



# Reduced Mid1 expression and delayed neuromotor development in daDREAM transgenic mice

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Downstream regulatory element antagonist modulator (DREAM) is a Ca<sup>2+</sup>-binding protein that binds DNA and represses transcription in a Ca<sup>2+</sup>-dependent manner. Previous work has shown a role for DREAM in cerebellar function regulating the expression of the sodium/calcium exchanger 3 (NCX3) in cerebellar granular neurons to control Ca<sup>2+</sup> homeostasis and survival of these neurons. To achieve a global view of the genes regulated by DREAM in the cerebellum, we performed a genome-wide analysis in transgenic cerebellum expressing a Ca<sup>2+</sup>-insensitive/CREB-independent dominant active mutant DREAM (daDREAM). Here we show that DREAM regulates the expression of the midline 1 (Mid1) gene early after birth. As a consequence, daDREAM mice exhibit a significant shortening of the rostro-caudal axis of the cerebellum and a delay in neuromotor development early after birth. Our results indicate a role for DREAM in cerebellar function.

**Keywords:** midline 1, cerebellar lobes, nuclear calcium, transcriptomic analysis

## INTRODUCTION

Downstream regulatory element antagonist modulator (DREAM), also named calsenilin or KChIP-3, is a Ca<sup>2+</sup>-binding protein of the neuronal calcium sensors family able to repress transcription of specific genes in a Ca<sup>2+</sup>-dependent manner (Carrion et al., 1999), to interact with presenilins and to modify APP processing (Buxbaum et al., 1998; Lilliehook et al., 2003) and to regulate the membrane expression and gating of Kv4 potassium channels (An et al., 2000; Ruiz-Gomez et al., 2007) and of voltage-dependent calcium channels (Thomsen et al., 2009; Anderson et al., 2010). In addition, DREAM modulates downstream signaling of different membrane receptors including NMDA (Wu et al., 2010; Zhang et al., 2010) and THSR (Rivas et al., 2009).

Transcriptional activity of DREAM is triggered by its sumoylation-dependent nuclear translocation (Palczewska et al., 2011), regulated by redox state (Rivas et al., 2011), and accomplished by its Ca<sup>2+</sup>-dependent specific binding to DNA and to other nucleoproteins, including CREM and CREB (Ledo et al., 2000, 2002; Rivas et al., 2004; Scsucova et al., 2005; Zaidi et al., 2006). In addition, high affinity binding of DREAM to DRE sequences in the DNA requires Ca<sup>2+</sup>-dependent DREAM oligomerization (Carrion et al., 1998; Osawa et al., 2001, 2005).

Downstream regulatory element antagonist modulator is widely expressed in the central nervous system as well as in the thyroid gland, testis, and the immune system (Carrion et al.,

1999). Tissue-specific target genes for DREAM regulation have been identified first *in vitro* (Carrion et al., 1999; Link et al., 2004) and more recently *in vivo*, using DREAM null mice (Cheng et al., 2002) and especially transgenic mice expressing a dominant active DREAM mutant (daDREAM; Gomez-Villafuertes et al., 2005; Savignac et al., 2005, 2010; Rivera-Arconada et al., 2010). Specifically, a significant increase in prodynorphin mRNA was found in spinal cord from DREAM null mice (Cheng et al., 2002) and a decrease in NCX3 and BDNF mRNA and protein levels were reported in hippocampus and cerebellum (Gomez-Villafuertes et al., 2005) and in spinal cord (Rivera-Arconada et al., 2010) respectively, from daDREAM mice. The regulatory effect of daDREAM on the expression of transcriptional targets was shown to be specific, since for instance no change in the expression of other members of the sodium/calcium exchanger family could be observed in hippocampus and cerebellum of daDREAM transgenic mice (Gomez-Villafuertes et al., 2005). As expected, transcriptional regulation by DREAM of various cellular targets has diverse and tissue-specific functional consequences. In the CNS, this includes changes in sensory noxious perception (Cheng et al., 2002; Rivera-Arconada et al., 2010),  $\beta$ -amyloid accumulation (Lilliehook et al., 2003), and learning and memory formation (Alexander et al., 2009), as well as modified T-cell proliferation and Ig production (Savignac et al., 2005, 2010) in the immune system.

Taking the cerebellum as an example, previous work using primary cultures of cerebellar granular neurons has shown the role of DREAM in the regulation of NCX3 expression, which in turn is fundamental for the maintenance of  $Ca^{2+}$  homeostasis and viability of the cerebellar granular neurons in the culture (Gomez-Villafuertes et al., 2005). In the present study, to further investigate the functional involvement of DREAM-mediated transcription in cerebellar function, we have performed a transcriptomic analysis of the cerebellum from daDREAM transgenic mice. We found that expression of the midline 1 gene (*Mid 1*) is repressed in daDREAM mice. Related to this, daDREAM mice exhibit a significant shortening of the rostro-caudal axis of the cerebellum and a severe delay in neuromotor development early after birth.

