A suppressor screen in Mecp2 mutant mice implicates cholesterol metabolism in Rett syndrome

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Mutations in MECP2, encoding methyl CpG-binding protein 2, cause Rett syndrome, the most severe autism spectrum disorder. Re-expressing Mecp2 in symptomatic Mecp2-null mice markedly improves function and longevity, providing hope that therapeutic intervention is possible in humans. To identify pathways in disease pathalogy for therapeutic intervention, we carried out a dominant N-ethyl-N-nitrosourea (ENU) mutagenesis suppressor screen in Mecp2-null mice and isolated five suppressors that ameliorate the symptoms of Mecp2 loss. We show that a stop codon mutation in Sqle, encoding squalene epoxidase, a rate-limiting enzyme in cholesterol biosynthesis, underlies suppression in one line. Subsequently, we also show that lipid metabolism is perturbed in the brains and livers of Mecp2-null male mice. Consistently, statin drugs improve systemic perturbations of lipid metabolism, alleviate motor symptoms and confer increased longevity in Mecp2 mutant mice. Our genetic screen therefore points to cholesterol homeostasis as a potential target for the treatment of patients with Rett syndrome.

Rett syndrome (RTT; MIM 312750) is an X-linked neurological disorder presenting with autistic features that afflicts approximately 1 in 10,000 females. After a few months or years of apparently normal postnatal development, progressive neurological manifestations of disease occur, including loss of speech and motor skills, stereotypic hand movements, difficulty walking, irregular breathing and seizures. Mutations in the X-linked gene MECP2 are the primary cause of RTT1. Hemizygous males with truncating or loss-of-function mutations typically die of encephalopathy, whereas mild mutations in either sex are associated with a variety of intellectual disabilities and autism2.

Mouse models recapitulate many of the symptoms of RTT, and their study has provided insight into the physiological basis of disease3–5. Although heterozygous female Mecp2/+ mice show phenotypic variance due in part to random X-chromosome inactivation, hemizygous male Mecp2/Y mice have a fully penetrant phenotype. Mecp2-null males are normal at birth and weaning but then develop limb clasping, tremors, lethargy and abnormal breathing, which progressively worsen until death between 6 and 16 weeks of age. Restoration of Mecp2 expression in mutant mice after the onset of symptoms rescues the neurological deficits, including motor function, and significantly prolongs survival6. Therefore, MECP2 has roles in the postnatal maturation and/or maintenance of neuronal properties and circuits6. These findings suggest that RTT may be ameliorated or even reversed by genetic or pharmacological means after symptom onset2–7. Mechanistically, MECP2 binds to methylated DNA to regulate gene transcription through repression or activation8. When MECP2 represses gene transcription, it associates with chromatin-remodeling complexes that contain type 1 histone deacetylases (HDACs)3,9. As an epigenetic factor, MECP2 expression is therefore critical within a relatively narrow range, making gene therapy a difficult approach for symptom rescue.

We reasoned that a genetic screen for suppressors of symptoms using a Mecp2 mouse model could identify pathways that are responsible for disease pathology and may thereby help pinpoint potential therapeutic targets. Genetic modifier screens are commonly applied to identify genes that act in developmental or biochemical pathways in fruit flies, worms and bacteria10; however, this approach is uncommon in mice11,12. Here we take advantage of advances in mutagenesis, sequencing and genotyping methods13 to identify five loci that suppress disease phenotypes in Mecp2-null mice. One suppressing mutation occurs in Sqle, which encodes squalene epoxidase, a rate-limiting enzyme in cholesterol synthesis. Mutation of a rate-limiting enzyme such as SQLE suggests that downregulation of the cholesterol synthesis pathway can ameliorate symptoms. In support of this idea, we show that administration of statins, which also inhibit the cholesterol synthesis pathway, improves motor performance and increases longevity in Mecp2 mutant mice. Cholesterol homeostasis may therefore be a therapeutic target for treating specific features of RTT pathology.

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RESULTS

ENU screen identifies five suppressors of MeCP2

We treated C57BL/6J male mice with ENU and mated them to female mice heterozygous for the Bird null allele of MeCP2 made congenic on a 129S6/SvEv genetic background (129.Mecp2tm1.1Bird/+). (Fig. 1a). We then screened first-generation (G1) Mecp2-null males (Mecp2tm1.1Bird/Y) for rescue of neurological defects using a health scoring system that included assessments of limb clasping, tremors, body size, cage activity and longevity, +/+ female wild-type mice; +/Y, male wild-type mice. (b) N2 mice from five lines (352 (Sum1m1Ju; green), 856 (Sum2m1Ju; blue), 895 (Sum3m1Ju; red), 1395 (Sum4m1Ju; brown) and 1527 (Sum5m1Ju; purple)) produced N3 offspring that showed increased longevity. Closed circles represent offspring of a male N2 parent, and open circles represent offspring of a female N2 parent. The longevity of the G1 founder of each line is indicated by the colored squares. Mecp2tm1.1Bird/Y mice rarely survive past 120 d (horizontal dashed line).

