration of Synap1 failed to reverse any behavioral phenotypes (31), highlighting the difficulty of reversing a congenital developmental disorder. In contrast, multiple disease features of Rett syndrome and MECP2 duplication syndrome mouse models can be rescued by postsymptomatic normalization of MeCP2 levels (32, 33). Similarly, adult restoration of Shank3 can rescue selective autistic-like symptoms in mice (24). Therefore, the reversibility of each disorder must be evaluated individually.

As an early onset syndromic ASD, the reversibility of SMS was previously unknown. Here, we uncovered a critical postnatal window to reverse social behavior deficits in a mouse model of SMS by normalizing the Rai1 level. Restoring the Rai1 level in early adolescence, after mice have already exhibited transcriptional and social interaction deficits, partially restores transcriptome and fully rescues the social interaction deficits. On the molecular level, delayed Rai1 restoration normalized the expression of Rai1 target genes involved in the functional development of the nervous system but not those involved in metabolic processes (Fig. 2F). This suggests that early Rai1 expression regulates specific neural developmental pathways. The inability of adult Rai1 re-expression to reverse behavioral deficits also suggests that the temporal window for Rai1 to normalize the transcriptional events causing social behavioral deficits closes during adolescence. Given the continuous expression of Rai1 in the adult brain (12), the functions of Rai1 in the mature nervous system remain unclear.

At the neural circuit level, we demonstrated that, even after that critical window (3–4 wk of age), optogenetic activation of mPFC provides an alternative treatment option to directly modulate neural circuit activity and correct the social interaction deficit. This suggests that the function of neural circuits underlying social interaction may not be permanently damaged by Rai1 haploinsufficiency. It is possible that reduced spine density in the mPFC of Rai1STOP+ mice contributed to a decreased thalamic input important for social interaction in the tube test (18). The partial rescue effect of mPFC optogenetic activation likely partially overcame this defect. The partial rescue also suggests that loss of Rai1 causes neural defects in other brain regions and/or cell types. Supporting this notion, we found that both excitatory and inhibitory neurons depend on the proper Rai1 level to control social interaction. Identifying the Rai1-dependent inhibitory neurons will shed light on the neural mechanism underlying social interaction in the tube test.

The different therapeutic potential of Rai1 reactivation in adolescent and adult stages suggests that Rai1 has different roles in early development and adulthood. Interestingly, in a mouse model of PTLS that overexpresses Rai1 in the forebrain neurons (34), it was found that reducing the Rai1 level either before or after symptom onset was insufficient to prevent or reverse the
PTLS-like phenotypes. Therefore, SMS and PTLS likely have distinct critical windows for therapeutic interventions. Our work lays the groundwork for future therapeutic strategies for designing clinical trials for SMS and potentially other genomic disorders.

Materials and Methods

Animals. All animal procedures followed animal care guidelines approved by Stanford University’s Administrative Panel on Laboratory Animal Care. F1 hybrids of C57Bl/6J and CD1 mice were used for all experiments. RaitCreERT2 mice were backcrossed onto a CD1 background for at least six generations, and all Cre mice were maintained in a C57Bl/6J background. Mice were housed in groups on an inverted 12:12 h light/dark cycle with ad libitum access to food and water. The UbcCreERT2, Thy1GFP, Vglut2CreERT2, and VgatCreERT2 mice were obtained from the Jackson Laboratory (23, 25, 28).

Tamoxifen Treatment. Tamoxifen (T5648; Sigma) was dissolved in corn oil at a concentration of 20 mg/mL by vortexing and heating to 50 °C. Tamoxifen was protected from light by aluminum foil, aliquoted, and stored at −20 °C for no more than 2 wk. Each mouse received an i.p. 100 μg/kg tamoxifen injection every alternative day for 10 d (five doses).

Dendritic Spine Analysis. Mice were transcardially perfused with 4% paraformaldehyde and sectioned into 60-μm slices. Immunofluorescent staining was performed using chicken anti-GFP antibody (ab13970; Abcam) for 24 h followed by 2 h of room temperature secondary antibody staining (Jackson Immunoresearch). Confocal images of mPFC (the layers II/III of the main trunk of intact layer V pyramidal neurons) were taken with a 20× objective. Spine density is an underestimation due to the inability to count spines pointing toward or away from the imaging plane from the main apical dendritic trunk. Six slices per mouse brain, two to three cells per slice, and three dendritic apical trunk segments (~35–3 × 35 μm) per cell were imaged. Spine images were acquired from three mice per genotype. Spine counts from different anterior–posterior axes were used. All quantifications were done with the experimenter blind to the mouse genotypes. Statistical analysis was performed with Student’s t test.

Tube Test. Animals used for the tube test were housed with mice with the same genotype and encountered unfamiliar mice in the tube test to avoid measuring social hierarchy established between cagemates. Mice were housed in cages in the testing environment for 1 d before training. In each of 2 training days, each mouse passed through the tube for 10 trials. In testing days, two mice of differing genotypes were placed at the two ends of the tube and released simultaneously to meet in the middle of the tube. The mouse that retreated first from the tube was designated as the loser. Experimenters were blinded to the genotypes of the mice in each trial. For the tube test using 3-wk-old mice, the diameter of the tube was reduced using clear vinyl so that the mice could not turn around. All mice participating in the tube test were of similar age and body weight.

Statistical Analysis. All data were statistically analyzed using Prism 7 (GraphPad) software, and P values less than 0.05 were considered significant. Statistical analysis was performed using Student’s t test or one- or two-way ANOVA with Bonferroni’s or Tukey’s post hoc comparison.

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