## RESULTS

### GENOME-WIDE ANALYSIS IN daDREAM CEREBELLUM

To search for DREAM transcriptional targets that could disclose a role for DREAM in cerebellar function, we performed genome-wide analysis in a line of transgenic mice previously reported to express daDREAM in the cerebellum (Gomez-Villafuertes et al., 2005). Comparison of basal gene expression in wild type and transgenic adult cerebellum, using cDNA microarrays, identified only 11 genes whose expression was significantly altered in daDREAM transgenic cerebellum (Gene Expression Omnibus accession number GSE34765; **Figure 1**). Up- and down-regulated genes are presented in **Table 1** and the bioinformatics analysis is shown in **Tables A1** and **A2** in Appendix.

Among the genes with modified expression (**Table 1**), we focused our attention on the significant down regulation of the *Mid 1* gene, an ubiquitin ligase specific for the protein phosphatase 2A (PP2A; Trockenbacher et al., 2001). Loss of function mutations in the *Mid1* protein cause the X-linked Opitz BBB/G syndrome, a congenital anomaly disorder characterized by developmental defects of midline structures (Opitz, 1987; Quaderi et al., 1997). Importantly, *Mid1* deficient mice mimic the hypoplasia of the anterior portion of the medial cerebellum (Lancioni et al.,

2010), a clinical feature present in Opitz patients, suggesting that DREAM, through the regulation of *Mid1*, might participate in cerebellar development. Real-time qPCR analysis confirmed the reduced level of *Mid1* mRNA in the cerebellum of adult transgenic mice as well as in the hippocampus (**Figure 2A**), areas with significant expression of daDREAM (**Figure 2B**). As reported for other DREAM target genes like prodynorphin in the hippocampus (Cheng et al., 2002) or *CANT1* in the cerebellum (Cali et al., 2012), probably due to functional compensation by other members of the KCHIP family, no change in the expression of *Mid1* was observed in cerebellum or hippocampus from DREAM knockout mice (**Figure 2C**). Western blot analysis of cerebellum and hippocampus from daDREAM mice confirmed the reduced levels of *Mid1* protein with respect to wild type mice (**Figure 2D**). Taken together, these results implicate endogenous DREAM/KCHIPs in the regulation of *Mid1* gene expression in the brain.

### DREAM REGULATES EARLY POSTNATAL *Mid1* EXPRESSION

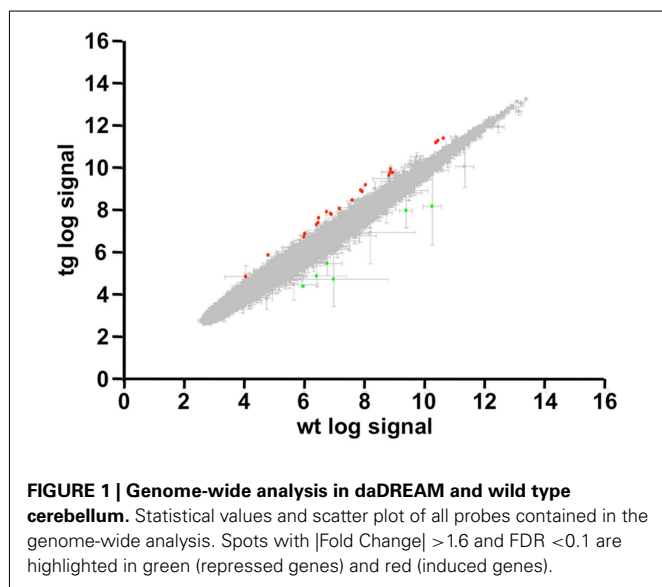
*Mid1* is widely expressed at early human embryonic stages, however during organogenesis the expression pattern becomes more restricted to the tissues affected in the Opitz syndrome (Pinson et al., 2004). The development of the cerebellum in mice is mostly postnatal and is complete after the second week of life (reviewed in Millen and Gleeson, 2008). To investigate a role for DREAM in the regulation of *Mid1* and cerebellar development, we next analyzed the expression of the daDREAM transgene during cerebellar development and the consequences on *Mid1* expression. Real-time qPCR analysis showed that the expression of daDREAM in the cerebellum was very high early after birth (P2) and got down at P7–P21 (**Figure 3A**) to values slightly higher than adult levels (see **Figure 2B**). Expression of *Mid1* in wild type cerebellum was high early after birth (P2–P7) and reached adult levels at P21 (notice the different scale in **Figure 2A**). Expression of daDREAM was associated with a significant reduction of *Mid1* mRNA levels in transgenic cerebellum (**Figure 3A**). Early postnatal expression of the transgene was not exclusive to the cerebellum and was observed as well in other brain areas, like the hippocampus (**Figure 3B**). Expression of daDREAM in the hippocampus was also associated with a significant reduction of *Mid1* mRNA levels (**Figure 3B**).