To identify a chromosomal location for the suppressing traits (where M is an unknown ENU-induced suppressing mutation), DNA from 7–12 long-lived Mecp2tm1.1Bird/Y, M/+ mice from each founder line were genotyped along with DNA from short-lived Mecp2tm1.1Bird/Y, +/+ N2 littermates using an Illumina medium-density SNP panel. We analyzed the resulting genotypes for heterozygous C57BL/6J linkage to the rescue phenotype according to haplotype assessment at the N3 generation. These data showed significant linkage for Sum1m1Ju on mouse chromosome 16 (log10 odds (LOD) score, 4.82), for Sum2m1Ju on chromosome 3 (LOD score, 3.61), for Sum3m1Ju on chromosome 15 (LOD score, 3.03) and for Sum4m1Ju on chromosome 7 (LOD score, 3.40). We confirmed linkage and achieved fine mapping by assessing markers within the putative map location for additional mice in the line. The maximum LOD score for Sum5m1Ju was 2.86; we did not confirm a map location in this line.

A stop-codon mutation confers rescue

We subjected genomic DNA from two third-generation Mecp2tm1.1Bird/Y; Sum3m1Ju/+ long-lived mouse cousins to exome capture and massively parallel sequencing. We found seven heterozygous, protein-altering variants in both mice but not in 15 control mouse exomes (a set including C57BL/6J and 129S6/SvEvTac DNAs; Supplementary Fig. 4a).

Table 1 Subjective health scores averaged for offspring assessed in various lines

| MeCP2m1.1Bird/Y, Summ1m1Ju/+ | MeCP2m1.1Bird/Y, Summ2m1Ju/+ | MeCP2m1.1Bird/Y, Summ3m1Ju/+ | MeCP2m1.1Bird/Y, Summ4m1Ju/+ | MeCP2m1.1Bird/Y, Summ5m1Ju/+ | MeCP2m1.1Bird/Y, +/+ |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Age (weeks)                | 8  | 20 | 8  | 20 | 8  | 20 | 8  | 20 | 8  | 20 | 8  | 20 |
| Clasping                   | 0  | 2  | 1  | 3  | 1  | 3  | 2  | 2  | 1  | 2  | 4  | N/A |
| Tremors                    | 0  | 1  | 1  | 1  | 2  | 2  | 2  | 2  | 1  | 2  | 3  | N/A |
| Body weight                | 0  | 1  | 0  | 2  | 1  | 1  | 0  | 0  | 1  | 2  | 3  | N/A |
| Lethargy                   | 0  | 1  | 0  | 1  | 0  | 1  | 0  | 1  | 1  | 2  | 4  | N/A |
| Inflammation               | 0  | 0  | D, | D, | D, | E  | D, | D, | E  | E  | 0  | N/A |
| Malocclusion               | 0  | 0  | 1  | 1  | 1  | 0  | 0  | 0  | 0  | 0  | 0  | N/A |

Shown are the average health scores for each parameter at 8 and 20 weeks of age. The health scores are as follows: 0, not present; 1, normal and similar to wild type; increasing to 5, near death and warranting euthanasia. D, dermatitis; E, eye inflammation. N/A, not applicable, as Mecp2-null males do not live to this age.
Only one of these variants ranked highly with respect to both overlap with Sum3m1Jus+/+ mapping data (chromosome 15: 34260887–95144876; Supplementary Fig. 4b) and predicted functional impact: a nonsense mutation (c.1195C>T, p.Arg399X) in Sqle (ENSMUSG000000022351), encoding squalene epoxidase, also known as squalene monoxygenase (Fig. 3a).

SQLE catalyzes the first oxygenation reaction in the committed production of cholesterol, an essential lipid either supplied by diet or synthesized from acetate by a complex pathway.24,25 Cholesterol homeostasis is maintained by negative feedback regulation of genes and their encoded enzymes in the pathway, including the rate-limiting enzymes 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductase (HMGCR) and SQLE20–22. The enzymatic reaction of SQLE requires a mitochondrial electron donor to produce 2,3-oxidosqualene, a transient intermediate that is cyclized to lanosterol by lanosterol synthase (LSS)23. SQLE is expressed in many tissues24 and is conserved throughout evolution; the mouse and human proteins are 84% identical at the amino acid level. Mice homozygous for SqleSum3Jus die before birth at embryonic day (E) 8.5, a phenotype that is consistent with that found in other mice with mutations affecting cholesterol synthesis25 (Supplementary Table 1). Two isoforms are annotated in mice: the SqleSum3Jus stop mutation identified here occurs in a highly conserved exon present in the long isoform, which, unlike the short isoform, is consistently translated to protein in mammalian species. The long isoform is absent in homozygous mutant SqleSum3Jus/SqleSum3Jus embryos, suggesting nonsense-mediated RNA decay, and the predicted short form is not upregulated in SqleSum3Jus/SqleSum3Jus embryos or SqleSum3Jus+/+ mice (Fig. 3b and Supplementary Fig. 5). Protein blot analysis of E8.0 SqleSum3Jus homozygous embryos showed that the expected 64-kDa protein was absent, as was a 36-kDa presumed degradation product, which is consistent with a null mutation (Fig. 3c). Only one other null eukaryotic mutation of Sqa1e has been identified, which results in an inability of plants to tolerate drought stress26,27.

Cholesterol synthesis takes place through complex pathways, all of which use the rate-limiting enzymes HMGCR and SQLE28. Previous analysis suggested that expression of Hmgcr and Sqle was predictive of the behavior of genes encoding intermediate enzymes in these pathways (data not shown). To determine the effect of the Sqa1e mutation on cholesterol biosynthesis, we assessed the expression of Hmgcr and Sqle in the brains and livers of SqleSum3Jus+/+ mice (Fig. 3d,e). Sqle expression was reduced by nearly 50% in SqleSum3Jus+/+ brains at P70, with no compensatory change in Hmgcr expression (Fig. 3d and Supplementary Table 2). Sqa1e expression was also reduced in the brains of 129.Mecp2tm1.1Bird/Y mice regardless of Sqa1eSum3Jus mutation status. Consistently, concentrations of the cholesterol precursors desmosterol and lanosterol were decreased in SqleSum3Jus+/+ brains at P70 (Fig. 3f and Supplementary Table 3a). Livers of SqleSum3Jus+/+ mice at P70 showed little decrease in Sqa1e or Hmgcr expression, although the 129.Mecp2tm1.1Bird/Y mice had increased expression of both genes regardless of Sqa1eSum3Jus mutation status (Fig. 3e). Likewise, these changes in expression are reflected in serum cholesterol concentrations (Fig. 3g). Together these data suggest that Sqa1eSum3Jus+/+ mice carry a loss-of-function allele of Sqa1e.

To determine which RTT-like traits were ameliorated in 129.Mecp2tm1.1Bird/Y, Sqa1eSum3Jus+/+ mice, we carried out a series of behavioral and breathing assessments out at the N7 backcross generation. In addition to increased longevity, these mice showed improved motor activity on a rotarod (Fig. 3h) and increased activity in the open field (Fig. 3i). However, they showed little improvement of irregular respiration as measured by plethysmography and no change in acoustic startle responses (Supplementary Fig. 6a–d). Thus, heterozygous loss of Sqle does not improve all the symptoms that are associated with Mecp2 mutation.