### CEREBELLAR MORPHOLOGICAL CHANGES IN daDREAM MICE

Absence of *Mid1* protein in *Mid1* null mice results in abnormal formation of the anterobasal cardinal lobe, which is evident already at P0–P2 (Lancioni et al., 2010). In adult *Mid1* null mice, although abnormal in shape, even the anterior vermal lobes maintain the correct layer organization and thickness (Lancioni et al., 2010). According to the significant reduction in *Mid1* expression, adult daDREAM mice showed a significant shortening in the antero-posterior axis but also in the left–right axis (**Figures 4A,B**). Sagittal sections from P15 wild type and daDREAM mice confirmed a correct layer organization of transgenic cerebellum and showed the shortening of the rostro-caudal axis (**Figure 4C**).

### IMPAIRED MOTOR DEVELOPMENT IN daDREAM MICE

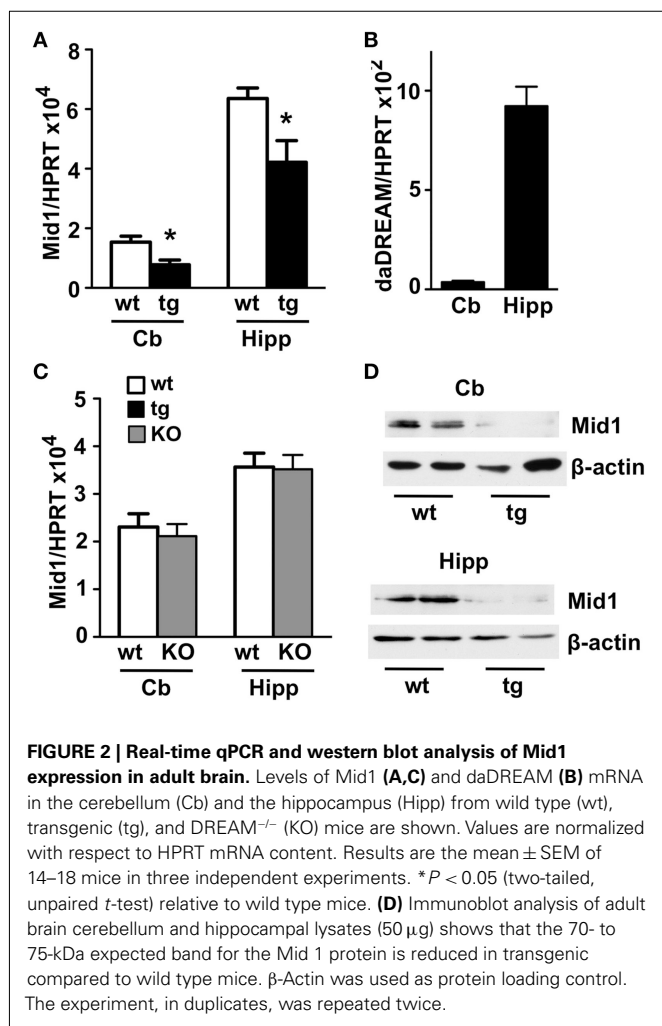
Absence of *Mid1* protein in *Mid1* null mice has been associated with impaired motor coordination in the adulthood. To assess possible changes during early postnatal development that could be



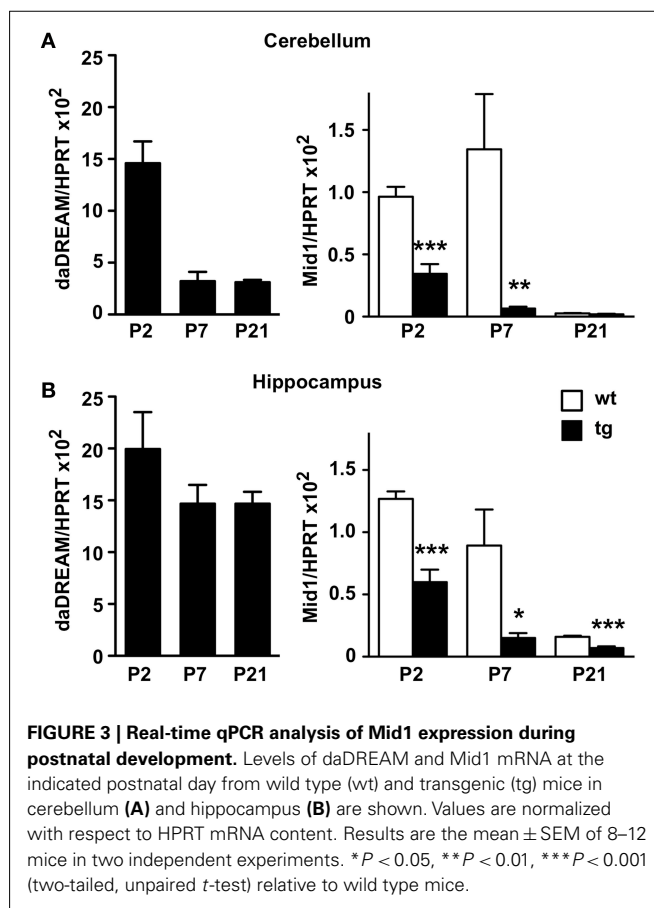
**Table 1 | List of induced and repressed genes in daDREAM vs. wild type cerebellum.**