### Mecp2-null male mice have abnormal cholesterol metabolism

We hypothesized that the heterozygous Sqle mutation ameliorates a previously unrecognized dysregulation of cholesterol metabolism in Mecp2-null mice. Cholesterol is a major and essential component of the brain, which must be produced there exclusively through synthesis, as cholesterol-rich lipoproteins cannot cross the blood–brain barrier. Therefore, we quantified cholesterol concentrations in the brains of 129.Mecp2tm1.1Bird/Y and 129.Mecp2tm1.1Bird/Y, SqleSum3Jus+/+ mice (Fig. 3j). As predicted, cholesterol levels were significantly increased in the brains of Mecp2-null mice, whereas they were reduced in the brains of mice with the SqleSum3Jus+/+ allele. We also measured cholesterol concentrations in the livers of these mice (Fig. 3k). The lack of increase in these mice suggests that Mecp2-null mice are using alternative pathways to maintain cholesterol homeostasis, although not necessarily synthesizing cholesterol in the liver. These results are consistent with the findings of previous studies that show decreased cholesterol uptake in Mecp2-null mice,29,30 and suggest that the SqleSum3Jus+/+ allele may be ameliorating this phenotype.
blood-brain barrier39. To facilitate proper neurotransmitter release and dendrite remodeling, neuronal membranes require constant turnover to eliminate cholesterol that has been adulterated by reactive oxygen species, which are byproducts of their high rate of metabolism30. When neurons require cholesterol turnover or accumulate too much cholesterol or its intermediates, the cytochrome P450 oxidase activity30. When neurons require cholesterol turnover or accumulate too much cholesterol or its intermediates, the cytochrome P450 oxidase (CYP27A1) hydroxylates cholesterol to produce 24-oxocholesterol, allowing for egress by one-way diffusion into the circulation across the blood-brain barrier (Fig. 4a)31. Two alleles of Mecp2 are primarily used as mouse models of RTT: Mecp2<sup>3a/1.1Bird</sup>, a null mutation3, and Mecp2<sup>2m1.1Ace</sup>, which expresses low levels of a truncated protein32. At postnatal day (P) 28, when Mecp2<sup>2m1.1Ace</sup> mice show mild symptoms, Cyp46a1 expression was increased 38% in 129.Mecp2<sup>2m1.1Bird/Y</sup> brains over wild-type brains (P = 0.02), with a similar trend seen in B6.Mecp2<sup>2m1.1Ace/Y</sup> brains, indicating a heightened need for cholesterol turnover by neurons in the early stages of disease (Fig. 4b). Notably, brains carrying two different Mecp2 alleles also showed higher concentrations of total cholesterol at P56 (129.Mecp2<sup>2m1.1Bird/Y</sup>, P = 0.05; B6.Mecp2<sup>2m1.1Ace/Y</sup>, P = 0.21; Fig. 4c). However, by P56, when mutant male mice are severely symptomatic, the cholesterol biosynthesis and turnover genes Hmgcr and Sqle and Cyp46a1 were downregulated in both alleles (Fig. 4b). Desmosterol is the most prevalent intermediate that is produced by the primary cholesterol synthesis pathway in the adult brain34. Notably, the amount of desmosterol was also decreased at P56 in 129.Mecp2<sup>2m1.1Bird/Y</sup> brains (P = 0.01), and the amounts of both lanosterol and desmosterol were decreased by P70. Furthermore, by P70, brain cholesterol concentrations had returned to wild-type levels, reflecting cholesterol biosynthesis pathway downregulation in the adult 129.Mecp2<sup>2m1.1Bird/Y</sup> brain (Fig. 4c and Supplementary Table 4a,b). Similarly, de novo cholesterol synthesis assayed by the in vivo incorporation of <sup>3</sup>H<sub>2</sub>O into sterols34 confirmed that sterol synthesis decreased in the adult brains of B6.Mecp2<sup>2m1.1Ace/Y</sup> mice (Fig. 4d and Supplementary Table 4a,b). Therefore, brain cholesterol metabolism is perturbed in two Mecp2 alleles on two different genetic backgrounds. Either increased or decreased amounts of steroids can impair neurological function, demanding a tight regulation of brain sterol synthesis35. In Mecp2 mutant mice, our data suggest that overproduction of cholesterol in the brain probably feeds back to decrease synthesis. Although RTT is primarily a neurological disease, most cholesterol synthesis studies have been carried out in the liver, which regulates the systemic release of cholesterol in conjunction with dietary consumption. The expression of Hmgcr and Sqle was unchanged at P28 and was significantly higher at P56 in 129.Mecp2<sup>2m1.1Bird/Y</sup> livers compared to wild-type livers (P ≤ 0.05; Fig. 4e). Although total liver cholesterol and phospholipid concentrations were unchanged, reflecting the transient nature and rapid packaging of newly synthesized cholesterol into lipoproteins for secretion, the concentration of the primary liver-storage lipids, triacylglycerides (TAGs), was greatly increased in 129.Mecp2<sup>2m1.1Bird/Y</sup> mice by P56 (P = 0.003; Fig. 4f and Supplementary Fig. 7). In contrast, B6.Mecp2<sup>2m1.1Ace</sup> mice showed no significant perturbations in gene expression at P28 or P56, a modest increase in de novo liver cholesterol synthesis and a modest increase in liver TAG concentration at P56 (Fig. 4e-g and Supplementary Table 4a,b). Consistently, total serum cholesterol, low-density lipoprotein (LDL) cholesterol and triglyceride concentrations were significantly elevated in 129.Mecp2<sup>2m1.1Bird/Y</sup> mice (P ≤ 0.01), but not
B6.Mecp2tm1.1Jae/Y mice, at P56 (Fig. 4h–j). The C57BL/6J inbred strain and the 129Sv substrains inherently manage peripheral cholesterol metabolism differently. Together these data suggest that perturbations in brain cholesterol synthesis are common in the two alleles in two different strain backgrounds; however, severe peripheral dysregulation of the pathway occurs only in the 129.Mecp2tm1.1Bird strain.

Statin ameliorate behavioral and metabolic symptoms

Aberrant cholesterol metabolism may therefore contribute to Mecp2-null symptoms. We reasoned that a pharmacologic inhibitor of cholesterol synthesis might produce an attenuation of symptoms comparable to that of the genetic inhibitor Sdq1Samp5/J in Mecp2-null mice. We treated wild-type and 129.Mecp2tm1.1Bird/Y mutant mice with statin drugs, which are commonly prescribed to reduce systemic cholesterol by interfering with cholesterol synthesis through competitive inhibition of HMGCR. As a preliminary trial, we treated age-matched littersmates with 3 mg per kg body weight of fluvastatin weekly starting at 5 weeks of age. This treatment lowered serum cholesterol concentrations and increased cage activity during a 3-week trial period (data not shown). Starting at week 8, we increased administration of the 3 mg per kg body weight dose of fluvastatin to three times per week (Supplementary Table 5). Fluvastatin treatment lowered serum cholesterol by interfering with cholesterol synthesis through competitive inhibition of HMGCR. As a preliminary trial, we treated age-matched littermates with 3 mg per kg body weight of fluvastatin weekly starting at 5 weeks of age. This treatment lowered serum cholesterol concentrations and increased cage activity during a 3-week trial period (data not shown). Starting at week 8, we increased administration of the 3 mg per kg body weight dose of fluvastatin to three times per week (Supplementary Table 5). Fluvastatin treatment lowered serum cholesterol.
cholesterol and total liver lipids, improved subjective health scores, rotarod performance and open-field activity and increased lifespan when compared with control mice receiving a sham dose (Fig. 5a–c) and was more effective at improving these parameters than the Sqle mutation. Similar to the Sqle mutation, fluvastatin treatment did not improve baseline breathing irregularity or acoustic startle responses (Supplementary Fig. 8a–d).

Markedly, brains of fluvastatin-treated 129.Mecp2tm1.1Bird/Y mice showed an increase in the amounts of cholesterol intermediates, particularly desmosterol, toward wild-type levels at P70 (Fig. 5f and Supplementary Table 3a). The cholesterol biosynthesis pathway is inhibited in brains of Mecp2-null mice, probably because aberrant turnover results in cholesterol accumulation33. Treatment starting at an early age may therefore maintain some amount of pathway activity in the brain to increase lifespan and ameliorate motor symptoms in Mecp2 mutant mice.

To determine whether the effects of fluvastatin were shared by other statin drugs, we treated 129.Mecp2tm1.1Bird/Y mice with lovastatin, which differs from fluvastatin in its hydrophobicity, pharmacokinetics, transport and metabolism37,38. Lovastatin was of particular interest because it is highly lipophilic, increasing the likelihood that it will reach the brain. Lovastatin also ameliorates neurological symptoms in mouse models of fragile X syndrome39 and is being used in clinical trials to ameliorate cognitive problems inchildren with neurofibromatosis type I40. Notably, the livers of female 129.Mecp2tm1.1Bird/Y mice had 164% higher lipid content per gram of tissue than those of wild-type littermates at 8 months of age, which is a substantial increase (Fig. 6e). Notably, although serum cholesterol concentrations were unchanged, fluvastatin treatment reduced lipid accumulation in the liver to wild-type levels in female mice assessed at the end of the 8-month trial (Fig. 6d,e).

DISCUSSION
Our data show that cholesterol metabolism is abnormal in mouse models of RTT and that genetic and pharmacologic interventions to lower cholesterol synthesis lead to improvements in symptoms. Given the diverse roles of cholesterol in the nervous system, including membrane trafficking, signal transduction, myelin formation, dendrite remodeling, neuropeptide formation and synaptogenesis35, abnormal brain cholesterol metabolism may be a crucial link between loss of MECP2 function and neuronal and glial dysfunction. Even small perturbations of cholesterol metabolism can have large effects on neuronal function, disrupting normal development41,42 and contributing to aging disorders, including Huntington and Alzheimer diseases43. Within a fixed window of time, it is probable that cholesterol synthesis in the brain varies among cell types, including neuronal subtypes in various states of differentiation and activity, as well as among non-neuronal cells, including astrocytes, oligodendrocytes and microglia. Whole-brain cholesterol synthesis was decreased in Mecp2-null mice by 23% per gram of tissue, a notable difference considering the variety of cell types present. The only other mouse mutant to show decreased brain cholesterol synthesis is the Cyp46a1 mutant mouse,
which lacks the 24S-hydroxycholesterol that is required for brain cholesterol turnover. The discovery that re-expression of MeCP2 in astrocytes contributes to the mitigation of RTT symptoms in a non-cell autonomous manner may be linked to cholesterol turnover, as astrocytes supplement neuronal cholesterol through lipoprotein transfer. Cholesterol turnover is also required to produce geraniol, a product of HMGCR upstream of SQLE that is essential for learning and synaptic plasticity, and is important for the interaction between neurons and astrocytes at the synapse. Furthermore, microglia, which function as brain macrophages, recycle neuronal cholesterol during dendrite pruning; replacing mutant with wild-type microglia through bone-marrow transfer ameliorates RTT symptoms in MeCP2-null mice. Modulating cholesterol homeostasis and turnover in the brain also ameliorates motor symptoms in MeCP2-null mice: fluvastatin treatment affects the abundance of desmosterol, which is produced primarily by astrocytes in the adult brain.