Fold change	<i>P</i> -value × 10 <sup>-5</sup>	FDR	Probe ID	Transcript ID	Gene symbol	Gene description
1.70	0.922	0.023	1440557_at	Mm.186257.1	lpw	Imprinted gene in the Prader-Willi syndrome region
1.75	1.792	0.038	1436733_at	Mm.40013.1	E130309F12Rik	RIKEN cDNA E130309F12 gene
1.86	0.914	0.026	1460049_s_at	Mm.213028.1	1500015O10Rik	RIKEN cDNA 1500015O10 gene
1.95	0.319	0.0160	1417654_at	Mm.3815.1	Sdc4	Syndecan 4
2.10	0	0	1449340_at	Mm.43375.1	Sostdc1	Sclerostin domain containing 1
-4.68	0.035	0.000	1436240_at	Mm.216255.1	B230214O09Rik	RIKEN cDNA B230214O09 gene
-4.17	0.177	0.016	1431214_at	Mm.157900.1	LOC433762	Hypothetical gene LOC433762
-2.95	0.133	0.027	1440139_at	Mm.174301.1	gb:BB729836	Moderately similar to S12207 hypothetical protein
-2.83	0.231	0.030	1438239_at	Mm.24820.1	Mid1	Midline 1
-2.60	0.408	0.037	1425545_x_at	Mm.33263.2	H2-D1	Histocompatibility 2, D region locus 1
-2.38	0.639	0.048	1419327_at	Mm.22635.1	Pdxdc1	Pyridoxal-dependent decarboxylase domain containing 1

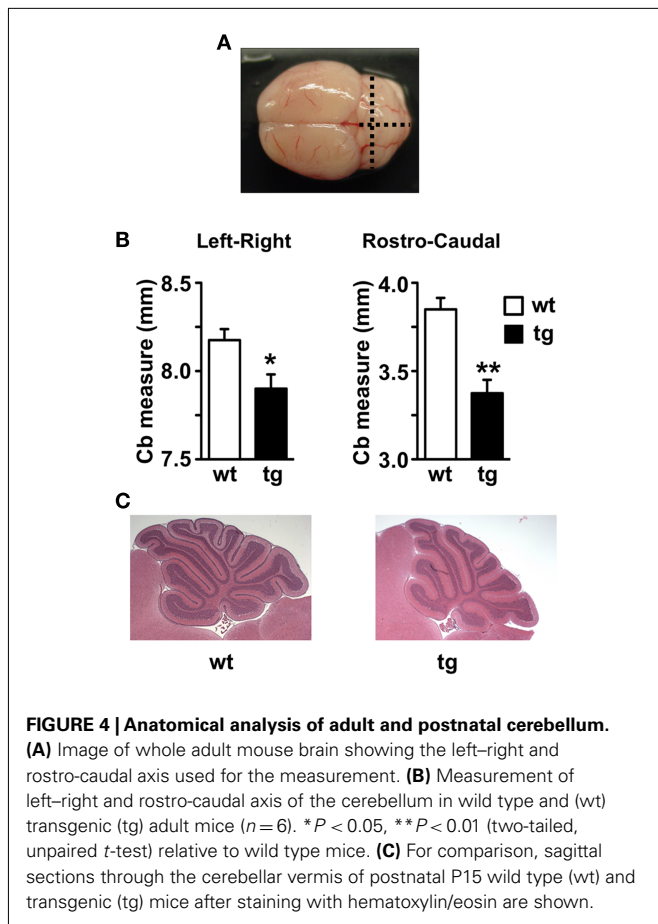
Genes with FDR < 0.05 were included in the list of induced or repressed candidates.



related to Mid1 down regulation in daDREAM mice, we employed a test battery consisting of somatometric, neurologic, and sensorial and motor tests. No differences in somatometric growth, or in the appearance of developmental landmarks, i.e., eye opening,