Perturbed lipid metabolism is evident in the livers as well as the brains of 129. MeCP2<sup>tm1.1Bird</sup> mice and is ameliorated by statin treatment. The precise mechanisms by which statin drugs alter brain cholesterol are unknown but probably include indirect effects of altered systemic lipid metabolism. The <sup>Sqle<sup>Sim3/+</sup></sup> mutation in the 129. MeCP2<sup>tm1.1Bird/Y</sup> genetic background rescued similar behavioral abnormalities as did statin treatment, although it did not change peripheral cholesterol concentrations. Furthermore, 129. MeCP2<sup>tm1.1Bird/Y; Sqle<sup>Sim3/+</sup></sup> brains showed different patterns of sterol changes than did those of statin-treated mice. Statins affect pathways upstream of cholesterol synthesis and are indiscriminate of cell type, whereas the effect of SQLE is limited to cholesterol synthesis in a subset of neurons and glial cells. Notably, statin treatment improved behavioral parameters better than the mutation on this thesis in a subset of neurons and glial cells. Notably, statin treatment was effective, whereas large doses were detrimental, demonstrating the importance of further studies to optimize treatment protocols. Although these findings do not comprise a preclinical trial, they point to mechanism-based strategies for treatment that may ameliorate a subset of symptoms and thereby improve quality of life for some patients with RTT. None of the suppressors eliminated symptoms entirely, supporting the idea that combination therapy will be required for thorough alleviation of RTT symptoms. The identification of additional suppressor loci may point to other potential targets.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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AUTHOR CONTRIBUTIONS
M.J.J conceived of the work, carried out the genetic screen and dissected embryos. J.S. and W.H. carried out the capture sequencing and analysis. C.M.B. confirmed map locations and lesions, performed statin injections and carried out behavior and plethysmography testing and quantitative RT-PCR (qRTPCR). S.M.K. performed protein blotting and liver histopathology. H.M.B. performed preliminary qRT-PCR. J.G.M., B.L. and S.D.T. analyzed steroids and performed synthesis studies. S.D.T. evaluated liver cholesterol and triglycerides. A.A.P. and D.M.K. provided Jaenisch mice and laboratory facilities. D.M.K. helped analyze plethysmography data. M.J.J., D.W.R., D.M.K., S.D.T., S.M.K. and C.M.B. wrote the manuscript with the input from the other coauthors.

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M.J.J conceived of the work, carried out the genetic screen and dissected embryos. J.S. and W.H. carried out the capture sequencing and analysis. C.M.B. confirmed map locations and lesions, performed statin injections and carried out behavior and plethysmography testing and quantitative RT-PCR (qRTPCR). S.M.K. performed protein blotting and liver histopathology. H.M.B. performed preliminary qRT-PCR. J.G.M., B.L. and S.D.T. analyzed steroids and performed synthesis studies. S.D.T. evaluated liver cholesterol and triglycerides. A.A.P. and D.M.K. provided Jaenisch mice and laboratory facilities. D.M.K. helped analyze plethysmography data. M.J.J., D.W.R., D.M.K., S.D.T., S.M.K. and C.M.B. wrote the manuscript with the input from the other coauthors.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Mouse strains and genetic screen. All animal experiments were conducted under protocols approved by local Animal Care and Use Committees in Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited animal facilities at BCM and the University of Texas Southwestern. Congenic 129.Mecp2tm1.1Bird/+ females and male mice were maintained by backcrossing females to males of the 129S6/SvEvTac strain. C57BL/6J males were imported from The Jackson Laboratory at 6 weeks of age and injected with three weekly doses of 100 mg per kg body weight ENU at 8 weeks of age as described58. After recovery of fertility, 60 ENU-treated males were mated to 129.Mecp2tm1.1Bird/+ females, and their G1 male offspring were genotyped for Mecp2 allele status according to The Jackson Laboratory standard protocols. The B6.Mecp2tm1.1Bird line was made in a hybrid 129/Ola C57BL/6 embryonic stem cell line52 and backcrossed to C57BL/6J for over ten generations. The line is maintained by additional backcrosses to C57BL/6J mice.

For each founder line, long-lived G1 males were mated to 129S6/SvEvTac females; G2 offspring were mated to 129.Mecp2tm1.1Bird/+ females or 129S6/SvEvTac males to produce N3 mice for linkage mapping. Fine structure mapping was achieved with informative MIT markers identified using the Mouse Genome Informatics website (http://www.informatics.jax.org). PCR (ABI) followed by gel electrophoresis with MetaPhor agarose (Lonza) on relevant primer pairs determined whether a given mouse carried heterozygous B6/129S6 or 129S6 homozygous DNA at each locus of interest. Primers are available upon request.

The investigator is blind to genetic mutation status when phenotyping in a genotype of mice. For each founder line, samples were obtained from mice in a fed state the late dark phase of a study, or other phenotypes.

Lipid and sterol analyses. 129.Mecp2tm1.1Bird/Y mice and age-matched +Y littermate controls housed in plastic Lab Products cages with corncob bedding in rooms, with alternating 13- and 11-h periods of light and dark, respectively, were provided with acidified water and a Harlan Teklad 2920x diet ad libitum (19.1% protein, 6.5% fat, 0% cholesterol). Gene expression, serum cholesterol concentrations (Cobas Mira clinical chemistry analyzer) and tissue lipids were assessed within a 2-h after-noon window after a 4–6 h fast at P28 and P56. Brain analyses were performed on the subcortical region, which contains the corpus callosum, striatum, thalamus, hypothalamus and hippocampus. Cholesterol intermediates were measured by tandem mass spectrometry following a previously published protocol38 after extraction from tissue of mice treated as described above. Before gas-liquid chromatography, lipids were isolated from tissue using CHCl3/CH2OH extraction, followed by dry- ing of the organic phase under N2 pressure. Tissue cholesterol concentrations were assessed by gas-liquid chromatography, and cholesterol synthesis was assessed from saponified tissue after the incorporation of 100 mcCi 1H2O after intraperitoneal injection as described38. For the in vivo cholesterol synthesis study, samples were obtained from mice in a fed state the late dark phase of a 12-h on/off light/dark cycle. These mice were adapted to individual housing and a Harlan Teklad 7001 rodent chow (low cholesterol (0.02%, wt/wt), low fat (4% wt/wt)) at P28 before analysis. The age of mice at sampling was P54–P56. All chemical assays were performed blinded to genotype and treatment group.

Real-time RT-PCR. Brain RNA was isolated using an RNeasy Lipid Tissue Mini Kit (Qiagen), and liver RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Liver RNA was treated with 1 IU DNase (Ambion Inc.) at 37 °C for 1 h per the manufacturer's instructions. First-strand cDNA was synthesized from 5,000 ng of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) per the manufacturer's instructions. RT-PCR was performed in triplicate for each sample on an ABI 7900 instrument (Applied Biosystems, CA, USA). Gene primers for qRT-PCR were designed against published mRNA sequences using Primer3 software and synthesized by Integrated DNA Technologies (Iowa, USA). Primer sequences are available by request. qRT-PCR was performed in triplicate on an ABI 7900 instrument (Applied Biosystems, CA, USA). Reactions contained cDNA from 10 ng total RNA, 0.1 µl forward and reverse primers, 5 µl Power SYBR Green Master Mix and water to a final volume of 10 µl. The PCR conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Single-product amplification was confirmed by disassociation curves and agarose gel electrophoresis. Gene expression was normalized to an Rpl19 (also known as L19) internal loading control, analyzed using the 2−∆∆CT method and expressed as either a raw 2−∆∆CT value or the expression of Mecp2/Y mice relative to that in wild-type mice60.

Exome sequencing. Genomic DNA was isolated from two N2 littermate females and five (female 5 and female 8) from line 895 (Sum10.0) and from C57BL/6J and 129S6/SvEvTac mice by standard methods. Mouse exome-capture reagents were designed to a 54.3-Mb target, including 203,225 exonic regions (C57BL/6J, NCBI37/mm9)17. Sequencing of enrichment shotgun libraries was performed on an Illumina GAx2 (paired-end, 76-bp reads) or Illumina HiSeq 2000 (paired-end, 100-bp reads). Sequence reads were mapped to the mm9 reference genome with BWA61, and variants were called with SAMTools62 requiring a minimum SNP quality of 20. Custom scripts were used to annotate variants with respect to their predicted impact on protein sequence. To remove indel strain polymorphisms as well as systematic sequencing artifacts, variants were removed from consideration if they were identified in any of 15 other mouse strains (including the parental strains, which were resequenced alongside the mutants), other lines from this study or other phenotypes.