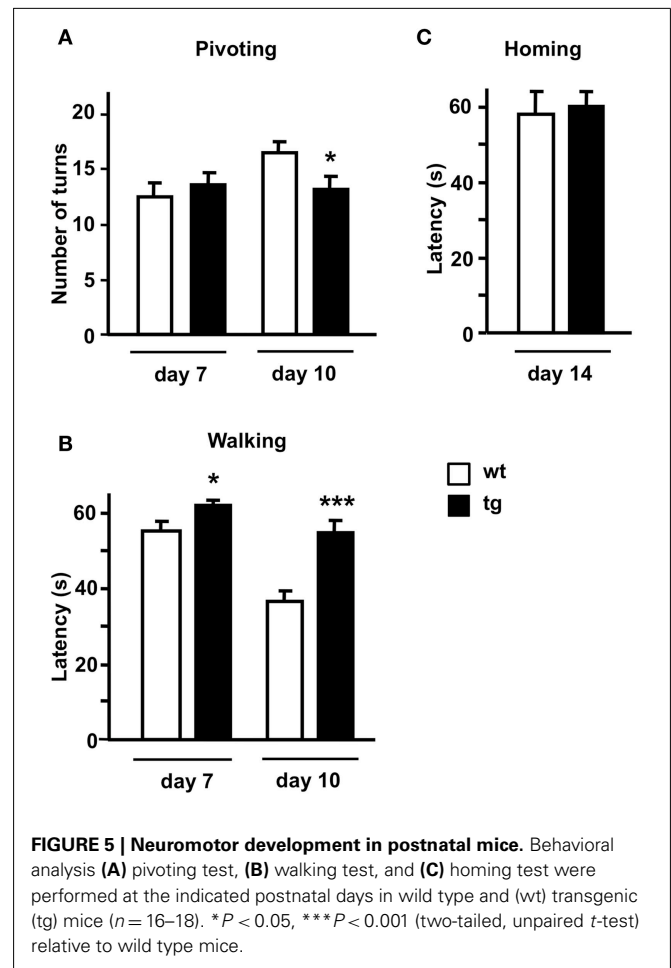
fur appearance, or incisor eruption, were detected in daDREAM mice. However, significant changes were observed in neuromotor development as assessed by the pivoting and the walking tests on postnatal days 7, 10, and 14. In the pivoting locomotion task, wild type, and daDREAM mice showed similar activity at P7. However, the normal age-dependent increase in activity, which is reflecting the adequate maturation of motor systems in wild type, was significantly reduced in daDREAM mice at P10 (Figure 5A).



Assessment of walking activity confirmed the hypoactive phenotype in daDREAM mice and, even at P7 the latency to walk in transgenic mice was significantly longer than in wild type mice (Figure 5B). At P10, the reduction in walking latency observed in wild type (33.6% of reduction, P7 vs. P10) was significantly less pronounced in daDREAM mice (10.8% reduction, P7 vs. P10; Figure 5B). This result, along with the affected pivoting activity in daDREAM mice, could suggest a supraspinal deficiency, indicating that the postnatal development of brain structures directly implicated in the motor function might be affected in daDREAM mice. This defect is specific of motor function, since daDREAM mice showed normal latency to reach their nest in the homing test compared to wild types (Figure 5C). This normal latency might suggest a normal development of other functional domains such as sensory perception.

## DISCUSSION

Genome-wide analysis of different tissues or brain areas from daDREAM transgenic mice has rendered discrete lists of potential DREAM regulated genes with some overlapping as well as tissue-specific targets (Savignac et al., 2010; Jose R. Naranjo, unpublished observations). In all cases, including the present data, these lists include both repressed and induced genes suggesting that besides the direct repressor action of DREAM, interactions between DREAM and other transcriptional regulators (Ledo et al.,



2002; Rivas et al., 2004, 2011; Scsucova et al., 2005; Zaidi et al., 2006) or indirect/secondary transcriptional effects might contribute to gene induction in daDREAM transgenic tissues. In the case of the daDREAM cerebellum, the list of genes with modified expression is especially short. This is not related to technical problems with the hybridization of the array since the bioinformatics analysis of the hybridization signals (see Figure 1) shows a perfectly normal distribution. Instead, the low number of hits might reflect the limitation of the technique, showing for instance a minor and not significant down regulation ( $-1.15$ -fold;  $P$ -value 0.06) of the *NCX3* gene (also named *Slc8a3*: solute carrier family 8) previously validated as a direct DREAM target and shown to be repressed by more than 50% in daDREAM cerebellum (Gomez-Villafuertes et al., 2005). Alternatively, the low number of targets may simply reflect the limited but specific functionality of DREAM in the cerebellum, a brain region where DREAM expression shows the lowest level among the different areas in the CNS (Carrion et al., 1999; Rhodes et al., 2004).

Among induced genes, syndecan 4 is a transmembrane proteoglycan for which there are no reports relating a function in brain or cerebellum. Sclerostin domain containing 1 (*Sostdc1*) is a secreted inhibitor of the Wnt and Bmp pathways, that antagonizes BMP signaling in the mesenchymal induction of teeth and orofacial morphogenesis, regulating also the spatial patterning of

teeth and hair. Deletion of *Sostdc1* leads to the full development of single extra incisors adjacent to the main incisors (Munne et al., 2009). On the other hand, induced expression of sclerostin domain containing 1 (*Sostdc1*) has been reported in the brain of mice deficient in phenylalanine hydroxylase (PAH), an animal model for the autosomal recessive disorder phenylketonuria (Park et al., 2009), though the functional meaning of this increased expression is not known. In daDREAM mice, however, induced levels of *Sostdc1* do not have an effect on dentation and hair appearance since both are normal.

Among repressed genes, the *Mid1* protein is biochemically well characterized and a role in brain development has been reported. Thus, early studies of *Mid1* function recognized its ubiquitin ligase activity and have identified its specific role in the degradation of protein phosphatase 2A by the proteasome (Trockenbacher et al., 2001). Importantly, PP2A is the major serine–threonine–tau phosphatase in the brain and dephosphorylates also other microtubule-associated proteins like 4EBP1 and p70S6K, two targets of the mTOR kinase (Nojima et al., 2003). Loss of function mutations of the *Mid1* protein are associated with developmental midline malformations present in Opitz syndrome patients (Opitz, 1987; Quaderi et al., 1997). Furthermore, cells derived from Opitz syndrome patients show decreased formation of mTORC1, the complex between mTOR and its interacting proteins Raptor and mLST8, as well as S6K1 phosphorylation, cell size, and cap-dependent translation. Expression of wild type *Mid1* or activated mTOR protein rescue the phenotype, suggesting that mTORC1 plays a key role in Opitz syndrome pathogenesis (Liu et al., 2011). In addition, a recent study has identified the binding of the cytoskeletal-associated *Mid1*/α4/PP2A complex to polyribosomes and mRNAs via a purine-rich sequence motif called MIDAS (MID1 association sequence) increasing the stability and translational efficiency of these mRNAs (Aranda-Orgilles et al., 2011). Notably, the MIDAS motif is frequent in mRNAs related to development and energy metabolism and mutated *Mid1* does not interact with MIDAS-containing mRNAs suggesting that defects associated with the Opitz syndrome could be produced by altered protein translation of some of these mRNAs (Aranda-Orgilles et al., 2011). About the mechanism by which *Mid1* could affect brain development it has been hypothesized that by controlling PP2A activity, *Mid1* is regulating the activity of *Gli3* (Krauss et al., 2008) a Wnt target gene related to the development of the hippocampus (Hasenpusch-Theil et al., 2012), which has been implicated in three human pathological conditions sharing common features with Opitz syndrome (Biesecker, 2006). Lack of the murine *Mid1* gene results in motor coordination defects as well as motor, non-associative, and procedural learning impairments that may correlate with the developmental delays of Opitz syndrome patients (Lancioni et al., 2010). The behavioral analysis in this study was done in adult mice with no specific focus on postnatal development. Our data report an early down regulation of *Mid1* expression in daDREAM mice and suggest a delayed neuromotor development in these mice. Down regulation of *Mid1* mRNA levels is not restricted to the cerebellum and is also observed in the hippocampus, a brain area with strong expression of the daDREAM mutant. Whether hippocampal morphology is altered in daDREAM mice and to what extent the reduction in other

genes like *NCX3* contribute to the morphological and neuromotor phenotypes remain to be investigated.

As expected for a protein with developmental functions, expression of *Mid1* in adult brain is reduced compared to postnatal levels. Interestingly, however, expression in the adult hippocampus is higher than in the adult cerebellum. This may suggest a specific function for *Mid1* in adult hippocampus and the existence of strong trans-activating mechanisms to compensate a presumably stronger repression due to higher levels of DREAM protein in hippocampus compared to cerebellum.

Analysis of the post-transcriptional regulation of the *Mid1* mRNA has shown that alternative use of different transcription start sites, alternative splicing, and the existence of different polyadenylation signal results in a wide variety of *Mid1* isoforms (Landry and Mager, 2002; Winter et al., 2004). Less is known, however, about the transcriptional mechanisms that regulate *Mid1* expression. Like in the case of the *BDNF* gene, another example of a gene with multiple transcription start sites, specific regulatory regions have been assigned to each transcription start site. Interestingly, *in silico* analysis of these different promoters, both in the human and the mouse genes, has identified putative DRE sites (Table 2), mostly located in the complementary strand, a circumstance previously reported for DRE sites in the ICER (Link et al., 2004) and the interferon  $\gamma$  (Savignac et al., 2005) genes. Nevertheless, *in vitro* analysis of the ability of these sites to bind recombinant DREAM and especially chromatin immunoprecipitation studies should confirm the *in silico* predictions.

In conclusion, the results from the genome-wide analysis of the cerebellum from daDREAM mice indicate that endogenous DREAM, in addition to regulate *NCX3*, might also contribute to cerebellar function through the regulation of *Mid1* gene expression.

## MATERIALS AND METHODS

### TRANSGENIC MICE

A cDNA encoding human DREAM with two amino acid substitutions at EF-hands 2, 3, and 4 and at the N-terminal LCD

**Table 2 | Downstream response elements, DREs, present in the 5' untranslated region of different *Mid1* isoforms.**

Species	Isoform	Position in mRNA	Sequence <sup>a,b</sup>	Orientation
Mouse	Var 1 <sup>c</sup>	155–164	tgatgacccc	←
	Var 2	68–78	cttgacattgt	←
	Var 2	261–270	gggtcatgga	←
Human	Var 1	317–328	gggtcatggga	→
	Var 2 <sup>d</sup>	161–173	ctgatgacaca	←
	Var 4	158–168	taatgactcca	←
	Var 4	259–268	aatgacgtaa	←

<sup>a</sup>The core sequence is underlined.