Drug administration. Fluvastatin (Selleckchem) was dissolved in sterile ultrapure water such that the desired dose for a 20 g mouse was given in 100 µl and was administered subcutaneously. Male mice were given a single 3 mg per kg body weight weekly dose at 5, 6 and 7 weeks of age and were then given three times weekly (Monday, Wednesday and Friday) 3 mg per kg body weight doses beginning at 8 weeks of age. Female mice were also given 3 mg per kg body weight doses but were treated only once per week beginning at 6 weeks of age. Lovastatin (Tocris Bioscience) preparation required activation in ethanol followed by adjustment to pH 7.2, as per the product information guidelines. The activated stock solution was diluted with ethanol to 20× the injected dose and kept at −20 °C for up to 1 month. The day of injection, a 1× working solution was prepared by diluting the stock solution in sterile saline such that the desired dose for a 20 g mouse was given in 100 µl. Male mice were injected subcutaneously with a twice-weekly 1.5 mg per kg body weight dose beginning at 5 weeks of age. The precise timing of the injections and phenotyping assays can be found in Supplementary Table 5.

For each of the two types of statin treatment, littermates of the same genotype were divided between the treatment and control groups such that the average and standard deviation of the starting weights and subjective health scores of the two groups were statistically indistinguishable. Cohorts of mixed genotypes in sample sizes of 10 per group were treated and assessed independently to ensure randomization and repeatability.

Assessments of behavior and breathing. Open-field locomotor activity was assessed using Versamax Animal Activity Monitors. Recordings were taken in a secluded room with dim light (20–25 lx) and artificial white noise (55–60 dB). Each mouse was placed in the center of the open-field chamber, and activity was recorded for 30 min. An aspect of motor performance was measured using the accelerating rotating rod (rotarod) (Stoelting). At 8 weeks (males) or 12 weeks (females) of age, mice were placed on a grooved rod rotating at a speed of 4 r.p.m. Over the course of a 5-min trial, the revolution rate increased steadily to a maximum of 40 r.p.m. The time each mouse was able to stay on the rod was recorded for eight trials, four on each of 2 consecutive days, with a minimum of 30 min between trials. A trial ended when the mouse fell off the rod, spun with the rod for two consecutive revolutions or successfully stayed on the rod for 5 min. Pre-pulse inhibition was measured using SR-Lab Starlette Chambers (San Diego Instruments). Unrestrained, whole-body plethysmography was carried out using the Buxco FinePointe system. Breathing parameters were recorded for 1 h after a 30-min acclimation period. Mice were monitored for activity. The data represent breathing during periods of stillness.

For fluvastatin treatment, subsequent to the pilot study that included 6 Mecp2−null mice per treatment group, all behavioral assessments were performed with the experimenter blinded to treatment group. For lovastatin treatment, all behavioral assessments were performed blinded to treatment group. For studies of the SgalSum line, although genotype information was obtained before the start of behavioral studies to ensure equal numbers

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NATURE GENETICS

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of each genotype were being assayed, this information was not linked to the mice until all behavioral assays were complete.

**Statistics.** LOD scores were generated using the R/qtl statistics package. Survival curves were compared using SPSS by Kaplan-Meier analysis followed by log-rank comparison. Statistical comparisons between two groups (wild-type mice compared to Mecp2 mutants) were performed in GraphPad Prism 5 using an unpaired, two-tailed Student’s *t* test; equal variances were not assumed, as the Mecp2 mutant group typically showed increased variability compared to the wild-type group. Statistical tests requiring multiple comparisons were analyzed in SPSS. Excepting the rotarod data, comparisons between multiple groups were analyzed by one-way ANOVA, and sphericity was not assumed; the Bonferroni adjustment was applied when comparing more than two genotypes, and the Dunnett post-hoc test was used to compare statin-treated groups with the vehicle-treated control group. Rotarod data were analyzed using repeated measures ANOVA.

For behavioral assays, sample sizes of at least 12 mice per group are recommended for preclinical assays. For chemical assays, sample sizes of 4–6 per group are usually sufficient to see significant differences. For the suppressor screen, we chose to screen one genome’s equivalent—approximately 700 gametes, when ENU is expected to produce one new mutation in a single gene in every 700 gametes screened. qPCR data were the only data in which outliers were observed. An outlier in triplicate repeats of a single sample was excluded if it was ≥2 s.d. above the mean for a given group.

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