<sup>b</sup>For comparison the mouse interferon  $\gamma$  DRE site in the complementary strand (←) is atcggctgacctagag and the human dynorphin forward element (→) is agc-cggagtcaaggag.

<sup>c</sup>The same element is present in mouse variant 2 at position 316–327.

<sup>d</sup>The same element is present in variants 1, 3, 6, 7, 8, and 9.

(daDREAM) was cloned downstream of the human CaMK-II $\alpha$  promoter (Mayford et al., 1995) in a bicistronic expression vector containing an IRES and the LacZ reporter gene. DREAM transgenic mice were prepared by pronuclear microinjection of this cassette in the C57BL/6x129/Ola hybrid background. Transgenic progeny were identified by Southern blotting and qualitative PCR of tail DNA using specific primers; forward 5'-TTGCAGTGCACGGCAGATACACTTGCTGA-3' and reverse 5'-CCACTGGTGTGGG CCATAATTCAATTCGC-3'. An amplified fragment of 326 bp indicated the presence of the transgene. Founder males were backcrossed to C57BL/6 females to generate lines that were maintained as heterozygous and age- and sex-matched littermates were used as controls. Of the different transgenic lines generated, in this study we used transgenic line 33 that shows expression of the transgene in different brain areas, including the cerebellum (Gomez-Villafuertes et al., 2005; Wu et al., 2010).

### MICROARRAY

RNA from whole cerebellum from wild type and transgenic mice was prepared using TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen). RNA was quantified and the quality was assessed with a 2100 Bioanalyzer (Agilent technologies). cDNA was synthesized from 4  $\mu$ g of total RNA using one-cycle target labeling and control reagents (Affymetrix) to produce biotin labeled cRNA. The cRNA preparation (15  $\mu$ g) was fragmented at 94°C for 35 min into 35–200 bases in length. Labeled cRNAs were hybridized to Affymetrix chips (GeneChip Mouse Genome 430 2.0 Array). Each sample was added to a hybridization solution containing 100 mM 2-(*N*-morpholino) ethanesulfonic acid, 1 M Na<sup>+</sup>, and 20 mM of EDTA in the presence of 0.01% of Tween-20 to a final cRNA concentration of 0.05  $\mu$ g/ml. Hybridization was performed for 16 h at 45°C. Each microarray was washed and stained with streptavidin-phycoerythrin in a Fluidics station 450 (Affymetrix) and scanned at 1.56  $\mu$ m resolution in a GeneChip® Scanner 3000 7G System (Affymetrix).

### MICROARRAY DATA ANALYSIS

Three biological replicates were independently hybridized for each cell type. GeneChip intensities were background-corrected, normalized and summarized by the RMA method (Irizarry et al., 2003) using the “Affy” package (Gautier et al., 2004) from Bioconductor. Rank Products method (Breitling et al., 2004) was applied to identify differentially expressed genes as implemented in the “RankProd” package (Hong et al., 2006) from Bioconductor. Genes with FDR <0.05 were included in the list of induced or repressed candidates. FIESTA viewer was used to facilitate the application of these numerical filters and the selection of candidate genes (Oliveros, 2007). For the functional annotation of the results GeneCodis 3.0 was used (Nogales-Cadenas et al., 2009) applying a hypergeometric distribution and a FDR threshold of 0.05 for detecting over-represented Gene Ontology terms in candidate genes.

### REAL-TIME QUANTITATIVE PCR

RNA was isolated from whole tissues using TRIzol (Invitrogen), treated with DNase (Ambion), and reverse transcribed using hexamer primer and Moloney murine leukemia virus reverse

transcriptase. To confirm the absence of genomic DNA, each sample was processed in parallel without reverse transcriptase. Real-time quantitative PCR (qPCR) for endogenous DREAM, and daDREAM was performed as described (Savignac et al., 2005). Validation of microarray up- or down-regulated genes was with specific primers and TaqMan MGB probes (Applied Biosystems). The results were normalized as indicated by parallel amplification of HPRT.

### WESTERN BLOT ANALYSIS

Fifty micrograms of total protein from cerebellum or hippocampus were resolved in SDS-PAGE and transferred to PVDF membranes (Millipore). Antibodies against Mid1 (Ab 70770, Abcam) and  $\beta$ -actin (Sigma) were used.

### ANATOMICAL ANALYSIS

For observation of adult cerebellum, 2-months-old wild type and transgenic mice were anesthetized and whole brains were carefully removed from the skull. Coronal and sagittal sections were taken and measurements of left–right and rostral–caudal axis were performed. For histological analysis, brains from 15-days-old pups (P15) were harvested and fixed in 4% paraformaldehyde for 48 h at 4°C. Brains were processed for paraffin embedding and microtome sectioning. Brain sagittal sections of 10  $\mu$ m were stained with hematoxylin/eosin using standard procedures.

### BEHAVIORAL ANALYSIS

Experiments were performed in newborn mice homozygous for the transgene and wild type littermates. Mice were initially housed in a temperature (21  $\pm$  1°C) and humidity (65  $\pm$  10%) controlled room with a 12/12-h light/dark cycle (lights on from 0800 to 2000 hours) with *ad libitum* food and water. Breeding pairs were formed and females visibly close to parturition were isolated. To avoid the effects of parity on behavioral ontogeny (Crusio and Schmitt, 1996) the first litter not used for the experiments. Testing involved approximately equal amounts of males and females. Experiments were carried out during the dark phase of the light–dark cycle. Behavioral tests and animal care were conducted according the EU and local ethical guidelines (EU directive 86/609 and Appendix A of the Council of Europe Convention ETS123, EU decree 2001-486 Decree 214/97 and Spanish law 32/2007) and approved by the local ethical committee (CEEA-PRBB). All behavioral experiments were carried out with the experimenter blind to the genotype. Developmental landmarks analyzed include: (i) assessment of body growth and body length were recorded from P1, the day of birth; (ii) fur appearance: beginning on P2 appearance of immature fur was observed and fur was defined as the mature hair being raised; (iii) incisor eruption: beginning on P7 pups were inspected daily for the emergence of both lower and upper incisor from the gingival; and (iv) eye opening, beginning on P9 pups were inspected daily for the complete opening of both eyelids. Neuromotor development was assessed on P7 and P10 by means of the pivoting and walking tests and general psychomotor development on P14 by means of the homing test.

In the pivoting locomotion test, the total number of degrees turned by the pup during a 60-s period was recorded. The test was performed on a flat surface covered with a paper on which lines

had been drawn to delineate four 90° quadrants. The number of degrees was scored only in completed 90° segments.

In the walking test, the latency for a mouse to lift up on all four legs and walk a distance exceeding its body length was measured on a flat surface covered with a green paper.

For the homing test, individual pups on postnatal day 14 were transferred to a cage containing new sawdust in 3/4 and 1/4 of sawdust of the home litter (“goal arena”). The pups were placed in the opposite side of the goal arena, near to the wall. The time taken to reach the home litter sawdust was recorded. A cut-off time of 180 s was applied.

Significance of the effects was assessed by two-tailed, unpaired Student’s *t*-test was used for comparisons between groups. Analysis was processed using the SPSS program.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Mara Dierssen, Britt Mellström, Marisa Brini, Ernesto Carafoli, and Jose R. Naranjo.

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## APPENDIX

**Table A1 | Functional annotation of over-represented candidate genes according to Gene Ontology (Cellular components).**

GO ID	Term	Probe ID	P-value	FDR
GO:0005925	Focal adhesion	1417654_at	0.0134524	0.045738
GO:0005615	Extracellular space	1449340_at, 1460049_s_at	0.0050619	0.0286841
GO:0015630	Microtubule cytoskeleton	1438239_at	0.0116782	0.0496325
GO:0043034	Costamere	1417654_at	0.0024424	0.0207604
GO:0042612	MHC class I protein complex	1425545_x_at	0.00211703	0.0359896

**Table A2 | Functional annotation of over-represented candidate genes according to Gene Ontology (Biological process).**

GO ID	Term	Probe ID	P-value	FDR
GO:0007389	Pattern specification process	1449340_at	0.0142579	0.0161589
GO:0016055	Wnt receptor signaling pathway	1449340_at	0.0262754	0.0262754
GO:0042475	Odontogenesis of dentin-containing tooth	1449340_at	0.00731229	0.0088792
GO:0006955	Immune response	1425545_x_at	0.0243609	0.0258834
GO:0032874	Positive regulation of stress-activated MAPK cascade	1438239_at	0.00211703	0.00514137
GO:0030514	Negative regulation of BMP signaling pathway	1449340_at	0.00471748	0.0066831
GO:0051894	Positive regulation of focal adhesion assembly	1417654_at	0.000977557	0.00415462
GO:0090398	Cellular senescence	1460049_s_at	0.00130323	0.00443099
GO:0051496	Positive regulation of stress fiber assembly	1417654_at	0.00227973	0.00484442
GO:0070314	G1 to G0 transition	1460049_s_at	0.000488878	0.00415546
GO:0045860	Positive regulation of protein kinase activity	1417654_at	0.00504214	0.00659357
GO:0007026	Negative regulation of microtubule depolymerization	1438239_at	0.00260505	0.00492065
GO:0019882	Antigen processing and presentation	1425545_x_at	0.00471748	0.0066831
GO:0008054	Cyclin catabolic process	1460049_s_at	0.000651793	0.00369349
GO:0002474	Antigen processing and presentation of peptide antigen via MHC class I	1425545_x_at	0.00195432	0.00553723
GO:0001916	Positive regulation of T-cell mediated cytotoxicity	1425545_x_at	0.00276768	0.00470505
GO:0002485	Antigen processing and presentation of endogenous peptide antigen via MHC class I via ER pathway, TAP-dependent	1425545_x_at	0.000488878	0.00415546